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REGULATIONS OF UREIDE BIOSYNTHESIS
GENES IN TROPICAL LEGUME ROOT NODULES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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****

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To my wife, Sou Hyun, and parents
ACKNOWLEDGEMENTS

I express sincere appreciation to Dr. Desh Pal S. Verma for his guidance and advice throughout this research. I would like to thank the other members of my advisory committee, Drs. L. Mark Lagrimini, Richard T. Sayre and Randall L. Scholl, for their suggestions and comments on this study. Gratitude is expressed to members of our lab for their friendship and encouragement. I offer sincere thanks for my wife, Sou Hyun, and my parents for their love, faith and standing with me through the long period of my endeavors. To my children, Cassidy and Lauren, I thank you for understanding my frequent absences during this study.
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CHAPTER I

TROPICAL LEGUME - RHIZOBIA SYMBIOSIS

Introduction

Soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively termed as rhizobia, induce root nodules on legume plants as a result of the symbiotic interactions. Within nodules, the bacteria become new intracellular organelles called bacteroids and reduce atmospheric nitrogen into ammonia, which is called symbiotic nitrogen fixation (Verma and Long, 1983; Long, 1989).

Although the morphology of legume root nodules shows much diversity (Corby, 1988), four anatomical features are considered to be the characteristic of legume nodule development (Brewin, 1991): induction of a new plant meristem in root cortical cells; tissue and cell invasion by rhizobia; development of a central tissue where the oxygen-sensitive nitrogen fixation reaction takes place; and development of peripheral vascular tissue located between the central mass of infected tissue and the nodule endodermis. To initiate the development of root nodules, sequential induction of many bacterial (Lerouge *et al.*, 1990; Kondorosi, 1992) and plant (Peters and Verma, 1990; Djordjevic *et al.*, 1987) genes is necessary, following signal exchange.

Based on the types of nitrogen reduced, legume plants are divided into two groups, one is amide-producer and the other is ureide-producer. Ureide-producers form determinate nodules whereas amide-producers form indeterminate nodules (see below).
Communication between Legume and Rhizobia

Leguminous plants release flavonoids, which are synthesized via phenylpropanoid pathway, into the rhizosphere, and rhizobia move towards specific flavonoids (Kape et al., 1991; Maxwell and Phillips, 1990). In Rhizobium, these signal molecules act as either nod gene inducers or inhibitors, depending on the specificity of NodD protein, a product of common nod gene (Kondorosi, 1992). The role of NodD is activating the expression of common nod genes in conjunction with plant flavonoids, and the fact that NodD proteins from different rhizobia recognize specific flavonoids is the basis of host-rhizobia specificity (Brewin, 1991; Tab. 1). The NodD protein binds to the nod box, a highly conserved 60-bp sequence present in front of all inducible nod transcriptional units (Kondorosi, 1992), with its DNA-binding helix-turn-helix domain (Shearman et al., 1986). It's been proposed that upon addition of the appropriate inducer, the plant signal molecules cross the outer membrane of the rhizobia and accumulate in the inner membrane. At this site, the nod box-bound NodD protein interacts with the inducer (flavonoids) and changes its conformation. Then NodD binds the RNA polymerase and activates transcription of nodABC and other inducible nod operons (Kondorosi, 1992). The NodD protein belongs to the LysR family of the procaryotic positive regulatory proteins (Henikoff et al., 1988). Rhizobium meliloti carries three variants of NodD, i.e. NodD1, NodD2 and NodD3, and each NodD has a different flavonoid specificity, resulting in a greater diversity of host specificity (Gyorgypal et al., 1988).

Upon induction, Rhizobium produces a nod factor, a return signal, and infects a susceptible host plant. NodRm-1, the factor produced by flavonoid-induced R. meliloti cultures (Lerouge et al., 1990), elicits root hair deformation and stimulates cortical cell division (Roche et al., 1991) when added in nanomolar concentrations to alfalfa.
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seedlings. NodRm-1 is a sulfated β-1,4-tetrasaccharide of D-glucosamine having three acylated amino groups. At the non-reducing end, the sugar contains an N-acyl C16 fatty acid group whereas the last sugars at the reducing end carries a sulfate group (Lerouge et al., 1990). Nod factors secreted by induced R. leguminosarum bv. viciae and R. sp. NGR234 cultures share structural similarities to NodRm-1 in that all are N-acetyl D-glucosamine oligomers, while nod factors secreted by other rhizobia contain different number of glucosamine residues (reviewed by Carson et al., 1994). Furthermore, R. leguminosarum bv. viciae Nod factors carry an O-acetyl group at the non-reducing end of the sugar residue and contain a fatty acid chain. R. sp. NGR234 nod factors have sulfate group. It has been shown that common nod genes, i.e. nod ABC, are responsible for the synthesis of core structure of nod factors while nodH and nodQ genes function as adding sulfate residue to this compound, resulting in the specific host-rhizobia interactions (Roche et al., 1991). The functions of other nod genes were reviewed in detail (Carson et al., 1994).

Communication between the host and rhizobia is a sequential process where the initiation of nodule primordium through nodule senescence needs discrete signal exchange. The purified bacterial signal molecules from culture can induce cortical cell division. Therefore, physical contact between rhizobia and host is not necessary for the initiation of nodule development. In legumes, the region just behind the apical meristem at the site of emerging root hairs is most susceptible to rhizobia infection (Bhuvaneswari et al., 1981), indicating those sites are most responsive to the morphogenetic signals (Verma, 1992). The fact that over-expression of bacterial nod genes decreases nodulation (Knight and Langston-unkefer, 1988) suggests that signal molecules are finely titrated by both partners. A model of communication by exchanging signals between legume and rhizobia is shown in Figure 1.
Figure 1. Early interactions and signal exchange(s) between legume plants and rhizobia leading to endocytosis and establishment of the symbiotic association (from Verma, 1992).
Root nodules are formed via three major stages: preinfection, infection and nodule formation, and nodule function (Sanchez et al., 1991). A set of host genes encoding nodule-specific host proteins, nodulins, are induced. Nodulins accumulate specifically in nodules and divided into two groups: early nodulins and late nodulins. Early nodulins are expressed during the infection and nodule formation stage while late nodulins are expressed during nodule functioning. In pea, the early nodulins *PsENOD3*, *PsENOD5*, *PsENOD12* and *PsENOD14* transcripts were localized in nodules of different developmental stages by *in situ* hybridization (Scheres et al., 1990b). *PsENOD12* mRNA is detected adjacent to the meristem in the invasion zone, while *PsENOD5* expresses highly in the early symbiotic zone where the first plant cells containing *Rhizobium* are present. *PsENOD3* and *PsENOD14* mRNAs are detected at a high level in the youngest layers of the late symbiotic zone. When *PsENOD3* and *PsENOD14* mRNA levels are decreasing, leghemoglobin transcript, the late nodulin, reaches its maximum level. Scheres et al. (1990b) concluded that the expressions of *PsENOD3*, *PsENOD5* and *PsENOD14* are only detected in the rhizobia-containing cells whereas *PsENOD12* transcript is present in all the cells of the invasion zone. *PsENOD12* is thought to be involved in the preparation of the cells for infection thread formation and growth (Scheres et al., 1990b). Although the amino acid sequence of the *PsENOD12* protein is homologous to other hydroxyproline-rich glycoproteins accumulating in wounded- or pathogen-attacked plant tissue, Scheres et al. (1990a) showed *PsENOD12* transcript is not accumulated in fungus-inoculated plants. The purified NodRm-2 factor induces root hair deformation, cortical cell division and the accumulation of *PsENOD5* and *PsENOD12* mRNA (Bisseling et al., 1990), indicating the bacterial nod factors induce these effects directly or by second signal(s) in a sequential manner elicited in host. The nod factors induce and control root cortical cell division (Verma, 1992).
Other factors affecting nodule development and function

It has been thought that endogenous phytohormones, especially auxins and cytokinines, control nodule development (Thimann, 1936; Libbenga et al., 1973). Van de Wiel et al. (1990) showed that the auxin transport inhibitors (ATIs) induce the development of nodule-like structures on alfalfa roots. In this study, ATIs induce the expressions of Nms30 and ENOD2 genes, which are associated with nodule morphogenesis. The ENOD2 nodulin was originally characterized in soybean nodule, and the deduced amino acid sequence of this nodulin shows a repetitive structure where two different pentapeptides alternate. ENOD2 is considered as a (hydroxy) proline-rich cell wall protein (Franssen et al., 1987). Cooper and Long (1994) observed that transzeatin, a cytokinin, induced cortical cell divisions that intimate some of the morphological and molecular events (ENOD2 expression) on alfalfa roots. Dobert et al. (1992) suggest that the rhizobial microsymbiont can influence on the levels of specific gibberellic acids (GAs) within nodule, and hypothesized that rhizobia induce alteration in GA balance in lima bean plants and cause stem elongation.

Both oxygen (Larsen and Jochimsen, 1987; Atkins et al.; 1990, Xue et al., 1991) and the availability of nitrate or ammonia (McNeil and Larue, 1984; Reynolds et al., 1990) affect the process of nitrogen assimilation in nodules. It has been known that the presence of combined nitrogen in the soil suppresses nodule induction. The host plants are assumed to play a role in this inhibition, and Delves et al. (1986) suggested that a shoot factor may involve in the nitrogen control of nodulation. Plant mutants that can nodulate in the presence of high nitrate concentrations were isolated (Jacobsen and Feenstra, 1984; Carroll et al., 1985). Recently R. meliloti nodulation genes were shown to be under nitrogen (NH$_4^+$) control (Dusha et al., 1989). Under the high ammonium concentration, the expression of inducible nod genes such as nodD3 remained low, but
the *nodD1* gene inhibition was not considerable. This fact implies that the ammonia effect is mediated via *nodD3*.

**Development of plastids in root nodules**

Plastids in legume root nodules have the metabolic importance in assimilating fixed-nitrogen. In ureide-transporting determinate nodules, plastids are the sites for *de novo* purine biosynthesis where enzymes necessary for catalyzing purine pathway as well as providing carbon to this pathway exist. The key feature of the ureide synthesis in determinate nodules is the compartmentation of metabolite flux between the infected and the uninfected cells. The microaerobic infected cells carry out nitrogen fixation and assimilation, while the uninfected cells are responsible for the oxidative catabolism of *de novo* synthesized purines to ureides. In amide-producing indeterminate nodules, plastids are the location for aspartate synthesis, one of the important amides produced in this type of nodules. Plastids in the uninfected cells of effective nodules and all cells of ineffective nodules contain large starch granules similar to amyloplasts and support the carbon requirement of nodules.

Recent advances in the studies of glutamine synthetase (GS) and glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) regulation using promoters of the genes encoding these enzymes showed that the expression of these genes is tightly controlled by the availability of the nitrogen-fixation products, ammonia and glutamine, respectively. For the understanding of the path of metabolites in root nodules, e.g. glutamine-importing and purine-exporting through plastid membrane, the studies performed using procaryotic and/or eucaryotic microorganisms may give us useful hints and tools. Understanding the regulation of *de novo* purine biosynthesis may allow us to
control the rate-limiting step(s) in this pathway and help eventually design more efficient nitrogen-assimilating plants.

**Effective vs. ineffective nodules**

As the rhizobia penetrate the root tissue via infection threads, cell division occurs in the cortex, forming a foci for nodule primordia (Long, 1989). Initially the developing nodule is a sink for nitrogen but, once nitrogen fixation begins it becomes a source for nitrogen and synthesize nitrogenous compounds which are transported to the other parts of the plant.

The nodules following normal development and nitrogen-fixing activity are termed as effective nodules. Based on the types of nitrogen compounds produced following nitrogen fixation, legume plants are divided into two groups, ureide-transporters and amide-transporters. The tropical legumes such as soybean, cowpea and bean primarily transport the ureides, allantoin and allantoic acid as fixed-nitrogen compounds, while the temperate legumes such as *Lotus* and alfalfa are amide-transporters, producing L-glutamine and L-asparagine as fixed-nitrogen compounds.

The ureide-producing nodules are usually determinate but all determinate nodules, such as in *Lotus*, are not ureide producers. The patterns of development and organization of these nodules are different from that in indeterminate nodules. In determinate nodules, a nodule meristem is induced in the root outer cortex, and the actively dividing, meristematic cells receive rhizobia (Newcomb et al., 1979). After successive cell divisions, the rhizobia-containing cells differentiate simultaneously into the nitrogen-fixing central tissue (Newcomb, 1981). Thus, in determinate nodules, the nodule growth is by the enlargement of the organ, and the nitrogen-fixing activity is dissociated from nodule growth. In indeterminate nodules, on the other hand, a nodule meristem is
induced in the inner cortex of the root. The indeterminate nodules are cylindrical in shape where new cells are constantly added to the meristematic end of the nodule, resulting in the elongation of the organ (Newcomb, 1976; Vasse et al., 1990).

The central infected region of nodule consists of two types of cells; those that are infected (bacteroid-containing) and the other that remain uninfected. In determinate nodules, the metabolic flow of fixed-nitrogen is compartmentalized in the organelles contained in either infected or uninfected cells (Newcomb and Tandon, 1981; Hanks et al., 1983; Shelp et al., 1983). The infected cells are generally microaerobic while the uninfected cells contain normal levels of oxygen.

In the infected cells of determinate nodules, the fixed nitrogen is incorporated into the amide position of L-glutamine by the cytosolic GS activity, which is then funnelled into the de novo purine biosynthesis pathway after being transported into the plastids. The purines produced are then transported to the uninfected cells of the nodules, and oxidatively catabolized to ureides (Schubert and Boland, 1990; Fig. 2). The uninfected cells occupy 21% of the total volume of the central infected region in soybean nodules grown without nitrate (Selker and Newcomb, 1985). Although the infected cells occupy more space than the uninfected cells, the number of the latter cells is much higher than that of the infected cells. Both infected and uninfected cells are spatially organized in a manner that the uninfected tissue produces large surface area which is in contact with infected tissue, making transport of the fixed nitrogen from the infected cells to the uninfected cells more efficient (Selker and Newcomb, 1985). When the nodule tissue is examined in the electron microscope, many plasmodesmata can be found between the infected and uninfected cells as well as between uninfected cells, indicating the possibility of symplastic transport between these cells.

For understanding the important role of each organelle, which is residing in both
Figure 2. The ureide biosynthesis pathway in determinate nodules. GS, glutamine synthetase; PRPP, phosphoribosylpyrophosphate; PRAT, PRPP amidotransferase; IMP, inosine 5'-monophosphate; ER, endoplasmic reticulum.
cell types, in metabolism, studying subcellular organization of root nodule is important. In the infected cells of determinate nodule, two prominent organelles, mitochondria and plastids, are congregated at the cell periphery, and the numbers of these organelles increase as the infected cells enlarge (Newcomb et al., 1985). Mitochondria in the infected cells have a much larger volume and number per unit cytoplasm than those in the uninfected cells. The plastids in the infected cells have a dense, granular matrix penetrated by a few cisternae and lamellae, and starch granules are usually absent (Newcomb et al., 1985). The shape of the plastids in these cells is variable. Small peroxisome-like structures are occasionally found in the periphery of the infected cell cytoplasm.

Unlike organelles in the infected cells, the plastids and mitochondria in the uninfected cells do not congregate along the plasma membrane (Newcomb et al., 1985). The plastids in uninfected cells usually contain large starch granules and depending on the amount of starch present, the size of plastids varies greatly (Selker and Newcomb, 1985; Newcomb et al., 1985). The differentiation of plastids in nodules is, therefore, cell-specific. Uninfected cells of soybean root nodules are highly vacuolated, and have large peroxisomes (microbodies) as well as a prominent system of tubular endoplasmic reticulum (Newcomb and Tandon, 1981). The enlarged peroxisomes are closely associated with the intercellular free spaces which may enable peroxisomes to uptake oxygen more easily from these spaces (Newcomb and Tandon, 1981).

