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UREIDE BIOSYNTHESIS IN LEGUME NODULES

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1. ABSTRACT

In tropical legumes like *Glycine, Phaseolus and Vigna* sp., ammonia as direct product of symbiotic nitrogen fixation is converted to ureides (allantoin and allantoic acid) and they were translocated to the shoots as nitrogen source. In the xylem sap of soybean in reproductive phase the ureides reached to 60-75% of soluble nitrogen. In nodules infected cells (plastid and mitochondria) and uninfected cells (peroxisome) shares *de novo* purine biosynthesis and urate oxidation to produce ureides respectively. Current research revealed unique feathers on this symbiotic metabolism, especially on regulation of purine biosynthesis, uricase gene expression and feedback inhibition of ureides to nitrogen fixing activity.

2. INTRODUCTION

In higher plants ureides (allantoin and allantoic acid) are detected in low concentrations in various tissues, but high concentration of ureides were detected only in restricted tissues like senesced leaves (1, 2). Unusual and significant ureide accumulation in legume plants was found first by Ishizuka in root nodule extract (3, 4). The concentration reached to ca. 7-9 mM in nodules and amount of synthesized ureide per day was supposed to be ca. 10-30 mg per plant, which is comparable to the case of human (700 mg urate excretion per day). After his finding was confirmed, systematic survey to detect ureide accumulation in various legume plants was performed by various researchers (5-11) and the resulting data indicated the unique nitrogen metabolism was limited in a group of legumes (*Glycine*, *Vigna* and *Phaseolus* sp.). Phylogenic tree of whole legumes using the chloroplast-encoded *rbc*L gene indicated these species are belonging to *Phaseoleae* which is called as tropical legume (12). Almost all other legumes employed amides like asparagines for translocation of fixed nitrogen. The fact that the tropical legumes all bear determinate nodules suggested their relationships between the nodule type and the compounds for translocation. However many legumes bearing determinate nodules do not accumulate ureide, and no correlation was suggested (12, 13).

The correlation of ureide accumulation with nitrogen fixation made this phenomenon a specific metabolism which was induced in symbiotic tissue, nodule. Physiological and biochemical research immediately revealed that these ureides are degradation products of purine base which is synthesized through *de novo* biosynthesis pathway from product of symbiotic nitrogen fixation (14, 15, 16). Due to current completion of genome sequencing of *Arabidopsis* (17), all genes for purine biosynthesis has been annotated and extensive reviews were described on the molecular mechanism for gene expression of legumes (18, 19), but in tropical legumes the molecular mechanism is still in obscure. In this review we describe the current knowledge on the metabolism.

3. UREIDE ACCUMULATION AND THE PHYSIOLOGICAL ROLE

3.1. Ureide accumulation

The ureides observed in legumes are allantoin and allantoic acids. In tropical legumes like soybean, various tissues were reported to accumulate ureides.

Matsumoto *et al.* reported his experimental data using isogenic mutant lines (3). Both nodulating (A62-1) and non-nodulating (A62-2) varieties of soybean accumulated ureides in seedling and leaves. In addition to seedling stage, some ureides were generated in leaves and were translocated to pods. Significant ureide accumulation was detected only in nodulating variety (A62-1). They appeared to be generated in nodules and accumulated in stems (12 mg allantoin/g fresh weight in soybean). The N-content in the compounds represented ca. 50-70% of fixed nitrogen. They concluded that seedling, leaves and nodules were tissues which synthesized ureides, but the site of biosynthesis for significant amount of ureides in soybean should be nodules (3).

In seedlings of these legumes, a completely different system to produce ureide was reported (20, 21). This biochemical system was confirmed in soybean seedlings to be through direct degradation of purine base by coupling reaction of peroxidase and diamine oxidase. This system was observed in short time in seedlings, and maybe corresponding to proliferation for tissue generation (20). Since soybean seedlings accumulate high concentration of a diamine (cadaverine), diamine oxidase can produce peroxide and a induced peroxidase can oxidize urate to produce ureide. Although their molecular mechanism is still in obscure this system would be operating specifically in seedlings or in proliferating cells.

