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Expression pattern of uricase II gene during root nodule development in *Phaseolus vulgaris*

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Abstract. A *Phaseolus vulgaris* uricase II cDNA clone has been isolated and sequenced. Comparison on the nucleotide level between this clone and the soybean uricase II clone revealed 88.8% identity. The in situ hybridization technique was used to follow the expression pattern in developing root nodules of *Phaseolus vulgaris*. The uricase II transcripts were localized only in the uninfected cells of the central tissue and mainly in the periphery of the cell. Uricase II mRNA is first detected in nodules 12 days after infection. A maximum level of transcripts is reached in 21-day-old nodules, followed by a considerable reduction in 28-day-old nodules.
Key words. Uricase II; *Phaseolus*; nodulin, nitrogen fixation.

In many legumes in which nitrogen transport from the roots is normally via amides, glutamine and asparagine, there is a switch to ureide sythesis when root nodules develop[1-3]. These plants are referred to as the 'tropical' legumes and belong mainly to the two tribes *Phaseoleae* and *Glycineae*. This switch from amide to ureide synthesis results in economies in carbon use: ureides use less organic carbon to transport the same amount of nitrogen than amides do[4]. The metabolic conversions required for the synthesis of ureides involve de novo purine biosynthesis and subsequent oxidative degradation of these purines[5-8]. In short, in active nodules of legumes, atmospheric N_2 is reduced by the bacteroids and the resulting ammonia[9] is assimilated into glutamine[10, 11] in the cytosol of the infected cells[12-14]. This glutamine is then channelled via serine/glycine and aspartate into purine biosynthesis which takes place in the plastids of the host cell[15]. The purine xanthine is exported to the cytosol where it is oxidized to uric acid, which is transferred to the peroxisomes of the adjacent uninfected cells[16, 17], and is converted to allantoin and other ureides before exported via the xylem. The conversion of uric acid to allantoin is carried out in the nodules of tropical legumes by a nodule-specific enzyme, namely uricase II[18, 19].

Uricase II is a tetrameric enzyme that is produced exclusively in the root nodules of legumes[20]. Uricase II constitutes approximately 2% of the soluble protein extracted from mature nodules of soybean and *Phaseolus*[21, 22]. In soybean, the uricase II gene has been isolated, and its product localized within the peroxisomes of unifected cells by immunogold-labelling analysis[23]. In the root nodules of *Phaseolus*, the purification of uricase II as well as the isolation of a cDNA clone cross-hybridizing with the soybean gene has been reported[24]. The accumulation of the protein starts 11 days after infection, which shows that *Phaseolus* uricase II is also a true nodulin, expressed just before the onset of nitrogen fixation[22, 25].

In this report we describe the isolation and nucleotide sequence of a *Phaseolus* uricase II cDNA clone, and in situ hybridization experiments designed to follow the expression of the uricase II gene in *Phaseolus* nodules at different development stages.

Materials and methods

Plant material. Growth conditions of bean plants (*Phaseolus vulgaris* cv. Tendergreen) and inoculation with *R. leguminosarum* biovar *phaseolii* were as described by Bisseling et al[26].

Isolation and sequence analysis of *Phaseolus* uricase II cDNA clone. The *Phaseolus vulgaris* cv. Tendergeen λgt11 cDNA library, made from polyA$^+$ RNA isolated from 21-day-old nodules, was generously provided by Dr. B. G. Forde (Rothamsted Experimental Station, UK). The screening of the library with a ^{32}P-labelled uricase II cDNA clone from soybean, phage purification, and subcloning of the positive clones into the pBluescript KS$^+$ vector (Stratagene Inc., USA) were done according to standard methods[27]. The sizes of the inserts of positive recombinant phages were determined by PCR using the forward and reverse λgt11 primers (Promega, USA). The nucleotide sequence of pPv-35.1 clone was determined using the dideoxy chain termination method[28]. Data were stored and analyzed by programs written by Staden[29] on a micro VAX/VMS computer.

In situ hybridization. Nodules harvested at different time intervals after inoculation with *Rhizobium* were

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Figure 1A.

