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Nucleotide Sequences and Expression of cDNA Clones Encoding Uricase II in *Canavalia lineata*

Kim, Ho Bang and Chung Sun An
(Department of Biology, Seoul National University, Seoul)

해녀콩 Uricase II의 cDNA 염기서열과 발현

金 昊 邦·安 正 善
(서울대학교 자연과학대학 생물학과)

ABSTRACT

Two full length cDNA clones encoding uricase II were isolated by plaque hybridization of a nodule cDNA library of *Canavalia lineata* with a uricase II cDNA clone from soybean as a probe. Clone pcClNUO-01 was consisted of 1,611 bp with one open reading frame (ORF) of 924 nucleotides (NT), while clone pcClNUO-02 was consisted of 1,024 bp with one ORF of 903 NT. Nucleotide sequences for ORFs of the two clones showed 88.9% and 89.3% homology, respectively, to that of soybean uricase II. Deduced amino acid sequence homologies to soybean uricase II were 84.1% and 85.4%, respectively. At 313 NT downstream of the termination codon in pcClNUO-01, putative signal (AATAAA) for poly(A) addition was found, and 17 residues of poly(A) was found further downstream of 21 NT. The peroxisome-targeting signal (Ser-Lys-Leu) was also found at the carboxyl terminal of the deduced amino acid sequences for both ORFs. Deduced amino acid composition of pcClNUO-01 and pcClNUO-02 shows that the ratios of basic amino acids (Arg, His, Lys) and acidic amino acids (Asp, Glu) are 46 to 35 and 47 to 35, respectively. This amino acid composition indicates a basic nature of uricase II enzyme. According to Northern analysis of different organs, uricase II gene was expressed only in root nodule. Genomic hybridization also revealed that the uricase II gene may be present as a small multigene family on the genome of *C. lineata*.

INTRODUCTION

Leguminous plants are able to utilize atmospheric nitrogen directly due to the complex symbiotic association with soil bacteria, *Rhizobium* (Sprent, 1979). Tropical legumes, such as soybean, assimilate symbiotically fixed nitrogen into ureides that are translocated to the shoot (Schubert, 1986). Ureides are formed in root nodules via *de novo* purine biosynthesis and oxidation. It has been suggested that the enzymes involved in purine biosynthesis are located in the proplastids of infected cells of the nodules, whereas the oxidation of purine occurs in the microbodies (peroxisomes) of uninfected cells of the nodules (Newcomb and Tandon, 1981).

Uricase (urate; oxygen oxidoreductase, EC 1.7.3.3) is a key enzyme involved in ureide production and catalyzes the conversion of uric acid to allantoin in the peroxisomes of uninfected cells of the nodules. This enzyme differs in all physicochemical properties from those in uninfected root or leaf, and has been termed uricase II (Bergmann *et al.*, 1983). Uricase II purified from nodules of soybean consists of four identical subunits whose molecular weight is 35 kD. N-35 is a subunit of uricase II and one of the major proteins in soybean root nodules (Bergmann *et al.*, 1983).

The soybean uricase II gene has seven introns and encodes 309 amino acids. But its amino terminal contains no signal sequence, therefore transit information must

occur elsewhere in the gene (Nguyen *et al.*, 1985). Southern hybridization of soybean genomic DNA with N-35 showed several *Eco*RI fragments hybridized with the probe, but it is not certain that how many genes encode uricase II (Nguyen *et al.*, 1985).

N-35 is translated on free polysomes in the host cell cytoplasm and posttranslationally translocated into the peroxisome without any apparent modification such as processing and glycosylation (Nguyen *et al.*, 1985). The posttranslational transport has also been observed for other peroxisomal proteins (Lazarow and Fujiki, 1985). Using a genetic approach, Gould *et al.* (1987) demonstrated that one animal peroxisomal enzyme, firefly luciferase (EC 1.13.12.7), contains the peroxisomal targeting signal at its carboxyl terminus. Miyazawa *et al.* (1989) proposed the importance of the three amino acid sequence, Ser-Lys-Leu-COOH, at the carboxyl terminus of these proteins for translocation to the peroxisomes.

Various researches were carried out with symbiotic association between *C. lineata* and its symbiont, *Rhizobium* sp. (Kim and An, 1989; Choi and An, 1991), but no plant genes associated with nitrogen assimilation have been studied. As a part of molecular biological study on nitrogen assimilation, we isolated two uricase II cDNA clones and determined their nucleotide sequences and tissue-specific expression.