There are many biochemical evidences that nitrogen for ureide synthesis in nodules is from the currently fixed nitrogen (22) and the accumulation is depending on plant type (23), not on rhizobia genotype. Since all of tropical legumes carry determinate nodules and they accumulate ureides, there was some misunderstanding that ureide accumulation corresponds to determinate type nodules. This type of nodules is usually round due to the localization of meristematic cells of nodules. In contrast, indeterminate nodules forms rod-shape nodules. Some legumes bearing determinate nodules like *Lotus japonicus* and *Lotus corniculatus* employ asparagine as a major translocation compound (21). Similar relationship is applicable to indeterminate nodules too. Almost all indeterminate nodules produce asparagines or glutamine, but an exception is cluster bean, *Cyamopsis tetragonolobus* (19). This legume accumulates ureide in addition to asparagine. The data suggested that ureide accumulation in nodules of a group of tropical legumes would be due to some metabolic conditions which are common between these species.

3.2. Physiological roles of ureide accumulation

Are there some benefits of the ureide accumulation for the plants? In contrast to tropical legume group, majority of legume nodules accumulates amide (mostly asparagines and glutamine) as the major nitrogenous compound to export fixed nitrogen to shoot. For long distance translocation, ammonia which is a direct product of nitrogenase is toxic and amino acids would be too physiologically active to transport through xylem system. Possibilities to oxidize ammonia to nitrate in nodules would be the last choice because of strategic location of those big energy consumers. One benefit to employ ureide is that one molecule contains four nitrogen atoms and so C/N ratio is higher than amide, although the solubility of ureides is less than amides (24).

In general ureide accumulation in such high concentrations and in large amount is very rare case in higher plants. Since purine biosynthesis is essential for all living cells to synthesis DNA, RNA, ATP, cytokinins and coenzymes, the formation of ureides through *de novo* purine biosynthesis is tightly regulated to maintain appropriate concentrations of purine nucleotides in the cells. In addition, plants usually recycle purine bases by translocation when the tissues were aged and withered. Mothes (1, 2) already reported the cases of significant accumulation of ureide, like in seedlings, withering leaves and cut leaves. In these tissues, global nucleotide degradation and biosynthesis are going on and exogenous C supply and energy status is limited. Root nodules might be under such metabolic conditions.

3.3. Parameter to estimate nitrogen fixation activity of legume crop

Ureides accumulation has been employed as a basement of non-destructive technique for assessment of nitrogen fixating activity of nodules of tropical legumes (25, 26). Since fixed nitrogen is an only major source for synthesize ureides in nodules of these legumes, ureides content in xylem sap has been reported to be well correlated to nitrogen fixation activity (acetylene reduction activity) of nodules, and simple colorimetric assay to determine ureides can be replaced of expensive gas chromatograph to determine acetylene reduction (ethylene formation). Nitrogen fixing activity of various legumes in the field condition has been a strong concern to estimate nitrogen input to crops. Ureide assay can estimate the approximate activities in time and cost saving way. This technique has been applied to various crops and is still in practice as a convenient field technique.

Other aspects for the role of ureides accumulation were on seed production and quality. Since *Phaseolus*, *Vigna* and *Glycine* sp contain many important crops for agriculture (like soybean; *Glycine max*, kidney bean; *Phaseolus vulgaris*, cowpea; *Vigna unguiculata*, azuki bean; *Vigna angularis* etc) the physiological role was surveyed extensively. First question is whether ureides can contribute more to seed production than amide or nitrate. In the case of Soybean (*Glycine max.*) which is a major crop in food industry in Japan and so the seed production and quality are big concerns for farmers and industry, the data using isogenic mutant lines of non-nodulating soybean and pot experiments carrying no nodules showed the plants can utilize ureides for seed production but there is no difference in seed quality and yield when they grow only with chemical nitrogen fertilizer, indicating ureidse production is not essential for completing legume plant life cycle (27). Rather accumulation of ureides was inhibitory for nitrogen fixation (28), and we describe this point below.

