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REGULATION OF THE ENZYMES OF THE UREIDE BIOSYNTHESIS IN TROPICAL VARIETIES OF LEGUMES

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EXECUTIVE SUMMARY

Project no 12 367

This work addressed the genetic regulation of one of the key enzymes of the ureide pathway in *Phaseolus vulgaris*, in order to increase bean productivity The overall aim of this project was to investigate the regulation of the uricase-II gene in *P vulgaris*

Uricase II is an enzyme highly induced in root nodules during the symbiosis established between tropical legumes and soil bacteria of the genus *Rhizobium* and *Bradyrhizobium* It participates in the biosynthesis of ureides, nitrogenous compounds transported from the nodules during nitrogen fixation During the course of this project, the nodule uricase II cDNA was isolated and characterized and was demonstrated that uricase II is encoded by a single gene in the *Phaseolus vulgaris* haploid genome During this investigation, it was published (Capote and Sanchez, Plant Physiology 115 1307-1317, 1997) that this gene is expressed in roots, stems and leaves of adult bean plants, and that its expression is induced in cotyledons, roots and hypocotyls during seedling establishment *In situ* hybridization and immunodetection experiments showed that uricase II mRNA and protein are localized in the vascular system of bean roots and hypocotyls, in phloem fibers and xylem-parenchyma cells, and in cotyledon

Additionally, 1572 bp of the regulatory region (promoter) from the common bean uricase-II gene was cloned and fused to a bacterial reported gene (GUS), in order to study the factors that control its expression This 1572 bp regulatory region was characterized (Cuernavaca and Maryland) The complete nucleotide sequence (Cuernavaca and Maryland) and putative regulatory motifs with discrete homology to other reported plant gene promoters, was obtained Transcriptional gene fusions (Cuernavaca), made from the promoter region (1572 bp) and its 5' serial deletions (constructed by Exo III and PCR) were cloned in phase to the GUS bacterial reporter gene in pBI121 for *Agrobacterium* mediated infection in transgenic

plants (unpublished results) Both, a PhD and a BS students graduated with this project Two papers with AID acknowledgments were published and several scientific communications were presented in national and international meetings

The support from AID was essential for the achievement of the mentioned goals

RESEARCH OBJECTIVES

This work focussed on the genetic regulation of the ureide pathway in *Phaseolus vulgaris* in order to increase bean productivity

Legumes, are a primary source of protein in the diet of the vast majority of the Latin American and African population Legume plants can be classified into amide or ureide transporters depending on the nitrogenous compounds that they export from the nodule to the shoot In tropical legumes such as bean, soybean and cowpea, a change in the transported nitrogen compounds from amides (glutamine and asparagine) to ureides (allantoin and allantoic acid) occurs when they are infected with efficient nitrogen-fixing bacteria (Matsumoto et al , 1977, Fujihara and Yamaguchi, 1980)

Root nodules are highly specialized symbiotic organs where the differentiated bacteria or bacteroids, are able to reduce atmospheric nitrogen into ammonia (Sánchez et al , 1991, Mylona et al , 1995) Ammonia ions are excreted into the plant infected cell cytoplasm, assimilated into glutamine and then incorporated into de novo purine biosynthesis (Schubert, 1986) In the nodule infected and non-infected cells, these purines are catabolized and consequently converted into ureides that are transported to the aerial parts of the plant (Matsumoto et al, 1977, Pate et al, 1980,) Xanthine dehydrogenase, uricase-II and allantoinase are the last three enzymes that participate in the ureide biosynthetic pathway and are mostly located in the nodule non infected cells (Atkins et al , 1980, Boland and Schubert, 1982)

Uricase II catalyzes the oxidative cleavage of uric acid into

allantoin and CO_2, liberating H_2O_2 The enzyme has been purified from cowpea (Ranbird and Atkins, 1981), soybean (Legocki and Verma, 1979, Lucas et al , 1983, Bergmann et al , 1983) and bean (Sanchez et al , 1987) nodules It is preferentially synthesized on free polysomes during nodule development and is localized in the peroxisomes of nodule non-infected cells (Hanks et al , 1981, Nguyen et al , 1985, Kouchi et al , 1989) In effectively nodulated bean plants, uricase II expression is highly induced before N_2 fixation starts It constitutes approximately 2% of the total soluble protein of the nodule (Sánchez et al , 1987) In empty nodules induced by *Rhizobium* mutants, the amount of uricase II mRNA is 10% in respect to those nodules infected with the wild type bacteria, suggesting that several events during nodule development (eg nodule induction, bacteria release, nitrogen fixation) are decisive for the regulation of uricase II gene
expression (Padilla et al , 1991)

Low uricase II activity has been detected in soybean callus tissue, where it is regulated by oxygen availability (Larsen and Jochansen, 1986) This enzyme has also been found in soybean cotyledons and hypocotyls during seed development and early germination (Tajima et al , 1991) In soybean cotyledons, uricase II has been immunocytochemically detected in the peroxisomes of this organ (Damsz et al , 1994) In this plant, uricase II is encoded by a small gene family that could have similar expression control mechanisms in nodules and developing seeds (Tajima et al ,1993) Another uricase activity (uricase-I) has been detected in soybean seed radicles, but this activity was due to a diamine oxidase/peroxidase system with different characteristics to nodule uricase (Tajima et al , 1983, Tajima et al , 1985)

Given the above background, the study of the genetic regulation of the uricase-II gene, a gene encoding for one of the key enzymes of the ureide pathway in *Phaseolus vulgaris*, was of central importance In order to gain incite into the molecular and physiological basis that could participate in the modulation of this metabolic pathway, the research objectives included the isolation and characterization of the bean nodule uricase II cDNA This objective was accomplished It became important to demonstrate if uricase II was encoded by a multigene family, as reported for

soybeans(REF) We concluded that a single copy of the uricase II gene is present in the *Phaseolus vulgaris* haploid genome Furthermore, beside the nodule, this gene is expressed in different organs of the seedling and the adult plant This study also indicted the temporal expression pattern of uricase II mRNA and protein in cotyledons, roots and hypocotyls during germination and seedling establishment We also show the in situ localization of the transcript and the histological immunodetection of the antigen in the mentioned seedling organs Additionally, uricase II protein was detected by western blot in Leucaena seedling organs, suggesting that this protein participates in seedling development in legumes that do not transport ureides The anti-uricase antibody also recognizes a 50-KDa peptide that is present in cotyledons, roots and hypocotyls but not in nodules (Capote and Sanchez, 1997)

Future directions

We are currently investigating the nature of this 50-KDa protein, our current hypothesis is that part of this enzyme is postranslational modified when expressed outside the nodule and probably this modification does not allow the enzyme to be transported into the peroxysomes in vascular bundles

RESEARCH ACCOMPLISHMENTS

During this period, two papers in international journals have been published, and 9 presentations in scientific meetings were presented Two students graduated (one Phd and a BS) Additionally, there is a plethora of unpublished results that will be polished in order to be submitted for publication

The unpublished data is presented in this section

The aim of the proposed research was to investigate the signals (bacterial, plant or environmental) which modulate the expression of the uricase-II gen in *Phaseolus vulgaris* nodules Therefore, in our laboratory we have subcloned and characterized a *Phaseolus vulgaris* uricase-II cDNA clone that was further used as a probe for the isolation of a uricase genomic clone from the lambda-EMBL3A genomic library (Cuernavaca, last year report) a 1572 bp regulatory region has been sequenced and characterized (Cuernavaca and Maryland) We have obtained its complete nucleotide sequence (Cuernavaca and Maryland) and the start ATG codon as well as the

transcriptional start site (+1) was located 20 bp from the ATG as determined by primer extension (Cuernavaca) Putative regulatory motifs with discrete homology to other reported plant gene promoters, were also identified (Maryland) The TATA box, OSE (Organ Specific Element) that contain the CTCTT and AAAGAT motives found in late nodulin gene promoters from bean Npv30, soybean N-23 and lbc3, an A/T rich regions conserved in other soybean and bean nodulin promoters and finally there are two putative G boxes

With the regulatory region we have tried to performed the following studies

-Cis regulatory regions studied in vitro To transiently express by electroporation into protoplasts of non-infected cells from bean nodules The regulatory sequences involved in the transcriptional regulation of a uricase-II promoter GUS Nos3' gene fusion

- Cis regulatory regions studied *in planta* Regulatory *in vivo* studies by the introduction of a uricase-II promoter GUS Nos3' chimeric gene and derivatives in transgenic *Lotus corniculatus* and hopefully *Phaseolus vulgaris* transformed by *Agrobacterium*

- Cis regulatory studies in cotyledons Transient gene expression in bean cotyledons by particle bombardment with the uricase-II promoter GUS Nos3' chimeric gene derivatives, constructed for the electroporation studies mentioned above

REGULATORY STUDIES IN ELECTROPORATED BEAN NODULE CELL PROTOPLAST

Transcriptional gene fusions, made from the promoter region (1572 bp) and its 5' serial deletions (constructed by Exo III and PCR) were cloned in phase the GUS bacterial reporter gene by subcloning the respective genomic fragment into the polylinker region of the GUS vector plasmids pBi121 (for *Agrobacterium* mediated infection in transgenic plants) and pBi426 (for the transient expression studies) With these molecular tools we will be able to electroporate protoplast isolated from fully infected or from non-infected nodules

In this respect, we have selected five pBi426 plasmid derivatives containing fragments of 72, 192, 334, 480, and 1572 bp transcriptionally fused to the reporter gen GUS This fragments include the following features form the promoter region pBi426-5, 72 bp(TATA box), pBi426-4,192 bp (TATA box, 3 OSE), pBi426-3, 334bp (TATA box, 3 OSE and a A/T-rich region), pBi426-2, 480 bp (TATA box, 3 OSE, A/T rich and 2 G boxes) and pBi426-1,1572 bp including 3 more OSE motifs

REGULATORY STUDIES IN TRANSGENIC PLANTS

Lotus corniculatus are been transformed by *Agrobacterium rhizogenes* according to Petit et al 1987 where an average time to obtain a complete transgenic plant is five months As an alternative, we are also following the protocol of Hansen J et al 1989 where the process is interrupted at the level of a chimeric plant (transformed roots and normal aerial parts) this process only requires about the half of the time In both cases the final objective is to generate transgenic nodules, the nodulation process in our conditions takes about 5-7 weeks This whole process has been labor intensive, taking more than 2 years to set up all the conditions to obtain *L corniculatus* transgenic nodules, in particular the transfer into Agrobacterium of different plasmid constructs, optimal seed germination and plant survival after transformation with the different *A rhizogenes* strains, and last excision of hairy roots and shoot regeneration We are in the process of quantifying the reporter enzymatic activity in the transgenic nodules We will switch to *Lotus japonicus* (Thykjaer et al, 1998) in the near future given all the advantages that this diploid plant has over Lotus corniculatus whis is tretraploid

During the last year we have started to attempt to transform *Phaseolus vulgaris* germinated seedlings under vacuum with either, *A tumefasciens* or *A rhizogenes* containing a 35S-GUS-Nos3' construct The infiltrated plants (germinating cotiledons) were inoculated with *Rhizobium etli* in order to avoid a hypersensitive response of common bean to *Agrobacterium* infection In the

survivors, localized foci of transformed cells were evident only in the roots of those bean plants transformed with *A tumefasciens* It has not been possible to obtain yet transgenic nodules in this plant In collaboration with Dr Miguel Lara's group we are improving the transformation procedure by trying to infect bean embryos and apical meristems and then follow the transformation in tissue culture conditions instead of vermiculite With these improvements we hope to increase the transformation rate and the amount of transformed zones (GUS blue spots) in order to introduce the uricase-II promoter (chimeric gene previously mentioned)

TRANSIENT EXPRESSION STUDIES IN BEAN COTYLEDONS BY PARTICLE BOMBARDMENT

Bean hypocotyls and leaf tissues have high levels of allantoinase and allantoinase It is likely that the substrates for both enzymes (allantoin and allantoic acid, respectively) come from nitrogen recycling in the cotyledons The enzyme responsible for the production of allantoin is uricase-II and in consequence it would be of interest to detect the expression of uricase-II transcript in the cotyledons By northern analysis we have been able to detect uricase-II expression in bean cotyledons of 1, 2, 4, 7 and 10 days after imbibition There was no detection of uricase II transcripts in dry seeds, but only in germinating cotyledons This expression was lower than in nodules(2-5%), reaching the maximum level of expression at the fourth day after imbibition (Capote and Sanchez, 1997)