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     R  D  D  N  S  D  I  V  A  T  D  T  M  K  N
P  CCGCGATGATAACTCTGACATCGTTGCCACTGACACCATGAAAAA   45
S  .........C.................T.....T...........  259

     T  V  Y  A  K  A  K  E  C  S     D  I  S  V
P  CACGGTGTATGCAAAAGCAAAGGAATGCTCGG--ATATCT-CTGT   87
S  ..............................T.AC...CTTT...C  304

     E  D  F  A  I  L  L  A  K  H  F  V  S  F  Y
P  CGAGGACTTTGCTATTCTACTTGCCAAGCACTTTGTATCATTTTA  132
S  ......G...........G.....T.....................  364

     K  K  V  T  G  A  I  V  N  I  V  E  K  P  W
P  CAAGAAGGTCACTGGTGCTATTGTGAATATCGTGGAAAAACCATG  177
S  .C.......T.....................T.............  414

     E  R  V  I  V  D  G  Q  P  H  Q  H  G  F  T
P  GGAGCGTGTCATTGTGGATGGTCAACCTCATCAACATGGTTTCAC  222
S  ...........C.....................G............A  464

     L  G  S  E  K  H  T  T  E  A  I  L  Q  K  S
P  ACTTGGGTCTGAGAAGCATACCACAGAGGCAATACTACAGAAGTC  267
S  ......................A..........G....G....A..  514

     G  S  L  Q  L  T  S  G  I  E  G  L  S  V  L
P  TTGTTCACTACAATTGACTTCTGGTATTGAAGGATTGTCAGTGTT  312
S  .........T..G.................................  564

     K  T  T  Q  S  V  F  E  N  F  I  R  D  K  Y
P  GAAGACAACCCAGTCTGGTTTTGAGAATTTCATTAGAGACAAGTA  357
S  ........................T.........A...........  614

     T  A  L  P  D  T  R  E  R  I  L  A  T  E  V
P  CACAGCACTTCCAGATACCCGCGAAAGGATTTTGGCAACAGAAGT  402
S  ............T.........T.........GG.A...........  664

     T  A  L  W  R  Y  S  Y  E  S  L  Y  N  L  P
P  AACTGCTCTGTGGAGGTATTCGTACGAATCGCTATACAACCTCCC  447
S  .....A...................T.........G..T.G......  714

     Q  K  P  L  Y  F  T  D  K  Y  L  E  V  K  K
P  TCAGAAGCCACTATACTTCACAGACAAGTATCTGGAAGTGAAAAA  492
S  .........G..T.....T.....A........A............  764

     V  L  A  D  T  F  F  G  P  T  K  Q  G  V  Y
P  AGTTCTGGCTGACACATTTTTTGGGCCAACCAAACAGGGGGTCTA  537
S  ...............T.........C...C.....GG...A.....  814

     S  P  S  V  Q  N  T  L  Y  L  M  A  K  A  T
P  TAGCCCATCTGTTCAAAACACACTCTACCTTATGGCAAAGGCCAC  582
S  ................................G..............  864

     L  N  R  F  P  D  I  A  Y  C  H  L  K  M  P
P  ACTGAACAGATTTCCTGACATTGCTTATTGCCATCTAAAGATGCC  627
S  .......................A.........AG............  914

     N  L  H  F  L  P  V  N  I  S  S  K  D  G  P
P  AAATCTTCATTTCTTACCAGTCAACATCTCAAGCAAGGATGGTCC  672
S  ..............A....T.....T.......A.C.......C..  964

     I  V  K  F  E  D  D  V  Y  L  P  T  D  E  P
P  AATTGTGAAGTTTGAGGATGATGTTTATTTACCAACGGACGAGCC  717
S  T.........................G..C..G.........T.....  1014

     H  G  S  I  E  A  S  L  L  Q  L  F  Y  I  L
P  TCATGGCTCAATTGAAGCAAGCTTATTACAACTTTTTTACATTTT  762
S  A.....G......C....T..T..GAGC.GC....            1054