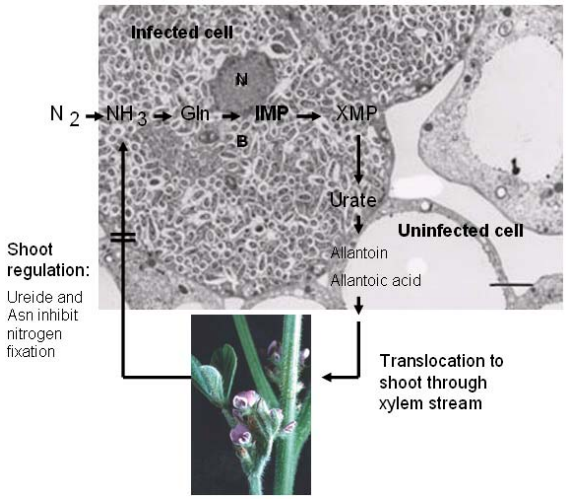

Figure 1. Ureide production in nodules and the feedback effect on nitrogen fixation. Ammonia assimilation and purine (IMP, XMP) biosynthesis occurs in infected cell and urate is supposed to be transferred to uninfected cells. Ureide is excreted from uninfected cell to xylem stream. Ureide is decomposed in shoot, especially in pods, and ureide or asparagines (Asn) in shoot can inhibit nitrogen fixing activity in nodules

3.4. Inhibitory effect of ureide accumulation in nitrogen fixation activity

On the way to elucidate the physiological role of ureides, an interesting finding was reported (28). It is the inhibitory effect of ureide to nitrogen fixation. When the plant was in water deficit, ureides were extensively accumulated in the shoots and the acetylene reduction acitivity of the nodules extensively reduced. An almost complete inhibition of acetylene reduction activity was observed when 10 mM ureide applied by stem infusion (29). In these experiment ureide concentrations increased in stems and not in nodules. The data suggested the presence of ureide feedback inhibition. Which compound is the cause of the inhibition? The data suggested asparagines or some intermediate compounds might be candidates to inhibit nitrogen fixation (30).

In the experiment supplying asparagines or ureides to plants, the inhibitory effect of ureide accumulation in leaves was suggested that both asparagines and ureides are involving in the mechanism, and when N-compound supply exceeds shoot requirement some shoot control operates to decrease nitrogen fixation activity (30). Carbon dioxide enrichment alleviates ureide-induced decline of nodule nitrogenase acitivity (31). In addition, feedback inhibition was observed in the case of transgenic plants which anti-sense uricase mRNA was expressing. Allopurinol which is a inhibitor of xanthine dehydrogenase and reduces urate formation also induced feedback inhibition (16). Although the molecular mechanism is not clear, their finding indicated ureides or their degrading products are inhibitory to nitrogen fixation and must be excreted from infected cell to keep the concentration low.

4. METABOLIC COMPARTMENT OF UREIDE PRODUCTION

4.1. Infected cells are hyperuricemia? Plastid or mitochondria?

One interesting feature of ureide production in nodules is that the metabolism is through the complex compartmentalization between organelles in infected cells and uninfected cells (32-35). As described in Figure 1, infected area of determinate type nodules contains two types of cells. One type is called as infected cell and contains many symbiosomes which have lysosome-like structure where bacteroids (differentiated form of rhizobia to fix nitrogen) are surrounded by membrane. Another type is uninfected cells which do not carry rhizobia. Uninfected cells are believed to be differentiated to support infected cells like companion cells. Plant gene expressions were quite different between these two cell types (34). For instance, free oxygen concentration in central part of nodules was reported to be extremely low, and especially in infected cells the value was reported to be ca. 10 nM (36). This micro-aerobic condition is believed to be essential for protecting nitrogenase, and would influence to ureide formation.