The central infected region of indeterminate nodules also consists of both infected and uninfected cells. The infected cells are the site of amide synthesis, but little is known concerning the role of the uninfected cells in indeterminate nodules. In the infected cells of alfalfa nodules, a large vacuole frequently occupies the center of the infected cells (Hirsch et al., 1983), and proplastids, small amyloplasts and the mitochondria are
displaced to the cell periphery (Hirsch et al., 1983). Small plastids were also observed in the infected cells of Lotus root nodules (Wood et al., 1985). In the infected host cells, the mitochondria are elongated, and the mitochondrial cristae are usually narrow, whereas the smaller, rounded mitochondria appear in the uninfected cells which may be hypertrophied (Hirsch et al., 1983). The rough endoplasmic reticulum (RER) and Golgi bodies are abundant in the infected cells at early stages, but the numbers of these organelles decrease as nodules mature (Verma et al., 1978).

The ineffective root nodules lack the ability to fix nitrogen, resulting in the formation of non-functional nodules. For this reason, the ineffective nodules have been used in studying the importance of ammonium in nodule-specific gene expression (Hirel et al., 1987; Dunn et al., 1988; Walker and Coruzzi, 1989; Cock et al., 1990). Ineffective nodules are mostly induced by mutant rhizobia (Hirsch et al., 1983; VandenBosch et al., 1985; Morrison and Verma, 1987; Grosskopf et al., 1993) which generally fail to fix nitrogen, or are sometimes induced on the roots without bacteria entering the host cell. The host controls the formation of nodules (Peterson and Barnes, 1981; Gresshoff and Delves, 1986; Egli et al., 1989).

The ineffective nodules are generally white in color due to the lack of leghemoglobin (VandenBosch et al., 1985). Small microbodies, but not enlarged peroxisomes, are present in the cortical cells of bean ineffective nodules (VandenBosch et al., 1985). As a consequence, cells in the central cortex contain large starch grains and these nodules contain significantly more starch (Forrest et al., 1987). The accumulation of starch grains in nodule cell is considered to be the indication of ineffective nodule formation (Nutman, 1959). In the ineffective indeterminate nodules, large amyloplasts are also found, and the numbers of RER, Golgi bodies (Hirsch et al., 1983) and
mitochondria (Mackenzie and Jordon, 1974) elevate, indicating the increased plant metabolic activity (Hirsch et al., 1983).

**Role of plastids in infected and uninfected cells of the root nodules**

Symbiotically-reduced nitrogen in root nodules is assimilated via the incorporation of NH$_4^+$ into glutamine in a reaction catalyzed by glutamine synthetase (GS). As mentioned earlier, in temperate legumes, the assimilated nitrogen is transported from the nodules in the form of amides whereas nodules of tropical legumes export ureides that are produced by oxidative catabolism of purine nucleotides synthesized de novo (Atkins, 1991). Accordingly, synthesis of purines is highly induced in nodules (Schubert, 1981).

In ureide-transporting nodules, the distribution of organelles and enzymes involved in the ureide biosynthesis between infected and uninfected cells has been studied through a diversity of approaches such as; ultrastructural (Newcomb and Tandon, 1981; Webb and Newcomb, 1987), biochemical (Hanks et al., 1983; Shelp et al., 1983) and immunocytochemical (Bergmann et al., 1983; Nguyen et al., 1985; VandenBosch and Newcomb, 1986; Verma et al., 1986; Webb and Newcomb, 1987) methods. The infected cells of cowpea nodules contain 83% of GS activity and 84% of the activities of enzymes involved in de novo purine biosynthesis (Shelp et al., 1983; Hanks et al., 1983). Verma et al. (1986) verified the cytoplasmic location of GS in the infected cells of soybean nodules using immunogold labeling. This was further confirmed by the induction of GS gene expression in transgenic *Lotus* nodules (Miao et al., 1991). Based on these results, the infected cells of determinate legume nodules are considered to be the primary site of NH$_4^+$ assimilation and de novo purine biosynthesis (Schubert and
Boland, 1984; Schubert, 1986). However, some NH$_4^+$ does reach the uninfected cells as shown by the expression of NH$_4^+$-induced GS promoter in transgenic *Lotus*.

Subcellular fractionation studies of nitrogen-fixing soybean (Boland et al., 1982; Boland and Schubert, 1983; Reynolds and Blevins, 1986) and cowpea (Shelp et al., 1983) nodules indicated that the enzymes involved in the entire pathway for purine nucleotide synthesis are contained in the proplastids. This enzyme group includes phosphoribosylpyrophosphate (PRPP) synthetase, PRPP amidotransferase (PRAT), aspartate aminotransferase (AAT), NADH-glutamate synthase, asparagine synthetase (AS), phosphoserine aminotransferase, serine hydroxymethyl transferase, phosphoglycerate dehydrogenase, methylene tetrahydrofolate oxidoreductase and triose-phosphate isomerase (see Figure 3).

PRAT catalyzes the first committed step of *de novo* purine biosynthesis, using PRPP as one of the substrates. Although PRPP synthetase activity was mostly detected in the soluble fraction and small amounts of this enzyme were found in the organelle fractions (Boland et al., 1982), there may be sufficient PRPP synthetase activity in the proplastid fraction to support a reasonable rate of *de novo* purine biosynthesis (Boland and Schubert, 1983).

AAT catalyzes a reversible reaction:

$$\text{glutamate} + \text{oxaloacetate} \leftrightarrow \text{aspartate} + \alpha\text{-ketoglutarate.}$$

AAT is important for both nitrogen and carbon metabolism in microbes, animals and plants (Ireland and Joy, 1985). One of the isoenzymes of AAT is located in the proplastids fraction of the root nodules (Boland et al., 1982). The detection of glutamate synthase activity in the proplastid fraction (Boland et al., 1982) is consistent with the observation in *Phaseolus vulgaris* nodules (Awonaike et al., 1981) and soybean root tissue (Suzuki et al., 1981) where this enzyme has been localized in the proplastids.
Figure 3. Proposed pathway and subcellular organization of the reactions involved in amide synthesis and ureide biogenesis in ureide-exporting legume nodules. Numbered reactions: 1, glutamine synthetase; 2, glutamate synthase; 3, PRPP synthetase; 4, PRAT; 5, PGA dehydrogenase; 6, phosphoserine aminotransferase; 7, phosphoserine phosphatase; 8, serine hydroxymethylase; 9, methylene FH dehydrogenase; 10, aspartate aminotransferase; 11, asparagine synthetase; 12, IMP dehydrogenase; 13, nucleotidase nucleosidase; 14, ribokinase; 15, xanthine dehydrogenase; 16, uricase; 17, allantoinase; 18, PEP carboxylase. LB, leghemoglobin; GLN, glutamine; GLU, glutamate; PRA, phosphoribosyl amine; G3P, glyceraldehyde-3-phosphate; PGA, phosphoglycerate; PEP, phosphoenol pyruvate; PSER, phosphoserine; SER, serine; GLY, glycine; OAA, oxaloacetate; mal, malate; fum, fumarate; ASN, asparagine; ASP, aspartate; XAN, xanthine; XMP, xanthine monophosphate; IMP, inosine monophosphate (From Schubert, 1986).
Asparagine synthetase, important enzyme in amide-transporting nodules, has been suggested to play a role in ureide-producing nodules as well, during early development (Reynolds et al., 1982), and has been detected in proplastids (Boland et al., 1982).

Along with the demand for assimilated nitrogen, the de novo purine biosynthesis pathway requires significant amounts of carbon in the forms of PRPP, methyl tetrahydrofolate (FH4), formyl-FH4, glycine and HCO3− (Reynolds and Blevins, 1986). Phosphoserine aminotransferase, serine hydroxymethyltransferase, phosphoglycerate dehydrogenase, methylene tetrahydrofolate oxidoreductase and triose phosphate isomerase are the key enzymes providing carbon for the de novo purine biosynthetic pathway, and the activity of these enzymes is detected in the proplastid factions of ureide-transporting legumes (see Figure 3; Boland et al., 1982; Boland and Schubert, 1983; Shelp et al., 1983; Reynolds and Blevins, 1986).

The enzymes catalyzing the oxidation of purines are located in the uninfected cells of this organ. Xanthine dehydrogenase (XDH) converts xanthine to uric acid, and greater activity of this enzyme was detected in the cytoplasm of uninfected cells (Datta et al., 1991). The uricase, catalyzing the conversion of uric acid to allantoin was localized in the peroxisomes of uninfected cells (Nguyen et al., 1985; Webb and Newcomb, 1987).

Compared to the ureide-transporting legumes, much less work on compartmentation of metabolite flow has been done in amide-transporting legumes. Aspartate aminotransferase is one of the well-characterized enzymes in the amide-transporting legumes. Gantt et al. (1992) isolated a nodule-enhanced AAT cDNA clone of alfalfa, and characterized it in detail. The N-terminal domain of AAT contains a putative plastid-targetting peptide, suggesting that this protein is localized in the plastid.
(Gantt et al., 1992). In effective symbiosis, the expression of this gene is increased during nodule development whereas the expression level is much lower in ineffective nodules (Gantt et al., 1992). Thus, effectiveness of nodules is one of the factors affecting regulation of AAT in temperate legume root nodules (Gantt et al., 1992).

**Nitrogen fixation and assimilation**

Bacteroids begin to fix nitrogen once the nodule structure is formed. Bacterial nitrogenase catalyzes the fixation of N₂ to NH₃. The stoichiometry and energy costs of nitrogen reduction are as follow:

\[ \text{N}_2 + 6 \text{Fd}_{\text{red}} + 12 \text{ATP} + 8 \text{H}^+ \rightarrow 2\text{NH}_4^+ + 12 \text{ADP} + 12 \text{Pi} + 6 \text{Fd}_{\text{ox}} \]
\[ 2 \text{H}^+ + 2 \text{Fd}_{\text{red}} + 4 \text{ATP} \rightarrow \text{H}_2 + 2 \text{Fd}_{\text{ox}} + 4 \text{ADP} + 4 \text{Pi}, \text{ where Fd = ferredoxin, red = reduced and ox = oxidized.} \]

**Amide synthesis and transport**

Nitrogenase consists of two components: dinitrogenase (component I) containing FeMo, and dinitrogenase reductase (component II) containing Fe (Gussin et al., 1986). Functional nitrogenase is a tetramer (α₂β₂) of about 220Kd, and encoded by bacterial nif genes (Gussin et al., 1986). Dinitrogenase reductase (component II) is highly oxygen labile and forms a homodimer whereas a single (4Fe-4S) cluster is present between the subunits. When ATP binds to this enzyme, the electron potential of (4Fe-4S) cluster is reduced, and as a consequence, electrons are transferred to the component I (Gussin et al., 1986, Vance, 1990). The dinitrogenase then transfers electrons to N₂ and H⁺. Since nitrogenase is an anaerobic enzyme, the physical location of bacteroids in the infected cells of legume root nodule (where O₂ level is low) provides an optimum condition for the proper functioning of this enzyme.
Based on the observation that isolated bacteroids excrete NH$_4^+$ (Bergersen and Turner, 1967), ammonium is considered to be assimilated in the host cell cytoplasm. GS and glutamate synthase are two major enzymes assimilating NH$_4^+$. Meeks et al. (1978) demonstrated that NH$_4^+$ is first incorporated into the amide position of glutamine (GLN) by the GS-catalyzed reaction using $[^{13}\text{N}]\text{N}_2$. This incorporation is blocked by methionine sulfoximine, a GS inhibitor, and the amide group is subsequently transferred to the 2-carbon of oxoglutarate by the activity of glutamate synthase. As mentioned earlier, GS is a cytosolic enzyme, and glutamate synthase exists in plastids.

The induction of these enzymes during nodule development is well documented (Robertson et al., 1975; Groat and Schrader, 1982; Reynolds et al., 1982; Atkins et al., 1984). Recently, more comprehensive studies of the GS induction have been performed using GS genes (Hirel et al., 1987, Miao et al., 1991). Using Northern hybridization, Hirel et al. (1987) showed that availability of ammonium ions [provided as NH$_4$NO$_3$ or (NH$_4$)$_2$SO$_4$] enhanced the expression of GS in root tissue within 2hrs, reaching a level similar to that in nodules by 8hrs, while KNO$_3$ had no effect during this period. When nitrogen fixation was prevented by replacing nitrogen with argon in the root environment or when nodules were formed by a Fix$^-$ mutant of Bradyrhizobium japonicum, the expression of GS did not increase over that in roots (Hirel et al., 1987). Thus, Hirel et al. concluded that GS is directly induced by the available ammonia. This was confirmed by isolating the GS gene promoter from soybean and fusing it with a reporter [β-glucuronidase (GUS)] gene (Miao et al., 1991). The GS-GUS fusion was introduced into a legume (Lotus corniculatus) and a nonlegume (tobacco) plant by Agrobacterioum-mediated transformations. This chimeric gene was expressed in a root-specific manner in both plants (Miao et al., 1991). Treatment with exogenous ammonia increased the expression of this chimeric gene in the legume background, while no induction was
observed in tobacco roots. These results suggested that the tissue-specificity of the soybean cytosolic GS gene is conserved in both tobacco and legume (*L. corniculatus*); however, this gene is directly regulated by externally provided or symbiotically-fixed nitrogen (Miao *et al.*, 1991) only in the legume background.

In tropical legume root nodule, the GLN formed in the cytosol of infected cells is transported to the plastids where the *de novo* purine biosynthesis pathway takes place. GLN is one of the substrates of PRAT, catalyzing the first step of *de novo* purine biosynthesis. It is likely that a plastid-specific GLN transporter is operating to uptake assimilated nitrogen from the cytosol in these nodules. GLN transporter gene has been cloned from *Escherichia coli* (Nohno *et al.*, 1986) yet no plant gene for this transporter has been isolated.

In soybean, the concentration of asparagine in nodules is increased especially during early nodule development (McClure and Israel, 1979; Schubert, 1981). The specific activity of asparagine synthetase (AS), which is located in plastids of tropical legume nodules (Boland *et al.*, 1982), has been measured (Reynolds *et al.*, 1982) and showed a rapid decline as nodules matured. Therefore, AS in tropical legumes plays a role only during early nodule development (Reynolds *et al.*, 1982). In amide transporting legumes, the activity of AS increases with the onset of nitrogen fixation (Scott *et al.*, 1976). CO₂ fixation in the dark, catalyzed by PEP carboxylase, is one possible source of carbon used for asparagine synthesis and transport. Thus, the PEP carboxylase activity is correlated with nitrogen-fixation activity in temperate legumes (Christeller *et al.*, 1977; Vance *et al.*, 1983).

**De novo purine biosynthesis**

The ureides, allantoin and allantoic acid, are the most efficient forms of nitrogen
transport compounds since they contain a higher molar ratio of nitrogen per carbon atom as compared to amides (Schubert, 1986). The production of ureides requires large amounts of purines, which are oxidatively catabolized to ureides (Atkins, 1981; Boland et al., 1982). Therefore, the rate of de novo purine biosynthesis in tropical legume nodules is highly induced (Kohl et al., 1988). Levels of PRAT mRNA in soybean and cowpea nodules were found to increase steadily as the nodule matures. Transcriptional data of PRAT gene show that this gene is induced prior to the commencement of nitrogen fixation (Nguyen et al., 1985; Kim et al., 1995a, 1995b) indicating a developmental control on the induction of this pathway, which is later used for assimilation of reduced nitrogen following the onset of nitrogen fixation. Glutamine produced as a result of assimilation of fixed nitrogen induces de novo purine biosynthesis (Kim et al., 1995b).