FUTURE WORK

-Trans regulation studies gel-retardation assay using nuclear extracts from roots, wild type and empty nodules to look for uricase promoter binding factors and by footprinting DNAse-protection assay to gain incite into the factors involved in the genetic control of the uricase-II gene in bean nodules In particular we have previously reported(Padilla, et al 1991) that uricase-II mRNA could be detected in bean nodules 2-3 days before nitrogen fixation and that about 10% of the maximal expression was present in empty nodules (

non-fixing nodules without rhizobial infections) suggesting a possible role of this gene during nodule development We will continue to try to answer this open and interesting question

-Peroxisome biogenesis As previously mentioned, there is a possibility that uricase-II could have a role in nodule development, besides the obvious role in ureide biogenesis In particular, Lee et al 1993, suggested that uricase-II could have a role in peroxisome biogenesis (uricase-II is a peroxisomal enzyme) because transgenic *Vigna* plants expressing an anti-sense mRNA, which arrest uricase-II expression, had the number and the shape of the peroxisomes affected In this regard we will transform *Lotus japonicus* (Thykjaer et al, 1998) plants with a full-lenght uricase-II cDNA clone under the control of the 35S and its own promoter Since Lotus is not a ureide-producing plant we expect that the over-expression of this gene in nodules and cotyledons will induce the biogenesis of peroxisomes The analysis will be performed by electron microscopy in nodule and cotyledons embedded tissues

Project Activities/Outputs

Graduated Students

- Nieves Capote a PhD student fully devoted to this project, obtained her degree in September 1997
  Elisa León obtained her BS degree in July 1996

Publications with AID acknowledgments

- Sanchez F, Capote N, Padilla J, Ortega JL, Kuin H, Lara M 1994 Genetic Regulation of the Ureide Biosynthetic Pathway in Tropical Legumes Bean Advance Biotechnology Research Network, BARN, (eds WM Roca et al) Centro Internacional de Agricultura Tropical (CIAT) Cali, Colombia pp 379-385

- Nieves Capote and Federico Sanchez 1997 Characterization of the Common Bean Uricase and its Expression in Organs than Nodules Plant Physiology 115 1307-1313

We expect to publish at least two more papers in international journals with the acknowledgment to the partial support of this AID project

Meeting Presentations

- N Capote, F Campos, J Padilla y F Sanchez
  Characterization of the uricase-II gene of Phaseolus vulgaris
  VII PAABS Congress XIX Sociedad Mexicana de Bioquimica
  Sociedad Española de Bioquimica Sept 27-Oct 2, 1992

- N Capote y F Sanchez
  Regulacion del gen de uricasa-II en nódulos de Phaseolus vulgaris
  VI Reunion Nacional de Bioquimica Vegetal
  Universidad Michoacana de San Nicolas de Hidalgo October 10-14, 1993

- Nieves Capote and Federico Sanchez
  Regulation of the Uricase-II gene in nodules of Phaseolus vulgaris
  4th International Congress of Plant Molecular Biology
  The International Society of Plant Molecular Biology (ISPMB) Amsterdam, The Nederlands June 19-24, 1994

- N Capote, E León, F Sanchez
  Regulación del gen de uricasa en nodulos de Phaseolus vulgaris
  Sociedad Mexicana de Bioquimica XX Congreso Nacional
  Universidad de Zacatecas Zacatecas, Zac October 30-Nov 4, 1994

- N Capote, M de J E León & F Sánchez
  Regulation of the Uricase-II gene in nodules of Phaseolus vulgaris 1er Simposium México Estados Unidos sobre Agrobiologia, fisiologia y biotecnologia de cultivos importantes para la agricultura mexicana Cocoyoc, Morelos, México November 5-9, 1995

- F Sanchez
  Simposio Biologia Molecular de Plantas Regulacion Genetica de la via de ureidos XIC Congreso de la Sociedad Española de Bioquimica y Biologia Molecular
  I Reunion con la Societé Française de Biochimie et Biologie Moléculaire Cordoba, España September 24-28, 1995 Invited Speaker

- M de Jesús Elisa Leon, Nieves Capote y Federico Sanchez
  Regulación de la expresión del gen de Uricasa II en plantas transgénicas de *Lotus corniculatus* y *Phaseolus vulgaris*
  XXI Congreso Nacional de Bioquimica Manzanillo, Col November 3-7, 1996

- Nieves Capote and F Sanchez
  Characterization of the Common Bean Uricase II and its

Expression in Organs other than Nodules
16th North American Conference on Symbiotic Nitrogen Fixation February 1-6 1998 Cancun, México

Travel
Nieves Capote spent one month in Dr Mauricio Bustos Lab to perform the particle bombardement experiments and to finish the DNA sequence of the uricase-II regulatory region

Personal Involved Two students, Nieves Capote (PhD) and Eliza León (BS) were full-time involved in this project(Cuernavaca) Also a technitian supported by the University of Mexico partially assisted in this project (MS, Juan Olivares)

Additional Support This project was additionaly supported by the University of Mexico, grant Direccion General de Asuntos del personal Académico IN 3000993, in fact the work never stoped during the period that funds were not available due to to the fact that it was very difficult to establish a sub-contract between the University of Mexico and the University of Maryland This contract agreement was not modified until 0/30/96 but funds were not available from 1994 up to beginning of 1997

Literature Cited

Atkins CA, Rainbird RM, Pate JS (1980) Evidence for a purine pathway of ureide synthesis in N_2-fixing nodules of cowpea *Vigna unguiculata* (L) Walp] Z Planzen Physiol **97** 249-260

Boland MJ, Schubert KR (1982) Purine biosynthesis and catabolism in soybean root nodules incorporation of ^{14}C into xanthine Arch Biochem Biophys **213** 486-491

Damsz B, Dannenhoffer JM, Bell JA, Webb MA (1994) Immunocytochemical localization of uricase in soybean cotyledons Plant Cell Physiol **35** 979-982

Fujihara S, Yamaguchi M (1980) Nitrogen fixation and allantoin formation in soybean plants Agric Biol Chem **44** 2569-2573

Hanks JF, Tolbert NE, Schubert KR (1981) Localization of enzymes of ureide biosynthesis in peroxisomes and microsoms of nodules Plant Physiol **68** 65-69

Hansen, J, Jorgensen JE, Stougaard J, Marker KA (1989) Hairy roots, a short cut to transgenic root nodules Plant Cell Rep 8 12-15

Kouchi H, Tsukamoto, Tajima S (1989) Differential expression of nodule specific (nodulin) genes in the infected, uninfected and cortical cells of soybean (Glycine max) root nodules J Plant Physiol **135** 608-617

Larsen K, Jochmsem BU (1986) Expression of nodule-specific uricase in soybean callus tissue is regulated by oxygen EMBO J **5** 15-19

Lee NG, Stein B, Suzuki H, and Verma DPS (1993) Expression of antisense nodulin-35 in *Vigna aconitifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plant Plant J **3** 599-606

Legocki RP, Verma DPS (1979) A nodule specific plant protein (Nodulin-35) from soybean Science **205** 190-192

Matsumoto T, Yatazawa M, Yamamoto Y (1977) Distribution and change in the contents of allantoin and allantoic acid in developing nodulating and non-nodulating plants Plant Cell Physiol **18** 353-359

Mylona P, Pawlowski K, Bisseling T (1995) Symbiotic nitrogen fixation The Plant Cell **7** 869-885

Nguyen T, Zelechowska M, Foster V, Bergmann H, Verma DPS

(1985) Primary structure of the soybean nodulin-35 gene encoding uricase II localized in the peroxisomes of uninfected cells of nodules Proc Natl Acad Sci USA **82** 5040-5044

Petit A, Stougaard J, Kuhle A, Marker KA, Tempé J (1987) Transformation and regeneration of the legume Lotus corniculatus a system for molecular studies of symbiotic nitrogen fixation Mol Gen Gent 207 245-250

Padilla JE, Miranda J and Sánchez F (1991) Nodulin regulation in commom bean nodules induced by bacterial mutants Mol Plant Microbe Interaction **4** 433-439

Pate JS, Atkins CA, White ST, Rainbird WR, Woo KC (1980) Nitrogen nutrition and xylem transport of nitrogen in ureide-producing grain legumes Plant Physiol **65** 961-965

Ranbird RM, Atkins CA (1981) Purification and some properties of urate oxidase from nitrogen-fixing nodules of cowpea Biochimica et Biophysica Acta **659** 132-140

Sánchez F, Campos F, Padilla J, Bonneville JM, Enriquez C, Caput D (1987) Purification, cDNA cloning and developmental expression of the nodule-specific uricase from *Phaseolus vulgaris* L Negro Jamapa Plant Physiol **84** 1143-1147

Sánchez F, Padilla JE, Pérez H, Lara M (1991) Control of nodulin gene in root-nodule development and metabolism Annu Rev Plant Physiol Plant Mol Biol **42** 507-528

Schubert KR (1986) Products of biological nitrogen fixation in higher plants synthesis, transport and metabolism Ann Rev Plant Physiol **37** 539-574

Schubert KR and Boland MJ (1990) In Academic Press,Inc The biochemistry of plants **16** 197-282

Tajima S, Ito H, Tanaka K, Nanakado T, Sugimoto A, Kouchi H, Okazaki K (1991) Soybean cotyledons contain a uricase that cross-reacts with antibodies raised against the nodule uricase (Nod-35) Plant Cell Physiol **32** 1307-1311

Tajima S, Kanazawa T, Takeuchi E, Yamamoto Y (1985) Characteristics of a urate-degrading diamine oxidase-peroxidase enzyme system in soybean radicles Plant Cell Physiol **26** 787-795

Tajima S, Kato N, Yamamoto Y (1983) Cadaverine involved in urate degrading activity (uricase activity) in soybean radicles

Plant Cell Physiol **24** 247-253

Tajima S, TanakaK, Takane K, Sugimoto A, Okazaki K, Kouchi H (1993) Soybean nodule uricase gene (nodulin-35) is expressed in cotyledons during seed development and early germination In R Palacios, J Mora, WE Newton, eds, New Horizonts in Nitrogen Fixation Kluwer, Dordretch, The Netherlands, p373

Thyjkaer T, Schauser L, Danielsen D, Finneman J, Stougard J (1998) Transgenic Plants Agrobacterium-Mediated Transformation of the Diploid Legume Lotus japonicus p 514-520 in Cell Biology A laboratory Handbook 2nd Edit Vol 3 Academic Press San Francisco

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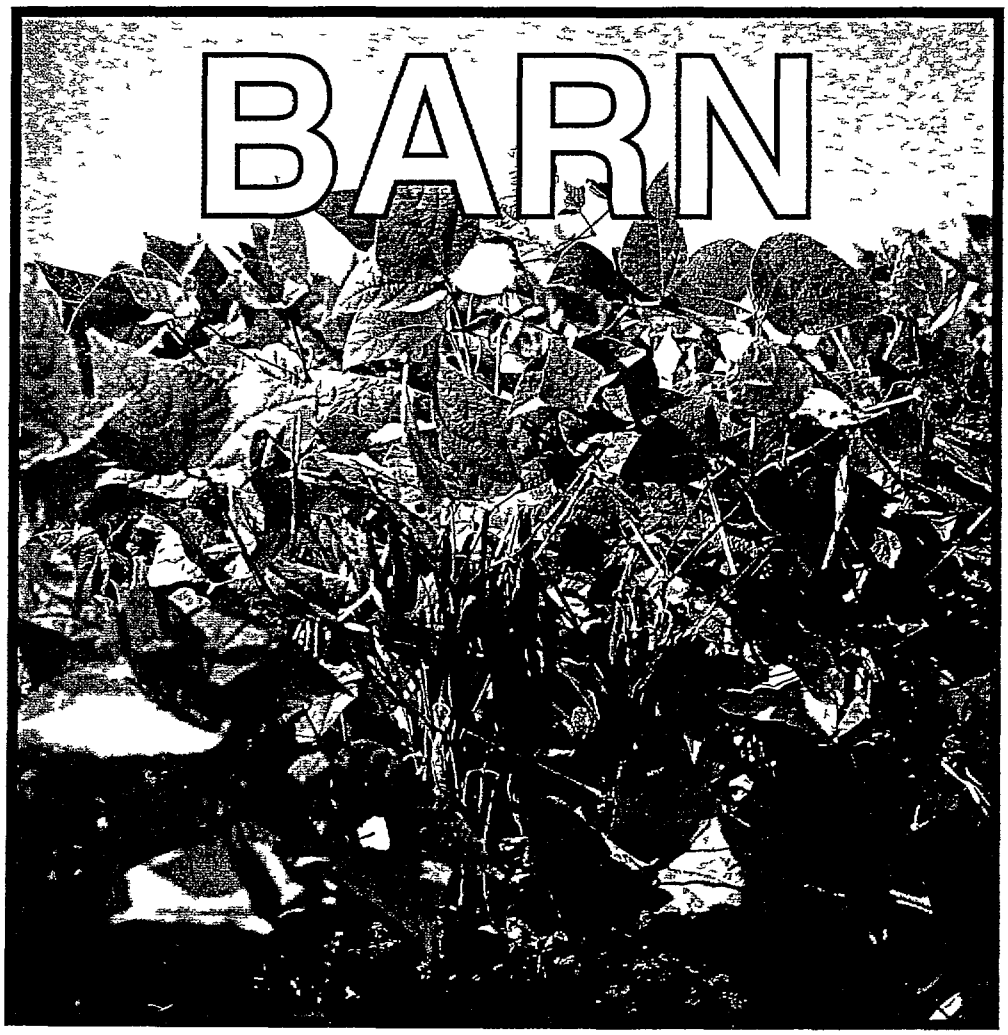