As Figure 1 indicated, ammonia which is produced by bacteroid nitrogenase will be excreted to cytoplasm of infected cell and believed to be utilized to synthesize purine nucleotides after ammonia assimilation. The enzyme activities contributing to purine synthetic pathway, xanthine oxidase, and xanthine dehydrogenease were detected in both infected cells and uninfected cells. However, uricase which catalyzes irreversible transfer of urate to allantoin was not detected in infected cells. The radioactive tracer experiment data suggested that currently formed purine nucleotides are immediately transferred to xantine or urate without forming high molecular weight DNA and RNA (37). These data suggested that urate generated in infected cells would be transported to uninfected cells and subjected to irreversible oxidation to form allantoin in peroxisome there.

There is still some argument which organelle between mitochondria and plastid, in infected cells is contributing mainly for purine biosynthesis (38, 39). High activity of enzymes for purine nucleotide biosynthesis has been reported first in plastids fraction in infected cells. Xanthine dehydrogenase that can convert xanthine to uric acid was observed by immunocytochemistry in soluble fractions of both infected and uninfected cells in the infected zone. Thus, it has been proposed that purine nucleotides are formed in plastids in infected cells. However, plastids are well developed in the uninfected cells in the infected zone, and substantial activities of enzymes involved in *de novo* purine biosynthesis were found in the uninfected cell fraction, although the contamination by infected cells could not be excluded completely in such experiment. In addition, mitochondria in infected cells also contained enzymes for purine biosynthesis. Import assay of ^{35}S-aminoimidazole ribonucleotide synthetase (AIRS) to each organelle suggested that product of one AIRS gene can be targeted to both plastid and mitochondria (40). The data supported the

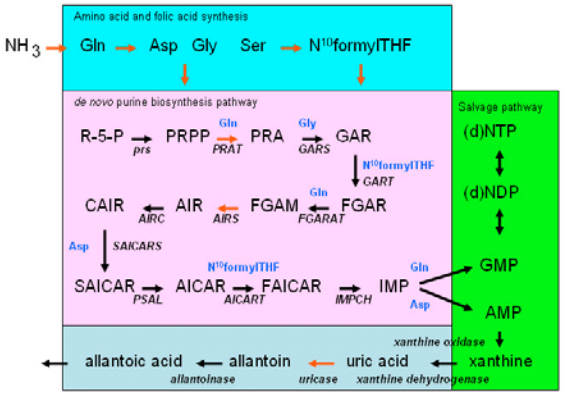

Figure 2. Ureide biosynthesis pathway and the possible regulations. Ureide synthesis pathway is divided into three parts. Red arrows indicate reported regulatory points. In metabolic pool of amino acids (blue box), glycine (Gly) and N^{10}-formyl tetrahydrofolate (N^{10}formylTHF) can be elevated. In *de novo* purine biosynthesis pathway (pink box), phosphoribosylpyrophosphate amidotransferase (PRAT), glycinamide ribonucleotide synthetase (GARS), glycinamide ribonucleotide transformylase (GART), formylglycinamide ribonucleotide amidotransferase (FGARAT), aminoimidazole ribonucleotide synthetase (AIRS), aminoimidazole ribonucleotide carboxylase (AIRC), succinoaminoimidazolecarboximide ribonucleotide synthetase (SAICARS), adenylosuccinate-AMP lyase (ASAL), aminoimidazolecarboximide ribonucleotide transformylase (AICART), inosine monophosphate cyclohydrolase (IMPCH) are enzymes for producing phosphoribosylamine (PRA), glycinamide ribonucleotide (GAR), formylglycinamide ribonucleotide (FGAR), formylglycinamidine ribonucleotide (FGAM), aminoimidazole ribonucleotide (AIR), carboximideaminoimidazole ribonucleotide (CAIR), succinoaminoimidazolecarboximide ribonucleotide (SAICAR), aminoimidazolecarboximide ribonucleotide (AICAR), formylaminoimidazolecarboximide ribonucleotide (FAICAR) from phosphoribosylpyrophosphate (PRPP)

idea that both plastid and mitochondria are operating for purine biosynthesis in infected cells, and resulting to excretion of large amount of urate to outside like hyperuricemia (41).