Figure 4 shows the general scheme of the de novo purine biosynthesis pathway, and Table 2 shows the de novo purine synthesis pathway genes isolated to date from different organisms.

Glutamine PRPP amidotransferase (PRAT) catalyzes the first committed step in de novo purine biosynthesis:

\[
glutamine + PRPP + H_2O \rightarrow \text{phosphoribosyl-1-amine} + \text{glutamate} + \text{PPi}
\]

PRAT is one of the at least 16 different amidotransferases catalyzing the incorporation of reduced nitrogen in the biosynthesis of cellular components such as nucleotide bases, amino acids and antibiotics (Zalkin, 1993). Most glutamine amidotransferases can use ammonia (NH₃) alternatively. The catalytic site for glutamine utilization contains cysteine residue whereas the site for PRPP binding and NH₃-utilization resides in a transferase domain (Smith et al., 1994). PRAT has the catalytic cysteine residue at its amino terminus. PRAT is subject to feedback regulation by adenine and guanine nucleotides (Zhou et al., 1993). GMP is the most potent nucleotide inhibitor of the E. coli PRAT
Figure 4. Pathway of de novo purine biosynthesis and its regulation. Enzymes of relevance are identified by their gene symbols and the genes cloned from legume nodules are marked by *: purF, phosphoribosylpyrophosphate (PRPP) amidotransferase; purD, glycineamide ribonucleotide (GAR) synthetase; purN,T, GAR transformylase; purL, formylglycinamidine ribonucleotide (FGAM) synthetase; purM, aminoimidazole ribonucleotide (AIR) synthetase; purE, AIR carboxylase; purC, phosphoribosylaminomimidazole carboxamide (SAICAR) synthetase; purB, adenylosuccinate lyase; purH, aminoimidazole carboxamide ribonucleotide (AICAR) transformylase / Inosine monophosphate (IMP) cyclohydrolase. PRA, phosphoribosylamine; CAIR, carboxyaminoimidazole ribonucleotide; AMP, adenosine monophosphate; GMP, guanosine monophosphate.
Table 2. *De novo* purine-synthesis genes and their identification in different organisms.

<table>
<thead>
<tr>
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<th>Coding Enzyme</th>
<th>Organisms</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>purF (1)</td>
<td>Phosphoribosyl-pyrophosphate/amidotransferase (PRAT)</td>
<td><em>E. coli</em></td>
<td>(1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. subtilis</em></td>
<td>(1983)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. cerevisiae(ade4)</em></td>
<td>(1984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. melanogaster</em></td>
<td>(1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chicken</em></td>
<td>(1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Rat</em></td>
<td>(1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Human</em></td>
<td>(1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Soybean</em></td>
<td>(1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Vigna</em></td>
<td>(1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arabidopsis</em></td>
<td>(1994)</td>
</tr>
</tbody>
</table>

| purD (2)         | Glycinamide ribonucleotide (GAR) synthetase | *E. coli*               | (1989) |
|                  |                                        | *B. subtilis*           | (1990) |
|                  |                                        | *S. cerevisiae(ade5)*   | (1986) |
|                  |                                        | *S. pombe(ade1)*        | (1987) |
|                  |                                        | *D. melanogaster*       | (1983) |
|                  |                                        | *D. pseudoobscura*      | (1987) |
|                  |                                        | *Chicken*               | (1990) |
|                  |                                        | *Human*                 | (1990) |
|                  |                                        | *Arabidopsis*           | (1994) |

| purN (3)         | GAR transformylase                     | *E. coli*               | (1987) |
|                  |                                        | *B. subtilis*           | (1993) |
|                  |                                        | *S. cerevisiae(ade5)*   | (1985) |
|                  |                                        | *D. melanogaster*       | (1986) |
|                  |                                        | *Chicken*               | (1990) |
|                  |                                        | *Human*                 | (1990) |
|                  |                                        | *Arabidopsis*           | (1994) |

| purT (3)         |                                        | *E. coli*               | (1993) |

<p>| purL (4)         | Formylglycinamidine ribonucleotide (FGAM) synthetase | <em>E. coli</em>               | (1989) |</p>
<table>
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<tr>
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<th>Organisms</th>
<th>Year</th>
</tr>
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<td>E. coli</td>
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<tr>
<td></td>
<td></td>
<td>S. cerevisiae (ade5)</td>
<td>(1986)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. pombe (ade1)</td>
<td>(1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. melanogaster</td>
<td>(1986)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>(1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human</td>
<td>(1990)</td>
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<tr>
<td></td>
<td></td>
<td>Arabidopsis</td>
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</tr>
<tr>
<td>purE (6)</td>
<td>AIR carboxylase</td>
<td>E coli (purEK)</td>
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</tr>
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<tr>
<td></td>
<td></td>
<td>S. cerevisiae (ade2)</td>
<td>(1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. pombe (ade6)</td>
<td>(1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>(1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vigna</td>
<td>(1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human (ade2)</td>
<td>(1990)</td>
</tr>
<tr>
<td>purC (7)</td>
<td>Phosphoribosyl aminimidazole carboxamide (SAICAR) synthetase</td>
<td>E. coli</td>
<td>(1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. subtilis</td>
<td>(1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. cerevisiae</td>
<td>(1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>(1990)</td>
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<tr>
<td></td>
<td></td>
<td>Vigna</td>
<td>(1994)</td>
</tr>
<tr>
<td></td>
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<td>(1990)</td>
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<td>purB (8)</td>
<td>Adenylosuccinate lyase (ASL)</td>
<td>E. coli</td>
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</tr>
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<td>B. subtilis</td>
<td>(1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>(1990)</td>
</tr>
<tr>
<td>purH (9,10)</td>
<td>Aminoimidazole carboxamide ribonucleotide (AICAR) transformylase / Inosine monophosphate cyclohydrolase</td>
<td>E. coli</td>
<td>(1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. subtilis</td>
<td>(1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>(1991)</td>
</tr>
</tbody>
</table>
whereas AMP is less inhibitory (Messenger and Zalkin, 1979). GMP plus AMP show synergistic inhibition. PRPP antagonizes the inhibition by AMP and GMP in a competitive manner (Zhou et al., 1993). In *E. coli* PRAT, two nucleotide sites were identified where Lys$^{326}$ and Tyr$^{329}$ are GMP-binding residues and Gly$^{331}$ and Asn$^{351}$ are AMP-binding amino acids (Zhou et al., 1993). The *Bacillus subtilis* PRAT contains a [4Fe-4S] cluster which is responsible for the oxygen-dependent proteolytic degradation of this enzyme (Bernlohr and Switzer, 1981). Oxygen sensitivity of the cluster is diminished by AMP (Smith et al., 1994). In *B. subtilis*, PRAT is inactivated and proteolytically degraded in nutrient-starved cells, and Fe-S center is thought to be involved in O$_2$-dependent inactivation of this enzyme (Makaroff et al., 1983).

**Regulation of purine gene expression**

In procaryotes, a large number of transcriptional regulators have been identified and grouped into three families based on homology: the LysR family (Henikoff et al., 1988), the DeoR family (van Rooijen and de Vos, 1990) and the GalR-LacI family (Weickert and adhya, 1992). The regulators belong to the GalR-LacI family are supposed to regulate transcription by forming a dimer of identical subunits (Weickert and adhya, 1992). Each subunit consists of three parts: an NH$_2$-terminal DNA binding domain containing helix-turn-helix motif which binds to the dyad symmetry sequence in an operator, a hinge helix DNA-binding motif and COOH-terminal corepressor-binding domain (CBD) that is responsible for corepressor binding and dimerization (Schumacher et al., 1995). These GalR-LacI family regulators require effectors for the regulation.

In *E. coli*, purR, a gene encoding the repressor protein, has been identified (Rolfes and Zalkin, 1988) and it has been shown that this repressor binds to the operators of pur genes including purF (He et al., 1990; Meng et al., 1990). PurR is a member of
LacI repressor family of proteins having N-terminal helix-turn-helix motif for DNA binding and C-terminal effector binding/oligomerization domain (Schumacher et al., 1993). The *E. coli* PurR is a dimeric protein, and the corepressors hypoxanthine and guanine bind cooperatively to the effector-binding domain resulting in a conformational change to the PurR (Choi and Zalkin, 1992; Schumacher et al., 1995). An excess amount of purines acts as an environmental signal to stop *de novo* purine biosynthesis. The structure of PurR shows substantial structural changes and hinge-bending rotations (of 17° and 23°) between the NH₂ and COOH subdomains of each monomer subunit relative to the corepressor-bound form, resulting in an open corepressor-binding domain (CBD; residues 60 to 341). (Schumacher et al., 1995). As a result of these rotations, the hinge helices, which are responsible for making key minor groove interactions and kinking the DNA, will be pulled apart and destabilized. These events result in the relocation of the helix-turn-helix motifs such that they will no longer bind in successive major grooves (Schumacher et al., 1995). The repressors of the LacI family have a similar mechanism of effector-regulated specific DNA binding. It is noteworthy that all genes of *E. coli* regulated by PurR contain a conserved sequence called PUR box, which is a PurR-binding domain consisting of 16 nucleotides (He et al., 1990; Meng et al., 1990). Two PurR-binding sites were also identified in the transcribed part of *purR* indicating *purR* expression is autoregulated. We isolated soybean PRAT promoter and identified a putative PUR box motif in this promoter which is located downstream of the TATA box, which indicated that the similar repressor-type regulation of this pathway may be operating in plants (Kim and Verma, 1996). Figures 5 and 6 show the consensus nucleotide sequence for GalR-LacI repressor-binding motifs in procaryotic cells and general feature of these regulator proteins, respectively.

We have also isolated the cDNA clones encoding 5-aminoimidazole
<table>
<thead>
<tr>
<th>Operator</th>
<th>Consensus</th>
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<tbody>
<tr>
<td>lacI/lacO</td>
<td>T T G T G A G C G G A T A A C A A</td>
</tr>
<tr>
<td>galR/galOE</td>
<td>G T G T A A A C . G A T T C C A A</td>
</tr>
<tr>
<td>/galOI</td>
<td>G T G G T A G C . G G T T A C A T</td>
</tr>
<tr>
<td>cytR/deoP2</td>
<td>A T T T G A A C C A G A T C G C A T</td>
</tr>
<tr>
<td>purR/purM</td>
<td>T C G C A A A C . G T T T G C T T</td>
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<tr>
<td>/purL</td>
<td>A C G C A A A C . G G T T T C G T</td>
</tr>
<tr>
<td>/purF</td>
<td>A C G C A A A C . G T T T T C T T</td>
</tr>
<tr>
<td>Consensus</td>
<td>— — G — - A - C G - T — - C — -</td>
</tr>
</tbody>
</table>

Figure 5. Comparison of putative operators for deoP2, purM, and purL with galOE, Gal OI, lac and purF operators (from Rolfes and Zalkin, 1988).
Figure 6. General structure of GalR-LacI family repressors.
ribonucleotide (AIR) carboxylase and 5-aminimidazole-4-N-succinocarboxamide ribonucleotide (SAICAR) synthetase, involved in de novo purine biosynthesis, from a cowpea (Vigna aconitifolia) library by complementation of E. coli purE and purC mutants, respectively (Chapman et al., 1994). Sequencing of these clones revealed that the two enzymes are distinct proteins in cowpea, unlike in animals where both activities are associated with a single bifunctional polypeptide. As is the case in yeast, the cowpea AIR carboxylase has a N-terminal domain homologous to the eubacterial purK gene product. This PurK-like domain appears to facilitate the binding of CO₂ and is dispensable in the presence of high CO₂ concentrations. Because the expression of the mothbean purE cDNA clone in E. coli apparently generates a truncated polypeptide lacking at least 140 N-terminal amino acids, this N-terminal region of the enzyme may not be essential for its CO₂-binding activity.

Export of purines from plastids and their oxidation to ureides

Following de novo purine synthesis in the infected cells of tropical legume root nodules, IMP or xanthine are transported to the uninfected cells where they are converted into ureides (Datta et al., 1991). The compartmentation of the purine and ureide biosynthetic pathways in different organelles of both infected and uninfected cell types is important for maintaining metabolite channeling as well as levels of key intermediates and effectors (Schubert and Boland, 1990). Oxygen seems to play an important role in the intercellular compartmentation of ureide biosynthetic enzymes. For example, PRAT is highly oxygen sensitive and is located in the infected cells where the oxygen level is low, while uricase that requires oxygen is located in the uninfected cells. Therefore, different steps of ureide biosynthetic pathway are operating in different types of cells in nodules.
(Schubert, 1986, Verma, 1989). The fact that purines inhibit uricase activity (Hurst et al., 1985) also indicates that the steps need to be compartmentalized.

**Purine transport from plastids**

The exact types of metabolites transported between infected and uninfected cells are not identified (Schubert and Boland, 1990). It has been speculated that plasma membranes are impermeable to nucleotides (Muller et al., 1982), and therefore, these nucleotides need to be converted to either nucleoside or free base to be translocated to the uninfected cells by active transport system (Schubert and Boland, 1990). However, results from the previous studies show that ATP and ADP may be translocated into chloroplasts effectively (Heldt, 1969; Robinson and Wiskich, 1977a, 1977b; Robinson, 1985). For example, addition of ATP to PPI-inhibited chloroplasts results in uptake of catalytic amounts of ATP and restoration of the internal adenine nucleotide pool, relieving the inhibition of CO2 fixation (Robinson and Wiskich, 1977). Heldt (1969) noted that the main role of the adenine nucleotide translocation in chloroplasts is to deliver ATP, synthesized by glycolysis or respiration. These results suggest the possibility that IMP, final product of *de novo* purine biosynthesis, may be transported from plastids in root nodules (Schubert and Boland, 1990). Considering the location of XDH in the uninfected cells, we can assume that XMP, xanthosine or xanthine, converted from IMP, may also be transported from plastids.

The ascomycete *Aspergillus nidulans* is one of the well-characterized systems where nitrogen metabolism has been studied in details (Romero et al., 1991). This fungus utilizes purines as a sole nitrogen source. Recently, two purine permease genes have been cloned from this microorganism (Gorfinkel et al., 1993; Diallinas et al., 1995). Uric acid-xanthine permease (UapA) is a highly hydrophobic protein with 12-14
putative transmembrane segments, and has no amino acid sequence homology to different permeases (Gorfinkiel et al., 1993). The transcription of this gene is inducible by 2-thiouric acid, and is highly repressible by ammonium (Gorfinkiel et al., 1993). Another type of purine permease in Aspergillus is a wide specificity purine permease (UapC), which contributes 30% and 10% to uric acid and hypoxanthine transport rates, respectively (Diallinas et al., 1995). The expression of uapC gene is weakly induced by 2-thiouric acid and repressed by ammonium. The UapC protein is also hydrophobic and contains 12-14 putative transmembrane segments (Diallinas et al., 1995). The UapC protein shows high similarity (58% amino acid identity) to the UapA protein and some similarity (23-34% amino acid identity) to several bacterial transporters (Diallinas et al., 1995). Along with uaZ, coding for uric acid oxidase, and hxA, coding for xanthine dehydrogenase, both uapA and uapC genes are regulated by UaY, the specific regulator of the purine utilization pathway in A. nidulans (Suarez et al., 1995). The results shown in this microorganism may be useful in searching the regulation of purine metabolism in legume plants. The Aspergillus mutant strains impaired in purine transport may provide tools for complementation study, or uap clones may be used as heterologous probes for isolating purine permeases from soybean or Vigna.