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Genetic regulation of the ureide biosynthetic pathway in tropical legumes

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ABSTRACT

Legume crops such as soybean cowpea and beans are a major source of protein for the population in developing countries Most of the sustainable agriculture on these crops depends almost completly on Biological Nitrogen fixation (BNF) This symbiosis is potentially capable of supplying the nitrogen requirements of the plant from atmospheric nitrogen hence the importance for understanding the basis for the stablishment and functioning of such interaction In the *Rhizobium*-legume interaction an exchange of molecular signals regulates the expression of genes essential for infection nodule development and function (Brewin 1991 Hirsch 1992) Effectively nodulated legumes fall within the classes of being either amide exporting or ureide exporting species (Atkins CA 1987) High concentrations of ureide nitrogen in the xylem stream is usually associated with effective nodulation and high rates of fixation in tropical legumes such as *Phaseolus vulgaris* (common bean) *Glycine max* (soybean)and *Vigna* (cowpea) (Pate JS Atkins CA *et al* 1980) This paper is an effort to study the genetic regulation of the ureide pathway in *Phaseolus vulgaris* in order to increase bean productivity

Legumes can fix nitrogen when nodulated by bacteria of the genera *Rhizobium Bradyrhizobium* and *Azorhizobium* Nitrogen-fixing root nodules are specialized organs that differentiate from normal root tissues as a consequence of the rhizobial infection The infection of plant roots by the rhizobia and susequent nodule development have been studied in considerable detail at the genetic cellular and molecular levels Progress in this area has been the subject of recent reviews Nap and Bisseling (1990) Sanchez *et al* 1991 Brewin (1991) Hirsch (1992) and de Bruijn and Schell (1993)

In general once ammonia has been produced by the bacterial nitrogensase and assimilated into glutamine, this aminoacid is used by the plant to produce de *novo* purines Ureides are produced by the catabolism of these de *novo* synthetized purines by the ureide pathway (Schubert KR and Boland MJ 1990) in root nodules of tropical legumes Ureides are a class of

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cyclic or acyclic nitrogenous organic compounds derived from or structurally related to urea Ureides found in plants include allantoic acid allantoine citruline uric acid hypoxanthine xanthine caffeine etc High concentration of ureides in the xylem sap is usually associated with effective nodulation and high rates of fixation although non nodulated or nodulated plants utilizing combined nitrogen as nitrate supplied to the roots sometimes contain some ureides in xylem In *Phaseolus vulgaris* however significant levels of xylem-borne ureides are found in nodulated plants supplied with ammonium or ammonium nitrate (Schubert KR and Boland MJ 1990) Allantoin and allontoic acid are the predominant organic nitrogenous compounds produced and exported from common bean root nodules during an effective BNF

Key enzymes of the ureide pathway are a nodule-enhanced xanthine dehydrogenase isoform (Nguyen J 1986) involved in the conversion of xanthine into uric acid uricase II a nodule-specific uricase an enzyme that converts uric acid into allantoin localized in peroxisomes of uninfected cells of the central tissue (Nguyen T *et al* 1985 and Sanchez *et al* 87) and allantoinase that catalyzes the final step in the ureide biogenesis pathway in nodules the conversion of allantoin into allantoic acid (Schubert K R and Boland, M J , 1990) Allantoinase activity has been associated to the ER of the nodule central tissue (Hanks J F *et al* 1981)

The initial expression of uricase II transcript in common bean nodules is not dependant on BNF and in empty nodules of *P vulgaris* it is already expressed (10%) as compaired to the wild type nodules (Padilla J E et al 1991) Although uricase II could be considered a nodule specific enzyme incubation of soybean callus tissue and root cultures at varying oxygen levels demonstrated that low oxygen concentration (4-5%) induces uricase-II activity and *de novo* synthesis (Larse, K and Jochimsen BU 1986) In this regard it has been recently reported that soybean cotyledons contained an uricase that cross-reacted with antibodies raised against the nodule uricase (Nod-35) Tajima, S *et al*, 1991)

In order to study the regulation of this gene, we have recently cloned the regulatory region (1 8 kb) and fussed it to the GUS bacterial reporter gene The spatial and temporal regulation of the uricase-II gene(s) from root nodules and cotyledons of beans is being analyzed by multiple approaches. The ultimate goal of this work is to set the rational molecular and genetical basis to increase ureide production in tropical legumes to impact legume crop yield

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Results and discussion

The spatial and temporal regulation of the uricase-II gene in root-nodules of *Phaseolus vulgaris*

Expression of uricase II genes in empty nodules of *Phaseolus vulgaris* induced by a mutant *Rhizobium* strain CFN4202 suggests that the regulation of this gene does not require bacterial release (Padilla et al , 1991) Further indications of the regulatory mechanisms of uricase II gene expression can be provided by morphological and *in situ* hybridization studies on the transcript in wild type and empty nodules of common bean H Kuin and T Bisseling (Wageningen) results corroborate the fact that bean uricase-II enzyme is located in uninfected cells of the central tissue (Nguyen et al , 1985) In this regard uricase-II transcript can be detected in wild type and mutant nodules, far more early than the onset ofr nitrogen fixation and the expression of other "late nodulins" such as leghemoglobin and nodulin-30 (Padilla *et al* , 1991) (Figure 1)

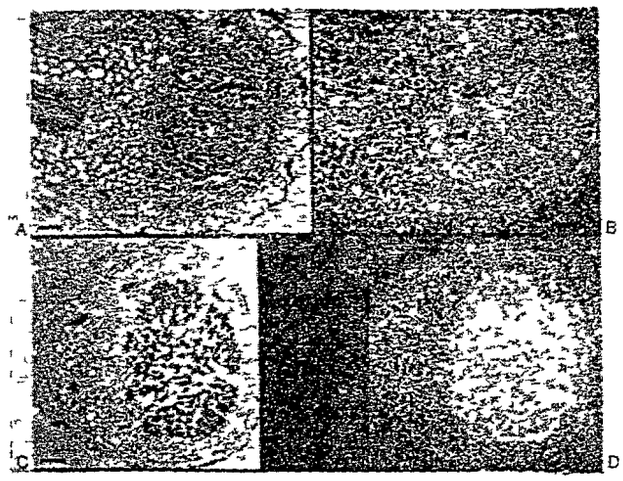

Figure 1 *In situ* localization of uricase II transcripts in cross sections of *P vulgaris* nodules A, C are bright field mic ographs B D are dark field micrographs in which the silver grains are vis ole as white dots A Uricase II cDNA clone was used to make an antisense probe with ^{35}S UTP A B stage 2 nodules (5 8 day old nodule) bar 250 m C D stage 4 (15 day old) bar 500 m

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Transcriptional regulation of the uricase-II gene

In order to study the transcriptional regulation of this gene, we have cloned genomic sequences from *P vulgaris* cultivar Negro Jamapa Recently we have partially characterized by nucleotide sequencing the promoter and 5' flanking region Analysis of the nucleotide sequence of the uricase promoter and upstream region have reveal important features, *i e* TATA box and some conserved elements such as the AT-rich enhancers and the OSE (Organ Specific Elements) found upstream of leghemoglobin and other nodulin genes (de Bruij and Schell, 1993)

Transcriptional gene fusions of the regulatory region (1 8 kb) with the GUS bacterial reporter gene have been created by subcloning appropriate genomic restriction fragments into the polylinker region of the GUS vector plasmid pBI121 (Jefferson, 1987) (Figure 2)

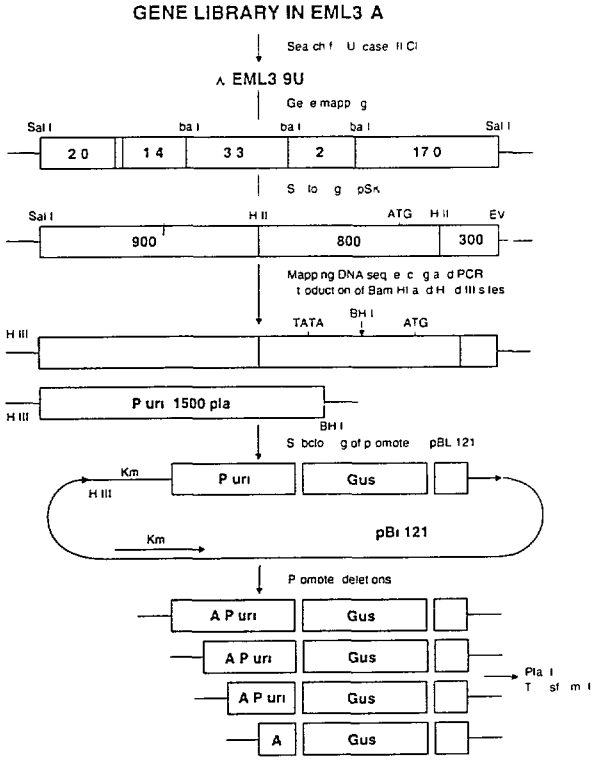

Figure 2 Uricase II gene promoter cloning strategy and transcriptional GUS gene fusions in pBI121

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Transgenic *Lotus corniculatus* plants are being generated with the 1 8 kb and serial deletions of the regulatory region Although root nodules of *Lotus corniculatus* induced with nodulating strains of *R loti* do not produce ureides and consequently, would not be expected to regulate uricase genes in exactly the same way as seen in bean, it could be important to test the function of uricase promoter elements in this background Such experiments could still yield interesting information concerning the degree of conservation of some components of the gene regulatory networks operating during nodule development in legumes

Future directions

Transient expression experiments are being performed in electrophorated and particle bombarded nodule slices of wild type and *Rhizobium* mutant induced nodules with the chimeric uricase promoter-GUS transcriptional fusions as well as the NOD-30 promoter-luciferase fusion, as an internal control (NOD30 is an infected cell specific gene) Cotyledonary tissues will be also analyzed in regard to environmental, developmental, and physiological effectors modulating uricase II gene regulation An internal control in this case is a transcriptional fusion with phaseolin (Bustos *et al* 1991) promoter and the luciferase reporter gene The rationale of these kind of experiments will be to attempt to identify the factors reponsible for the initial induction and subsequent maintenance of uricase II, and for the induction of other enzymes in the pathway biosynthesis of ureides The regulation of this metabolic pathway seems to be an efficienty marker, not only to monitor fundamental nodule functions such as assimilation and transport of nitrogeneous compounds but also to reveal novel aspects of nodule development, *eg* peroxisomes biogenesis, infected and uninfected cells ontogeny

Acknowledgments

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References

Atkins C A 1987 Metabolism and translocation of fixed nitrogen in the nodulated legume Plant Soil 100 157 69

Bresin N J 1991 Development of the legume root nodule Annu Rev Cell Biol 7 191 226

Bustos M M Begum D Kalkan F A Batraw M J and Hall T C 1991 The EMBO Journal 10 1469 1479

De Bruijn F J and Schell J 1993 Regulation of Plant genes specifically induced in developing and mature nitrogen fixing nodules Cis acting elements and trans acting factors In Control of Plant Gene Expression CRC Press Boca Raton Fl 1993

Hanks J F Tolbert N E and Schubert K R 1981 Localization of Enzymes of Ureide Biosynthesis in Peroxisomes and Microsomes of Nodules Plant Physil 68 65 67

Hirsch A M 1992 Developmental biology of legume nodulation New Phytol 122 211 237

Larsen K and Jochimsen B U 1986 Expression of nodule specific uricase in soybean callus is regulated by oxygen EMBO J 5 15 19

Nap J P and Bisseling T 1990 Developmental biology of a plant procaryote symbiosis the legume root nodule Science 250 948

Nguyen J 1986 Xanthine dehydrogenase its distribution properties and function Physiol Veg 24 263-281