MATERIALS AND METHODS

Plant materials. *C. lineata* seedlings were inoculated with *Rhizobium* sp. SNU003 (Kim and An, 1989) and grown in a greenhouse under natural light. Nodules were harvested 3~4 weeks after inoculation and stored at −70℃. Roots and primary leaves were obtained from non-inoculated plants grown for three weeks.

Enzymes and chemicals. Enzymes and chemicals were purchased from NEB (restriction enzymes), USB (DNA sequencing kit), Promega (nick-translation kit), Amersham (nylonmembrane, [α-^{32}P]-dCTP, [^{35}S] dATP-αS) and Sigma (other chemicals).

DNA isolation. Plasmid DNA was isolated from *E. coli* according to Brush *et al.* (1985). Phage DNA was prepared as previously described by Sambrook *et al.* (1989). Genomic DNA was purified from leaves of *C. lineata* according to Ausubel *et al.* (1987).

RNA isolation and Nothern hybridization. Total RNA was isolated by the method of Davis *et al.* (1986). Total RNA of 20 μg was size-fractionated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. Northern hybridization was carried out as previously described by Sambrook *et al.* (1989).

Preparation of the probe. Uricase II cDNA clone of soybean (pNOD35) was used as a probe (Nguyen *et al.*, 1985). Probe DNA was labelled with [α-^{32}P]-dCTP by nick-translation system (Promega).

Screening of a cDNA library. About 5×10^4 bacteriophages were screened according to Benton and Davis (1977) from λgt10 cDNA library of *C. lineata* nodule (Kim, 1993).

Molecular cloning. Standard procedures were used for the recombinant work as previously described by Sambrook *et al.* (1989). cDNA inserts of phage clone were subcloned into *Eco*RI site of pUC19 and pBSKS using *E. coli* strain JM101 as a host. Subclones were identified by digestion with *Eco*RI.

DNA sequencing. The nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Plasmid DNA was deleted unidirectionally with exonuclease III and S1 nuclease by using the Erase-a-base system based on the protocol of Henikoff (1984). Plasmid DNAs with serial deletion of about 200 bp in size were chosen and used as templates for DNA synthesis after denaturation. The reaction products were separated in 6% polyacrylamide-urea sequencing gels. Autoradiograms were obtained by exposure to X-ray films and used for reading the nucleotide sequence.

Genomic hybridization. Genomic DNA of *C. lineata* was digested with several restriction enzymes and separated on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized with uricase II cDNA of soybean (Southern, 1975).

RESULTS AND DISCUSSION

Selection of phage clone containing uricase II-gene from a nodule cDNA library. A nodule cDNA library of *C. lineata* constructed at our laboratory (Kim, 1993) was screened with uricase II cDNA clone of soybean (Nguyen *et al.*, 1985) by plaque hybridization. About 50 phage clones were selected from the first screening. After the second screening, eight clones were selected, and phage DNA was isolated from each clone. Digestion of the purified phage DNA with *Eco*RI showed that the insert size was various from 0.6 kb to 1.6 kb (Fig. 1A). Southern hybridization showed that all the inserts were hybridized with the probe (Fig. 1B). The inserts of the eight phage clones were subcloned into pUC19 or pBSKS and their partial nucleotide sequence were determined. Two

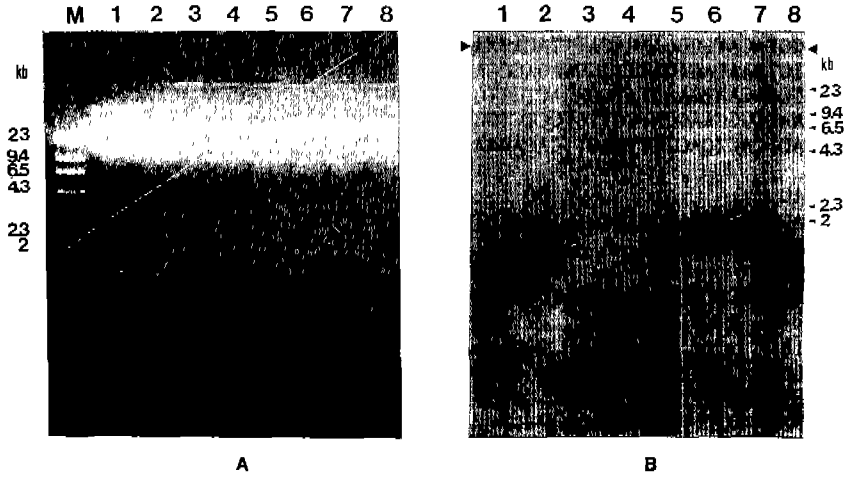

Fig. 1. Electrophoresis of *Eco*RI-digested DNAs from positive phage clones in a 0.7% agarose gel (A) and corresponding blot hybridized with soybean uricase II-probe, pNOD35 (B). M: λ/*Hind*III size marker. Upper numbers are phage clone numbers.