**Purine oxidation**

After transport to the uninfected cells, the purines are catabolized to uric acid by XDH. The uric acid is further oxidized to allantoin by uricase, present in peroxisome. Soybean peroxisomal uricase (nodulin-35) gene has been isolated and characterized (Nguyen et al., 1985). Vigna nodulin-35 cDNA has also been cloned, and introduced into the plant in the antisense orientation driven by cauliflower mosaic virus 35S promoter (Lee et al., 1993). Nodules formed on the resulting transgenic hairy roots were
found to be reduced in size than normal nodules and the plants exhibited a nitrogen
deficiency phenotype (Lee et al., 1993). Uricase activity in these nodules was reduced
(50-60% reduction), and peroxisomes were reduced in size (Lee et al., 1993). These
results were in accordance with the yellowing of plants treated with allopurinol (Atkins et
al., 1988), a uricase inhibitor, and indicate amides do not compensate for the nitrogen
requirement of tropical legumes blocked in ureide biosynthesis (Lee et al., 1993).

The peroxisomally-produced allantoin is further oxidized to allantoic acid by
allantoinase localized in the endoplasmic reticulum (ER) (Schubert and Boland, 1990).
Both allantoin and allantoic acid are transported to other parts of the plants and used as
source of nitrogen in tropical legumes.

Regulation of nitrogen flux through purine/ureide pathway

In eucaryotes, nitrate serves as a major nitrogen source which needs to be reduced
to nitrite by nitrate reductase (NR). Nitrite is reduced to ammonium by nitrite reductase
(NiR) and to glutamate. The reaction catalyzed by NR is considered to be a rate-limiting
step in the overall nitrate assimilation, and thus, NR is highly regulated by the factors
such as light and nitrogen availability (Duke and Duke, 1984; Melzer et al., 1989).
Regulation of NR by the end products of the nitrogen assimilation, e.g. glutamine and
other amino acids, has been reported (Smarrelli, Jr. et al., 1987).

In soybean, three isoforms of NR have been identified and characterized (Streit
and Harper, 1986; Santucci, D., Haas, B. and Smarrelli, Jr., 1995). These enzymes
have different substrate specificity, kinetic parameters, pH optimum and metabolic
regulation. Two NR isoforms are constitutively expressing enzymes, whereas the third
NR isoform, which is inducible, is active only when nitrate is provided exogenously
(Curtis and Smarrelli, Jr., 1986). However, ammonium in the form of NH₄Cl has no
effect on NR induction, indicating nitrogen nutrition and nitrogenous metabolites play important roles in the regulation of NR activity. Recently cDNA clones of two inducible NRs have been isolated and characterized from soybean (Wu et al., 1995).

As mentioned earlier, GS catalyzes the initial step of the nitrogen assimilatory pathway in legume root nodules. The expression of GS is enhanced greatly by the availability of ammonium ions, whereas nitrate has no effect on GS induction (Hirel et al., 1987; Miao et al., 1991). These results indicate that leguminous plants operate two comparable nitrogen-assimilating systems. When legumes are growing on nitrogen-rich environment, especially in the form of nitrate, they reduce the nitrate through NR and utilize it as a major nitrogen source, while under the nitrogen-limiting environment, they develop root nodules to fix nitrogen. In this organ, bacteroid-excreted ammonium, a starting substrate for the purine/ureide pathway, acts as an inducer of GS gene, and is incorporated into glutamine. Glutamine, then, induces the PRAT expression which catalyzes the first committed step of the de novo purine biosynthesis pathway. It has been known that nitrate inhibits the synthesis of ureides (McNeil and LaRue, 1984), and high levels of nitrate almost block the ureide synthesis in soybean and cowpea and switch these legumes to the amide metabolism (Schubert and Boland, 1990).

**Role of PRPP synthetase in de novo purine biosynthesis**

5-Phosphoribosyl 1-pyrophosphate (PRPP) synthetase (ATP: D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) catalyzes the reaction, D-ribose 5-phosphate + ATP —> PRPP + AMP. PRPP is the substrate for purine, pyrimidine and pyridine nucleotide synthesis (Konberg et al., 1955; Flaks et al., 1957) where phosphoribosyl transferases convert the bases of these nucleotides to the corresponding ribonucleoside monophosphates (Hove-Jensen et al., 1986). Since pentose phosphate
shunt, producing D-ribose 5-phosphate, is linked to nucleotide biosynthesis by the action
of PRPP synthetase, the regulation of this enzyme is considered to be under the strict
metabolic control including both enzyme activity and gene expression (Shimada et al.,
1990). PRPP itself acts as an important control factor for the de novo synthesis of
purines (Fox and Kelley, 1971b; Holmes, 1978; Bagnara and Finch, 1974) and
pyrimidines (Tatibana and Shigesada, 1972; Tatibana, 1978; Keppler and Holstege,
1982). In microorganisms and plants, PRPP is also utilized as a precursor of histidine
and tryptophan biosynthesis. Therefore, PRPP is an important precursor for the
synthesis of nucleic acids, proteins and NAD(P) (Jensen, 1983).

PRPP synthetase (PRS) has been purified from several organisms including
_Salmonella typhimurium_ (Schubert et al., 1975; Switzer and Gibson, 1978), _E.
coli_ (Hove-Jensen et al., 1986), _Bacillus subtilis_ (Arnvig et al., 1990), human
erythrocytes (Fox and Kelley, 1971a) and rat liver (Roth et al., 1974; Kita et al., 1989)
where this enzyme exists as an oligomeric complex. In rat liver, two highly homologous
isoforms, PRSI and PRSII with the size of 34kDa, form complex aggregates with other
39- and 41-kDa components at an approximate ratio of 3 : 1: 0.15 (Kita et al., 1989).

The gene encoding PRS has been isolated from human (Sonoda et al., 1991;
Iizasa et al., 1989; Becker et al., 1990), rat (Taira et al., 1987; Kita et al., 1994; Shimada
et al., 1990), _Candida albicans_ (Payne and Calderone, 1995), _Leishmania donovani_
(Hendrickson et al., 1993) and _E. coli_ (Hove-Jensen, 1985). In _Candida_, this gene is
involved in nitrogen signalling (Payne and Calderone, 1995). Recently in _Arabidopsis
thaliana_, it has been shown that blocking histidine biosynthesis with a specific inhibitor
of imidazole glycerol phosphate dehydratase caused increased accumulation of purines
(Guyer et al., 1995).
Inhibition study of PRS have been done in *E. coli* and human using analog binding method. The *E. coli* PRS was irreversibly inactivated on exposure to the affinity analog 2', 3'-dialdehyde ATP (Hilden *et al.* 1995). By isolating radioactive peptides from the enzyme modified with radioactive 2', 3'-dialdehyde ATP, followed by Edman sequencing, Hilden *et al.* (1995) identified Lys181, Lys193, Lys230 and possibly Cys229 as probable sites of analog reaction. Lys193 is conserved within the PRS family, and Lys181 is found at a position in the sequence where the cognate amino acid (Asp181) in human PRSI has been implicated in the enzyme regulation (Hilden *et al.* 1995).

6-Methylmercaptopurine ribonucleoside (MeSno) monophosphate inhibits both the first step of *de novo* purine synthesis and PRPP synthesis. It turns out that the inhibition of PRPP synthesis is due to MeSno monophosphate inhibition of PRS as shown in human fibroblasts (Yen *et al.*, 1981). This study identify the mechanism of 6-thiohypoxanthine (6-mercaptopurine) and MeSno action in inhibiting the growth of animal tumor cells where the MeSno monophosphate is metabolically generated from these compounds (Paterson and Tidd, 1975; Allan *et al.*, 1966; Paterson and Wang, 1970; Bennett and Allen, 1971).

So far thirteen gln PRAT gene sequences have been characterized (Kim *et al.*, 1995). *B. subtilis* PRAT has been studied by X-ray crystallography (Smith *et al.*, 1994). Like other PRATs, this enzyme contains N-terminal domain active site cysteine residue which is essential for the transfer of the glutamine, one of the substrates of PRAT, to the second substrate. The C-terminal domain catalyzes the NH3-dependent synthesis of PRA and containing end product inhibition domain by AMP and GMP (Smith *et al.*, 1994). According to Zalkin (1993), this feature is structurally similar to other "PurF
(PRAT)" subfamily of amidotransferases such as asparagine synthetase and glucosamine 6-phosphate synthase.

**Proposed Research**

The major goal of my research is to study the regulation of ureide biosynthesis genes in tropical legume root nodules, especially the regulation of *de novo* purine biosynthesis. PRAT catalyzes the first committed step of *de novo* purine biosynthesis, and produces 5-phosphoribosyl-1-amine from PRPP and L-glutamine. End-product inhibition of this enzyme is considered to play a key role in regulating the metabolic flux of carbon and nitrogen through this pathway.

In searching of the nitrogen-regulation of the *de novo* purine biosynthesis pathway in legume nodules, I propose to study the transcriptional regulation of PRAT by L-glutamine produced as a result of assimilation of fixed nitrogen. First, I propose to isolate the PRAT cDNA clone from soybean nodule library and characterize it. I also propose to isolate and characterize the soybean PRAT promoter to further understand the regulation of this gene, and identify the important *cis*-regulatory elements of this promoter. By transforming soybean and tobacco with PRAT-GUS (β-glucuronidase) construct, I will use the transgenic plants in determining the mechanism of L-glutamine induction of PRAT gene. Along with these experiments, PRAT induction by L-glutamine will be studied in mothbean, which is also tropical legume, by using *Vigna* PRAT cDNA. The results obtained from both legume plants will give us general idea in the regulation of nitrogen metabolism in tropical legume root nodules.

Finally, I propose to determine the coordination of regulation of ureide biosynthesis genes (GS, uricase and PRAT) in soybean Fix+ and Fix− nodules at different developmental stages, and follow the trends of these gene expressions with the
onset of nitrogen fixation. This study will enable us to understand the nitrogen-regulation of ureide biosynthesis genes in tropical legume-\textit{Rhizobium} symbiosis.
CHAPTER II

INDUCTION OF GLUTAMINE PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE GENE AND CHARACTERIZATION OF ITS cDNA FROM SOYBEAN

Abstract

A soybean (Glycine max) cDNA clone, encoding glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) which is the first enzyme of the de novo purine biosynthesis pathway, was isolated from nodule-specific cDNA library. The amino acid sequence deduced from soybean clone showed > 85% similarity to the PRAT sequence of mothbean and 33 to 47% similarity to those of bacteria, yeast, chicken, rat and human. The soybean clone encodes a protein with an N-terminal sequence resembling a plastid-targeting peptide. Downstream from this peptide is a sequence similar to the 11-amino acid propeptide found in the Bacillus subtilis, chicken, rat and human PRAT proteins. The soybean clone encodes characteristic cysteine residues that are known to be involved in the assembly of a [Fe-S] cluster near the C-terminus of this protein. Levels of PRAT mRNA in mothbean nodules were found to increase steadily as the nodules matured from 13 days to 23 days. PRAT mRNA was not detectable in uninfected root tissue but a low level of transcript was detected in leaves. Treatment of uninfected root with L-glutamine induced the PRAT mRNA transcript
suggesting that glutamine produced as a result of assimilation of fixed nitrogen controls the expression of this pathway in root nodules.

Introduction

Symbiotically reduced nitrogen in legume root nodules is assimilated via the incorporation of $\text{NH}_4^+$ into the amide position of glutamine in a reaction catalyzed by glutamine synthetase (GS). In temperate legumes, the assimilated nitrogen is transported from the nodules in the form of amides (glutamine and asparagine) whereas nodules of tropical legumes export ureides (allantoin and allantoic acid) that are produced by oxidative catabolism of purine nucleotides suggested to be synthesized de novo (Atkins, 1991). Accordingly, synthesis of purines is highly induced in nodules following commencement of nitrogen fixation (Schubert, 1981).

Our understanding of the de novo purine biosynthesis and its regulation is derived largely from the characterization of the pathways in bacterial and animal systems (Ebbole and Zalkin, 1987; Henikoff, 1987; Neuhard and Nygaard, 1987; Rolfes and Zalkin, 1988; Zhou et al., 1990). In all organisms thus far characterized, ten enzymatic reactions convert the activated ribose precursor, 5-phosphoribosyl-1-pyrophosphate (PRPP), to the purine nucleotide, inosine 5'-monophosphate (IMP). The first step in this pathway, involving the synthesis of 5-phosphoribosyl-1-amine from PRPP and glutamine, is catalyzed by glutamine PRPP amidotransferase (PRAT). End-product inhibition of this enzyme in bacteria (Messenger and Zalkin, 1979; Meyer and Switzer, 1979) and animals (Caskey et al., 1964; Holmes et al., 1973) as well as in plants (Reynolds et al., 1984) is thought to play a key role in regulating the metabolic flux of carbon and nitrogen through this pathway and in controlling the level of purine synthesis.
In plants, at least four enzymes of the pathway, PRAT, glycinamide ribonucleotide (GAR) synthetase, GAR transformylase and adenylosuccinate-AMP lyase, have been identified and partially purified from soybean and cowpea root nodules (Atkins, 1991; Reynolds et al., 1984). In nodules, most of these enzymes are localized in the plastids of infected cells (Atkins, 1991; Boland and Schubert, 1983; Shelp et al., 1983).

IMP or Xanthine appears to be transported to the uninfected cells before being oxidized to uric acid (Datta et al., 1991) which in turn is oxidized to allantoin by a nodule-specific uricase localized in peroxisomes (Nguyen et al., 1985). The latter is hydrolyzed to allantoic acid by allantoinase. Nodule-specific uricase from soybean (Nguyen et al., 1985) and mothbean (Lee et al., 1993) have been characterized in our lab, and it has been demonstrated that a block in uricase production by the expression of antisense uricase cDNA causes a reduction in the flux of nitrogen through this pathway (Lee et al., 1993).

Using genetic complementation of appropriate E. coli auxotrophic mutants with cDNA expression libraries (Delauney and Verma, 1990b), we isolated several soybean and mothbean cDNA clones encoding nodule enzymes (Delauney and Verma, 1990a; Delauney et al., 1993; Hu et al., 1992; Miao et al., 1991). Avian clones for purine biosynthetic enzymes have been isolated by complementation in E. coli (Aimi et al., 1990a; Aimi et al., 1990b; Chen et al., 1990; Ni et al., 1991), though this strategy was reportedly not successful for the isolation of PRAT clones (Zhou et al., 1990). Human and rat PRAT clones have been isolated recently by cDNA library screening using rat PRAT cDNA as a hybridization probe and polymerase chain reaction (PCR), respectively (Iwahana et al., 1993a; Iwahana et al., 1993b). We have also succeeded in isolating mothbean cDNA clones encoding AIR carboxylase, 5-aminoimidazole-4-N-
succinocarboxamide ribonucleotide (SAICAR) synthetase (Chapman et al., 1994) and PRAT. 5'-Phosphoribosyl-5-aminomimidazole (AIR) synthetase cDNA clone has been isolated from *Arabidopsis* and characterized in detail (Senecoff and Meagher, 1993).

We characterized the soybean PRAT cDNA clone and determined the expression levels of the PRAT gene at different stages of nodule development. Using mothbean PRAT cDNA clone, we searched the possible mechanism of induction of PRAT in root tissue by treatment with exogenous L-glutamine. Comparison of expression levels of PRAT and SAICAR synthetase genes in *Vigna* nodules suggested that PRAT transcript is induced prior to that of SAICAR synthetase (see Chapman et al., 1994) and PRAT expression level is higher than that of the latter enzyme.