Nguyen T Zelechowska M G Foster V Bergamn H Verma D S P 1985 Primary structure of the soybean nodule 35 gene encoding nodule specific uricase-II localized in the peroxisomes of uninfected cells of soybean Proc Natl Acad Sci USA 82 5040-5044

Padilla, J E Miranda J and Sanchez F 1991 Nodulin regulation in common bean nodules induced by bacterial mutants Molecular Plant Microbe Interactions 4 433-439

Pate J S et al 1980 Nitrogen nutrition and wylem transport of nitrogen in ureide producing grain legumes Plant Physiol 65 961 965

Sanchez F Padilla J E Perez H and Larra, M 1991 Control of nodulin genes in root nodule development and metabolism Annu Rev Plant Phys Plant Mol Biol 42 507 528

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Sanchez, F, Campos, F, Padilla, J, Enriquez, C, Caput D and Nonneville, J M 1987 Purification, cDNA cloning and developmental expression of the nodule-specific uricase form *Phaseolus vulgaris* L Plant Physiol 84 1143-1147

Schubert, K R and Boland, M J 1990 The Ureides The Biochemistry of Plants Vol 16 p/ 197-282 Academic Press

Tajima, S, Hideki I, Tanaka, K, Takashi, N, Sugimoto, A and Kouchi, H 1991 Soybean Cotyledons contain a uricase that croos-reacts with antibodies raised against the nodule uricase (Nod-35) Plant Cell Physiol 32 1307-1311

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Plant Physiol (1997) 115 1307–1317

Characterization of the Common Bean Uricase II and Its Expression in Organs Other than Nodules[1]

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Uricase II is a purine metabolic enzyme highly induced in root nodules during the symbiosis established between legumes and bacteria of the genera *Rhizobium* and *Bradyrhizobium* Here we describe the characterization of bean (*Phaseolus vulgaris*) nodule uricase II cDNA and show that uricase II is encoded by a single gene in the bean genome This gene is also expressed in cotyledons, roots, and hypocotyls during bean seedling establishment, and an anti uricase antibody recognizes the protein in different seedling organs Uricase II has also been found in *Leucaena leucocephala* seedlings, suggesting that it participates during seedling establishment in le gumes that do not transport ureides A 50 kD polypeptide that is detected by the anti uricase antibody is found in cotyledons during seedling development This higher molecular mass form is also de tected in developing roots and hypocotyls but not in nodules In situ hybridization experiments in root seedlings showed uricase II tran scripts in the metaxylem parenchyma cells and phloem fibers of the vascular system

Ureides are important components of N_2 metabolism in a wide variety of higher plants (Pate et al 1980 Thomas and Schrader 1981 Reynolds et al 1982) including the Leguminosae Boraginaceae Platanaceae Hippocastanaceae, and Aceraceae families (Mothes, 1961) Legumes can be classified into amide or ureide transporters depending on the nitrogenous compounds that they export from the nodule to the shoot In tropical legumes such as bean (*Phaseolus vulgaris*) soybean (*Glycine max*), and cowpea (*Vigna aconitifolia*) a change in the transported N_2 compounds from amides (Gln and Asn) to ureides (allantoin and allantoic acid) occurs when they are in association with N_2-fixing bacteria of the genera *Rhizobium* and *Bradyrhizobium* (Matsumoto et al 1977 Fujihara and Yamaguchi 1980)

Root nodules are highly specialized symbiotic organs in which differentiated bacteria or bacteroids reduce atmospheric N_2 to NH_3 (Sanchez et al 1991 Verma et al 1992 Mylona et al 1995) NH_4^+ ions are excreted into the plant s cytoplasm assimilated into Gln, and then incorporated into de novo purine biosynthesis (Schubert, 1986) In the infected and uninfected cells of the nodule, these purines are catabolized and consequently converted into ureides that are transported to the aerial parts of the plant (Matsumoto et al 1977 Pate et al 1980) Xanthine dehydrogenase uricase II and allantoinase the last three enzymes in the ureide biosynthetic pathway are predominantly located in the uninfected cells of the nodule (Atkins et al 1980 Hanks et al 1981 Bergmann et al 1983 Schubert 1986, Schubert and Boland 1990)

Uricase II (EC 1 7 3 3) catalyzes the oxidative cleavage of uric acid into allantoin and CO_2 liberating H_2O_2 This enzyme has been purified from cowpea (Rainbird and Atkins 1981) soybean (Legocki and Verma 1979 Bergmann et al 1983 Lucas et al 1983) and bean (Sanchez et al 1987) nodules It is preferentially synthesized on free polysomes during nodule development and is localized in the peroxisomes of noninfected cells of the nodule (Hanks et al 1981 Nguyen et al 1985 Van den Bosch and Newcomb 1986, Kouchi et al 1989) In effectively nodulated bean plants uricase II expression and activity are induced to high levels before N_2 fixation begins (Sanchez et al 1987 Padilla et al 1991) and the enzyme constitutes approximately 2% of the total soluble protein of the nodule (Sanchez et al 1987) In empty nodules induced by *Rhizobium* mutants the amount of uricase II mRNA is 10% of that observed in nodules infected with wild-type bacteria suggesting that events during nodule development (e g nodule induction bacteria release and N_2 fixation) are important for the regulation of uricase II gene expression (Padilla et al 1991)

Low-uricase II activity has been detected in soybean callus tissue where it is regulated by O_2 availability (Larsen and Jochimsem 1986) Uricase activity has also been found in soybean cotyledons and hypocotyls during seed development and early germination (Tajima et al 1991) The uricase II protein has been immunocytochemically detected in the peroxisomes of soybean cotyledon cells (Damsz et al 1994) In this plant uricase II is encoded by a small gene family (Tajima et al 1993) Uricase activity (uricase I) has also been detected in soybean seed radicles but this activity was found to be due to a diamine oxidase/peroxidase system with characteristics different from nodule uricase (Tajima et al 1983 1985) Uricase II cDNA clones have been isolated from soybean nodule (Bergmann et al 1983 Nguyen et al 1985) and cotyledon (Takane et al 1997) libraries and from cowpea (Lee et al 1993)

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Abbreviations AP alkaline phosphatase DIG digoxigenin

jackbean (*Canavalia liniata*) (Kim and An 1993) and bean (Sanchez et al 1987 Papadopoulou et al 1995) nodule libraries

In this paper we describe the isolation and characterization of the bean nodule uricase II cDNA and demonstrate that a single copy of the uricase II gene is present in the bean genome In addition to the nodule this gene is expressed in different organs of the seedling and the adult plant We have also investigated the patterns of uricase II mRNA and protein in cotyledons roots and hypocotyls during germination and seedling establishment In bean organs other than nodules a 50 kD polypeptide immunologically related to uricase II was detected and its initial characterization is reported Finally, we show the in situ localization of the uricase transcript in the seedling roots and discuss the biochemical and developmental significance of these results

MATERIALS AND METHODS

Growth of Plants

Bean (*Phaseolus vulgaris* cv Negro Jamapa) seeds were surface sterilized in 10% (v/v) commercial sodium hypochlorite for 10 min and then rinsed several times with sterile water Germination was carried out on moist paper towels in sterile trays at 28°C in the dark Four days postimbibition the seedlings were grown in an approximately 12-h light/12-h dark photoperiod The plants were not inoculated with *Rhizobium* bacteria Cotyledons roots and hypocotyls were harvested at different times postimbibition (0 h 12 h 1 d 2 d 3 d, 4 d 7 d, and 10 d) frozen immediately in liquid N_2 and stored at −70°C Germination and growth conditions were the same for *Leucaena leucocephala* and maize (*Zea mays*)

Isolation and DNA Sequencing of the Uricase II cDNA Clone

A bean uricase II clone (pSKuri) was isolated from a λ-Zap cDNA library constructed from 20-d-old bean nodules using an incomplete uricase II cDNA (Sanchez et al 1987) as a probe The nucleotide sequence of the uricase II cDNA clone was determined by the dideoxy chain termination method (Sanger et al 1977) using the Sequenase 2 0 kit (United States Biochemical) The nucleotide and deduced amino acid sequence were compared with the soybean (*Glycine max*) and cowpea (*Vigna aconitifolia*) uricase II using the GeneWorks computer program version 2 3 1 and associated databanks (Intelligenetics Mountain View CA)

Genomic Southern Analysis

DNA was extracted from etiolated bean hypocotyls and digested with *Eco*RI *Eco*RV *Hin*dIII and *Xba*I restriction enzymes Twenty-five micrograms of each restricted DNA was loaded per lane and fractionated on a 0 8% (w/v) agarose gel (Maniatis et al 1982) Southern analysis was performed as described by Maniatis et al (1982) with an *Eco*RI *Nco*I fragment from pSKuri (Fig 1A) labeled with [α-^{32}P]dCTP (Multiprime DNA labeling system kit Amersham) as a probe The hybridized filter was washed from 2× SSC and 0 1% (w/v) SDS at 55°C to 0 1× SSC and 0 1% (w/v) SDS at 65°C in three steps and exposed to Kodak X-Omat film for 3 d

RNA Isolation and Northern Analysis

Plant material previously harvested and frozen as described above was ground in a chilled coffee mill Total RNA from cotyledons uninfected roots and hypocotyls was extracted according to the procedure described by de Vries et al (1988) Thirty micrograms of total RNA was loaded per lane in 1% (w/v) agarose gels containing 2 2 M formaldehyde as described previously (Campos et al 1987) After transfer to a nylon membrane (Hybond N^+ Amersham) the RNA was hybridized with the uricase II cDNA labeled with [α ^{32}P]dCTP using the multiprime DNA labeling system kit To ensure that equal amounts of RNA were loaded the gels were stained with ethidium bromide and photographed In addition the filters were hybridized with a 28S rRNA probe The hybridized filters were washed from 2× SSC and 0 1% (w/v) SDS at 55°C to 0 1× SSC and 0 1% (w/v) SDS at 65°C in three steps and exposed to Kodak X-Omat film The hybridization signals were quantified using the one dimensional analysis program (Bio-Image Products Millipore)

Protein Extraction and Western Analysis

Frozen cotyledons uninfected roots or hypocotyls were ground in a coffee mill and incubated in 2 5 volumes of extraction buffer (30% [w/v] Suc 30 mM Tris-HCl pH 7 4 and 2% [w/v] polyvinylpolypyrrolidone) for 10 min at 4°C The extracts were centrifuged at 12 000*g* for 25 min at 4°C The supernatant fluids were filtered through two layers of Miracloth (Calbiochem) and precipitated with 60% (v/v) acetone Total soluble proteins were determined by the method of Bradford (1976) The samples were analyzed by SDS-PAGE using the Laemmli (1970) discontinuous buffer system For western analysis proteins were electroblotted onto nitrocellulose membranes (Immobilon P Millipore) as described by Maniatis et al (1987) blocked in 5% (w/v) commercial nonfat dry milk in TBST (10 mM Tris-HCl, pH 8 0 150 mM NaCl and 0 05% [v/v] Tween 20) incubated with anti uricase antibodies (1 1000) and detected with goat anti-rabbit antibodies coupled to horseradish peroxidase (1 5000 Amersham) using the enhanced chemiluminescence detection kit (Amersham) as indicated by the suppliers The exposure time for the western analysis of the cotyledons was longer than that required for the roots and hypocotyls For protein visualization the gels were stained with Coomassie brilliant blue (Merril 1990) or the nitrocellulose filters were stained with Ponceau S Anti-uricase is a rabbit polyclonal antibody obtained against uricase II which was purified to homogeneity from common bean nodules (Sanchez et al 1987)