different clones (Fig. 1, lanes 3, 7) were identified from the partial sequencing whose sizes were about 1.0 kb and 1.6 kb, respectively (two arrow marks in Fig. 1). The two subclones, pcClNUO-01 (Fig. 1, lane 7) and pcClNUO-02 (Fig. 1, lane 3), were used for determining their nucleotide sequences.

Analyses of nucleotide sequence and deduced amino acid sequence. Nucleotide sequences of pcClNUO-01 and pcClNUO-02 were determined (Figs. 2, 3). The two clones, pcClNUO-01 and pcClNUO-02, were 1,611 bp and 1,024 bp and contained ORF of 927 NT and 903 NT encoding 303 and 301 amino acids, respectively. With comparison of soybean uricase II cDNA, nucleotide sequences for the ORFs of the two clones showed 88.9% and 89.3% homologies and their deduced amino acid sequence homologies were 84.1% and 85.4%, respectively.

A putative translation start site of higher plant genes, 5′-AACAATGGC-3′, proposed by Lutcke *et al.* (1987) was found at the corresponding site of the two clones (pcClNUO-01; 5′-AACGAAGATGGC-3′, pcClNUO-02; 5′-AACGAAAGATGGC-3′). At 313 NT downstream of the termination codon in pcClNUO-01, putative signal (AATAAA) for poly(A) addition was found and 17 residues of poly(A) was also found further downstream of 21 NT.

Table 1 shows the deduced amino acid compositions of pcClNUO-01, pcClNUO-02 and pNOD35. The ratios of basic amino acids (Arg, His, Lys) to acidic amino acids (Asp, Glu) are 46 to 35, 47 to 35 and 45 to 36, respectively. This result indicates the basic nature of uricase II enzyme and is in accordance with the fact that uricase was eluted from the core of rat liver peroxisomes under alkaline conditions (Watanabe *et al.*, 1977). Ito *et al.* (1988) also reported that the amino acid composition of the purified rat liver uricase was consistent with that predicted from the nucleotide sequence.

Hydropathy profile (Kyte and Doolittle. 1982) of the ORF from pcClNUO-01 (Fig. 4) was very similar to that of pcClNUO-02, and there was no hydrophobic amino acid stretches to suggest membrane-spanning sequences. This is consistent with the biochemical and histochemical observations that uricase II is a peroxisomal protein (Nguyen *et al.*, 1985).

Peroxisomal proteins are synthesized on free ribosomes in the cytosol and transported post-translationally into the organelle (Borst, 1986). Unlike many precursor proteins destined for other cellular organelles, most peroxisomal proteins are synthesized at their final sizes and do not seem to contain cleavable targeting sequence at their N-termini (Borst, 1986). Miyazawa *et al.* (1989) proposed that several peroxisomal proteins had a targeting signal (Ser-Lys-Leu-COOH) at their C-terminal. This putative peroxisome targeting signal was also present at C-terminal of the two clones (Figs. 2, 3). However, when expressed in *E. coli*, the soybean uricase II cDNA produces an active soluble cytoplasmic enzyme which correctly assembles into a tetramer (Suzuki and Verma, 1991). Thus, the presence of peroxisomes does not appear to be essential for the proper assembly of the holoenzyme