**Materials and Methods**

**Isolation of RNA**

Three-week-old soybean nodules (10 g) were ground in a Waring blender in 20 ml of buffer A (0.2 M Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM KCl, 20 mM Mg acetate, 0.5% (w/v) SDS). The homogenate was extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and centrifuged at 10,000 g for 10 min. An equal volume of chloroform was added to the aqueous phase and vortexed. The RNA was precipitated by adding 2.5 volumes of ethanol to the supernatant and keeping at -80°C for 30 min. After centrifugation at 10,000 g for 10 min, the total RNA was dissolved in 3 ml of buffer B (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.4 M NaCl, 0.5% (w/v) SDS). Poly (A)+ RNA was obtained by chromatography on oligo (dT)-cellulose. 100 mg of oligo (dT) cellulose was resuspended in 1 ml of 0.1 M NaOH and the suspension was transferred to a plastic BioRad column. The packed column was
washed with 10ml H2O followed by 10ml of binding buffer [0.12M NaCl, 0.01M Tris-HCl pH 7.5, 0.001M EDTA]. Total RNAs were adjusted to 0.5mg/ml with 10ml of elution buffer [0.01M Tris-HCl pH 7.5, 0.001M EDTA] and heated at 68°C, 5 min. followed by sitting on ice. After adding NaCl (final concentration = 0.12M) to the RNA sample, RNA solution was loaded on column. The column was washed 4 times with 5ml of binding buffer. Poly (A)+ RNA was eluted with 4ml elution buffer and precipitated with 0.4ml 3M NaOAc and 10ml ethanol at 4°C, overnight. Poly (A)+ RNA was collected by centrifugation (10,000 r.p.m., 30 min.) and dried after decanting ethanol. The poly (A)+ RNA pellet was dissolved in water.

For the isolation of mothbean total RNA, tissues (7-day-old leaf, 3-day-old root and 13-, 16-, 19- and 23-day-old nodules) were collected from Vigna, and RNA was prepared from 10g of each tissue by hot phenol treatment (Vries et al., 1990).

For the induction of PRAT gene, Vigna seedlings were supplemented with 20 mM NH4NO3 or 10 mM L-glutamine daily after 3 days of sowing, and RNA, isolated from root tissue (7-day-old), was used for primer extension.

**RNA polymerase chain reaction**

RNA PCR was performed using the GenAmp PCR kit (Perkin Elmer Cetus, Norwalk, CT) with minor modifications to the manufacturer’s instructions. First strand of cDNA was synthesized using 1 μg of poly (A)+ RNA, 2.5 μM oligo (dT)16 and 2.5 units of MMLV reverse transcriptase at 42°C for 30 min. The reaction mixture was heated at 99°C for 5 min and then placed on ice. Two degenerate primers were synthesized for PCR. Primer I was 5’-AA(AG)AA(CT)(AC)GXTA(CT)GT(GT)GGX(AC)GXAC (ACT)TT (CT)ATC-3’ and Primer II was 5’-(AG)(GT)GTTGT(AC)CC(CG)C(GT)(AC) AC(AG)ATGA(AG)TC(AG)TC(AC)AC-3’ where X=ACGT. The PCR was initiated
with denaturation at 94°C for 1.5 min followed by 35 cycles at 94°C for 40 sec, 45°C for 2 min, 72°C for 3 min and a final incubation at 72°C for 7 min. An amplified DNA fragment of ~150 bp was isolated from an 1% agarose gel, blunt-ended with Klenow enzyme and subcloned into the \textit{Sma} I site of pUC19.

\textbf{Hybridization screening of \lambda \textit{phage library}}

A unidirectional cDNA library, constructed from RNA from 3-week-old soybean nodules in \lambda Zap II (Delauney and Verma, 1990b), was screened using the \textsuperscript{32}P-labeled PCR product described above as a hybridization probe. Hybridization was carried out in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M Na citrate) containing 0.5% SDS, 0.5% non-fat dried milk and the probe (3.6 x 10^5 cpm/ml) at 55°C for 10 h. Filters were washed 3 times in 2 x SSC, 0.1% SDS at room temperature for 5 min, then washed twice in 0.2 x SSC, 0.1% SDS at 55°C for 1 h. Positive clones were converted into recombinant pBluescript phagemids by \textit{in vivo} excision (Short \textit{et al.}, 1988).

\textbf{DNA sequencing}

DNA sequencing was carried out on double-stranded plasmid templates by the dideoxynucleotide chain termination method using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH). CsCl-purified DNA was denatured by incubation in 0.2N NaOH and 0.2mM EDTA, and neutralized by 0.3M sodium acetate (pH 4.5). The DNA was precipitated with 2.5 volumes of ethanol at -20°C for 15 minutes, pelleted in a microfuge tube, resuspended in distilled water and annealed with appropriate sequencing primers. Both strands of PRAT clones were sequenced. Plasmid deletions were constructed by restriction enzyme digestion and by treatment with Exonuclease III and S1.
nuclease (Erase-a-base system, Promega, Madison, WI). Several oligonucleotides were also synthesized and used as sequencing primers.

**Primer extension**

Oligonucleotide primers 5'-CAGAGGCAGAAATGTTTGTGG-3' and 5'-GGAAGTGCGAATGATGGGTG-3' corresponding to 5' end of *Vigna* PRAT (see Fig. 7) and SAICAR synthetase (Chapman et al., 1994) cDNAs, respectively, were synthesized. Primer extension was performed using modifications of the AMV Reverse Transcriptase Primer Extension System (Promega, Madison, WI). Fifteen μg of total RNA from mothbean tissues and 400 fmol/ml of the 32P-labeled primer were annealed at 30°C for 1 h, followed by reverse transcription at 42°C for 1 h. The reaction product was electrophoresed on a 8% polyacrylamide gel which was subsequently dried and exposed to X-ray film or analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Results**

**Isolation of soybean PRAT cDNA clone**

The PRAT from *E. coli* (Tso et al., 1982), *B. subtilis* (Makaroff et al., 1983), yeast (Mäntšälä and Zalkin, 1984), chicken (Zhou et al., 1990), human (Iwahana et al., 1993a) and rat (Iwahana et al., 1993b) contain several regions of significant sequence homology and these regions were also found to be conserved in *Vigna*. Based on the two most strongly conserved regions near the carboxy termini, degenerate PCR primers were synthesized. Soybean nodule poly (A)+ RNA was reverse transcribed into cDNA which was then amplified by PCR. The PCR product revealed a single DNA band of the expected size of ~150 bp, that was subcloned into pUC19. The nucleotide sequences of
the two clones analyzed were found to be identical except for a single nucleotide mismatch (data not shown). The deduced amino acid sequence of the PCR product showed significant homology to PRAT enzymes from other organisms. This PCR fragment was used as a hybridization probe to screen a soybean nodule cDNA library to isolate a full-length clone.

Approximately 60,000 plaques of soybean cDNA library were screened. Among 9 plaques showing positive signals after secondary screening, 5 were converted into phagemids by \textit{in vivo} excision. The longest insert in these clones was of 1.96 kb (pGF1), and the complete nucleotide sequence of this insert was determined.

Mothbean PRAT cDNA was also isolated in this lab. by genetic complementation of purine auxotrophy in an \textit{E. coli} purF mutant (Ashton and Verma, see Kim \textit{et al.}, 1995). The mutant was transformed with pooled plasmid DNAs from a mothbean cDNA library and transformants were plated onto selective medium lacking purines. The sequence of the cDNA insert complementing the \textit{E. coli} purF mutant, pVF5, was determined (Fig. 7).

\textbf{Primary structures of soybean and mothbean PRAT}

The nucleotide sequences of the soybean PRAT cDNA in pGF1 and that of \textit{Vigna} (pVF5) are shown in Fig. 7. The soybean sequence contained a single large open reading frame (ORF) which encodes a 62.2 kDa polypeptide assuming that translation is initiated at the first ATG codon. The primary structure of this polypeptide shows partial homology (see Fig. 8) with the PRAT enzymes of \textit{E. coli} (37 \% amino acid identity), \textit{B. subtilis} (47 \%), yeast (33 \%), chicken (40 \%), rat (38 \%) and human (38\%), but the plant enzyme extends by 69 amino acids beyond the N-termini of the avian, \textit{B. subtilis}, rat and human enzymes.
Figure 7. Comparison of cDNA and deduced amino acid sequences of soybean (Glycine max, pGFl) and mothbean (Vigna aconitifolia, pVF5) glutamine PRPP amidotransferases. The DNA sequence in lower case at the 5' end of the mothbean PRAT comprises the junction of the cloning vector and the linker used for insertion of the cDNA. The putative Shine-Dalgarno (SD) sequence, possible initiation codon (gtg) and in frame lacZ' termination codon(taa) are shown in lower case underlined. The sequence used for primer extension is indicated by underline. The non-homologous amino acids and nucleotides in both cDNA sequences are in bold type. The consensus polyadenylation signals are boxed.
The *Vigna* cDNA lacks a translation initiation codon but contains a large ORF encoding a 53.3 kDa PRAT polypeptide. The pVF5 cDNA shows 83% homology to the pGFl cDNA in the coding region and the deduced amino acid sequences of the mothbean and soybean PRAT enzymes share 85% amino acid identity (Fig. 8).

**Expression of the PRAT gene in different *Vigna* tissues**

Primer extension was used to determine the approximate transcriptional start site of the *Vigna* PRAT gene, the levels of PRAT mRNA in different tissues and the induction of PRAT mRNA by L-glutamine. The optimum conditions for primer extension were determined empirically. Annealing temperature affected the primer extension, and annealing at 30°C gave the best results. Primer extension was repeated for each experiment, and the same result was obtained. The primer extension product synthesized from mothbean nodule RNA was of about 250 nucleotides long indicating that ~220 bp were missing from the 5' end of the cDNA insert in pVF5. Levels of PRAT mRNA were approximately 5-fold higher in 13-day-old nodules than in leaves, and progressively increased in 16-, 19- and 23-day-old nodules with the levels in the latter being ~17 times higher than in leaves (Fig. 9). PRAT mRNA levels in uninfected mothbean roots were below the detection limits of the primer extension method used.

The primer extension products synthesized from mothbean root RNA isolated from tissue treated with 20 mM NH4NO3 or 10 mM L-glutamine were of the same size as that of nodule, and glutamine-treated roots contained about 7 times higher PRAT mRNA than those treated with NH4NO3. Although both levels were lower than that in nodules where *de novo* purine biosynthesis activity is very high (Fig. 10), these data clearly demonstrate that PRAT gene is induced in response to increased level of glutamine (amides). The glutamine level in tropical legume root nodules was not measured so far.
Figure 8. Comparison of PRAT amino acid sequences of soybean and Vigna with those from other organisms. The alignment was made using GCG Pile-Up program. Conserved amino acids are indicated in bold. (a) Amino acid sequences of putative transit peptide and propeptides in different PRAT sequences. PP, propeptide sequence; Underlined region, transit peptide of soybean. (b) Amino acid sequences of PRATs downstream of putative transit peptide and propeptides. Regions I and II, sequences used for designing degenerate primers for PCR; +, cysteine residues involved in the assembly of [Fe-S] cluster; *, N-Terminal cysteine residue. Gaps introduced into the sequences to maximize homology are indicated by dots. Sequences in (a) and (b) are contiguous.
Figure 9. PRAT mRNA levels in different tissues of mothbean measured by primer extension and calculated by PhosphorImager. R, root; L, leaf; ND, not detected.
Figure 10. PRAT mRNA levels in mothbean water-treated control root, root treated by 20mM NH$_4$NO$_3$ and 10mM L-glutamine, and 19-day-old nodule (positive control), measured by primer extension and calculated by PhosphorImager. ND, not detected.
However, considering the high production of NH$_4$NO$_3$ and L-glutamine in nodules, relatively higher concentration of these chemicals (20mM for NH$_4$NO$_3$ and 10mM for L-glutamine) were used throughout this study.

**Comparison of PRAT and SAICAR synthetase gene expression in *Vigna* nodules**

Since part of the purine biosynthesis pathway also contributes to thiamine synthesis, we explored the relative level of expression of PRAT and the SAICAR synthetase mRNAs encoding the enzymes catalyzing the first and seventh step of the pathway, respectively. Expression levels of PRAT and SAICAR synthetase genes were measured in actively nitrogen-fixing (19-day-old) *Vigna* nodules by primer extension method. The isolation and characterization of SAICAR synthetase cDNA has been published recently (Chapman *et al.*, 1994). Considering the size of primer extension products, the levels of PRAT mRNA was at least 4.4 times higher (based on PhosphorImager data) than that of SAICAR synthetase mRNA reflecting the high demand for PRAT expression in root nodules (Fig. 11). Moreover, expression of SAICAR synthetase gene seems to be delayed during nodule development (Chapman *et al.*, 1994). To avoid errors in quantification, reaction mixture was prepared in one tube including template, then equally divided into two fractions before adding labelled primers.

**Discussion**

**Structure and subcellular location of PRAT in nodules**

We have isolated an almost full-length PRAT cDNA clone from mothbean nodule
Figure 11. PRAT and SAICAR synthetase mRNA levels in 19-day-old mothbean nodules. F, PRAT; C, SAICAR synthetase.
cDNA expression library by functional complementation of an *E. coli purF* purine auxotroph. A soybean clone was also isolated by soybean nodule cDNA library screening using a cloned PRAT PCR fragment as probe. Alignment of the deduced amino acid sequence of the soybean PRAT with the sequences of human, rat, avian and microbial PRATs revealed the presence of an N-terminal domain rich in basic amino acids. This sequence resembles a presequence required for transit across the plastid membranes (Keegstra and Olsen, 1989; von Heijne *et al.*, 1989). The presence of this putative presequence is consistent with reports that PRAT activity is located in the plastid fraction in root nodules (Boland and Schubert, 1983; Shelp *et al.*, 1983).

The mothbean PRAT cDNA clones isolated all lacked an initiation codon and most of the region encoding the transit presequence. The presence of a stop codon in the polylinker attached to the cDNAs in the mothbean library would terminate translation of the *lacZ*’ peptide (see Fig. 7); thus, the PRAT enzyme encoded by the complementing mothbean cDNA clones are not synthesized in *E. coli* as fusion proteins. Instead, it is likely that a GTG codon within the vector cDNA (Fig. 7) served as the translation initiation codon and allowed formation of a functional PRAT enzyme; this codon is preceded by a putative Shine-Dalgarno sequence (Gold, 1988). Since these clones were selected by functional expression of PRAT activity in *E. coli*, the lack of complete 5' terminal sequences in all 13 clones isolated may indicate that the transit peptide interferes in some way with the PRAT enzyme activity in *E. coli* cytoplasm, or it may export the enzyme out of the cell. A similar failure to recover cDNA clones encoding a complete plastid-targeting presequence has been observed with the mothbean AIR carboxylase and SAICAR synthetase cDNA clones isolated by functional complementation of *E. coli purE* and *purC* mutants respectively (Chapman *et al.*, 1994).
The *B. subtilis*, avian, rat and human PRAT enzymes contain an N-terminal 11-amino acid propeptide which undergoes post-translational proteolytic processing to expose an N-terminal, active-site cysteine residue in the mature protein (Zhou et al., 1990). Apart from the initiation methionine, three glutamate and one glycine residues are conserved in the propeptides of chicken, *B. subtilis*, rat and human. Alignment of the legume PRAT sequences indicates that even though the *Vigna* enzyme lacks two amino acids in the propeptide region, the same three glutamate residues are conserved in the nodule PRAT polypeptides (Fig. 8(a)), suggesting that a similar propeptide is present in the plant enzymes. Excision of this putative propeptide may also expose a cysteine residue in the mature PRAT of plants. In the *E. coli* enzyme, the N-terminal cysteine acts in conjunction with histidine-100 and aspartate-29 to form a catalytic triad that has been implicated in the glutamine amide transfer reaction (Mei and Zalkin, 1989). The histidine residue is conserved in the legume enzymes and the aspartate residue is conservatively substituted by a glutamate residue, as occurs in the *B. subtilis* and avian enzymes (Fig. 8(b)), emphasizing the essential role of these residues for enzyme activity.