     L  *
P  ATTGTGAAATTGGGTCGCGTTTCATACGATTCGGTTATGCCATAG  807
P  ACGCGTAAAGGTTTTTAATGGTTTCCCGTAACCAGACGATCTTCG  852
P  GATTATGGCTGTTCCGTTTATGCCACAGCAGGGTAAAAGGAACTT  897
P  CCAGCTTTTTTTGCCTGGCTTTCGTCAAACGGGAGAGGAAGAAGC  942
P  AACCAACGGCAGTTGATGGAGTTATTGTAGTACTGACAGTAGCGC  987
P  GGCCGGTCGGCCAGTAGCAGATTGTCGGGTTGCGCTGCCATAAAC  1032
P  AGTGACTGGCTCGAATTC                             1050
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fixed in 4% paraformaldehyde supplemented with 0.25% glutaraldehyde in 10 mM sodium phosphate buffer for 4 h[39]. Fixed nodules were dehydrated and embedded in paraffin and sections 7–10 µm thick were cut as described by Yang et al.[30]. The pPv-35.1 clone was transcribed from the T3 promoter. Antisense RNA probes

Figure 1B.

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P  RDDNSDIVATDTMKNTVYAKAKECSDI-SVEDFAILLAKHFVSFYKKVTGA   50
S  RD-NSKIVATDTMKNTVYAKAKECSKILSAEKFAILLALHFVSFYKKVTGA  104

P  IVNIVEKPWERVIVDGQPHQHCFTLGSEKHTTEAILQKSGSLQLTSGIEG  100
S  IVNIVEKPTERVINDGQPHEHGFKLGSEL-TTEAIVQLSGSLQLTSGIEG  153

P  LSVLKTTQSVFENFIRDKYTALPDTRERILATEVTALWRYSYESLYNLPQ  150
S  LSVLKTTQSFGVNFIRDKYTALPDTRERILATEVTALWRYSYESQYSLPQ  203

P  KPKYFTDKYLEVKKVLADTFFGPTKQGNYSPSVQNTLYLMAKATLNRFPD  200
S  KPFYFTEKYQEVKKVLADTFFGPPNGGVYSPSV-NTLYLMAKATLNR-PD  251

P  IAYCHLKMPNKHFLPVNIS--SK-DGPIVKFEDDVYLPTDEPHGSIEASL  247
S  IAYVSLKMPNLHFLPVNISNISNQDGPIVKFEDDVYLPTDEPHGSIQNSL  301

P  LQLFYILL*                                           255
S  SRTLSKL*                                            308
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Figure 1.
A Homology of the nucleotide sequences between the soybean (S) and *Phaseolus* (P) cDNAs coding for uricase II. Nucleotides are numbered to the right of the sequence. Dots indicate identical nucleotides, and dashes missing bases in both sequences. The predicted amino acid sequence of the longest ORF of the *Phaseolus* clone is shown in standard single-letter code above the nucleotide sequence. The amino acids are positioned above the first nucleotide of the triplet. The termination codon is indicated with asterisk (*). *B* Comparison of the amino acid sequences deduced from the *Phaseolus* (P) and soybean (S) uricase II clones. The number of amino acids is indicated on the right. Identical amino acids are linked by vertical lines (|), missing amino acids are denoted by dashes (–).

labelled with ^{35}S-UTP (Amersham, UK, 1000–1500 Ci/mmol), or (DIG)-11-rUTP (Boehringer, Germany) were obtained[31] and hybridized to the tissue sections, according to a protocol[32] derived from a method described by Cox and Goldberg[33]. Sections, coated with Kodak NTB2 nuclear emulsion, were exposed for 2 to 4 weeks and stained with toluidine blue O. The non-radioactive hybridization was performed as described by Kouchi and Hata[34]. The sections were stained with safranin.

Results and discussion

Isolation of the uricase II cDNA clone pPv-35.1. A *P. vulgaris* var. Tendergreen nodule-specific cDNA library constructed in a λgt11 vector was screened using the soybean uricase II cDNA clone as a probe. A striking difference in the abundance of uricase II mRNA in *Phaseolus* compared to soybean is reflected in the relative number of positive clones obtained during the library screening procedure. Several cDNA clones were easily isolated in *Phaseolus*, as was expected since in this plant uricase II is the second most abundant protein in nodules. In soybean, on the other hand, it has been reported[23], and verified by our own results (an average of 2 positive clones in 6×10^4 screened plaques), that the mRNA of uricase II is present in low concentration. In

soybean, the abundance of the protein is attributed to its accumulation in the peroxisomes of unifected cells[23]. The discrepancies in the number of cDNA clones present in the two libraries may reflect differences either in the transcription rates or/and in the stability of the uricase II mRNAs in the nodules of these

