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Nodulin Regulation in Common Bean Nodules Induced by Bacterial Mutants

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Nodulin expression was evaluated in nodules of common bean (*Phaseolus vulgaris*) induced by *Rhizobium phaseoli* mutants and an *Agrobacterium* transconjugant. Either noninfected, slow-developed, or ineffective nodules, or nodules arrested after bacterial release were formed. The expression levels of ENOD2, uricase-II, leghemoglobin (Lb), and nodulin-30 (Npv-30) transcripts were compared in nodules induced by wild type and mutant strains at initial (12 d) and terminal (21 d) developmental stages. Uricase-II mRNA was detected in "empty" nodules produced by three different mutants, suggesting an additional regulation of this nodulin during the early stages of nodulation. Accumulation of ENOD2 and uricase-II transcripts were observed in slow-developed but not in arrested nodules. Npv-30 and Lb mRNAs were only found in nodules containing infected cells; however, their relative levels differ depending on the nodule-inducing mutant. Ineffective strains produced nodules with similar initial development and nodulin gene expression, but decreased amounts of late nodulin transcripts at the terminal stage. Correlations that suggest conditions for the initial and coordinated regulation of nodulin expression in determinate-type nodules are discussed.

Additional keywords: nitrogen fixation, nodule development, nodulin genes, *Rhizobium*-legume symbiosis.

Rhizobium-legume interactions result in the formation of nitrogen-fixing nodules. The nodulation process is regarded as a suitable model to study plant development, and has been analyzed in terms of differential gene expression (Nap and Bisseling 1990), morphogenesis (Sprent 1989; Scheres *et al.* 1990), and metabolic compartmentalization (Dilworth and Glenn 1984; Kouchi *et al.* 1988). The exchange of molecular signals between the bacteria and the host-plant are decisive for nodule organogenesis and gene regulation (Long 1989; Bisseling *et al.* 1990; Sánchez *et al.* 1991)

Recent reviews describe the differential expression of nodulin genes during nodule development (Verma and Delauney 1988; Nap and Bisseling 1990; Sánchez *et al.* 1991). Nodulins can be classified as early and late, based on the temporal course of their expression. Some nodulins can be used as phenotypic markers because they are involved in organogenesis and/or physiological specialization of nitrogen-fixing nodules (Scheres *et al.* 1990; Sánchez *et al.* 1991). We have studied several of these "indicative" nodulins in common bean nodules (Padilla *et al.* 1987; Campos *et al.* 1987; Sánchez *et al.* 1987; Sánchez *et al.* 1991). They include: ENOD2, which encodes for a hydroxyproline-rich protein associated with nodule induction (Franssen *et al.* 1987) and is expressed in nodule parenchyma, a region of the nodule recognized as a diffusion barrier to oxygen (van de Wiel *et al.*1990; Layzell *et al.* 1990); leghemoglobins, which are oxygen-binding proteins with a role in regulating O_2 diffusion for bacterial respiration and nitrogenase protection and are found in the cytosol of infected plant cells (Appleby 1984); uricase-II (nodule-specific urate oxidase), which is preferentially localized in the peroxisomes of uninfected nodule cells of ureide-producing tropical legumes (Bergman *et al.* 1983; Nguyen *et al.* 1985); nodulin-30 (Npv-30), which includes a gene family that encodes transcripts abundantly expressed in effective nodules of *Phaseolus vulgaris* L. (Campos *et al.* 1987) and has common features to a nodulin gene family previously described in soybeans (F. Campos, C. Carsolio, M. Rocha-Sosa, and F. Sánchez, in preparation; Verma and Delauney 1988; Sánchez *et al.* 1991); and glutamine synthetases (GS), which participate in ammonia assimilation. Data concerning the regulation of the GS gene family in common bean nodules have been recently published (Cock *et al.* 1990).