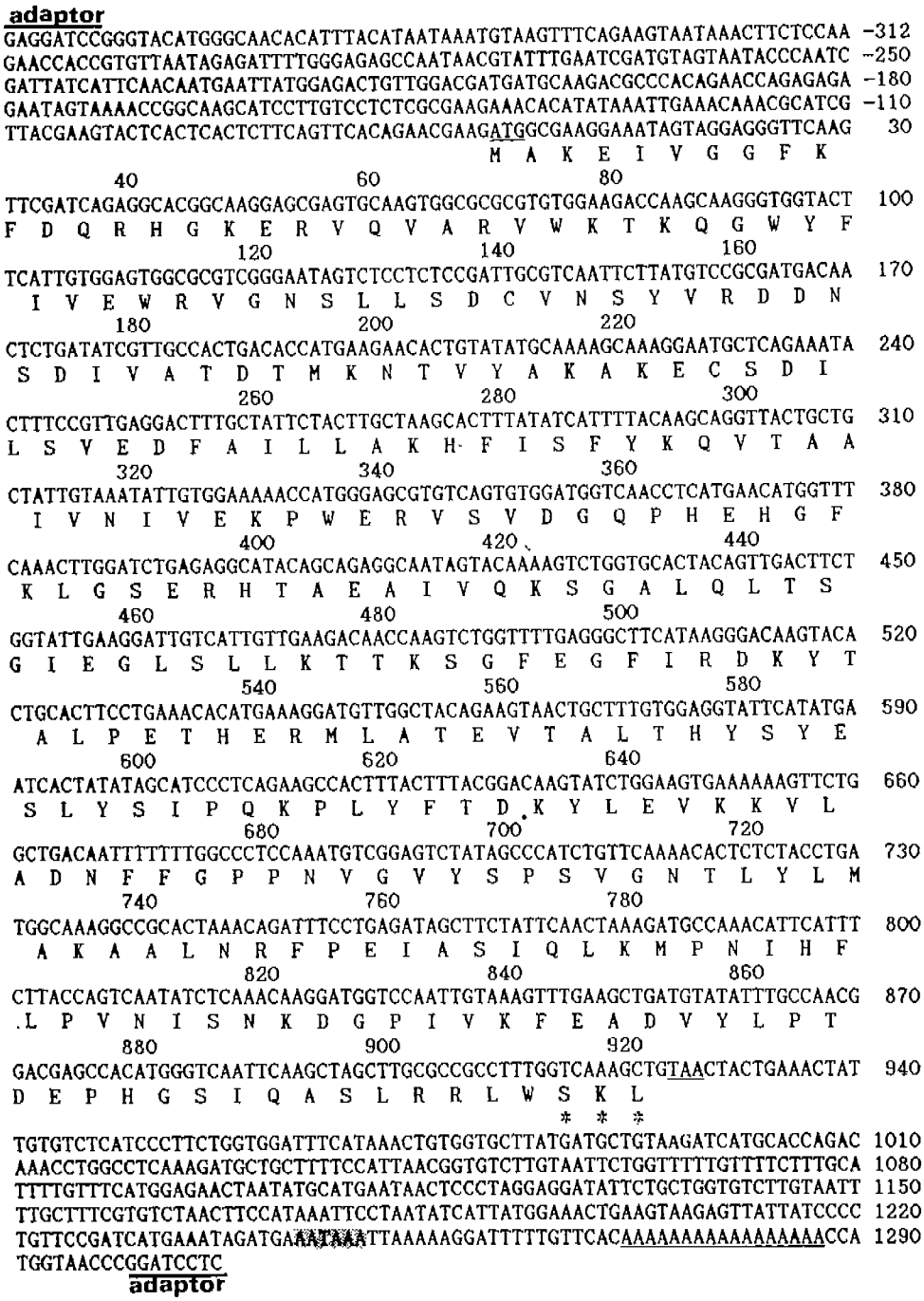

Fig. 2. Nucleotide sequences and deduced amino acid sequences of the cDNA clone, pcCINUO-01. The translation start and termination codon are underlined. The eukaryotic poly(A) addition signal (▨) and putative peroxisome targeting signal (***) is present at 3'-noncoding region and carboxyl terminal, respectively. Poly(A) residues of 17 NT is also underlined at 21 NT downstream from poly(A) addition signal. EMBL Data Bank accession number is X76286.