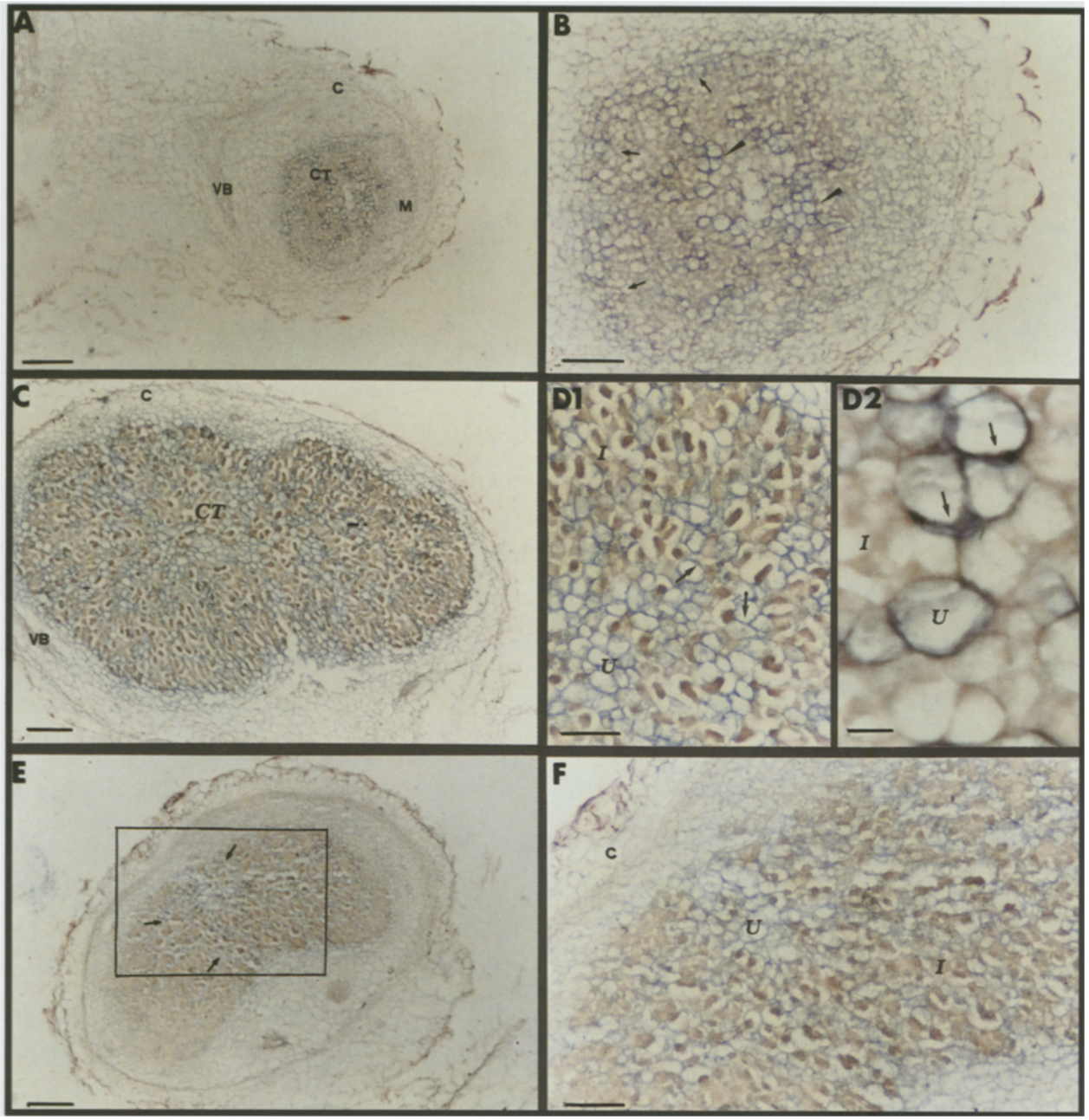

Figure 2. In situ localization of uricase II transcripts in developing root nodules of *P. vulgaris*. Sections were hybridized with DIG-11-rUTP labelled antisense pPv-35.1 RNA. Positive hybridization signals are visible as blue colour development.
CT: central tissue, C: cortical cells, VB: vascular bundle, M: meristematic cells, U: uninfected cells, I: infected cells.
A Section of a 15-day-old nodule. Hybridization signal is detected only in the central tissue of the nodule. Bar represents 100 μm.
B The central tissue of 14-day-old nodule in a higher magnification. The signal is detected in the peripheral uninfected cells of the proximal part of the central tissue (arrows) and in the uninfected cells residing among infected cells (arrowheads). Bar represents 50 μm.
C Section of a 21-day-old nodule. The expression of the uricase II gene is at maximum level. This is reflected in an intense colour precipitate in all the uninfected cells of the central tissue. Bar represents 100 μm.
D1–D2 Magnification of the central tissue of a 21-day-old nodule. In several cells the signal is detected mainly in the periphery of the uninfected cells (arrows). Bar presents 50 μm in D1 and 10 μm in D2.
E Section of a 28-day-old nodule. The signal is detected in the uninfected cells in the most central part of the central tissue (arrows). Bar represents 100 μm.
F Detail of *E*. The intensity of the hybridization signal of the uninfected cells compared to *D1* is clearly reduced. Bar represents 100 μm.

plants. This question requires further experimental investigation.

Nucleotide sequence determination of pPv-35.1. The uricase II clones isolated from *Phaseolus* were plaque-purified, and their sizes were determined using the PCR technique. The largest clones were subcloned into the pBluescript KS^+ vector. Northern blot analysis has revealed that the uricase II cDNA in bean hybridizes with an mRNA of about 1.3 Kb in length[24] (Papadopoulou, unpubl. data). None of the isolated clones was a full-sized clone, and further attempts to obtain one were unsuccessful. Therefore, the nucleotide sequence of the largest available clone, pPv-35.1 (1048 bp) was determined by the dideoxy chain termination method[28], and the sequence was compared with that of published soybean cDNA clone[23]. As was expected, the first 54 amino acids of the ORF are absent. The overlap between the two clones begins at the nucleotide 214 of the soybean clone, revealing 88.8% identity in the coding region (fig. 1A). As can be seen by comparing the amino acid sequences of the soybean and *Phaseolus* clones, a number of amino acids have been replaced while others have been added or omitted (fig. 1B).