The use of bacterial mutants that impair symbiosis has contributed to understanding the role of various bacterial genes in nodule development and plant-gene expression (Govers *et al.* 1986; Morrison and Verma 1987; Sánchez *et al.* 1991). Mutations that affect nodulation and nitrogen fixation of *Rhizobium leguminosarum* bv. *phaseoli* Jordan, were obtained by insertional mutagenesis linked to purine auxotrophy (Noel *et al.* 1988), lipopolysaccharide synthesis (Noel *et al.* 1986), and respiratory phenotypes (Soberón *et al.* 1990; M. Soberón, G. R. Aguilar, J. E. Padilla, and F. Sánchez, unpublished). Alternative strategies for the analysis of common bean-*R. l.* bv. *phaseoli* interactions, included genetic complementation of pSym-cured strains for nodulation (Cevallos *et al.* 1989), site-directed mutagenesis of *nifHDK* operons, (Romero *et al.* 1988), and *Agrobacterium* pSym transconjugants (Martínez *et al.*

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1987; Brom *et al.* 1988).

This paper reports an analysis of nodulin gene expression in common bean nodules induced by mutant strains. Quantitative data on transcript levels of four indicative nodulins at two nodulation stages are presented. Results show diverse and unexpected patterns of nodulin expression. We discuss some conditions that might participate in the regulation of these nodulin genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this work are listed in Table 1. Yeast extract-mannitol (YM) medium was described elsewhere (Diebold and Noel 1989). Antibiotics were used at the following concentrations (in $\mu g\ ml^{-1}$): rifampycin (Rif), 50; tetracycline (Tc), 5; kanamycin (Km), 30; spectinomycin (Sp), 100; chloramphenicol (Cm), 15; nalidixic acid (Nal), 10; and streptomycin (Str), 100.

Strain construction and DNA manipulations. Strain CE330 was used as wild type. It forms nonmucoid colonies (Exo^-) on agar plates, and nodulates effectively (Diebold and Noel 1989). Mutation Exo^- was introduced into strain CFN2001 (a pSym-cured CFN42; Palacios *et al.* 1983) by pJB3-mediated conjugal transfer as described (Noel *et al.* 1984). The resulting strain (JP032) was used as a plasmid receptor to obtain strains JP32C and JP32F (Table 1). Nodules formed by these Exo^- strains allowed a more efficient nodule RNA isolation. DNA was transferred to a pTi-cured *Agrobacterium tumefaciens* strain (Brom *et al.* 1988) to look for nodulin expression induced by transconjugants. Matings for plasmid transfer and marker selection were done as described by Cevallos *et al.* (1989).

Plant growth and inoculation. *P. vulgaris* cv. Negro Jamapa seeds (ProNaSe, México D.F.) were surface-sterilized in commercial bleach and germinated on moist sterile filter paper. A pair of 3-day-old seedlings were transferred to sterile "Tetra-Brick" milk packings (9 × 6 × 18 cm) and inoculated with saturated bacterial suspensions grown in YM medium. Plants were grown hydroponically under greenhouse conditions as reported earlier (Padilla *et al.* 1987).

Analysis of nodulation and nodule harvest. Nodulation was scored at varying times after inoculation. Nodule size, color, and proportion of infected zone were examined and compared to data from original references (Table 1). Sample nodules were checked for recovery of the appropriate strain as reported (Martínez *et al.* 1987). Two stages of development (12 and 21 days after inoculation) were selected to estimate relative levels of nodulin expression. Nodules were harvested and stored as described (Padilla *et al.* 1987).