4.2. Peroxisome as ureide producing organelle

Human can not degrade urate in blood, but uninfected cells have been believed to be able to degrade urate in peroxisome. This reaction is catalyzed by a kind of oxygenase, uricase (EC 1.7.3.3). There are many evidences about the localization of uricase in nodule cells. The location was reported as the data of differential centrifugations, immuno-gold electron microscopy, and the data clearly indicated the location is on peroxisome in uninfected cells. Because uricase is a core protein in peroxisome, *in situ* hybridization of uricase mRNA also very clearly indicated the location in peroxisomes in uninfected cells. When infected cells and uninfected cells were separated by preparing protoplast, uricase activity was detected in uninfected cells. Such location of uricase is predictable because uricase needs oxygen for the reaction and higher free oxygen concentrations were reported in uninfected cells.

For degradation of allantoin, allantoinase (EC 3.5.2.5) and allantoate amidohydrolase (EC 3.5.3.4) were detected in various plant tissues (42). By these two enzyme reactions, allantoin was degraded to ureidoglycolate. Next reaction was catalyzed by a currently reported enzyme, ureidoglycolate urea-lyase which forms glyoxylate and urea (42). The ureidoglycolate urea-lyase activity was higher in shoot, especially in pod. Seedlings also accumulate ureide transiently in the cotyledons. In various plant tissues such pathway would be operative. *Arabidopsis* plants are able to use allantoin as sole nitrogen source and so enzymes for the metabolism can be studied in detail.

4.3. Urate and allantoin transporter

Since flows of ureides from infected cells to uninfected cells and from uninfected cells to xylem are in large scale, presence of effective transporters should be suggested. Although allantoin and uric acid transporters have not been cloned in tropical legumes, a novel superfamily of transporter (*AtUPS1*) for allantoin and other oxo derivatives of nitrogen heterocyclic compounds were cloned already in *Arabidopsis* (43). They cloned the gene by functional complementation of a yeast mutant defective in allantoin uptake. AtUPS1 transported uric acid, xanthine and allantoin. Other amide transporter (AMT), nucleotide transporter (NT) or ureide transporter (UT) as reported in yeast has not been reported yet in plants.

5. GENE REGULATION OF UREIDE PRODUCTION

For purine supply in the cell there are two sources, purine biosynthesis and salvage pathway. Since there are evidences that ureide from the nodules are through *de novo* purine biosynthesis, we focus on the regulation of *de novo* biosynthesis. Biosynthesis of ureide is more complex than that of amides. In the case of asparagine synthesis it needs 4 enzymes from ammonia (GOGAT, GS, asparagine synthetase, aspartate aminotransferase. However, for *de novo* purine biosynthesis it needs 20 enzymes from ammonia assimilation (Figure 2). Why tropical legumes employ such complex pathway? In the beginning, profiles of metabolic regulation will be described.

5.1. Amino acid and folate synthesis

One possible regulatory point is elevation of pool size of glycine, serine and folate derivatives. Purine ring is consisted of aspartic acid, glutamine, two derivatives of teterahydroformic acid (THF) and glycine. Aspartic acid and glutamine are present in high concentrations in nodules, and glycine and folate derivative supply can be a regulating point for purine ring synthesis (Figure 2). Le *et al.* detected by proteomic analysis that P-protein of glycine cleavage system (GCS), an enzyme complex to reversibly synthesize glycine, were significantly up-regulated and L-protein of GCS was down-regulated in nodule mitochondria comparing to root mitochondria (44). This would result in elevation of 5, 10-methylene-THF pool

(44). In human, hyperglycinemia is a syndrome which glycine concentration in the cell is extensively elevated due to supression of one protein of GCS (45). Such elevated glycine supply might be a regulatory point of ureide accumulation in nodules. Biochemical study on this point is necessary to elucidate this mechanism.