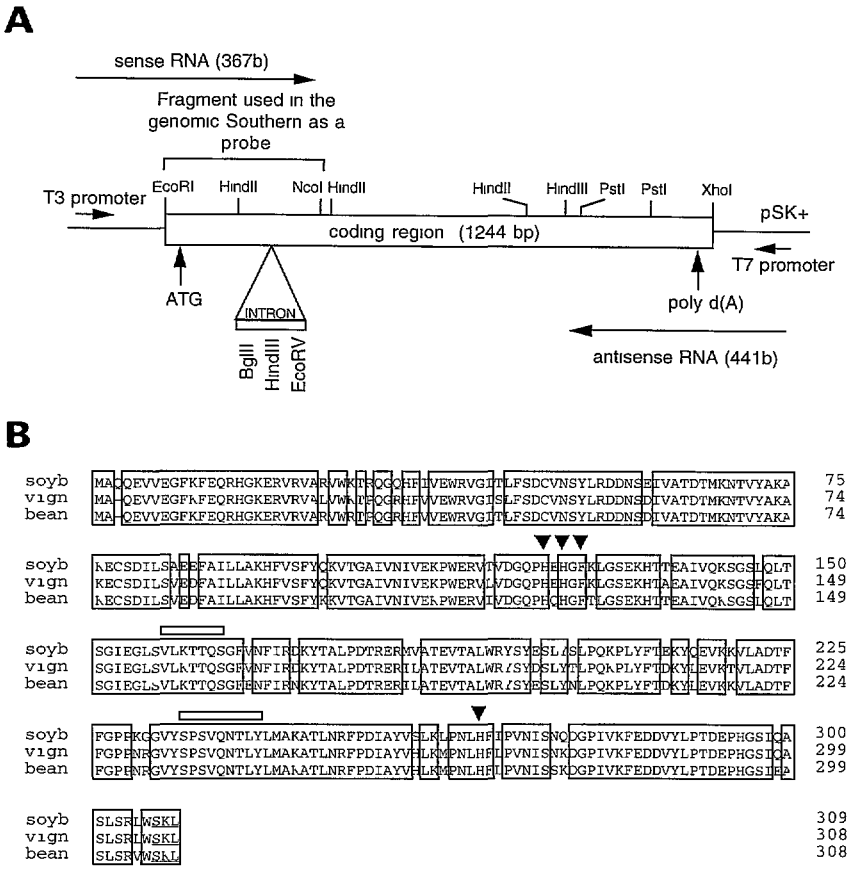

Figure 1 A Restriction map of the uricase II cDNA clone T7 and T3 promoters from which transcripts for the in situ hybridization experiments were initiated are indicated B Alignment of the deduced amino acid sequences from bean soybean (soyb) and cowpea (vign) uricase II Arrowheads indicate the His and Phe amino acid residues proposed by Wu et al (1989) as the copper binding site of the protein The two conserved motifs of unknown function are indicated with open boxes above the sequence The SKL motif for protein import into peroxisomes is underlined

V8 Protease Digestion

V8 protease digestion of the crude extracts from 4-d-old (days postimbibition) bean seedling roots was made in polyacrylamide gels as previously described (Cleveland et al 1977) Anti uricase (1 1000) and sheep anti-rabbit-AP (1 5000 Boehringer Mannheim) antibodies were used to detect the resulting peptides

Urate Oxidase Activity

Two grams of various bean seedling organs was homogenized with 6 mL of extraction buffer (50 mM of potassium phosphate buffer containing 50 mM KCl 5 mM Mg acetate 1 mM DTT 1 mM EDTA and 4% [w/v] polyvinylpolypyrrolidone) The extracts were centrifuged at 12 000*g* for 25 min at 4°C The supernatants were filtered through glass fiber and centrifuged at 175 000*g* for 15 min The resulting supernatants were precipitated with 60% (v/v) acetone resuspended in 2 mL of 85 mM Gly, pH 10 and passed through a Sephadex G-25 column (0 7 × 26 cm) using 85 mM Gly pH 10 as the eluent Urate oxidase activity was measured as described previously (Bergmann et al 1983) except that 85 mM Gly pH 10 buffer was used Units of uricase activity are in nanomoles of oxidized uric acid per minute per milligram of protein Protein concentration was determined by the Bradford (1976) procedure

In Situ Hybridization

Uninfected roots from 4-d-old bean seedlings and 21 d old nodules were harvested and fixed at room temperature overnight in 4% (w/v) paraformaldehyde and 0 25% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer pH 6 8 supplemented with 1 M NaCl with the 1st h under vacuum Fixed tissues were dehydrated and embedded in paraffin following the method described by Van de Wiel et al (1990) Seven-micrometer-thick sections were cut in a manual microtome The uricase II cDNA clone (pSKuri) linearized with *Hin*dIII was transcribed from the T7 promoter and a 441-base antisense RNA labeled with (DIG)-11-ribonucleotide UTP (Boehringer Mannheim) was obtained (Fig 1A) The same plasmid digested with *Nco*I was transcribed from the T3 promoter to obtain a 367-base sense RNA labeled by the same method (Fig 1A)

To estimate the yield of the assay the transcripts were analyzed by agarose gel electrophoresis and ethidium bromide staining The amount of labeled RNA generated was measured by direct detection dilutions of the labeled transcripts and a control DIG-dUTP labeled DNA (5 μg/mL) were blotted on a nylon membrane (Hybond N^+) and detected with the anti DIG AP antibody (1 15000 Boehringer Mannheim) and the AP substrates 4-nitroblue tetrazolium chloride and 5 bromo-4-chloro 3 indolyl-phosphate Sense and antisense RNAs were hybridized to the

tissue sections according to the protocol described by McKhann and Hirsch (1993) Equal amounts of labeled sense and antisense probes were added to the correspond ing sections The hybridization and antibody incubation times were the same for the sense and antisense experi ments Probed slides were dried, mounted in Poly Mount (Polysciences Warrington PA) examined under a microscope (Zeiss) and photographed with ×16 ×40 and ×100 objectives using 100 ASA 35-mm film (Kodak)

RESULTS

Isolation and Characterization of a Uricase II cDNA Clone

From a λ Zap common bean cDNA library constructed from 20 d old nodule RNA a uricase II clone (pSKuri) was isolated using an incomplete nodule uricase II cDNA clone (Sánchez et al 1987) as a probe This clone contains the entire uricase II coding region Its nucleotide and deduced amino acid sequences were obtained and compared with the nodule soybean and cowpea uricase II sequences (Fig 1B) The uricase II-coding regions share a high percentage of identity at the nucleotide (90% with soybean 94% with cowpea) and amino acid levels (91% with soybean 95% with cowpea) The bean uricase II cDNA encodes a protein of 308 amino acids that contains the four conserved motifs present in all urate oxidases described previously the V-L K-T-T-Q S motif (Bairoch 1991) and the S P-S-V-Q-K/H/N-T L Y motif, each of unknown function the H X-H-X F motif that joined to a third His (residue 266 in bean uricase) forms the protein copper-binding site proposed by Wu et al (1989) and the tripeptide S-Basic-L (S-K-L for bean soybean and cowpea uricase II), the signal for peroxisome entry localized at the carboxyl terminus of the protein (Motojima and Goto, 1989 Wallrath et al 1990 van den Bosch 1992 Miura et al 1994 Fig 1B) The 3 noncoding region of 294 bp contains a putative polyadenylation signal sequence (AATAAA) located 67 nucleotides from the poly(A^+) RNA

Genomic Southern Analysis

To estimate the complexity of uricase II genes in the *P vulgaris* genome, we analyzed total bean DNA digested with several restriction enzymes using Southern analysis The *Eco*RI *Nco*I uricase II cDNA fragment was used as a probe (Fig 1A) We detected a single hybridization band in DNAs restricted with *Eco*RI and *Xba*I and two bands in DNAs restricted with *Hin*dIII and *Eco*RV (Fig 2) The latter are restriction sites located at the first intron of the uricase II gene (N Capote-Mainez and F Sanchez unpublished results Fig 1A) We conclude that unlike in soybean in which it has been proposed that uricase II is encoded by a small gene family (Tajima et al 1993) in the bean genome this gene exists as a single copy

Expression of Uricase II mRNA in Different Organs of Adult Bean Plants

To determine whether the single bean uricase II gene was expressed in a nodule-specific manner we analyzed the expression of this gene in organs other than nodules using northern analysis We found an approximately 1 2-kb transcript in roots stems and leaves of uninfected adult bean plants (Fig 3) of the same size as the one found in nodules (Sanchez et al 1987) indicating that uricase II expression was not restricted to this organ However, transcript levels in N_2-fixing nodules were much higher than those of the other organs examined

Expression Pattern of Bean Uricase II mRNA during Seed Germination and Seedling Establishment

N_2 is actively mobilized during seedling development (Polayes and Schubert 1984) Because uricase II is a key enzyme in N_2 metabolism we investigated the expression of the uricase II gene in different seedling organs during seed germination and seedling establishment

Total RNA from cotyledons roots and hypocotyls of germinated bean seedlings was analyzed by northern blots using the nodule uricase II cDNA as a probe During seed germination (0 4 8 and 12 h postimbibition) uricase II mRNA was not detected in the cotyledons At 1 d postimbibition when the radicle had emerged and seedling growth had started uricase II mRNA was first detected

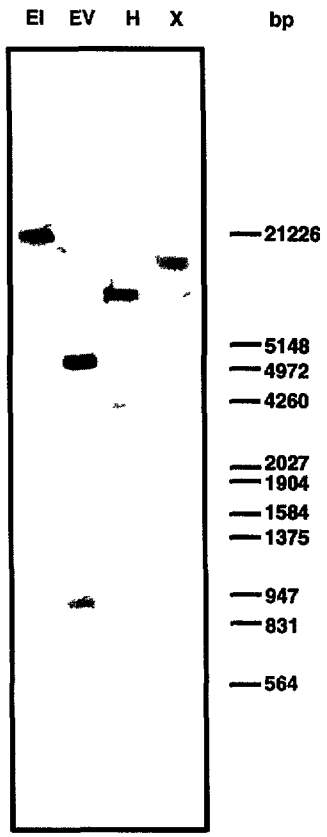

Figure 2 Southern analysis of bean DNA restricted with *Eco*RI (EI) *Eco*RV (EV) *Hin*dIII (H) and *Xba*I (X) restriction enzymes Twenty five micrograms of each restricted DNA was loaded per lane The blot was probed with an *Eco*RI *Nco*I fragment from a pSKuri clone (Fig 1A) Molecular size markers are indicated

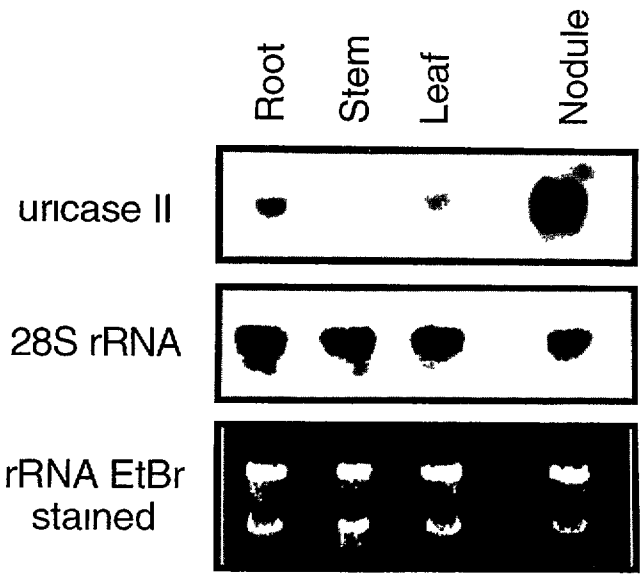

Figure 3 Expression of uricase II gene in different organs of adult plants Total RNA (25 μg) from roots stems leaves and 21 d old nodules was analyzed by northern blots using the uricase II cDNA as a probe To verify that equal amounts of RNA were loaded the gels were stained with ethidium bromide (EtBr) and the filters were hybridized with a 28S rRNA probe

reaching a maximum at 4 d postimbibition (Fig 4A) Uricase II transcripts were not detected in the dry embryo radicle or in radicles 12 h postimbibition In developing roots the uricase II transcript is detected 1 d postimbibition showing a similar induction pattern as that of cotyledons (Fig 4B) Constant uricase II mRNA levels were maintained in hypocotyls from 3 to 10 d postimbibition (Fig 4C)

Expression Pattern of Bean Uricase II Protein during Seed Germination and Seedling Establishment

Uricase II protein was also detected in the cotyledons roots, and hypocotyls of developing seedlings (Fig 5 A B and C respectively) Western analysis showed not only the 35-kD polypeptide corresponding to the uricase II subunit (Sanchez et al 1987) but in addition a novel 50-kD polypeptide not found in nodules The 35-kD polypeptide was detected in dry seeds and cotyledons at 12 h 1 d and 2 d postimbibition whereas the 50-kD cross-reacting polypeptide was visible in proteins isolated from cotyledons 2 to 10 d postimbibition (Fig 5A) In roots (Fig 5B) and hypocotyls (Fig 5C) both polypeptides appeared in similar proportion and at the same time in development which was approximately coincident with the detection of the uricase II transcript in those organs Both forms were maintained at constant levels during seedling establishment Although the same amount of protein was loaded in the three experiments the exposure times used in the root (Fig 5B) and hypocotyl (Fig 5C) western analyses were considerably shorter than those used for the cotyledon western analysis (Fig 5A) These data indicate that the amount of uricase II protein with respect to the total soluble protein is higher in roots and hypocotyls than in cotyledons

Uricase II Is Present in Amide Transporting Legumes

We analyzed total proteins from *L leucocephala* seedlings (a legume that preferentially transports amides as nitrogenous compounds during symbiotic conditions) and from a nonleguminous plant maize (*Zea mays*) by SDS-PAGE and immunochemically with an anti-uricase II antibody We