RNA extraction. Total RNA was extracted from 10-day-old roots, 0.5-cm long root meristems, or nodules by grinding the respective tissue in liquid N_2. A guanidine-HCl/phenol procedure as described by Logeman *et al.* (1987) was used with minor modifications. After purification, total RNA was quantitated spectrophotometrically, diluted to 0.1 $\mu g\ ml^{-1}$ in 70% (v/v) ethanol and stored at −20° C until used.

cDNA probes and northern analysis. Nodulin cDNA clones used to probe nodule RNAs are described in Table

Table 1. Bacterial strains, plasmids, and cDNA probes used in this study

Strain	Origin/genotype, characteristics, or insert	Relevant Phenotypes[a]	Reference
Rhizobium phaseoli			
CE3	CFN42 *str-1*	Exo^+ Nal^r Sm^r Nod^+ Fix^+	Noel *et al.* 1984
CE330	CE3 *exo330*::Tn*5*	Exo^- Str^r Km^r Nod^+ Fix^+	Diebold and Noel 1989
CFN2001	CFN42 cured of p42a and p42d(Sym)	Rif^r Nal^r Nod^-	Palacios *et al.* 1983
JP032	CFN2001 *exo330*::Tn*5*	Rif^r Km^r Nod^-	This study
JP32C	JP032(pNC206::pSM991.25)	Exo^- Rif^r Tc^r	This study
JP32F	JP032(p42d.2210)	Exo^- Rif^r Sp^r	This study
CE106	CE3 *pur106*::Tn*5*	Str^r Km^r Pur^-	Noel *et al.* 1984
CE109	CE3 *lps109*::Tn*5*	Str^r Km^r Lps^-	Noel *et al.* 1984
CE110	CE3 *pur110*::Tn*5*	Str^r Km^r Pur^-	Noel *et al.* 1984
CFN031	CE3 with a Tn*5* insertion	$TMPD^{++}$ Str^r Km^r Cyt o^-	Soberón *et al.* 1990
CFN4202	CE3 with Mu-dI insertion	$TMPD^-$ Str^r Km^r Cyt c^-	M. Soberón (Cuernavaca)
Agrobacterium tumefaciens			
AT42D	GMI9023(pCFN42d)	Rif^r Km^r	Brom *et al.* 1988
AC991	GMI9023(pNC206::pSM991.25)	Rif^r Tc^r	This study
Plasmids			
pCFN42d	pSym::Tn*5* *mob*		Brom *et al.* 1988
p42d.2210	pCFN42d *nifH*a::Sp *nifD*b::Km		Romero *et al.* 1988
pSM991.25	18.5 Kb from pSym containing *nod* regions I & II		Cevallos *et al.* 1989 Vázquez *et al.* 1991
pNC206	IncP1 Cb^r Km^r		A. Pühler (Bielefeld)
cDNA Probes			
pGmENOD2	ENOD2 (Ngm-75) from soybean		Franssen *et al.* 1987
pNF-Lb01	Leghemoglobin (Lb) from common bean		Campos *et al.* 1987
pNF-N30-1	Member of Npv-30 family from common bean		Campos *et al.* 1987
pNF-UR07	Uricase-II from common bean		Sánchez *et al.* 1987
pZm5Sr	5S rRNA from maize		V. Walbot (Stanford)

[a] Pur = purine; Lps = lipopolisaccharide; TMPD = N,N,N',N'-tetramethyl-*p*-phenylenediamine; Cyt = cytochrome.

1. Northern blots were prepared by electrophoresis of 5 μg of RNA in 1.5% agarose gels containing 2.2 M formaldehyde as described (Campos *et al.* 1987). Gels were blotted onto nylon membranes (Hybond-N+, Amersham Corp., Arlington Heights, IL) by alkaline transfer as indicated by the supplier. To estimate nodulin transcript levels, up to 4 μg of RNA was denatured in formaldehyde and formamide at 55° C for 5 min and adjusted to 10× SSC (1× = 0.15 sodium chloride, 0.015 M sodium citrate, pH 7.0). Eight serial dilutions (ranging between 4 and 10^{-2} μg of RNA) were applied by vacuum aspiration with a Slot-blot apparatus (Mani-Fold II, Schleicher and Schuell, Keene, NH). Purified inserts of cDNA clones were ^{32}P-labeled by Multi-Prime Extension (Du Pont, Wilmington, DE), separated from free label, on which at least 0.5 × 10^6 disintegrations per minute per slot were applied. Membranes were hybridized in the presence of 50% (v/v) formamide, in a rotating oven at 42° C (Bachofer, Reutlingen, FRG) after hybridization they were subjected to high-stringency washings (0.1× SSC, 0.1% sodium dodecyl sulfate, 65° C for 20 min, twice). Wet filters were exposed to X-ray film (Kodak) at −70° C with intensifying screens. Conditions of the manufacturer were followed for reprobing of filters.