5.2. Purine biosynthesis pathway

In purine biosynthetic pathway, which enzyme is a regulatory point in ureide formation in legumes? After completion of genomic sequencing of *Arabidopsis*, extensive studies on regulation of purine biosynthesis were reported (46-55). Figure 2 shows the summarized gene sequences in the purine synthetic pathway. In animal system, glutamine phosporibosylpyrophosphate amidotransferase (PRAT, EC. 2.4.2.14) is reported to be a key regulatory enzyme. Similar result was obtained using legume plant (52, 53), showing the significantly increased activity of PRAT during nodule formation. In contrast, Atkins *et al.* observed a case of feedback control that AIRS (aminoimidazole ribonucleotide synthetase; EC. 6.3.3.1) was a regulatory gene when nitrogen fixation was ceased (54). They water-cultured soybeans with bubbling air and then pulsed Ar/O_2 (80%:20%) instead of air. After several hrs' incubation with Ar/O_2, AIRS gene expression was significantly suppressed (54). The northern blot data showed AIRS gene expression was strongly suppressed. The data indicated AIRS would be a possible feedback regulatory point in purine biosynthesis in nodules. In Ar/O_2 bubbling condition, nodule infected zone was aged very quickly and this was a reason that they employed short time pulse of Ar/O_2 gas instead of long time exposure (19, 54). They also observed that uricase activity in the nodules was also suppressed in such condition. In western blot analysis, the uricase protein content did not change in that condition, suggesting inhibitory uricase protein modification occurred then (54).

5.3. Uricase gene regulation

Uricase is an enzyme which catalyzes urate to form allantoin. The gene expression of uricase has been studied extensively as a direct regulatory point of ureide accumulation. Uricase gene expression was detected first as a nodule specific gene from soybean and designated as Nod-35 (32, 56-59). Although uricase gene over-expression in soybean nodules was well confirmed to occur only in uninfected cells (33), the enzyme induction mechanism is still in obscure (58). We describe our current knowledge on the molecular mechanism of the gene expression.

Amino acid sequences of uricase genes of various legumes were surveyed (59, 60). All uricases contained signal amino acid sequence at 3' terminus to target to peroxisome. When a phylogenic tree was drawn from the deduced amino acid sequences of uricases, ureide transporting legumes made one sub-group which is different from non-ureide legume (60). In the case of *L. japonicus*, the uricase does not belong to the subgroup of tropical legumes and appeared to be an intermediate type, comparing to amide type legumes. When nitrogenous compound in the xylem sap of *L. japonicum* was analyzed it was mainly asparagine, but the content of ureides was higher than that in *Medicago sativa* (59). These data suggest that phylogenic data of uricase amino acid sequence corresponded to ureide content in the xylem sap. Ureide-transporting legumes would have evolved separately from amide-transporting legumes, and suggested the presence of common molecular mechanism for gene expression like cis-element in the upstream region of genomic uricase gene.

For analyzing promoter activity for uninfected cell specific expression of uricase, *in situ* hybridization analysis of uricase was surveyed in *Glycine max*., *Lotus japonicus* (57, 60) and *Medicago sativa* (59). The uricase expression was detected slightly in nodule parenchyma, vascular bundles and root tips. However, the expression was clearly localized in uninfected cells, corresponding to the data of immuno-electron microscopy and organelle enzyme localization.

Survey of the *cis*-element motif in the promoter region of the uricase gene has been studied in our group. Since regeneration of transgenic plant using *Agrobacterium tumefaciens* was unstable in soybean plant, we employed hairy root transgenic system with promoter:GUS chimera DNA and *Agrobacterium rhizogenes*. Promoter:GUS construct of uricase gene was introduced to transgenic hairy root following to inoculation with *Bradyrhizobium japonicum*. Figure 3A shows typical uninfected cell specific expression of GUS in transgenic nodules. For obtaining candidate nucleotide sequences for DNA motif, DNA sequences of 5'-upstream region of uricases from various legumes were compared, and sequences which are common among uricase genes which showed uninfected cell specific expression. The final candidate of nucleotide sequence (GTAATG) showed promoter activity for GUS expression like Figure 3A. In addition, gel shift assay of nucleus proteins indicated the presence of a protein which bind to the candidate DNA fragment (Figure 3B). The molecular evolution of the motif can be compared with that of infected cell expression motif (AAGAG)in leghemoglobin promotor analysis that has been investigated using nonlegume hemoglobin genes (61).