The *B. subtilis* PRAT enzyme undergoes a second post-translational modification involving the assembly of a [4Fe-4S] cluster (Makaroff et al., 1983) in which cysteine residues at positions 382, 434, 437 and 440 serve as ligands. Three of these at positions 382, 437 and 440 (Fig. 8(b)) are conserved in the legume PRAT enzymes suggesting that a similar type of iron-sulfur component, possibly [3Fe-3S], may also be assembled in the legume PRAT enzymes.

Based on the primer extension products, the 5' end of the PRAT transcript in mothbean leaves is identical to that in nodules. Since the nodule PRAT enzyme has an N-terminal presequence, a similar or identical sequence is likely to be present in the leaf PRAT, suggesting that the latter may be targeted to the chloroplasts. The localization of
PRAT activity in pea chloroplasts has previously been reported (Doremus and Jagendorf, 1984).

**Induction of de novo purine biosynthesis genes in nodules**

Synthesis of purines has previously been shown to increase sharply with nodule development (Atkins et al., 1982) and appears to be dependent on the export of NH$_4^+$ from the bacteroids (Atkins et al., 1984). Levels of PRAT mRNA in mothbean nodules were substantially higher than in leaves and roots reflecting the increased production of purines in these organs (Fig. 9). Thus increase in PRAT mRNA levels in nodules during development was found to parallel the nitrogen fixation activity of the nodule which in soybean begins ~12 days after infection and reaches a peak after ~24 days (Schubert, 1981). However, significant expression of PRAT mRNA occurred prior to nitrogen fixation (8 to 10-day-old nodules), and the PRAT mRNA levels are also found to be high in Fix$^-$ nodules (see Chapter III) suggesting that purine synthesis pathway opens, prior to, and independent of the nitrogen fixation activity. The high levels of purines may be required for endoreduplication of DNA in infected cells (see below). Once the pathway is opened, glutamine from assimilation of fixed nitrogen is funneled into purine synthesis and excessive purines are then oxidized to ureides. The DNA synthesis declines (as measured by S phase-specific gene expression) in nodules when nitrogen fixation commences (see Chapter III).

The actively growing tissues should contain enzymes for the de novo purine biosynthesis required for nucleic acid and ATP production. However, using primer extension assay, PRAT gene expression was undetectable in the meristematic tissue of 3-day-old roots. Although the purine synthesis activity may be very high in the root meristem cells, the proportion of these cells is small in the root, and therefore, it is
difficult to detect the PRAT mRNA in this tissue. In nodules DNA synthesis is enhanced due to endoreduplication resulting in a ploidy level in an infected cell of up to 64N (Mitchell, 1965). The fact that expression of the PRAT gene in mothbean roots was induced by treatment with L-glutamine (10mM) (Fig. 10) suggests that glutamine produced due to nitrogen fixation may constitute one of the signals for activating PRAT gene expression (Fig. 12). Since L-glutamine induced an approximately 7-fold more PRAT mRNA than NH$_4$NO$_3$, the \emph{in vivo} conversion of NH$_4^+$ to glutamine by glutamine synthetase may be a prerequisite to the efficient stimulation of PRAT gene expression.

Because purine biosynthesis pathway is also shared by thiamine synthesis, we determined the expression levels of PRAT and SAICAR synthetase genes in 19-day-old \textit{Vigna} nodules. The fact that mRNA expression levels of PRAT was higher than that of SAICAR synthetase suggests at least two possibilities. First, since the thiamine synthesis is branched out from AIR which is formed prior to the SAICAR synthesis in the pathway, high expression of upstream purine biosynthesis pathway genes may be necessary to accommodate the high demand of precursor for AIR. Second, expression of PRAT promoter may be generally higher than that of SAICAR synthetase promoter. In a recent study (Brayton \textit{et al}, 1994), expression of luciferase reporter from the human glutamine phosphoribosylpyrophosphate amidotransferase promoter was found to be approximately 3 to 4 fold higher than from the promoter of bifunctional 5'-phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth and seventh steps in the purine pathway to form 5-amino-4-imidazolecarboxylate ribonucleotide and SAICAR, respectively.

In mature determinate nodules, the purines are exported from the infected to the uninfected cells for ureide production. The increased levels of PRAT mRNA in nodules
Figure 12. Role of glutamine from nitrogen fixation in the enhanced induction of de novo purine biosynthesis pathway, the key route to ureide production in tropical legume nodules. GLN, L-glutamine.
suggests that one of the control mechanisms of de novo purine synthesis in legume plants involves regulation of PRAT activity at the level of transcription. Such regulation likely acts in concert with allosteric regulation of the PRAT enzyme (Reynolds et al., 1984). In addition, it has been suggested (Zhou et al., 1990) that the presence of the N-terminal propeptide and the iron-sulfur component in the avian and B. subtilis amidotransferases contribute another mechanism for regulation of this enzyme activity. The fact that purines are exported from infected to uninfected cells for ureide production may help in avoiding the feedback control of the pathway and further increases purine synthesis (Fig. 12). The availability of the nodule PRAT cDNA clones and the expression of nodule PRAT in E. coli will allow further investigation into the regulation of the plant enzyme at both the gene and protein levels. We have recently isolated a soybean PRAT gene and in-detailed characterization of this promoter and its possible interaction, directly or indirectly, with glutamine may reveal how the de novo purine biosynthesis is regulated at the transcriptional level in tropical legume nodules. Opening of this pathway is essential for the assimilation of symbiotically reduced nitrogen in these legumes (Lee et al., 1993) and any improvement in the regulation of this pathway may enhance nitrogen availability to the plant.
CHAPTER III
REGULATION OF UREIDE BIOSYNTHESIS GENES

Abstract
To elucidate the temporal regulation of ureide biosynthesis genes in tropical legume root nodules, we used glutamine synthetase (GS), glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) and uricase cDNAs, key genes for ureide biosynthesis in these plants, as probes to hybridize to poly (A)+ RNAs prepared from 3-day-old soybean root elongation zone, root tips, and Fix+ and Fix− nodules of different developmental stages. The DNA synthesis activity was measured by histone H4 gene expression. GS gene was constitutively expressed in the root elongation zone and root tips, and this gene expression was highly increased as the nodules mature, particularly after nitrogen fixation begins, indicating the role of this gene for ammonia assimilation in nodules.

Uricase gene was also expressed in root tips and elongation zone, but the expression level was lower than that of GS in these tissues. Similar to GS, the uricase gene expression was highly induced following the onset of nitrogen expression. Increased level of uricase gene expression is also detected in 6- and 8-day-old nodules, indicating the developmental control of this gene.

PRAT gene expressions in root elongation zone and root tips were very low, but high level of this gene expression was detected following the commencement of nitrogen
fixation, including 8-day-old nodules. The expression of histone H4 and cyc07 genes were high in young nodules (6- and 8-day-old) as well as root tissues suggesting that DNA synthesis activity is high during nodule organogenesis, but the activity is almost shut down following the onset of nitrogen fixation. These results indicate the major role of de novo purine biosynthesis in ureide biosynthesis.

The expression of GS, PRAT and uricase gene were higher in Fix+ nodules especially in mature nodules (16-day-old) than Fix- nodules indicating the role of nitrogen fixation in providing the metabolic substrate for ureide biosynthesis pathway in this organ.

Introduction

Ureide biosynthesis in tropical legumes takes place via three major steps: 1) ammonia assimilation, 2) de novo purine biosynthesis and 3) oxidative purine catabolism. These steps are compartmentalized between the infected and uninfected cells in nodules. Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme for the ammonia assimilation. Several different isoforms of GS, located in different compartments of cell such as cytosol and chloroplast and in different tissues and organs of plants, function for the ammonia assimilation in different physiological conditions (Awonaike et al., 1981; Oaks and Hirel, 1985; Cullimore and Bennett, 1989). The chloroplastic GS is responsible for the assimilation of ammonia produced from photorespiration in leaves whereas cytosolic form of GS assimilates ammonia absorbed by roots (Oaks and Hirel, 1985). In both temperate and tropical legumes, nodule-specific GS assimilates the symbiotically fixed nitrogen (Lara et al., 1983; Forde et al, 1989; Dunn et al., 1988; Konieczny et al., 1988; Sengupta-Gopalan and Pitas, 1986). Hirel et al. (1987) have found that in soybean nodules, the ammonia produced as a result of nitrogen fixation,
stimulate the expression of nodule-specific GS isoforms suggesting the expression of GS is under the tight control of the substrate availability.

Peroxisomes are specialized organelles present in all eucaryotic cells and contain enzymes that plays various metabolic roles from β-oxidation to ureide production (Trelease, 1984; Lazarow and Fujiki, 1985; Borst, 1989). In tropical legume root nodules, proliferation of peroxisomes can be observed in the uninfected cells where the oxidation of uric acid to allantoin and allantoic acid, final step of ureide biosynthesis, is completed. Nodulin-35 (N-35), a subunit of nodule-specific uricase (uricase II) of soybean, is preferentially synthesized on free polysomes during nodule development and is compartmentalized in peroxisomes of the uninfected cells of this organ (Nguyen et al., 1985). Antisense N-35 experiment in Vigna (Lee et al., 1993) demonstrated that amides do not compensate for the nitrogen requirement of tropical legumes blocked in ureide biosynthesis. Uricase is apparently induced in root nodules along with de novo purine biosynthesis. Nguyen et al. (1985) showed that mRNA for the N-35 appears in nodules between 6 and 9 days after Rhizobium infection.

Levels of PRAT mRNA in tropical legume nodules were increased steadily as the nodules mature. We have observed that PRAT is induced prior to the onset of nitrogen fixation as well (Kim et al., 1995a, 1995b) indicating a developmental control on the induction of this pathway, which is later used for the assimilation of reduced nitrogen following the commencement of nitrogen fixation. L-Glutamine, which is produced by GS and one of the substrates of PRAT, is supposed to act as an inducer of de novo purine biosynthesis (Kim et al., 1995b).

Histone H4 gene is specifically expressed during S-phase of cell division, and can therefore be used as a marker for DNA synthesis activity. The histone H4 gene isolated from different plants show high sequence homologies in the coding region (77-62).
97% amino acid sequence homology) (Chaboute et al., 1987). We used the Arabidopsis histone H4 cDNA to detect the DNA synthesis activity in nodules. Along with the histone H4 gene, soybean partial fragment of cyc07 gene has been isolated by PCR and used as a homologous probe to confirm the results by histone H4 gene. To date, homologs of this gene, such as rat v-fos transformation effector gene (fte-1) (Kho and Zarbl, 1992) and yeast mitochondrial fusion targeting gene (MFT I) (Garrett et al., 1991), have been isolated from different organisms (Kidou et al., 1994).

Fix+ nodules are also termed as effective nodules which show normal development and nitrogen-fixing activity. In Fix+ nodules, ureide biosynthesis activity takes place following the onset of nitrogen fixation. Fix- nodules are induced by either mutant rhizobia or mutant legume hosts. Since Fix- nodules cannot produce ureides, the legumes forming Fix- nodules on the roots show nitrogen deficiency phenotype. Figure 13 shows the differences of cells between Fix+ (normal rhizobia infection) and Fix- (no rhizobia infection due to failure in bacterial endocytosis) nodules.

Materials and Methods

Plant Materials and Bacterial Strains

Soybeans (Glycine max var. Prize) were grown in controlled environmental growth chambers at 26 °C. The plants were watered once a day with nitrogen-free Hoagland's solution. To induce Fix+ nodules, soybeans were inoculated with Bradyrhizobium japonicum strain 61A76 and grown in vermiculite. B. japonicum strain T8-1 (Morrison and Verma, 1987) was used to induce Fix- nodules on soybean roots. Fix+ nodules were harvested 6, 8, 10, 16, 22 and 28 days after inoculation while 10- and 16-day-old nodules were harvested in case of Fix- nodules. Control roots were obtained
Figure 13. Light microscopy of nodules induced by mutant (A) and wildtype (B) rhizobia (from Morrison and Verma, 1987)
from 3-day-old soybean seedlings.

**Isolation of RNA**

Total RNA from soybean tissues were prepared by hot phenol treatment (Verwoerd *et al.*, 1989). Plant tissues were ground in liquid nitrogen, and a fine powder was transferred to 30ml of hot extraction buffer (80°C) [Phenol-0.1M LiCl, 100mM Tris-HCl pH8.0, 10mM EDTA, 1% SDS (1:1)]. The mixtures were homogenized by shaking for 3 minutes, and 15ml of chloroform-isoamylalcohol (24:1) was added and mixed well. After centrifugation (10,000 r.p.m., 1hr.), the water phases were removed and mixed with one volume of 4M LiCl. RNAs were precipitated overnight and collected by centrifugation (10,000 r.p.m., 30 min.). The pelletes were dissolved in water, then 0.1 volume of 3M NaOAc (pH5.2) was added and RNAs were precipitated by adding 2 volumes of ethanol. After centrifugation, the RNA pelletes were washed with 70% ethanol and dried. Poly (A)+ RNAs were obtained by chromatography on oligo (dT)-cellulose.

**Northern Blot Analysis**

The DNA probes (*Arabidopsis* histone H4, soybean GS, PRAT, uricase and *cyc07*) were labelled with α-32p-dATP and used for hybridization. The hybridization was carried out in 0.5M Na2HPO4·7H2O, 7% (w/v) SDS, 0.5% (w/v) nonfat dried milk and 1mM EDTA (pH8.0) at 65°C. Filters were washed in 100mM Na2HPO4·7H2O, 5% SDS, 0.5% nonfat dried milk and 1mM EDTA (pH8.0) at 65°C for 1hr., then washed in 40mM Na2HPO4·7H2O, 1% SDS and 1mM EDTA (pH 8.0) at 65°C for 1hr. Filters were exposed to X-ray films at -80°C and analyzed by PhosphoImager (Molecular Dynamics, Sunnyvale, CA).
Single membrane filter was used to hybridize with *Arabidopsis* histone H4, soybean PRAT, uricase, GS and cyc07 genes. A probe was removed from the filter after each hybridization, and the same filter was rehybridized with the next probe. To remove radiolabelled probes, filters were boiled twice in 0.05X SSC, 0.01M EDTA (pH 8.0) and 0.1% SDS for 30min., then were rinsed briefly in 0.01X SSC. To calculate the expression level of each gene, the value analyzed by PhosphoImager was normalized by the expression level of mitochondrial ATPase in each lane.

**Isolation of soybean cyc07 partial clone**

Soybean cyc07 partial clone was isolated by RNA PCR as described in chapter II. Two degenerate primers were synthesized for PCR. Primer I was 5'-GGXAA(AG)AC(ATC)(TC)TXGT(TCG)AC(ATC)(AC)GXACXCA(AG)GGT -3' and primer II was 5'-(AG)TCXGT(ATG)GT(TC)TXAC(AG)TC(AGC)AC(AG)TG(ATG)GC(TC)TC-3' where X=ACGT. An amplified DNA fragment of ~260bp isolated from an 1% agarose gel was blunt-ended with Klenow enzyme and subcloned into the SmaI site of pUC19 for sequencing. DNA sequencing was carried out on double-stranded templates by the dideoxy nucleotide chain termination method using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH).