Determination of relative level of nodulin transcripts. Two or three probes were assayed in the same filter series to exclude differences between them. Whole-root and root meristem RNA were used as negative controls. All lanes and slots were finally reprobed with a maize ribosomal cDNA clone, and the corresponding hybridization signals were used as internal standards. The intensity of spots in autoradiograms was quantitated with a Zenieh SLR 1D/2D Model Soft Laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA). Two-dimensional images were integrated and, by linear regression analysis of the spot intensity vs. RNA dilution, the slope value was calculated for each eight-slot sample within the linear range. These values were normalized with their corresponding 5S rRNA levels for comparisons. Repetitions were averaged and relative increments/decrements of nodulin mRNA levels with respect to 21-day-old wild type nodules were plotted.

RESULTS AND DISCUSSION

In this report, we have used *Rhizobium* symbiotic mutants and *Agrobacterium* transconjugants to correlate distinct blockages of nodule development and the expression of indicative nodulin genes. This correlation is important to reveal particular conditions that regulate plant gene expression in *P. vulgaris* nodules, as has been defined in other systems (Fuller *et al.* 1984; Dickstein *et al.* 1988; Nap and Bisseling 1990).

Developmental expression of indicative nodulins. The size of these four nodulin transcripts in wild type common bean nodules is presented in Figure 1A. A single hybridization band (lane EN2) homologous to the soybean ENOD2 probe was observed, regardless of nodule age and inducing strain. The relative levels of all these mRNAs at 8-, 12-, 21-, and 30-day-old nodules were estimated by a slot-blot hybridization analysis of RNA dilutions (see Materials and Methods; Fig 1B). Maximal accumulation of all indicative nodulin mRNAs was observed at 30 days, except for the early nodulin ENOD2 (12 days). Moreover, the relative level at 8 days compared to maximum was, for ENOD2, 27%; uricase-II, 3.8%; Lb, 0.14%; and Npv-30, 0.015%. Thus, the kinetics of induction in wild type nodules indicates a distinctive temporal pattern for each nodulin.

Bacterial mutants and symbiotic phenotypes of nodules. All strains tested here were derived from the chromosomal background or pSym regions from *R. l.* bv. *phaseoli* strain CE3 (Noel *et al.* 1984; Table 1). Symbiotic phenotypes of nodules formed by these strains were classified in four groups as presented in Table 2.