6. CONCLUSION AND FUTURE PROSPECTS

Ureide metabolism has been described well in animal as an excretion metabolism which is evolutionally modified in different species for adapting their environment. In higher plants it is an essential but minor metabolism to recruit nucleotide nitrogen to growing point of the plant. Only in seedlings or developing seeds, the metabolism is one of major ones because other tissues send ureides through xylem stream. As described in this review, plant cells in infected region of nitrogen fixing nodules exposed their ability to generate and excrete large amount of ureide continously. Possible regulation points in the metabolism were described in this review. However, molecular mechanism to activate the regulation points is still in obscure. Since 1) Arabidopsis can grow with ureide as sole nitrogen source; 2) tropical legumes can grow well without ureides, 3) transgenic plant of antisense uricase mRNA showed nitrogen deficiency with nodules (62), the key would be in the process of plant-microbe communication.

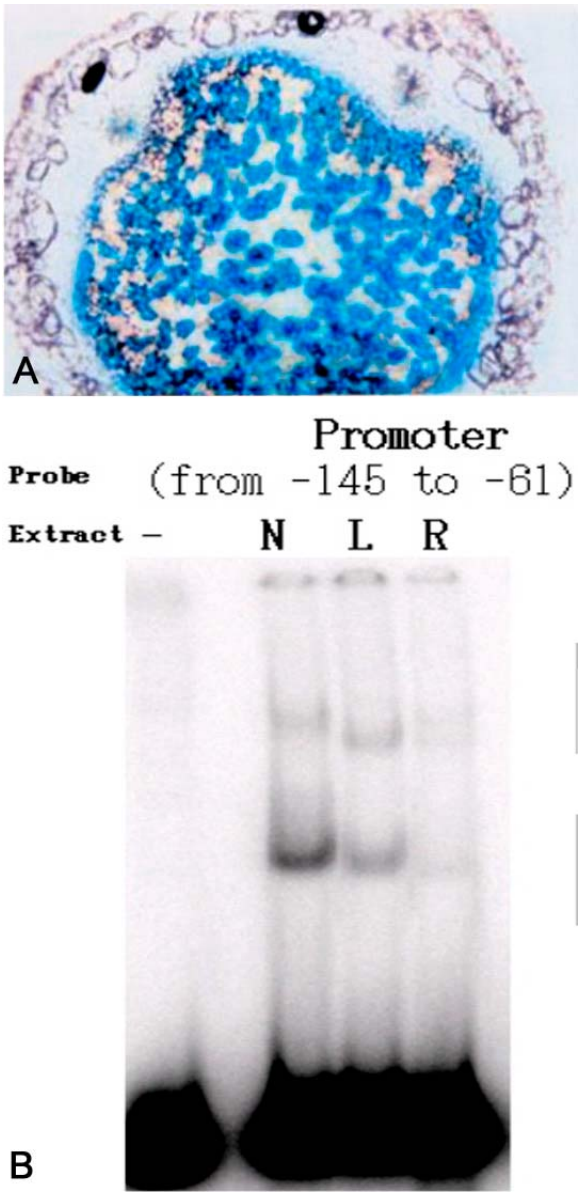

Figure 3. Detection of cis-element in 5'-upstream region of uricase. Promoter:GUS reporter gene assay indicated uninfected specific activity (Figure 3A). In gel shift assay (Figure 3B), a nucleus protein which binds to the proposed promoter region may be present mainly in nodules (N). The content in leaves (L) and roots (R) would be much lower

In animal system inhibition of purine metabolism influences to cell cycle because purines control both G1 to S phase transition and progression (63). Further research effort is necessary to resolve the question in gene control level. Genome sequencing or full-length cDNA library data of tropical legumes is desirable, and regulation of ureide metabolism in tropical legumes would connect to technologies for production of purine alkaloids or maintain high nitrogen fixation activity of nodules throughout crop growing period.

7. ACKNOWLEDGMENTS

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