**Results**

**DNA synthesis and endoreduplication in root nodules**

*Arabidopsis* histone H4 cDNA was used to monitor the DNA synthesis activity in soybean root and nodule tissues. As shown in figure 14 and 15, the expression of this gene was well-detected in 3-day-old root tissues, reflecting the high demand for DNA
synthesis during root development. The histone H4 gene expression was highly induced in 6- and 8-day-old nodules, but the level of this gene expression was sharply decreased following the onset of nitrogen fixation (around 12 days after Rhizobium infection), indicating the DNA synthesis activity in tropical legume nodules is high during early nodule development. To confirm this result, a partial clone of soybean cyc07 which expresses specifically in S-phase, was isolated by PCR, and used as a homologous probe to monitor the DNA synthesis activity in soybean root and nodule tissues. The soybean cyc07 PCR fragment shows high amino acid sequence homology to periwinkle cyc07 and rat fte-1 genes (Fig. 16). Figure 17 shows the expression pattern of soybean cyc07 gene in different tissues of plant, and the trend of cyc07 expression is very similar to that of histone H4 expression.

**Expression of soybean PRAT gene**

The expression of soybean PRAT was well-detected in 8- and 10-day-old nodules where DNA synthesis activity is highly induced due to endoreduplication resulting in a ploidy level in an infected cell of up to 64N (Mitchell, 1965). PRAT mRNA levels in 3-day-old root tip and elongation zone and 6-day-old nodule tissues, however, were very low (detected by Phospholmager) in this experiment, which is similar to the result of Vigna primer extension experiment. The expression level of PRAT gene in soybean nodules was highly induced following the commencement of nitrogen fixation (Fig. 14), which is consistent with the pattern of Vigna PRAT gene expression (see Chapter II).

**Expression of soybean uricase gene**

The expression of soybean uricase gene was hardly detected in 3-day-old root tissues, while this gene expression was well-observed in 6-, 8- and 10-day-old nodule
Figure 14. Expression of ureide biosynthesis genes in soybean root and nodule tissues. H4, histone H4; PRAT, PRPP amidotransferase; GS, glutamine synthetase. Numbers are days after rhizobia infection.
Figure 15. Trends of the expressions of ureide biosynthesis genes during soybean nodule development. Each value needs to be compared to others of different developmental stages.
Figure 16. Alignment of amino acid sequences for soybean and periwinkle Cyc07, and rat Fte-1.
Figure 17. The expression pattern of soybean cyc07 in root and nodule tissues. RT, root tip; R, root elongation zone. Numbers are days after *Rhizobium* infection.
tissues, indicating the role of this gene during early nodule development. This result is consistent with the previous study (Nguyen et al., 1985) where the mRNA for the N-35 was detected in 6- and 9-day-old nodules by dot blotting. It is noteworthy that the timing of the expression of uricase, catalyzing the step downstream of PRAT action in ureide biosynthesis, coincides with that of PRAT expression. The expression of uricase gene was highly induced after nitrogen fixation takes place.

**Expression of GS gene in soybean root nodules**

The soybean GS gene is expressed in 3-day-old root tissues, and then, the GS expression level was slightly decreased during early nodule development. Like ureide biosynthesis genes, GS expression was highly induced following the onset of nitrogen fixation. In the previous study, Hirel et al. (1987) suggested that GS genes in soybean may be regulated by the availability of ammonia, either provided externally or from nitrogen fixation. The high expression of GS in the young root and nodule tissues indicates the important role of GS in general nitrogen metabolism.

Figure 15 shows the trends of the expression levels of various genes involved in nitrogen assimilation in soybean root nodules. As indicated above, PRAT, uricase and GS expressions were highly induced following the commencement of nitrogen fixation whereas the expression of histone H4 (and cyc07) was induced during root and early nodule development, but was sharply decreased after nitrogen fixation commenced.

**Levels of expression of nitrogen assimilation genes in Fix+ and Fix- nodules**

To test the effect of nitrogen fixation on the ureide biosynthesis, the expression levels of soybean GS, PRAT and uricase genes were measured between Fix+ and Fix-
nODULES. Figure 18 and 19 show that the mRNA levels of these genes in Fix\(^+\) nodules (both 10- and 16-day-old) were much higher than in Fix\(^-\) nodules, although the 16-day-old Fix\(^+\) nodules showed sharp increases in these gene expressions (1.7X in GS, 3.4X in PRAT and 4.1X in uricase), suggesting that the ureide synthesis in tropical legume nodules is regulated by the availability of symbiotically reduced nitrogen. The high expression of uricase gene is also observed in both 10- and 16-day-old Fix\(^-\) nodules, supporting the idea that uricase plays an important role in development. The histone gene expression in both Fix\(^+\) and Fix\(^-\) nodules was decreased as the nodules mature.

**Discussion**

**PRAT is expressed during DNA synthesis and nitrogen fixation**

Using Northern hybridization, we studied the expression patterns of PRAT during nodule organogenesis. The DNA synthesis activity was monitored by measuring histone gene expression. *De novo* purine biosynthesis has been shown to increase sharply with nodule development (Atkins *et al.*, 1982) and \(\text{NH}_4^+\) exported from the bacteroids may contribute to this increment (Atkins *et al.*, 1984). Figure 14 shows that PRAT expression levels increased in parallel to the nitrogen fixing activity of the nodule which in soybean starts \(~12\) days after rhizobia infection and reaches its maximum after \(~24\) days (Schubert, 1981). However, the PRAT mRNA was also well-detected in 8- and 10-day-old nodules (Fig. 14) due to endoreduplication resulting in a ploidy level in an infected cell of up to \(64\)N (Mitchell, 1965). Considering the fact that the expression of histone H4 was highly induced in young nodules (6- and 8-day-old) but was decreased sharply after the start of nitrogen fixation, we conclude that *de novo* purine biosynthesis...
Figure 18. Expression patterns of ureide biosynthesis genes in soybean Fix^+ and Fix^- nodules (10- and 16-day-old). H4, histone H4; GS, glutamine synthetase; PRAT, PRPP amidotransferase; ATPase, mitochondrial ATPase.
Figure 19. Quantification of the expression levels of ureide biosynthesis genes in soybean Fix+ and Fix- nodules (10- and 16-day-old).
in root nodules contributes to the DNA synthesis during organogenesis as well as plays a key role in assimilation of fixed nitrogen in mature nodules.

The results showing that PRAT expression level is very low in 3-day-old root and 6-day-old nodule tissues suggest two possibilities. First, as observed in the *Vigna* PRAT expression study (Kim et al., 1995), the proportion of the root meristem cells (where purine synthesis activity is supposed to be very high) is small in the root, hence detection of the PRAT mRNA is very difficult. Second, it can be assumed that the salvage pathway may partially contribute to the high demand for purines at these developmental stages (young roots and nodules). Precise determination of PRAT expression in these tissues needs to be done by developing transgenic nodules containing PRAT-GUS construct (see Chapter IV).

**Coordination of increase in the level of GS and uricase activities**

The expression level of uricase compartmentalized in peroxisomes of the uninfected cells of this organ was very low in 3-day-old root tissues, but was gradually increased during early nodule development (Fig. 14) indicating the important role of this enzyme during nodule organogenesis. The high expression of uricase gene in 10- and 16-day-old Fix^- nodules (Fig. 18) lacking ureide-synthesizing metabolism supports this idea. The fact that the timing of this gene expression is similar to that of PRAT indicates that the activities of de novo purine biosynthesis and purine oxidation may be tightly linked together, so that the de novo-synthesized purines can be oxidized by uricase expressed in this organ. In this way, the increased amount of purines synthesized following the onset of nitrogen fixation can be oxidized effectively. In order to oxidize purines, peroxisomes are proliferated and purines act as peroxisome proliferator signals. In tropical legume nodules, proliferation of peroxisomes can be observed in the
uninfected cells, and it has been recently shown in our lab that xanthine affects the proliferation of this organelle (Wu and Verma, unpublished data).

It has also been shown that GS is the key enzyme for the ammonia assimilation and in root nodules, NH$_4^+$ directly stimulate the expression of nodule-specific GS isoforms suggesting that the expression of GS is under the tight control of the substrate availability. Figure 14 shows that GS was constitutively expressed in legume tissues (including Fix$^-$ nodules), and its expression level is increased after nitrogen fixation starts. This result support the study of Hirel et al. (1987), and the glutamine produced by GS acts as one of the signals to induce a PRAT gene expression.
CHAPTER IV
INDUCTION OF PRAT GENE IN TRANSGENIC PLANTS

Abstract

To elucidate the regulation of de novo purine biosynthesis, we have isolated a soybean gene encoding glutamine phosphoribosylpyrophosphate amidotransferase (PRAT) catalyzing the first step of the pathway, and characterized it. The promoter of PRAT contains putative CAAT and TATA boxes 214bp and 175bp from ATG codon, respectively, and an ~1.45kb-long intron is located between the 5' untranslated region and propeptide sequence. The putative PUR box motif, where the repressor of pur genes binds in procaryotes, is found downstream of the TATA box, indicating the similar repressor-type regulation of this pathway in plants. The consensus sequences of nodule-specific cis-acting regulatory elements, AAAGAT and CTCTT, are also located in the upstream region of CAAT box. The soybean PRAT promoter was transcriptionally fused to β-glucuronidase (GUS) gene, and introduced into both tobacco and soybean by Agrobacterium tumefaciens and A. rhizogenses infection, respectively. The GUS expression was specifically detected in tobacco roots and soybean hairy roots. The transgenic roots from both plants provided 10mM L-glutamine showed higher GUS expression than control roots, indicating glutamine, one of the substrates for PRAT, acts as an inducer of this gene. This result is in accordance with the idea that the ureide
biosynthesis in determinate nodules is tightly regulated by the availability of glutamine formed by symbiotically reduced nitrogen.

Introduction

In tropical legume root nodules, GS assimilates the symbiotically reduced nitrogen by incorporating NH$_4^+$ into the amide position of glutamine, which is further assimilated into purines via the de novo purine biosynthesis pathway. Accordingly, purine synthesis activity is very high in nodules following nitrogen fixation (Schubert, 1981). PRAT catalyzing the first committed step in this pathway is inhibited by the end-product, nucleotide, but activated by PRPP, one of the substrate synthesized via pentose phosphate pathway. Therefore, this enzyme is thought to play a key role in regulating both carbon and nitrogen metabolism through this pathway, and in controlling the level of purine synthesis (Caskey et al., 1964; Holmes et al., 1973; Messenger and Zalkin, 1979; Meyer and Switzer, 1979; Reynolds et al., 1984). In mature determinate nodules, the purines are exported from the infected to the uninfected cells for ureide production via oxidative catabolism of purines. This may help in avoiding the feedback control of the pathway and further increases purine synthesis in the infected cells.

The second regulation point of de novo purine biosynthesis is a transcriptional regulation of purine-synthesizing genes. In E. coli, purR, a gene encoding the repressor protein, has been isolated and characterized (Rolfes and Zalkin, 1988). This protein binds to the operators of pur genes including purF, and regulates the purine biosynthesis through repression/derepression mechanism (He et al., 1990; Meng et al., 1990). The E. coli PurR is a dimeric protein, and the corepressors hypoxanthine and guanine bind cooperatively to the effector-binding domain (C-terminal) resulting in a conformational change to the PurR (Choi and Zalkin, 1992; Schumacher et al., 1995). Therefore, the
helix-turn-helix motifs at the N-terminus no longer bind in successive major grooves (Schumacher et al., 1995).

The N-terminal propeptide and the iron-sulfur component in the avian and B. subtilis PRAT contribute to the third mechanism for regulation of this enzyme activity. The processing of the propeptide generates a functional protein by exposing the first cysteine residue, which is responsible for the utilization of L-glutamine as a substrate. Under adverse condition, the iron-sulfur cluster is supposed to be the site for oxygen-binding, resulting in the proteolytic degradation of this anaerobic enzyme.

In chapter II, we have observed that the mRNA level of Vigna PRAT was increased by exogenous L-glutamine. Soybean PRAT expression level was also elevated following the onset of nitrogen fixation (Chapter III), indicating one of the control mechanisms of de novo purine synthesis in legume plants involves regulation of PRAT activity at the level of transcription. The soybean PRAT promoter was transcriptionally fused to GUS gene, and introduced into both tobacco and soybean. Since the GUS expression was specifically detected in tobacco roots and soybean hairy roots, the L-glutamine inducibility of PRAT promoter was tested in both transgenic roots. Along with the previous work (Kim et al., 1995b), the results obtained in this experiment support the idea that the ureide biosynthesis in determinate nodules is tightly regulated by the availability of glutamine formed by symbiotically-reduced nitrogen.

**Materials and Methods**

**Plant Materials and Bacterial Strains**

Soybean (*G. max* var. Prize) and tobacco (*Nicotiana tabacum* var. Xanthi) were maintained as described in Chapter II.
E. coli K-802 from Clontech, Palo Alto, CA, was used as the host for screening a soybean genomic library. Agrobacterium rhizogenes K599 and A. tumefaciens LBA 4404 containing PRAT-GUS constructs in pBI101 (Clontech) were used for soybean hairy root formation and tobacco transformation, respectively.

**Isolation of Soybean PRAT Genomic Clone by Library Screening**

A soybean (G. max var. Resnik) genomic library constructed in EMBL3-SP6/T7 vector (Clontech) was screened by plaque hybridization at 55°C using a full-length PRAT cDNA (see Chapter II) as a probe. The DNA probe was labelled with α-32P-dATP to a specific activity of 4.1 x 10^6 cpm/μg DNA using multiple primer labelling system (Amersham). 10 positive clones were isolated and restriction maps of 3 positive clones were constructed. The HindIII fragments (3.2, 1.7 and 0.9kb) of one of the positive clones were subcloned in pUC19 plasmid and sequenced. The locations of 5' and 3' ends of PRAT gene were mapped based on Southern hybridization and DNA sequencing.

**DNA Sequence Analysis**

DNA sequencing of the appropriate subclones of genomic DNA in pUC19 and pBluescript was carried out by the dideoxy chain termination method. Different deletions of PRAT genomic clone and synthetic oligonucleotide primers were used for the sequencing.

**Construction of PRAT promoter and GUS Reporter Gene Fusions**

The fragment containing the promoter and 5' end of coding region of PRAT was sequenced following appropriate subcloning. The ~3.2kb promoter region located between XhoI site and HindIII site in the 5' noncoding region (see Fig. 21) was
subcloned into pBluescript, and then transferred to pBI101 (Fig. 20). The HindIII site chosen is near to the first ATG, avoiding the possibility of deleting important motif(s) necessary for the promoter functioning. This construct was introduced into soybean root and tobacco via Agrobacterium-mediated transformations. Cauliflower mosaic virus 35S-GUS gene fusion (pBI121) (Jefferson et al., 1987) was used as a control.

Transgenic Plants

Soybean transgenic hairy roots were obtained using A. rhizogenes-mediated transformation. G. max seeds were germinated and grown on vermiculite. Three-day-old plants were infected on the hypocotyls with A. rhizogenes containing the PRAT-GUS construct or control plasmids (pBI121). Hairy roots were formed on the surface of infected stems around 12 days after inoculation. Transgenic hairy roots were identified by staining with X-gluc.

Tobacco transformation was performed by the leaf disc transformation (Horsh et al., 1984). The PRAT-GUS construct in pBI101 plasmid was directly introduced into A. tumefaciens LBA4404 by electroporation using an ECM 600 apparatus (BTX, San Diego, CA). The transformants were selected on kanamycin-containing YMB medium, and then were used to inoculate leaf discs of tobacco. Transformed callus and shoots were selected on MS (Murashige and Skoog, 1962) medium containing kanamycin (200μg/ml). Roots from the transformed shoots were induced on MS medium without any hormone. After rooting, the transgenic plants were transferred to vermiculite and grown in controlled growth chambers. The primary transgenic plants were self-fertilized, and the mature seeds were collected and germinated on kanamycin-containing MS agar medium. The kanamycin-resistant seedlings were used for further study.
Figure 20. PRAT-GUS (β-glucuronidase) transcriptional fusion construct in pBI101 used for plant transformation. NPTII, neomycin phosphotransferase type II; NOS-Pro, nopaline synthase promoter; NOS-ter, nopaline synthase terminator sequence; RB, right border; LB, left border.
Induction of PRAT by Glutamine

The hairy roots induced on soybean stems were collected, and washed in distilled water overnight, with shaking to deplete nitrogen sources in the roots. Water was replaced once during incubation. One group of the roots were then incubated in 10mM L-glutamine solution, and the other group of the roots were continuously maintained in fresh distilled water. Roots were incubated in each solution for 20hrs.