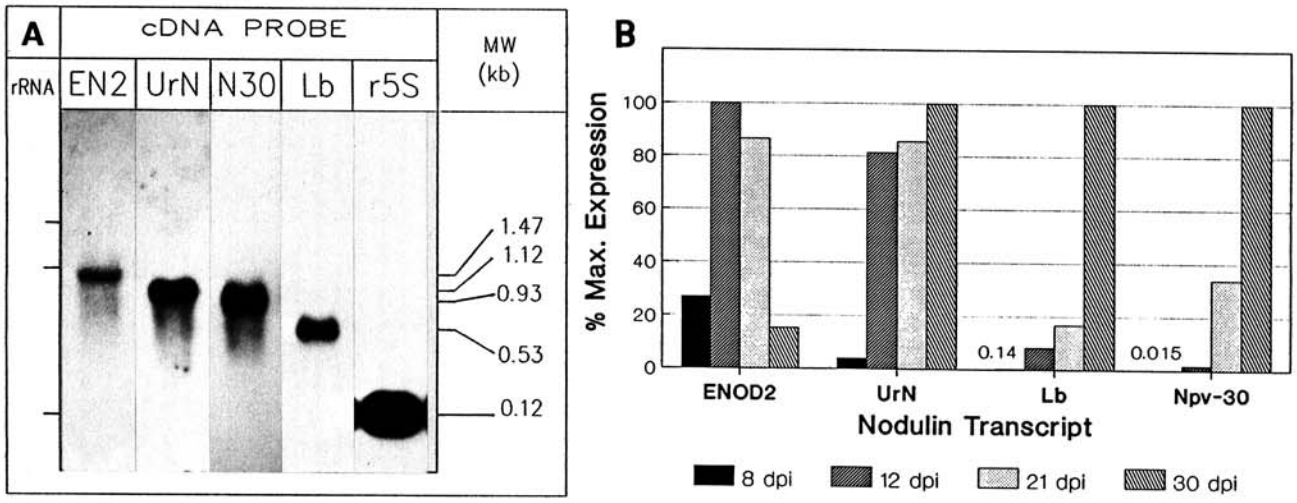

Fig. 1. Expression of four indicative nodulins from common bean nodules. **A,** Northern blot of 21-day-old wild type nodule RNA hybridized against the following cDNA probes (Table 1): ENOD2 (EN2), uricase-II (UrN), nodulin-30 (N30), leghemoglobin (Lb), and 5S ribosomal DNA (r5S). Position of rRNA bands at left, estimated MW of nodulin transcripts at right. **B,** Developmental expression of four indicative nodulins. Relative levels were quantitated after scanning densitometry of autoradiograms from slot-blot hybridization of serial RNA dilutions (see Materials and Methods; Fig. 2B).

An *A. tumefaciens* strain carrying cloned *R. l.* bv. *phaseoli* nodule-inducing genes (Cevallos *et al.* 1989; Vazquez *et al.* 1991) failed to produce even pseudonodules. This result differs with respect to other *Rhizobium* spp. *nod* regions in this background in that they can induce empty nodules and early nodulins on their legume hosts (Govers *et al.* 1986; Dickstein *et al.* 1988; Nap and Bisseling 1990) In contrast, this same strain containing the entire pSym from CE3, does induce nodules with a low level of nitrogen fixation (Table 2; Brom *et al.* 1988). This suggests that other regions from pSym of *R. l.* bv. *phaseoli* strain CE3 are important to induce nodulation by *Agrobacterium*.

Group I includes noninfected (empty) nodules produced by three different strains (Table 1). These mutations have been shown to block bacterial invasion (Noel *et al.* 1986; Vandenbosch *et al.* 1985; M. Soberón, G. R. Aguilar, J. E. Padilla, and F. Sánchez, unpublished) and, as reported in other systems (Govers *et al.* 1986; Dickstein *et al.* 1988; Bisseling *et al.* 1990), transcription of ENOD2 is activated in connection to nodule organogenesis. Unexpectedly, uricase-II, considered as a late nodulin gene, was expressed in all these cases (see below), whereas Lb and Npv-30 mRNAs were undetectable (Fig. 2B).

Group II includes those phenotypes associated with limited infection or bacterial development (Table 2). The respiratory mutant CFN031 formed small and green nodules, containing few bacteroids (Soberón *et al.* 1990). The deficiency of cytochrome *o* in this strain was proposed to restrain the intracellular proliferation of bacteria, causing an interrupted development and premature senescence (Soberón *et al.* 1990). A purine auxotroph (CE106) of *R. l.* bv. *phaseoli* was reported as a noninfective mutant (Noel *et al.* 1984; Vandenbosch *et al.* 1985). In our hands, this strain produced small nodules with a "leaky" and delayed infection phenotype. The correct genetic markers were present in bacteria recovered from nodules (CE106N), but the appearance of revertants was not ruled out. Nodule development in plants inoculated with the transconjugant AT42D was noticeably delayed. Twelve-day-old nodules were white and small; however, at 21 days, various pink nodules showing low nitrogenase activity were present