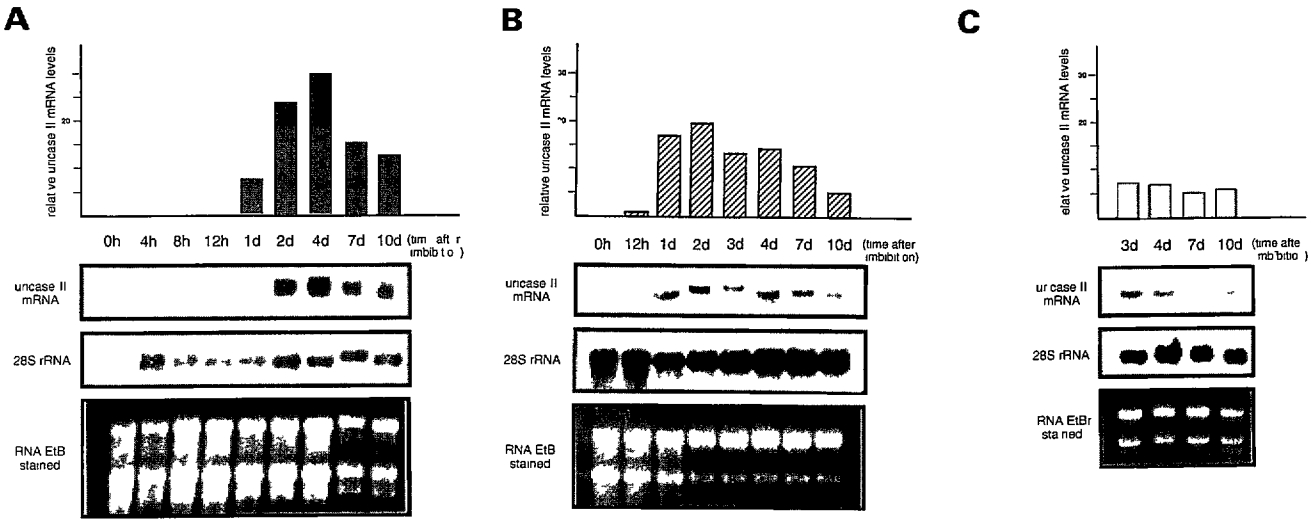

Figure 4 Expression of uricase II gene during bean seed germination and seedling establishment Northern blot analysis of total RNA from cotyledons (A) roots (B) and hypocotyls (C) Twenty five micrograms of total RNA was loaded per lane Uricase II cDNA was used as a probe in all experiments The gels were stained with ethidium bromide (EtBr) and the filters were hybridized with a 28S rRNA probe The experiments were performed two times with identical results The mRNA levels were quantified using the one dimensional analysis program (Bio Image)

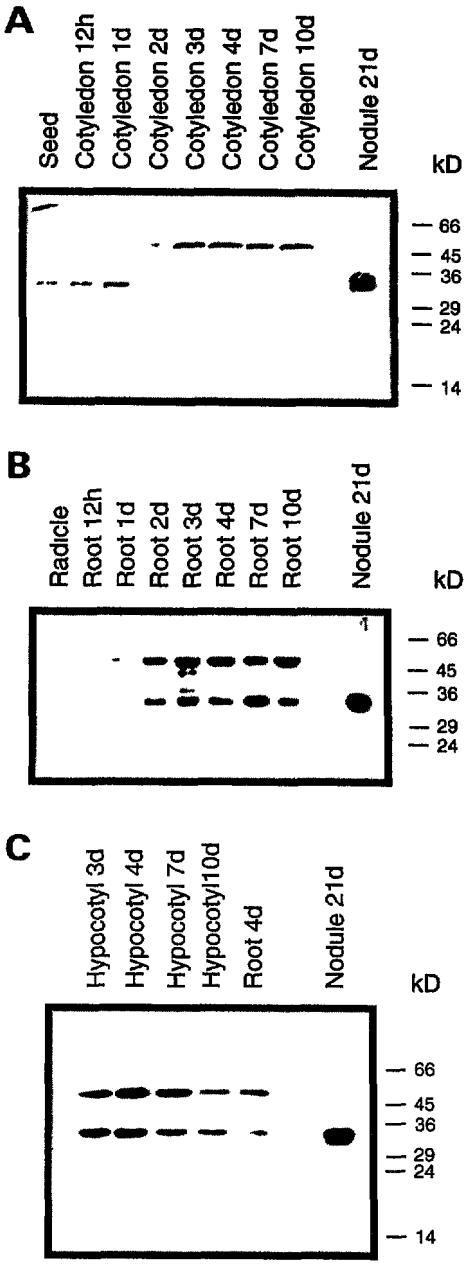

Figure 5 Expression of uricase II protein during bean seed germination and seedling establishment Immunodetection of uricase II protein in cotyledons (A) roots (B) and hypocotyls (C) All experiments had a protein sample from 21 d old nodules as a control Crude extracts of proteins (20 μg per lane except the nodule extract which was 2 μg) were analyzed by SDS PAGE and immunoblotting with anti uricase (1 1000) and anti rabbit coupled to horseradish peroxidase (1 5000 Amersham) antibodies The exposure time in A was longer than in B and C Molecular mass standards are indicated

detected the uricase II subunit (the 35-kD polypeptide) and the 50-kD protein in 4 d-old roots and hypocotyls from *L leucocephala* seedlings but only the 50-kD form in 4-d-old cotyledons (Fig 6) the same pattern found in bean seedlings In 4-d-old maize seedlings no cross-reacting bands were detected in any organ analyzed

Immunological and Enzymatic Analysis of the 50 kD Polypeptide

To determine whether the 50-kD polypeptide was a modification product of the 35-kD protein or a different but immunologically related protein, we digested the 35- and 50 kD polypeptides with V8 protease, separated the reaction products on an SDS-PAGE gel and detected the resulting peptides with an anti-uricase antibody The 50 kD protein digestion yielded three peptides of 34 7 9 and 7 3 kD (Fig 7) and all digestion products were recognized by the anti-uricase polyclonal antibody supporting the idea that the 50-kD protein could be a modified form of the 35-kD uricase

V8 proteolysis of the 35 kD protein yielded four different peptides of approximately 11 7 6 6 9, and 5 8 kD, all of which were recognized by the anti uricase antibody In a second experiment (Fig 8) we measured uricase specific activity in cotyledon extracts from dry seeds up to 10 d postimbibition We detected similar uricase-specific activity levels along all stages of cotyledon development Cotyledons from 3 to 10 d postimbibition, containing only the 50 kD polypeptide (Fig 5A) had urate oxidase activity These extracts were precipitated with 15% (v/v) TCA and analyzed by western blot, corroborating the presence of the 50-kD but not the 35-kD protein (data not shown) We also measured the uricase-specific activity in other bean seedling organs and mature nodules These results are shown in Table I A cotyledon extract from maize in which no protein immunorelated to bean uricase was detected (Fig 6), was also included

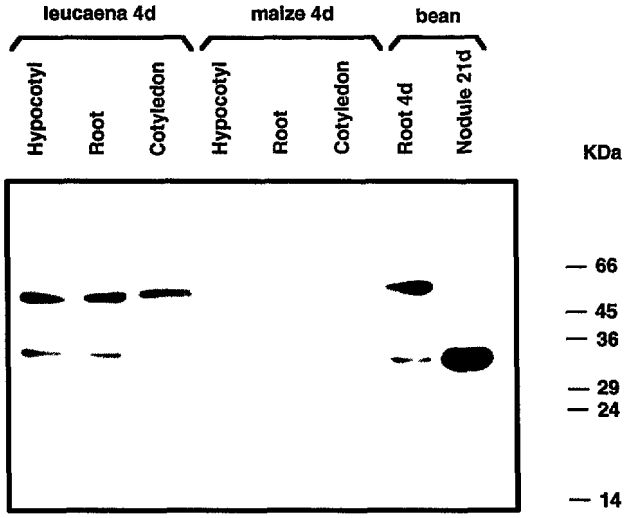

Figure 6 Detection of uricase II protein in hypocotyls roots and cotyledons of *L leucocephala* Crude extracts of proteins (20 μg per lane except the nodule extract which was 2 μg) were analyzed by SDS PAGE and immunoblotting with anti uricase (1 1000) and anti rabbit horseradish peroxidase (1 5000) antibodies Seedling organ extracts from maize 4 d postimbibition were also included Molecular mass standards are indicated

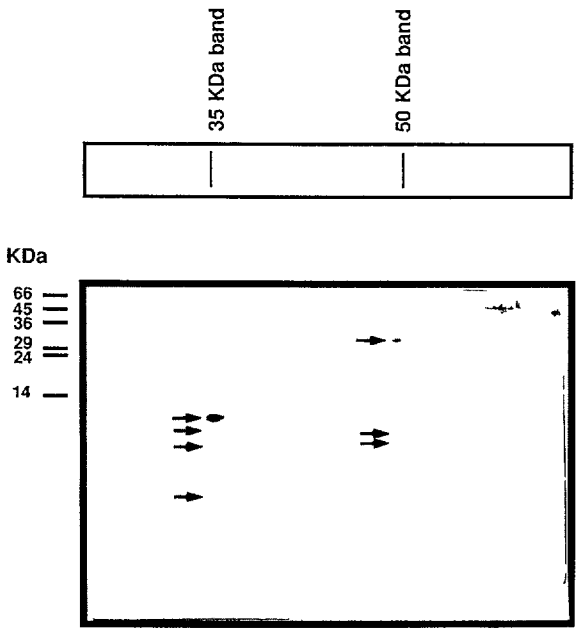

Figure 7 Immunoblot analysis of peptides generated from the 35 and 50 kD proteins by V8 hydrolysis The two bands that cross reacted with the anti uricase antibody were digested in the gel with V8 protease loaded in SDS PAGE and immunodetected with anti uricase (1 1000) and anti rabbit AP (1 5000 Boehringer Mannheim) antibodies Arrows indicate the resulting peptides Molecular mass standards are indicated

Spatial Localization of Uricase II Transcripts in the Roots of Developing Bean Seedlings

To determine the localization of the uricase II mRNA in developing bean roots 4-d-postimbibition roots were collected and examined by in situ hybridization We used antisense and sense (as a control) DIG-labeled mRNAs as probes (see Materials and Methods) Uricase II transcripts were detected in the root vascular tissue (Fig 9) In the xylem the signal accumulated in the cytoplasm of the parenchyma cells that surround the metaxylem vessels (Fig 9 A and C) These cells have a living protoplasm at maturity, as shown in the micrographs (Fig 9E) Some of the cytoplasm is so closely appressed to the cell wall that the signal seems to be over the wall In the phloem the signal was confined to the cytoplasm of phloem fibers (Fig 9 A and D) Although fibers are usually dead late in development the fibers we analyzed had not lost their cytoplasm at this stage of development (4 d postimbibition Fig 9F) We also performed in situ hybridization experiments on 21 d old nodule sections as a positive control We detected the uricase II mRNA not only in the uninfected cells of the central tissue as previously reported (Tate et al 1994 Papadopoulou et al 1995) but also in the nodule parenchyma cells and in the vascular tissue (Fig 9G) No signal was detected in the infected cells or in the outer cortex of the nodule Sense controls did not exhibit any hybridization signal (Fig 9 B and H)

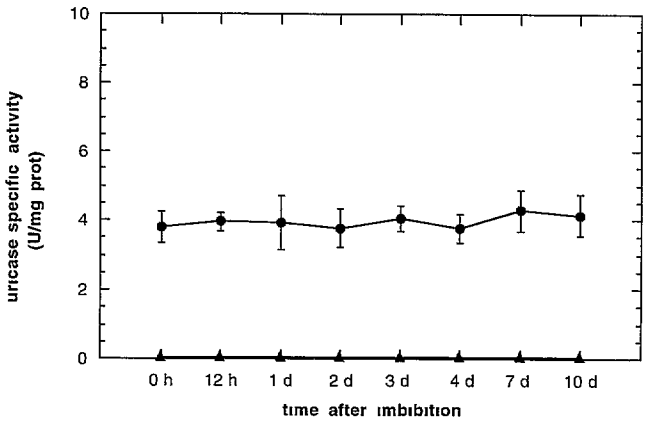

Figure 8 Uricase specific activity in the cotyledons of developing bean seedlings Five micromolar uric acid (●) or no uric acid (▲) was used in the reactions as a substrate Each value is the mean ± SD of three experiments Each experiment included triplicate samples Units (U) of uricase activity are in nanomoles of oxidized uric acid per minute under the conditions of the reaction