The transgenic tobacco roots induced on MS agar medium were washed as described above, then one part of the roots from plant were induced in 10mM L-glutamine, and the other part of the roots from the same plant were maintained in fresh distilled water. L-glutamine induction period was 20hrs. Roots (L-glutamine-induced and uninduced) were used for GUS assay.

Histochemical Assay of Roots

Histochemical GUS assay was carried out using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) as a substrate (Jefferson et al., 1987). L-Glutamine-induced and uninduced roots were incubated in 1mM X-gluc in 50mM sodium phosphate buffer (pH7.2) for 5hr at 37°C. The GUS-stained roots were maintained in 70% ethanol, and used for photomicrography.

Results

Isolation and Characterization of a Soybean PRAT Genomic Clone

Soybean PRAT genomic clone was isolated by screening the genomic library with a full-length PRAT cDNA probe, which is considered to be nodule-specific since the expression level of this gene was highly induced in nodule (Chapter II and III). Three
positive clones were characterized by restriction mapping and Southern hybridization using 5'- and 3'-specific probes of PRAT cDNA. Although these clones have the fragments commonly hybridized to the specific probes, the restriction patterns were not identical each other, and therefore, these clones were considered to be members of PRAT gene family. Since the amino acid sequence of PRAT is highly conserved among different organisms, the PRAT probe used might recognize other members of multi-gene family. The number of PRAT gene in soybean needs to be determined by Southern hybridization. The HindIII fragment (~0.95kb) commonly hybridized to soybean PRAT cDNA from these clones were subcloned into pUC19 and sequenced. λ14, one of the three clones characterized, showed higher sequence homology to PRAT cDNA than the other two clones, and therefore, used for further characterization.

A 5kb XhoI fragment of λ14 contains the partial coding and promoter regions (Fig. 21) of PRAT. This fragment was subcloned into pBluescript and characterized further. The HindIII sub-fragment of ~1.7kb from this XhoI fragment was subcloned into pUC19 and sequenced. As shown in Figure 21, this fragment contains the sequence encoding propeptide found in many PRATs so far isolated, followed by cysteine residue which is responsible for the utilization of L-glutamine as a substrate. Compared to the propeptide sequence of PRAT cDNA clone, the genomic clone lacks 2 amino acids (E and Q) in this sequence. Upstream of this propeptide sequence is a plastid targeting sequence (see Chapter II). In PRAT genomic clone, the amino acid sequence upstream of this propeptide is different from that of cDNA. However, the amino acids in this upstream region are mostly basic, indicating this region is involved in plastid targeting. The differences in the coding region found in soybean PRAT gene and cDNA may be due to the differences in soybean variety (G. max var. Prize for cDNA; G. max var. Resnik for genomic DNA).
Figure 21. 5'-Flanking sequence of the soybean PRAT gene.
Upstream of these basic amino acids region is a putative intron where two alternating poly (T/A) nucleotides are located (Fig. 21). This poly (T/A) nucleotide sequence is frequently found in plant introns (Goodall and Filipwicz, 1989). Two identical 21bp-long nucleotide sequences (marked by dots) were also found in the downstream of the second poly (T/A) region, although the function or importance of these sequences is not known. The putative 3' intron/exon junction (AG) is boxed, and the consensus nucleotide sequence (TGCTAAT) located in the upstream of the 3' junction AG is underlined. The consensus sequences in vertebrates and yeast are CTPuAPy (Pu=purines and Py=pyrimidines) and TACTAAC, respectively.

The putative translational start codon (ATG) is boxed (Fig. 21). A translational stop codon TAA, was found in-frame with the ATG codon in the upstream sequence. This is a frequently observed phenomenon in other plant genes. The CAAT and TATA box-motifs were found 214bp and 175bp from ATG codon, respectively, and two G-box core motifs (ACGT) and flanking nucleotides are marked by asterisks. The G-box is known to be responsive to diverse environmental stimuli (Brunelle and Chua, 1993). The putative PUR box motif, where the repressor of pur genes binds in procaryotes, is located downstream of the TATA box, indicating the similar repressor-type regulation of the de novo purine biosynthesis pathway may be operating in determinate nodules. Interestingly, three nucleotides in the putative PUR box and the second G-box motif are overlapped (see Fig. 21). AAAGAT and CTCTT, important nodule-specific cis-acting regulatory elements (Stougaard et al., 1987; Stougaard et al., 1990), were also located in the upstream of CAAT box. The putative 5' intron/exon junction GT is also boxed, and HindIII site used for constructing PRAT-GUS transcriptional fusion is underlined.
Figure 22. L-Glutamine induction of PRAT in soybean hairy root (A) and tobacco root (B). a, not induced; b, induced; B, induced.
L-glutamine induction of PRAT promoter in roots

Figure 22 shows the expression of β-glucuronidase (GUS) gene, which is under the control of soybean PRAT promoter, in both tobacco root and soybean hairy root. The GUS was specifically expressed in root tissues (data not shown), indicating that the PRAT promoter is root- and nodule-specific. Without L-glutamine induction, the GUS expression was confined to the root tips of both plants. However, with the L-glutamine induction, the GUS expression was detected in both root tip and root elongation zone, confirming the previous results (Kim et al., 1995) where the exogenously-provided L-glutamine increased the level of PRAT mRNA in Vigna roots. Roots containing cauliflower mosaic virus 35S-GUS construct showed the constitutive expression of β-glucuronidase, and basically no difference was observed between with and without glutamine induction (data not shown).

Discussion

Isolation and characterization of PRAT promoter

The soybean PRAT genomic DNA was isolated by genomic library screening. The 5kb XhoI fragment from λ14 clone contains the sequence encoding propeptide found in several PRATs from other organisms, followed by N-terminal cysteine residue responsible for the utilization of L-glutamine as a substrate. In B. subtilis, avian, rat and human PRAT enzymes, this propeptide need to be processed proteolytically to expose an N-terminal cysteine (Zhou et al., 1990). Upstream of this propeptide region is a plastid targeting sequence where mostly basic amino acids are located. The amino acid sequence differences between PRAT genomic DNA and cDNA in this region may be explained by
the difference in soybean variety. This phenomenon was observed between soybean N-35 genomic (*G. max* var. Dare) and cDNA (*G. max* var. Prize) clones (Suzuki and Verma, 1991) as well as nodulin-26 genomic (*G. max* var. Dare) and cDNA (*G. max* var. Prize) clones (Miao and Verma, 1993).

A putative intron region could be identified based on the two alternating T/A nucleotide sequence upstream of propeptide and targeting sequences. Two direct repeat nucleotide sequences (TATCTTTAGTTATAATTTATC) were located near the poly (T/A) region, but the function of this repeating sequence is not clear. The PRAT gene promoter region revealed a CAAT and TATA boxes located 214bp and 175bp from ATG codon, respectively. Several motifs involved in plant gene regulation were also identified. Two putative G-box motifs (GAACGTCA, ACACGTAG) were located in the upstream of ATG codon. The second G-box is very similar to the sequence of abscisic acid (ABA) responsive elements found in maize catalase (CACGTAAC; Guan *et al.*, 1996) and wheat *Em* genes (CACCTTGGC; Guiltinan *et al.*, 1990). The existence of putative PUR box motif located downstream of the TATA box suggests the procaryotic-type repression/derepression mechanism in *de novo* purine biosynthesis may be operating in legume nodules. The existence of repressor in purine metabolism was shown in *Drosophilla* (Dutton and Chovnick, 1990). Alignment of the putative PUR boxes of *E. coli* and soybean shows they share 7 conserved nucleotides in this 16bp-long region, and especially *E. coli* PurR2 and soybean PUR boxes have 10 identical nucleotides in this motif (Fig. 23). It is noteworthy to indicate that three nucleotides in the PUR box and the second G-box (possibly ABA response element) are overlapped, suggesting a fine-tuning regulation may have occurred in this region. AAAGAT and CTCTT, nodule-specific cis-acting regulatory elements, were found in upstream of CAAT box, and these elements are supposed to be involved in, or required for, the organ-specific regulation of nodulin...
Figure 23. Alignment of putative pur regulon control sites (PUR Box) of E. coli and soybean. Each colon represents the center of a dyad symmetry. Consensus nucleotides conserved in the majority of the operators are asterisked. Nucleotides in soybean PUR box appearing in other operators at the same positions are underlined. spurF, soybean purF (PRAT) gene.
genes (Stougaard et al., 1987). Supporting this idea is the fact that GUS expression under the control of PRAT promoter is specifically observed in roots.

**L-Glutamine acts as an inducer for PRAT**

The L-glutamine induction of PRAT gene in tobacco root and soybean hairy root, monitored by GUS expression, supports the previous study by Kim et al. (1995), and suggests that de novo purine biosynthesis in determinate nodules is tightly regulated by the availability of glutamine synthesized from symbiotically reduced nitrogen. GS is obviously the major contributor in providing L-glutamine substrate to this pathway. The GUS expression in the tips of tobacco root and soybean hairyroot without L-glutamine induction indicates the high level of nitrogen flux in this developmentally-active region. Further characterization of PRAT promoter in searching of additional signals for the induction of this gene will enable us to understand the in-detailed regulation of nitrogen metabolism in legumes.
CHAPTER V

SUMMARY AND CONCLUSIONS

The development of nitrogen-fixing nodules induced on the roots of legume plants by rhizobia is mediated by many signal exchanges between both organisms. The symbiotically reduced nitrogen is assimilated either as amides or via ureides such as in tropical legumes.

In ureide-transporting determinate nodules, plastids are the sites for \textit{de novo} purine biosynthesis where enzymes necessary for catalyzing purine pathway as well as providing carbon to this pathway exist. The key feature of the ureide synthesis in determinate nodules is the compartmentation of metabolite flux between the infected and the uninfected cells. The infected cells that are microaerobic carry out nitrogen fixation and assimilation, while the uninfected cells are responsible for the oxidative catabolism of \textit{de novo} synthesized purines to ureides. In amide-producing indeterminate nodules, plastids are the location for aspartate synthesis, one of the important amides produced in this type of nodules. Plastids in the uninfected cells of effective nodules and all cells of ineffective nodules contain large starch granules similar to amyloplasts and support the carbon requirement of nodules.

In order to study the regulation of ureide biosynthesis genes in tropical legume root nodules, I isolated soybean (\textit{Glycine max}) cDNA clone encoding glutamine
phosphoribosylpyrophosphate amidotransferase (PRAT), the first enzyme of the de novo purine biosynthesis pathway, from nodule cDNA library and characterized it. The amino acid sequence deduced from soybean clone showed > 85% similarity to the PRAT sequence of mothbean and 33 to 47% similarity to those of bacteria, yeast, chicken, rat and human. The soybean clone encodes a protein with an N-terminal sequence resembling a plastid-targeting peptide, which supports the previous study showing the activities of de novo purine-synthesizing enzymes were detected in the plastid fractions. Downstream from this peptide is a sequence similar to the 11-amino acid propeptide found in the Bacillus subtilis, chicken, rat and human PRAT proteins. This propeptide needs to be processed proteolytically to expose the N-terminal cysteine residue. The soybean clone also encodes characteristic cysteine residues that are known to be involved in the assembly of a [Fe-S] cluster near the C-terminus of this protein, and this cluster, possibly [3Fe-3S] in soybean, seems to be involved in the oxygen-dependent proteolysis of this enzyme under unfavorable conditions. Supporting this idea is the location of PRAT in the infected cells, which is microaerobic, of nodule. Levels of PRAT mRNA in mothbean nodules were found to increase steadily as the nodules matured from 13 days to 23 days. PRAT mRNA was not detectable in uninfected root tissue but a low level of transcript was detected in leaves. I studied the transcriptional regulation of PRAT gene in the tropical legume systems. Treatment of uninfected root with L-glutamine induced the PRAT mRNA transcript suggesting that glutamine produced as a result of assimilation of fixed nitrogen is funnelled into the de novo purine biosynthesis and controls the expression of this pathway in root nodules.

To further elucidate the regulation of de novo purine biosynthesis, I isolated a soybean PRAT promoter and characterized it. The promoter contains putative CAAT and TATA boxes 214bp and 175bp from ATG codon, respectively, and an ~1.45kb-long
intron is located between the 5' untranslated region and propeptide sequence. The putative PUR box motif, where the repressor of pur genes binds in procaryotes, is found downstream of the TATA box, indicating the similar repressor-type regulation of this pathway may be operating in plants. AAAGAT and CTCTT, the consensus sequences of organ(nodule)-specific cis-acting regulatory elements, are also located in the upstream of CAAT box, supporting the observation that this gene was specifically expressed in roots (see below). The soybean PRAT promoter was transcriptionally fused to β-glucuronidase (GUS) gene, and introduced into both tobacco and soybean by Agrobacterium tumefaciens and A. rhizogenes infection, respectively. The GUS expression was specifically detected in tobacco roots and soybean hairy roots. The transgenic roots from both plants provided by 10mM L-glutamine showed higher GUS expression than control roots, confirming the previous study where glutamine, one of the substrates for PRAT, acts as an inducer of this gene. This result indicates that the ureide biosynthesis in determinate nodules is tightly regulated by the availability of glutamine formed by symbiotically reduced nitrogen.

To search the temporal regulation of ureide biosynthesis genes in tropical legume root nodules, I used GS, PRAT and uricase cDNAs, key genes for ureide biosynthesis in these plants, as probes to hybridize to poly (A)+ RNAs prepared from 3-day-old soybean root elongation zone, root tips, and Fix+ and Fix- nodules of different developmental stages. The DNA synthesis activity was measured by Arabidopsis histone H4 gene expression. GS gene was constitutively expressed in the root elongation zone and root tips, and this gene expression was highly increased as the nodules mature especially after nitrogen fixation takes place, indicating the role of this gene for ammonia assimilation in nodules.
Uricase gene was also expressed in root tips and elongation zone, but the expression level was lower than that of GS in these tissues. Similar to GS, the uricase gene expression was highly induced following the onset of nitrogen fixation. However, increased level of uricase gene expression was also detected in 6- and 8-day-old nodules, indicating the developmental control of this gene.

PRAT gene expression in root elongation zone and root tips was very low, but a high level of this gene expression was detected following the commencement of nitrogen fixation. In fact PRAT gene was induced prior to the commencement of nitrogen fixation, i.e. 8-day-old nodules. The expression of histone H4 and cyc07 genes was high in young nodules (6- and 8-day-old) as well as root tissues suggesting that DNA synthesis activity is high during nodule organogenesis, but the activity was almost shut down following the onset of nitrogen fixation. These results indicate the major role of de novo purine biosynthesis in ureide biosynthesis.

The expression of GS, PRAT and uricase genes was higher in Fix+ nodules especially in mature nodules (16-day-old) than Fix- nodules indicating the role of nitrogen fixation in providing the metabolic substrate for ureide biosynthesis pathway in this organ.

These results indicate that the expression of these genes is tightly controlled by the availability of ammonia and glutamine, the nitrogen-fixation products, respectively. For the understanding of the path of metabolites in root nodules, glutamine-importing and purine-exporting through plastid membrane may give us useful hints and tools. Understanding the regulation of de novo purine biosynthesis may allow us to control the rate-limiting step(s) in this pathway and help eventually design more efficient nitrogen-assimilating plants in future.
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