Taking into account that the two plants belong to different tribes, the conservation of the primary structure of the enzymes may be characterized as relatively high. This fact could be explained if the function of the enzyme in the special ureide-catabolism pathway is considered. This pathway exists in both plants when they are nodulated.

In situ hybridization at late stages. We were interested to determine the type of cell in which the uricase II gene is induced, and the timing of its expression. We therefore localized the uricase II transcripts in developing *Phaseolus* nodules by in situ hybridization. Nodules from *Phaseolus* plants infected with *Rhizobium leguminosarum* biovar *phaseolii* were collected at different developmental stages: 14, 21, 28 and 35 days after infection. Thin sections were taken and hybridized to anti-sense RNA for uricase II.

At 14 days after infection the nodule is not fully formed, although the central tissue, consisting of infected and unifected cells, can be distinguished. Infected cells are still of about the same size as unifected ones, but show a more dense cytoplasmic content. In this developmental stage the hybridization signal is detected as a blue precipitate in the uninfected cells, both in cells at the periphery of the central tissue and in uninfected cells between patches of infected cells (figs 2A, 2B).

In 21-day-old nodules the central tissue consists of clearly distinguished infected and uninfected cells and is surrounded by a layer of uninfected cells which forms the boundary layer[35]. At this stage the uricase II transcripts are detected in the uninfected cells which extend into the whole central tissue of the nodule among larger infected cells (figs 2C, 2D1).

In 28-day-old nodules the spatial expression pattern of the uricase II gene is not significantly changed. The signal is detected again in the uninfected cells, though its intensity is considerably reduced (figs 2E, 2F). In 28-day-old nodules the signal appeared only after a 16-hour incubation of the colour reaction. In contrast, the signal in 14-day- and 21-day-old nodules appeared within the first 6 hours of the colour reaction. This is consistent with the temporal expression profile of uricase II mRNAs reported before[22, 24], which shows that in 28-day-old nodules the level of the uricase II transcripts declines.