Table 1 *Uricase specific activity from different plant organs*

Plant Organ	Uricase Specific Activity
	units mg⁻¹ protein
Bean cotyledons 4 d old	3 74 ± 0 40
Bean roots 4 d old	15 40 ± 0 84
Bean hypocotyls 4 d old	13 33 ± 1 61
Bean nodules 21 d old	1790 ± 380
Maize cotyledons 4 d old	0 00

[a] Units of uricase activity are in nanomoles of oxidized uric acid per minute under the conditions of the reaction

DISCUSSION

Uricase II has been commonly studied in tropical legume nodules because of its participation in ureide biosynthesis during the symbiosis these plants establish with bacteria of the genera *Rhizobium* and *Bradyrhizobium* In this work we have demonstrated that this enzyme is also expressed in other bean organs (roots stems and leaves Fig 3) and that its expression is probably modulated when the plant needs to mobilize reduced N_2 i e during seedling establishment The presence of a single uricase II gene in the bean genome (Fig 2) suggests that different regulatory mechanisms for the uricase II gene rather than differential regulation of several genes, account for its expression in response to the different stimuli of symbiosis and development

Bean plants associated with N_2-fixing bacteria mainly transport nitrogenous compounds as ureides However even in nonnodulated bean plants the transport of ureides constitutes about 13 to 42% of the total organic N_2 in the xylem sap (Thomas and Schrader 1981) The uricase detected in the seedling (Fig 5) and adult plant organs (Fig 3) probably maintains this ureide transport rate during seedling and plant development On the other hand plants are C-starved during seed germination and seedling establishment, before photosynthesis starts (Schubert and Boland 1990) In these conditions the biosynthesis of ureides

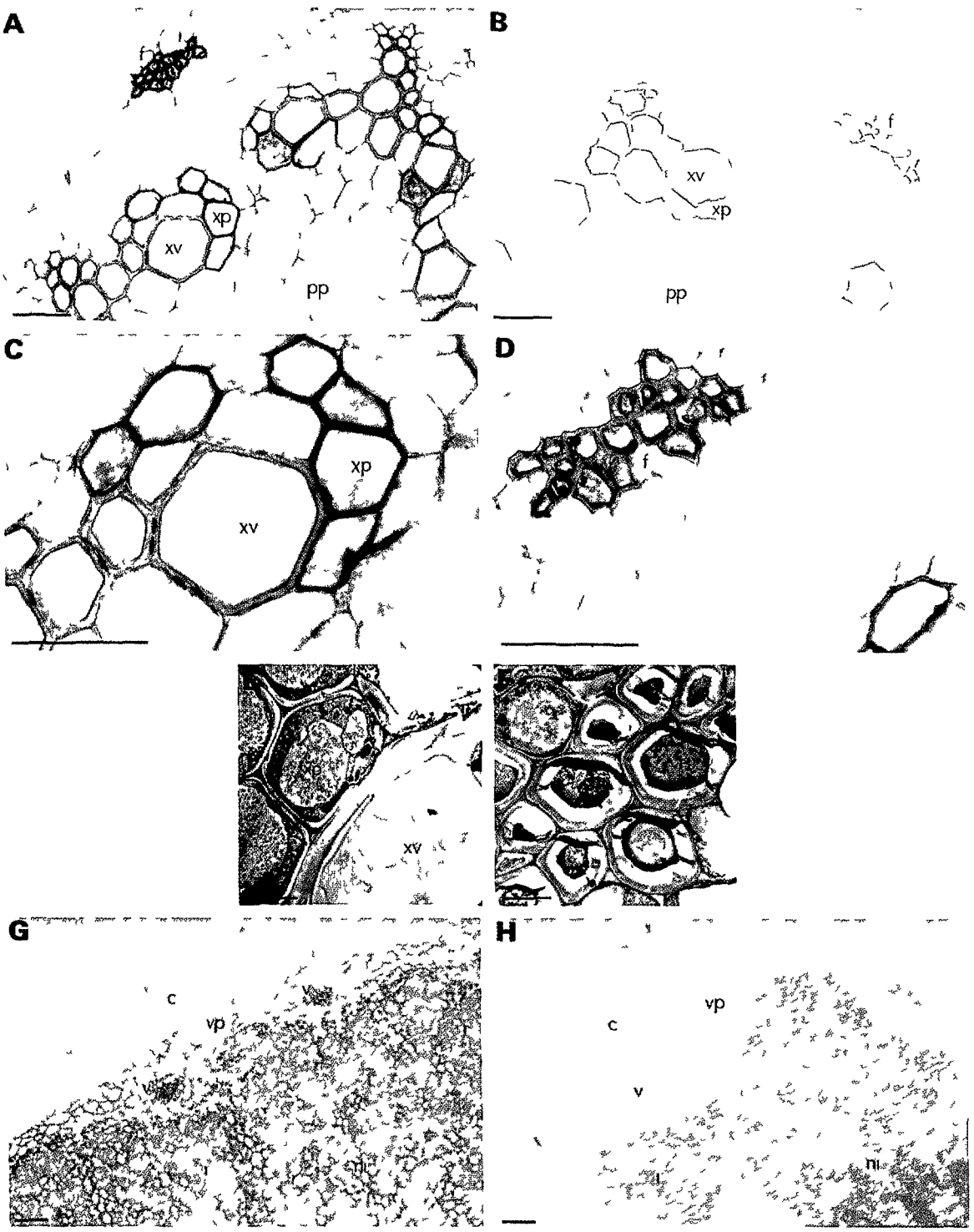

Figure 9 Localization of uricase II transcripts in 4 d postimbibition roots of bean seedlings by in situ hybridization Tissues were fixed embedded in paraffin cut into 7 μm sections and hybridized with DIG labeled sense and antisense probes Anti DIG AP (1 2500 Boehringer Mannheim) and the substrates 4 nitroblue tetrazolium chloride and 5 bromo 4 chloro 3 indolyl phosphate were used to detect hybridization Photographs were taken with ×16 ×40 and ×100 objectives Bars represent 10 μm (1 μm for electron micrographs) A C and D Root cross sections hybridized with antisense uricase II RNA B root cross sections hybridized with sense uricase II RNA E and F electron micrographs showing the metaxylem parenchyma cells (E) and the phloem fibers (F) of the vascular tissue from 4 d postimbibition bean roots f Phloem fibers pp pith parenchyma xp metaxylem parenchyma cells and xv metaxylem vessels G Nodule cross sections hybridized with antisense uricase II RNA H nodule cross sections hybridized with sense uricase II RNA c Cortex i infected cells ni noninfected cells v vascular tissue and vp vascular parenchyma

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in which uricase II participates, may be a strategy for energy conservation (Mothes 1961) The detection of the uricase II protein in *L leucocephala* seedlings (Fig 6), an amide-transporter legume suggests that this legume uses a similar strategy to improve the plant C economy during seedling development Although we could not detect any uricase II antigen or urate oxidase activity in maize seedlings under our experimental conditions, we cannot discard the possibility that this plant contains uricase II Alternatively maize plants could use a strategy other than the transport of ureides to improve C metabolism during seedling establishment

The expression of the uricase II gene is induced in bean cotyledons at the onset of seedling development, approximately 1 d postimbibition, coinciding with the beginning of reserve mobilization (Fig 4A) A massive hydrolysis of DNA and RNA takes place in the seed during germination elevating the levels of purines which are substrates for ureide biogenesis (Polayes and Schubert, 1984) and may also participate in uricase II induction The presence of uricase II transcripts in developing bean roots and hypocotyls (Fig 4, B and C) suggests that these organs are also able to synthesize ureides

In addition to the uricase II subunit (a 35-kD polypeptide) we detected a uricase-related protein of 50 kD in developing cotyledons roots and hypocotyls of bean seedlings that, interestingly, is not found in nodules Tajima et al (1991) reported the detection of a less abundant band of high molecular weight by immunoblot analysis of soybean cotyledon proteins with anti-uricase antibody They suggested that this band could be the dimeric form of the uricase protein Damsz et al (1994) also reported the detection of uricase II and two high-molecular-weight polypeptides in proteins extracted from 28-d-old soybean nodules However, the molecular mass of the 50-kD polypeptide (Fig 5) does not correspond to a dimer of a 35-kD monomer and the three peptides derived from its digestion with V8 protease have sizes different from those derived from the 35-kD protein (Fig 7) This supports the idea that the 50-kD polypeptide we detected is not a dimeric form of the enzyme

The 50-kD polypeptide may be a modified version of the 35-kD protein, which could give rise to different-sized peptides after V8 proteolysis However these putative modifications do not seem to be due to ubiquitination of the 35-kD uricase II subunit as a signal for protein degradation because anti-ubiquitin antibodies did not cross-react with the 50-kD band (data not shown) It is possible that a differential splicing of the uricase II mRNA in cotyledons roots, and hypocotyls could give rise to a higher-molecular-weight protein but northern analysis consistently showed only a single 1 2-kb uricase II transcript in all organs including nodules (Fig 3) Alternatively, the 35- and 50-kD polypeptides may represent related but distinct proteins encoded by different genes The 50-kD protein could share reactive epitopes with the 35-kD uricase protein but the nucleotide sequence of the corresponding genes would not have enough identity to be detected by nucleic acid hybridization experiments This possibility was explored by using less stringent hybridization and washing conditions in the Southern and northern analyses No differences were found from the results presented (data not shown)

Extracts from seedling cotyledons containing only the 50-kD polypeptide had urate oxidase activity (Fig 8) suggesting that the immunologically related protein has uricase activity Uricase-specific activity was maintained in bean cotyledons during all the stages of bean seedling development (from dry seeds to 10 d postimbibition Fig 8) Furthermore, we also detected a higher uricase-specific activity in roots and hypocotyls from 4-d-postimbibition bean seedlings (Table I), in which both polypeptides were present In contrast, Tajima et al (1991) reported the presence of uricase activity in cotyledons from soybean seedlings, but this activity decreased by 5 d postimbibition Additionally they reported lower uricase levels in developing soybean hypocotyls which disappeared rapidly by 2 d postimbibition

The 35-kD uricase II polypeptide is present in the dry seed and remains in the cotyledon 12 h and 1 d postimbibition (Fig 5A) although uricase II mRNA is not present at these stages of development (Fig 4A) It is likely that the uricase II protein that participates in ureide biosynthesis during the seed-filling period but not its mRNA is maintained in the cotyledon during desiccation and early germination The detection of the uricase II transcript coincides with the progressive disappearance of the 35 kD protein and the accumulation of the 50 kD polypeptide further supporting the hypothesis that the 50 kD polypeptide could be a modified version of the 35-kD protein We are continuing with these investigations

Uricase II transcripts have been localized by in situ hybridization in the uninfected cells of the central tissue of bean nodules (Tate et al 1994 Papadopoulou et al 1995) However we have also detected uricase mRNA in the vascular tissue that traverses the nodule as well as in nodule parenchyma cells (Fig 9G) confirming the results of Vaughun and Stegink (1987) who found uricase II in cells of the vascular parenchyma of effective nodules In this work we have extended these localization studies to developing bean roots 4 d postimbibition Here the signal for uricase II transcript was primarily detected in the metaxylem parenchyma cells that surround the large xylem vessels (Fig 9, A and C) and in the phloem fibers (Fig 9 A and D)

Electron microscopy showed that the phloem fibers from young roots contain a living cytoplasm as so do the parenchyma cells in which the uricase II signal is detected (Fig 9 E and F) The metaxylem parenchyma cells are considered to be active in the transport of solutes because they contain abundant mitochondria and ER (Chonan et al 1981) Uricase II likely synthesizes ureides in the metaxylem-parenchyma cells and transports them to the xylem vessels through the abundant plasmodesmata that interconnect both cell types The ureides would be distributed from the xylem vessels to the whole plant The presence of uricase II in the phloem fibers however is not easily explained These cells serve mainly as support and have a very thick cell wall Sakurai et al (1996) detected cytosolic Gln synthetase (EC 6 3 1 2) in the phloem fibers and xylem parenchyma cells from nongreen leaves of de

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LITERATURE CITED

Atkins CA, Rainbird RM Pate JS (1980) Evidence for a purine pathway of ureide synthesis in N fixing nodules of cowpea [*Vigna unguiculata* (L) Walp] Z Pflanzenphysiol **97** 249–260

Bairoch A (1991) The SWISS PROT protein sequence data bank (supplement) Nucleic Acids Res **19** 2241–2246

Bergmann H Preddie E Verma DPS (1983) Nodulin 35 a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules EMBO J **2** 2333–2339

Bradford MM (1976) A rapid and sensitive method for the quantification of micrograms quantities of protein utilizing the principle of protein dye binding Anal Biochem **72** 248–254