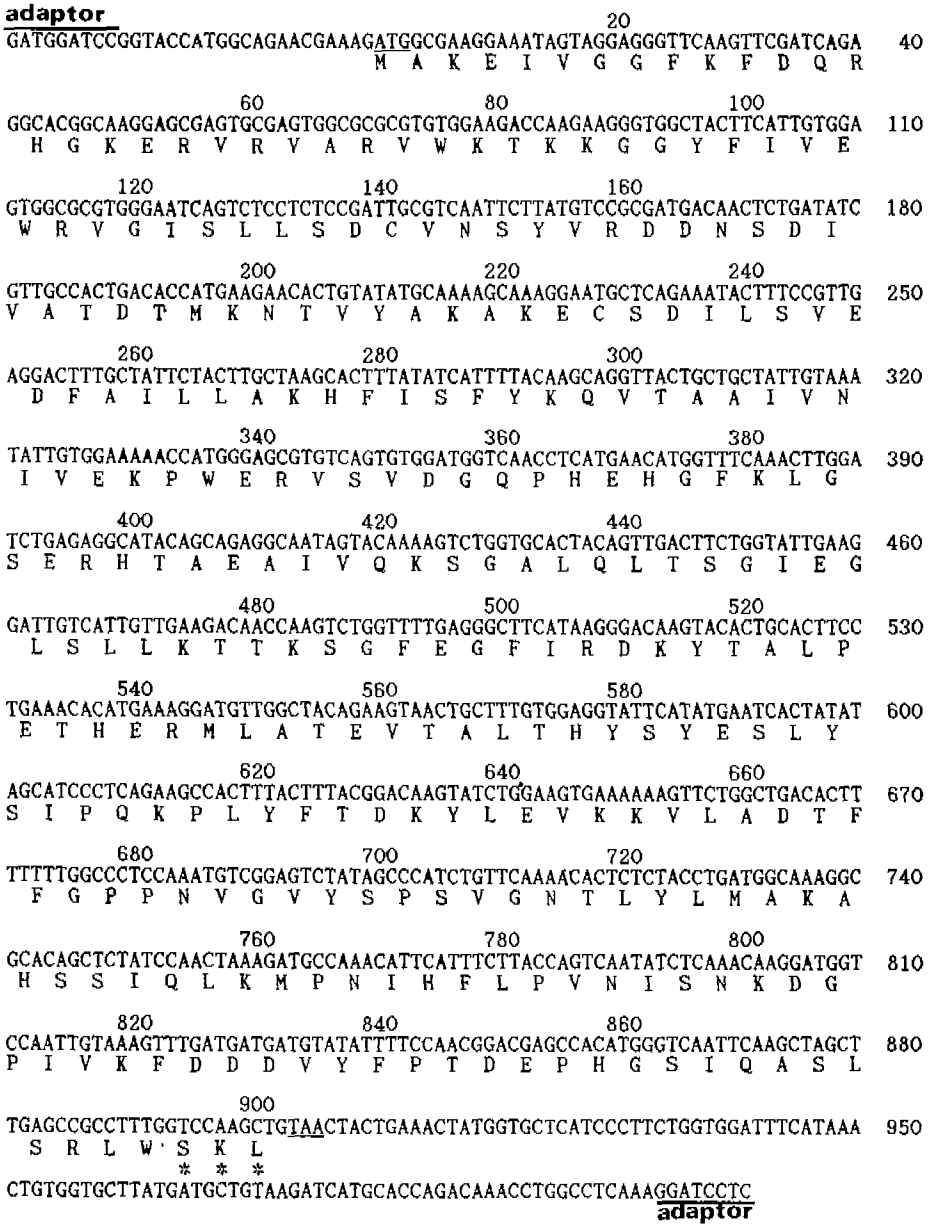

Fig. 3. Nucleotide sequences and deduced amino acid sequences of the cDNA clone, pcClNUO-02. The translation start and termination codon are underlined. The putative peroxisome-targeting signal (***) is present at carboxyl terminal. EMBL Data Bank accession number is X76287.

in *E. coli*.

On the basis of the deduced amino acid sequence, uricase II subunit of *C. lineata* had molecular weight of 33~34 kD similar to that of soybean (Bergmann *et al.*, 1983). Two spots with M.W. of 33~34 kD on the 2-D gel for the total soluble nodule proteins (Choi and An, 1991) may be uricase II subunit on the basis of their molecular weight and abundance. However, purification of uricase II from the nodule of *C. lineata* and expression of the cDNA in *E. coli* will provide a clear evidence whether uricase II of *C. lineata* is heterotetramer or homotetramer.

