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

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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 legumes 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 detected in developing roots and hypocotyls but not in nodules. In situ hybridization experiments in root seedlings showed uricase II transcripts in the metaxylem parenchyma cells and phloem fibers of the vascular system.

Ureides are important components of N₂ 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 N₂ compounds from amides (Gln and Asn) to ureides (allantoin and allantoic acid) occurs when they are in association with N₂-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 N₂ to NH₃ (Sánchez et al., 1991; Verma et al., 1992; Mylona et al., 1995). NH₄⁺ 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 CO₂, liberating H₂O₂. 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 (Sánchez et al., 1987) nodules. It is preferentially synthesized on free polypeptides 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 N₂ fixation begins (Sánchez et al., 1987; Padilla et al., 1991), and the enzyme 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% of that observed in nodules infected with wild-type bacteria, suggesting that events during nodule development (e.g. nodule induction, bacteria release, and N₂ 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 O₂ availability (Larsen and Jochimsen, 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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1 This work was supported by grants from Dirección General de Asuntos para el Personal Académico-Universidad Nacional Autónoma de México, nos. IN300993 and USAID12367.

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Abbreviations: AP, alkaline phosphatase; DIG, digoxigenin.
Jackbean (Canavalia liniata) (Kim and An, 1993), and bean (Sánchez 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 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 N₂, 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 (Sánchez et al., 1987) as a probe. The nucleotide sequence of the uricase II cDNA clone was determined by the dideoxy chain termination method and growth conditions were the same for A-Zap cDNA library constructed from 20-d-old bean nodules. The nucleotide sequence of the uricase II cDNA clone was determined by the dideoxy chain termination method and growth conditions were the same for A-Zap cDNA library constructed from 20-d-old bean nodules. The nucleotide sequence of the uricase II cDNA clone was determined by the dideoxy chain termination method and growth conditions were the same for A-Zap cDNA library constructed from 20-d-old bean nodules.

**Genomic Southern Analysis**

DNA was extracted from etiolated bean hypocotyls and digested with EcoRI, EcoRV, HindIII, and XbaI 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 EcoRI-NcoI fragment from pSKuri (Fig. 1A) labeled with [α-32P]dCTP (Multiprime DNA-labeling system kit, Amersham) as a probe. The hybridized filter was washed from 2X SSC and 0.1% (w/v) SDS at 55°C to 0.1X 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-32P]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 2X SSC and 0.1% (w/v) SDS at 55°C to 0.1X 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 (Sánchez et al., 1987).
Common Bean Uricase II and Its Expression

A

sense RNA (367b)
Fragment used in the genomic Southern as a probe
EcoRl Hindll Ncol Hindll Hindlll Pstl Pstl Xhol
pSK+
coding region (1244 bp)
ATG
t
4
T7 promoter
4
antisense RNA (441b)

B

soyb

bean

vign

soyb

bean

vign

soyb

bean

vign

soyb

bean

vign

soyb

bean

vign

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 12,000g for 25 min at 4°C. The supernatants were filtered through glass fiber and centrifuged at 175,000g 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 X 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 HindIII 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 Ncol 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 corresponding sections. The hybridization and antibody incubation times were the same for the sense and antisense experiments. 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' non-coding 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 EcoRI-NcoI uricase II cDNA fragment was used as a probe (Fig. 1A). We detected a single hybridization band in DNAs restricted with EcoRI and XbaI and two bands in DNAs restricted with HindIII and EcoRV (Fig. 2). The latter are restriction sites located at the first intron of the uricase II gene (N. Capote-Mainéz and F. Sánchez, 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 (Sánchez et al., 1987), indicating that uricase II expression was not restricted to this organ. However, transcript levels in N2-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

N2 is actively mobilized during seedling development (Polayes and Schubert, 1984). Because uricase II is a key enzyme in N2 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.
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 (Sánchez 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
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 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.

**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.
Common Bean Uricase II and Its Expression

Table 1. Uricase-specific activity from different plant organs

<table>
<thead>
<tr>
<th>Plant Organ</th>
<th>Uricase-Specific Activity</th>
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<tbody>
<tr>
<td>Bean cotyledons, 4-d-old</td>
<td>3.74 ± 0.40 units mg⁻¹ proteina</td>
</tr>
<tr>
<td>Bean roots, 4-d-old</td>
<td>15.40 ± 0.84</td>
</tr>
<tr>
<td>Bean hypocotyls, 4-d-old</td>
<td>13.33 ± 1.61</td>
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<tr>
<td>Bean nodules, 21-d-old</td>
<td>1790 ± 380</td>
</tr>
<tr>
<td>Maize cotyledons, 4-d-old</td>
<td>0.00</td>
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</table>

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

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.

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.

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).

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 N₂, 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 N₂-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 N₂ 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 Boocock, 1978). Even during the initial steps of 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 (1:5000, 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.
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 35-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 (Taté 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 cystolic Gln synthetase (EC 6.3.1.2) in the phloem fibers and xylem parenchyma cells from nongreen leaves of de-
veloping rice plants. They proposed that this enzyme might participate in the assimilation of NH$_4^+$ derived from the Phe ammonia-lyase (EC 4.3.1.5) reaction, the key enzyme for biosynthesis of lignin polymers in the cell wall. The NH$_4^+$ assimilated by the Gin synthetase may be incorporated, as in nodules, to the synthesis of purines and their conversion into ureides, with the subsequent participation of the uricase II enzyme. Experiments are in progress to establish the role of uricase II in the vascular tissue of bean seedlings.

ACKNOWLEDGMENTS

We are greatly indebted to Drs. José M. Colmenero, Manuel Pineda, and Shirley Gil for helpful discussions and to Drs. M. Lara, M. Rocha, A. Covarrubias, and M. Soberón for critical reading of the manuscript. We also thank A. Pichardo and S. Trujillo for photographic assistance and L. López for technical assistance with the microscopy.

Received May 14, 1997; accepted August 25, 1997.

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