Campos F Padilla J Vázquez M Ortega JL Enriquez C Sanchez F (1987) Expression of nodule specific genes in *Phaseolus vulgaris* Plant Mol Biol **9** 521–532

Chonan N Kaneko M Kawahara H Matsuda T (1981) Ultrastructure of the large vascular bundles in the leaves of rice plants Jpn J Crop Sci **50** 323–331

Cleveland DW Fisher SG, Kirschner MW Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis J Biol Chem **252** 1102–1106

Damsz B Dannenhoffer JM Bell JA Webb MA (1994) Immunocytochemical localization of uricase in soybean cotyledons Plant Cell Physiol **35** 979–982

de Vries S Hoge H Bisseling T (1988) Isolation of total polysomal RNA from plant tissues Plant Molecular Biology Manual B6 Kluwer Academic Publishers Dordrecht The Netherlands pp 1–13

Fujihara S Yamaguchi M (1980) Nitrogen fixation and allantoin formation in soybean plants Agric Biol Chem **44** 2569–2573

Hanks JF Tolbert NE Schubert KR (1981) Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules Plant Physiol **68** 65–69

Kim HB An CS (1993) Nucleotide sequences and expression of cDNA clones encoding uricase II in *Canavalia lineata* Korean J Biol **36** 415–423

Kouchi H Tsukamoto M Tajima S (1989) Differential expression of nodule specific (nodulin) genes in the infected uninfected and cortical cells of soybean (*Glycine max*) root nodules J Plant Physiol **135** 608–617

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature **227** 680–685

Larsen K Jochimsem BU (1986) Expression of nodule specific uricase in soybean callus tissue is regulated by oxygen EMBO J **5** 15–19

Lee N Stein B Suzuki H Verma DPS (1993) Expression of antisense nodulin 35 RNA in *Vigna aconitifolia* transgenic root nodules retards peroxisome development and affects nitrogen availability to the plants Plant J **3** 599–606

Legocki RP Verma DPS (1979) A nodule specific plant protein (Nodulin 35) from soybean Science **205** 190–192

Lucas K, Boland MJ Schubert KR (1983) Uricase from soybean root nodules purification properties and comparison with the enzyme from cowpea Arch Biochem Biophys **226** 190–197

Maniatis T Fritsch EF Sambrook J (1982) Molecular cloning A Laboratory Manual Cold Spring Harbor Laboratory Press Cold Spring Harbor NY

Matsumoto T Yatazawa M Yamamoto Y (1977) Distribution and change in the contents of allantoin and allantoic acid in developing nodulating and non nodulating plants Plant Cell Physiol **18** 353–359

McKhann HI Hirsch AM (1993) *In situ* localization of specific mRNAs in plant tissues *In* BR Glick JE Thompson eds Methods in Plant Molecular Biology and Biotechnology CRC Press Boca Raton FL pp 179–205

Merril CR (1990) Gel staining techniques Methods Enzymol **182** 477–488

Miura S Oda T Funai T, Ito M Okada Y Ichiyama A (1994) Urate oxidase is imported into peroxisomes recognizing the C terminal SKL motif of proteins Eur J Biochem **223** 141–146

Mothes K (1961) The metabolism of urea and ureides Can J Bot **39** 1785–1807

Motojima K Goto S (1989) Cloning of rabbit uricase cDNA reveals a conserved carboxy terminal tripeptide in three species Biochim Biophys Acta **1008** 116–118

Mylona P Pawlowski K Bisseling T (1995) Symbiotic nitrogen fixation Plant Cell **7** 869–885

Nguyen T, Zelechowska M Foster V Bergmann H Verma DPS (1985) Primary structure of the soybean nodulin 35 gene encoding uricase II localized in the peroxisomes of uninfected cells of nodules Proc Natl Acad Sci USA **82** 5040–5044

Padilla JE Miranda J Sanchez F (1991) Nodulin regulation in common bean nodules induced by bacterial mutants Mol Plant Microbe Interact **4** 433–439

Papadopoulou K Roussis A Kuin H Katinakis P (1995) Expression pattern of uricase II gene during root nodule development in *Phaseolus vulgaris* Experientia **51** 90–94

Pate JS Atkins CA White ST Rainbird WR, Woo KC (1980) Nitrogen nutrition and xylem transport of nitrogen in ureide producing grain legumes Plant Physiol **65** 961–965

Polayes DA Schubert KR (1984) Purine synthesis and catabolism in soybean seedlings Plant Physiol **75** 1104–1110

Rainbird RM Atkins CA (1981) Purification and some properties of urate oxidase from nitrogen fixing nodules of cowpea Biochim Biophys Acta **659** 132–140

Reynolds PHS Boland MJ Blevins DG Randall DD Schubert KR (1982) Ureide biogenesis in leguminous plants Trends Biochem Sci **7** 366–368

Sakurai N Hayakawa T Nakamura T Yamaya T (1996) Changes in the cellular localization of cytosolic glutamine synthetase protein in vascular bundles of rice leaves at various stages of development Planta **200** 306–311

Sanchez F Campos F Padilla J Bonneville JM Enriquez C Caput D (1987) Purification cDNA cloning and developmental expression of the nodule specific uricase from *Phaseolus vulgaris* L Negro Jamapa Plant Physiol **84** 1143–1147

Sanchez F Padilla JE Perez H Lara M (1991) Control of nodulin gene in root nodule development and metabolism Annu Rev Plant Physiol Plant Mol Biol **42** 507–528

Sanger F Nicklen S Coulson AR (1977) DNA sequencing with chain terminating inhibitors Proc Natl Acad Sci USA **74** 5463–5467

Schubert KR (1986) Products of biological nitrogen fixation in higher plants synthesis transport and metabolism Annu Rev Plant Physiol **37** 539–574

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LITERATURE CITED

Atkins CA Rainbird RM Pate JS (1980) Evidence for a purine pathway of ureide synthesis in N fixing nodules of cowpea [*Vigna unguiculata* (L) Walp] Z Pflanzenphysiol **97** 249–260

Bairoch A (1991) The SWISS PROT protein sequence data bank (supplement) Nucleic Acids Res **19** 2241–2246

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Cleveland DW, Fisher SG Kirschner MW Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis J Biol Chem **252** 1102–1106

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de Vries S Hoge H Bisseling T (1988) Isolation of total polysomal RNA from plant tissues Plant Molecular Biology Manual B6 Kluwer Academic Publishers Dordrecht The Netherlands pp 1–13

Fujihara S Yamaguchi M (1980) Nitrogen fixation and allantoin formation in soybean plants Agric Biol Chem **44** 2569–2573

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Maniatis T Fritsch EF Sambrook J (1982) Molecular cloning A Laboratory Manual Cold Spring Harbor Laboratory Press Cold Spring Harbor NY

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McKhann HI Hirsch AM (1993) *In situ* localization of specific mRNAs in plant tissues *In* BR Glick JE Thompson eds Methods in Plant Molecular Biology and Biotechnology CRC Press Boca Raton FL pp 179–205

Merril CR (1990) Gel staining techniques Methods Enzymol **182** 477–488

Miura S Oda T Funai T Ito M Okada Y Ichiyama A (1994) Urate oxidase is imported into peroxisomes recognizing the C terminal SKL motif of proteins Eur J Biochem **223** 141–146

Mothes K (1961) The metabolism of urea and ureides Can J Bot **39** 1785–1807

Motojima K Goto S (1989) Cloning of rabbit uricase cDNA reveals a conserved carboxy terminal tripeptide in three species Biochim Biophys Acta **1008** 116–118

Mylona P Pawlowski K Bisseling T (1995) Symbiotic nitrogen fixation Plant Cell **7** 869–885

Nguyen T Zelechowska M Foster V Bergmann H Verma DPS (1985) Primary structure of the soybean nodulin 35 gene encoding uricase II localized in the peroxisomes of uninfected cells of nodules Proc Natl Acad Sci USA **82** 5040–5044

Padilla JE Miranda J Sanchez F (1991) Nodulin regulation in common bean nodules induced by bacterial mutants Mol Plant Microbe Interact **4** 433–439

Papadopoulou K Roussis A Kuin H Katinakis P (1995) Expression pattern of uricase II gene during root nodule development in *Phaseolus vulgaris* Experientia **51** 90–94

Pate JS Atkins CA White ST Rainbird WR Woo KC (1980) Nitrogen nutrition and xylem transport of nitrogen in ureide producing grain legumes Plant Physiol **65** 961–965

Polayes DA Schubert KR (1984) Purine synthesis and catabolism in soybean seedlings Plant Physiol **75** 1104–1110

Rainbird RM Atkins CA (1981) Purification and some properties of urate oxidase from nitrogen fixing nodules of cowpea Biochim Biophys Acta **659** 132–140

Reynolds PHS Boland MJ Blevins DG Randall DD Schubert KR (1982) Ureide biogenesis in leguminous plants Trends Biochem Sci **7** 366–368

Sakurai N Hayakawa T Nakamura T Yamaya T (1996) Changes in the cellular localization of cytosolic glutamine synthetase protein in vascular bundles of rice leaves at various stages of development Planta **200** 306–311

Sanchez F Campos F Padilla J Bonneville JM Enriquez C Caput D (1987) Purification cDNA cloning and developmental expression of the nodule specific uricase from *Phaseolus vulgaris* L Negro Jamapa Plant Physiol **84** 1143–1147

Sanchez F Padilla JE Perez H Lara M (1991) Control of nodulin gene in root nodule development and metabolism Annu Rev Plant Physiol Plant Mol Biol **42** 507–528

Sanger F Nicklen S Coulson AR (1977) DNA sequencing with chain terminating inhibitors Proc Natl Acad Sci USA **74** 5463–5467

Schubert KR (1986) Products of biological nitrogen fixation in higher plants synthesis transport and metabolism Annu Rev Plant Physiol **37** 539–574

Schubert KR Boland MJ (1990) The ureides *In* PK Stumpf EE Conn eds The Biochemistry of Plants Vol 16 Academic Press New York pp 197–282

Tajima S Ito H Tanaka K Nanakado T Sugimoto A Kouchi H Okazaki K (1991) Soybean cotyledons contain a uricase that cross reacts with antibodies raised against the nodule uricase (Nod 35) Plant Cell Physiol **32** 1307–1311

Tajima S Kanazawa T Takeuchi E Yamamoto Y (1985) Characteristics of a urate degrading diamine oxidase-peroxidase enzyme system in soybean radicles Plant Cell Physiol **26** 787–795

Tajima S Kato N Yamamoto Y (1983) Cadaverine is involved in urate degrading activity (uricase activity) in soybean radicles Plant Cell Physiol **24** 247–253

Tajima S Tanaka K Takane K, Sugimoto A Okazaki K Kouchi H (1993) Soybean nodule uricase gene (nodulin 35) is expressed in cotyledons during seed development and early germination *In* R Palacios J Mora WE Newton eds New Horizons in Nitrogen Fixation Kluwer Academic Publishers Dordrecht The Netherlands p 373

Takane K Tanaka K Tajima S Okazaki K Kouchi H (1997) Expression of a gene for uricase II (nodulin 35) in cotyledons of soybean plants Plant Cell Physiol **38** 149–154

Tate R Patriarca EJ Riccio A Defez R Laccarino M (1994) Development of *Phaseolus vulgaris* root nodules Mol Plant Microbe Interact **7** 582–589

Thomas RS Schrader RE (1981) Ureide metabolism in higher plants Phytochemistry **20** 361–371

Van den Bosch KA (1992) Biochemistry of peroxisomes Annu Rev Biochem **61** 157–197

Van den Bosch KA Newcomb EH (1986) Immunogold localization of nodule specific uricase in developing soybean root nodules Planta **167** 425–436

Van de Wiel C Scheres B Franssen H Van Lierop MJ Van Lammeren A Van Kammen A Bisseling T (1990) The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules EMBO J **9** 1–7

Vaughun KC Stegink SJ (1987) Peroxisomes of soybean *Glycine max* root nodule vascular parenchyma cells contain a nodule specific urate oxidase Physiol Plant **71** 251–256

Verma DPS Hu CA Zhang M (1992) Root nodule development origin function and regulation of nodulin genes Physiol Plant **85** 253–265

Wallrath LL Burnett JB Friedman TB (1990) Molecular characterization of the *Drosophila melanogaster* urate oxidase gene an ecdysone repressible gene expressed only in the Malpighian tubules Mol Cell Biol **10** 5114–5127

Wu XW Lee CC Muzny DM Caskey CT (1989) Urate oxidase structure and evolutionary implications Proc Natl Acad Sci USA **86** 9412–9416