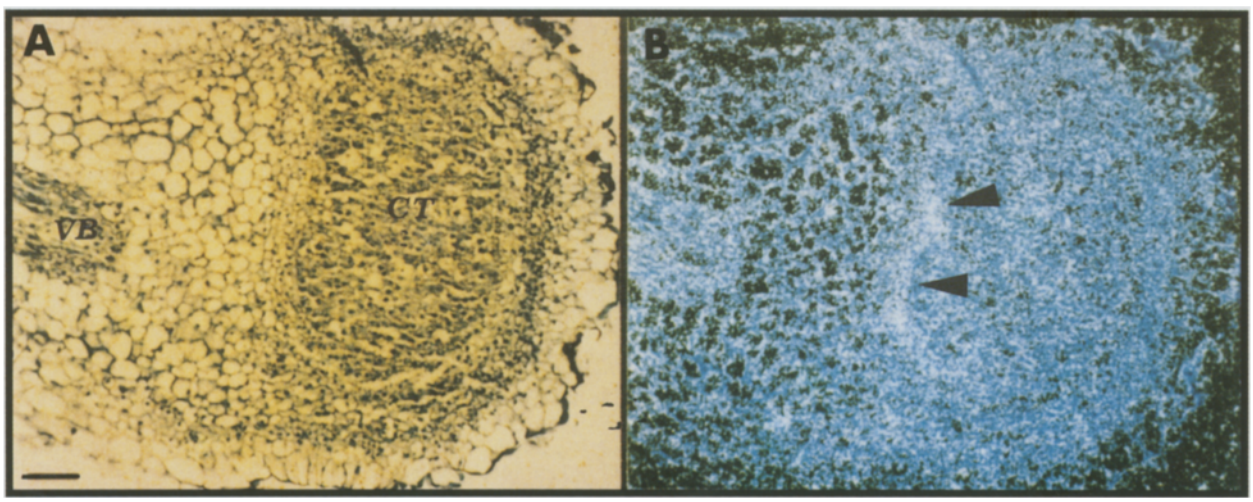

Figure 3. Section of a 12-day-old root nodule of *P. vulgaris* hybridized to ^{35}S-labelled antisense pPv-35.1 RNA. CT: central tissue, VB: vascular bundle.
A is a bright field micrograph in which the silver grains are visible as black dots.
B is a dark field micrograph in which the hybridization signal is represented by white dots. The localization of the uricase II transcripts is detected in the proximal part of the developing central tissue (arrowheads). Bar represents 100 μm.

In the last developmental stage studied, that is in 35-day-old nodules, no signal was detected in any attempts (data not shown). This could mean that the uricase II mRNAs do not exist any more in nodules of that age, probably because the senescence of the nodules is at a very advanced stage and the metabolism of the nodule is altered, leading to suppresion of the transcription of the uricase II gene.

It should be pointed out that in many cases the colour precipitate is detected in the periphery of the uninfected cells, where the peroxisomes are localized in these cells[36] (figs 2D1, 2D2).

In situ hybrization at early stages. Since we were interested in the early stages of development and the exact localization of the appearance of the uricase II transcripts at these stages, one more set of in situ hybridization experiments was performed using the non-radioactively labelled uricase II antisense RNA. However, in these experiments a problem of high background arose, possibly due to the low transcription level of the uricase II gene. As a solution, the uricase II antisense RNA was labelled with ^{35}S-UTP and hybridized to sections of 6, 9, 10- and 12-day-old nodules. These developmental intervals were chosen taking into account previous reports according to which the *Phaseolus* uricase II transcripts begin to accumulate from day 11 after inoculation[4, 31] and the soybean uricase II mRNA appears between 6 and 9 days after infection[26].

The first developmental stage at which the uricase II mRNA can be detected is in 12-day-old nodules. The hybridizing signal is present in the proximal part of the central tissue, and is specifically limited to a narrow area containing uninfected cells (fig. 3). The proximal area of the central tissue contains 'older' infected cells; that is, the first cells being invaded by rhizobia. The appearance of uricase II transcripts in this area can be related to the production of an intermediate compound in the pathway of ureide synthesis in the infected cells that triggers the activation of the uricase II gene. No signal was detected in any of the earlier stages studied.

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