Table 1. Deduced amino acid composition of pcClNUO-01, pcClNUO-02 and pNOD35

Residue	Number of residues		
	pcClNUO-01	pcClNUO-02	pNOD35
Ala	22	19	18
Arg	13	12	14
Asn	12	9	11
Asp	14	16	16
Cys	2	2	2
Gln	11	9	15
Glu	21	19	20
Gly	17	18	17
His	8	9	8
Ile	18	18	15
Leu	27	25	25
Lys	25	26	23
Met	5	5	4
Phe	15	15	17
Pro	14	13	14
Ser	23	25	23
Thr	16	17	20
Trp	6	5	5
Tyr	13	13	13
Val	26	26	29
Total residues	309	308	301

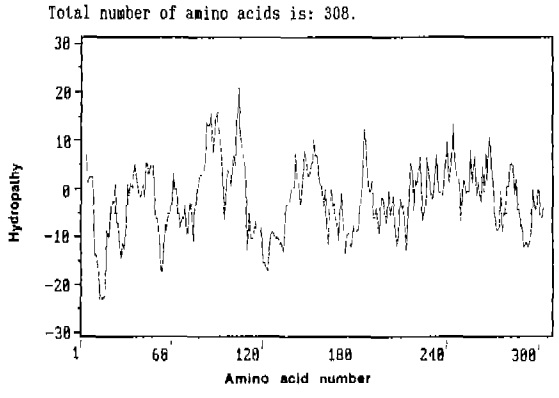

Fig. 4. Hydrophobicity profile of ORF from pcClNUO-01. Hydropobicityprofile of pcClNUO-02 is very similar to Fig. 4.

Tissue-specific expression of uricase II from *C. lineata*. Uricase is a key enzyme involved in ureide production, and the nodule form of this enzyme (uricase II) is different in all physicochemical properties from that found in the uninfected root or leaf (Bergmann *et al.*, 1983).

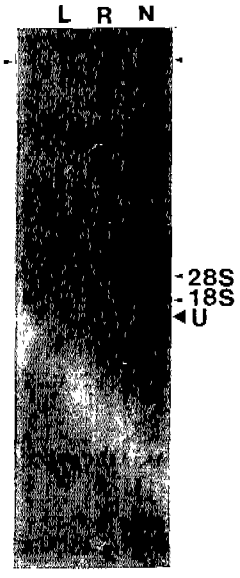

Fig. 5. Northern analysis with 1.6 kb insert of pcClNUO-01 as a probe to determine tissue-specific expression of uricase II gene in *C. lineata*. L, R and N indicates leaf, root and nodule, respectively. 28S and 18S indicate 28S rRNA and 18S rRNA. U indicates uricase II transcript hybridized with the probe.

Northern analysis showed that uricase II gene in *C. lineata* was expressed only in the nodule with no cross-hybridization with RNAs from uninfected root and leaf (Fig. 5). The size of uricase II transcripts was about 1.6 kb. Nodule-specific expression of uricase II gene was also reported in soybean (Nguyen *et al.*, 1985) and french bean (Sanchez *et al.*, 1987).

Our preliminary data showed uricase activity even in uninfected root, although 14 times less than that of the root nodule. This uricase activity in uninfected root was thought to be due to the product of a different gene (Nguyen *et al.* 1985) and/or a different enzyme using the same substrate (Tajima *et al.*, 1985).

Late nodulins are induced shortly before nitrogen fixation (Nap and Bisseling, 1990; Verma and Delauney, 1988). They include enzymes involved in nitrogen assimilation, carbon metabolism and amide and urcide biogenesis (Schubert, 1986; Egli *et al.*, 1989). Initial expression of uricase II transcripts in soybean and french bean nodules is not dependent on active nitrogen fixation (Larsen and Jochimsen, 1986; Sanchez *et al.* 1987). To determine the time of appearance of uricase II mRNA, total RNA from nodule or nodulated root was slot-blotted and hybridized with pcClNUO-01 (Fig. 6). The result showed that

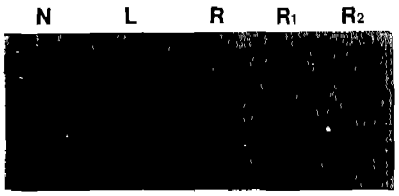

Fig. 6. Slot-blot hybridization with 1.6 kb insert of pcCl-NUO-01 as a probe to determine expression time of uricase II gene in *C. lineata* root nodule. L, leaf; R, unioculated root; R1, roots, 7 days after inoculation; R_2, roots, 10 days after inoculation; N, nodules, 21 days after inoculation.

mRNA for uricase II appeared in about 7 days after infection. This result is similar to that of soybean, in which uricase II mRNA appeared between 6 and 9 days after infection (Nguyen *et al.*, 1985). Northern analysis using the leghemogloin gene as a probe and measurement of nitrogenase activity during nodule development will determine whether uricase II is induced before or after the start of nitrogen fixation.

Genomic Southern hybridization. Many proteins are encoded in the plant chromosomes by multiple copy genes. cDNA and genomic clones for uricase II have been obtained from soybean (Nguyen *et al.* 1985) and cDNA clones from *P. vulgaris* (Sanchez *et al.* 1987). However, it is still not clear how many genes encode uricase II. Southern hybridization of *Eco*RI digests of soybean genomic DNA with uricase II probe suggested that there may be multiple genes for this enzyme (Nguyen *et al.*, 1985), but there was also a possibility that these hybridization band pattern was due to allelic variants rather than separate genes.

Southern hybridization of *C. lineata* genomic DNA, digested with several restriction enzymes, also revealed three to five restriction fragments hybridizing with pcCl-NUO-01 probe (Fig. 7). Southern hybridization data and the fact that two different cDNA clones were isolated from the nodule cDNA library suggests that uricase II gene may be present as multiple genes on the genome of *C. lineata*, but isolation of genomic clones will provide more accurate information.

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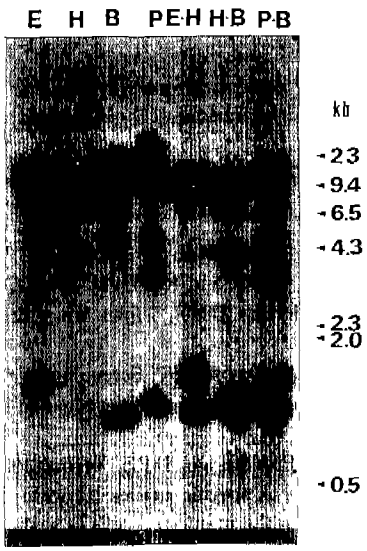

Fig. 7. Southern hybridization of *C. lineata* genomic DNA with 1.6 kb insert of pcClNUO-01 as a probe. M, λ/*Hin*dIII size marker; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; P, *Pst*I.

적 요

대두의 uricase II cDNA를 탐침으로 plaque 혼성화 방법에 의해 해녀콩의 뿌리혹 cDNA library로부터 두 개의 phage 클론(λClNUO-01, λClNUO-02)을 선별하였다. 두 phage 클론은 약 1.6 kb와 1.0 kb의 insert를 갖고 있었으며 이들의 염기서열을 결정하기 위하여 pUC19과 pBSKS vector에 subclonig(pcCLNUO-01, pcCLNUO-02)하였다. Sanger법에 의해 염기서열을 결정한 결과, 두 클론은 각각 1,611 bp와 1,024 bp로 이루어져 있었으며 pcClNUO-01은 308개의 아미노산, pcClNUO-02는 301개의 아미노산을 암호화하는 open reading frame(ORF)을 갖고 있었다. 두 클론의 ORF의 염기서열은 대두의 uricase II와 각각 88.9%, 89.3%의 상동성을 보여주었으며, 아미노산 서열은 84.1%, 85.4%의 상동성을 보여주었다. pcClNUO-01의 경우, 종결코돈으로부터 313 NT 하류쪽에 진핵생물의 poly(A) 첨가 신호인 AATAAA 서열이 존재하였으며 이로부터 21 NT 하류쪽에 17 잔기의 poly(A)가 존재하였다. 두 클론의 염기서열에서 추정된 아미노산 서열의 카르복시 말단에는 세포질에서 합성된 몇몇 단백질들이 peroxisome으로 수송되는데 필요한 신호서열인 Ser-Lys-Leu-COOH 서열이 존재하고 있었다. 두 클론의 염기서열을 토대로 아미노산 조성을 살펴본 결과, 염기성 아미노산(Arg, His, Lys)과 산성 아미노산(Asp, Glu)이 각각 46 대 35, 47 대 35의 비를 보여주었는데 이는 uricase II 단백질의 염기성 성질을 보

여주는 결과로 추정된다. Northern 혼성화 결과 해녀콩에서 uricase II는 뿌리혹에서만 특이적으로 발현됨을 볼 수 있었고, 게놈 혼성화 반응 결과는 uricase II 유전자가 해녀콩 게놈상에 유전자 가족으로 존재할 수 있음을 보여주었다.

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