Table 2. Symbiotic phenotypes of mutant-induced nodules

Group	Aspect of nodules (days)	Strain	Symbiotic phenotype
0	No nodulation	AC991	Nod^-
I	Empty nodules		
	Uninfected, white and small nodules (21)	CE109	Nod^- Inf±
	Uninfected, white and small nodules (21)	CE110	Nod^+ Inf^-
	Uninfected, white and small nodules (21)	CFN4202	Nod^+ Inf^-
II	Arrested or slow development		
	Poorly infected, small and green nodules (15–25)	CFN031	Inf^+ Bad^-
	Small, white to light pink nodules (21)	CE106N[a]	Inf^+ Bar±
	Delayed nodulation (8–18), pink nodules (21)	AT42D	Bad? Fix±
III	Ineffective nodules		
	Half-sized (18), green and pink nodules (21)	JP32C	Bad± Fix^-
	Developed (18), large, pink nodules (21)	JP32F	Bad^+ Fix^-
Control	Developed (18), large, dark pink nodules (21)	CE330	Fix^+

[a]Designation used in this work (see text).

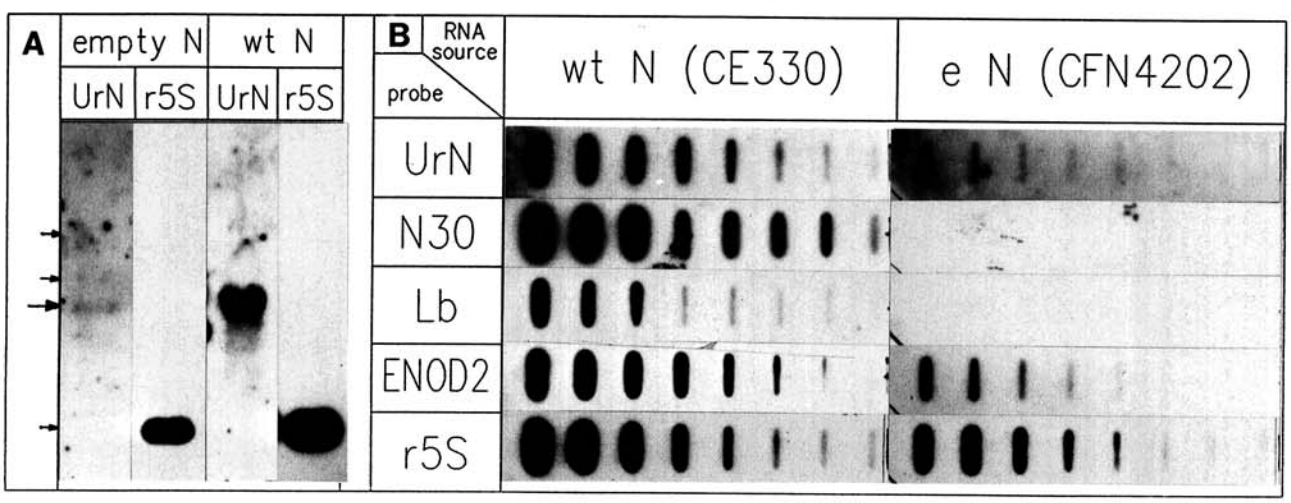

Fig. 2. Expression of uricase-II in noninfected nodules of *Phaseolus vulgaris*. Total RNA from empty nodules (eN) and wild type nodules (wtN) was separated by electrophoresis in denaturing conditions (**A**), or serial dilutions blotted by vacuum aspiration (**B**). Membranes were hybridized with the indicated ^{32}P-labeled probe and washed at high stringency. Arrows at left of A indicate position of ribosomal and uricase-II RNA bands; legends as in Figure 1.

(Brom *et al.* 1988).

In group III nodules, nitrogenase expression is blocked (Cevallos *et al.* 1989; Romero *et al.* 1988). At the initial stage, strains JP32C and JP32F induced normally developed nodules, but after 21 days, external differences with respect to wild type nodules were observed (Table 2).

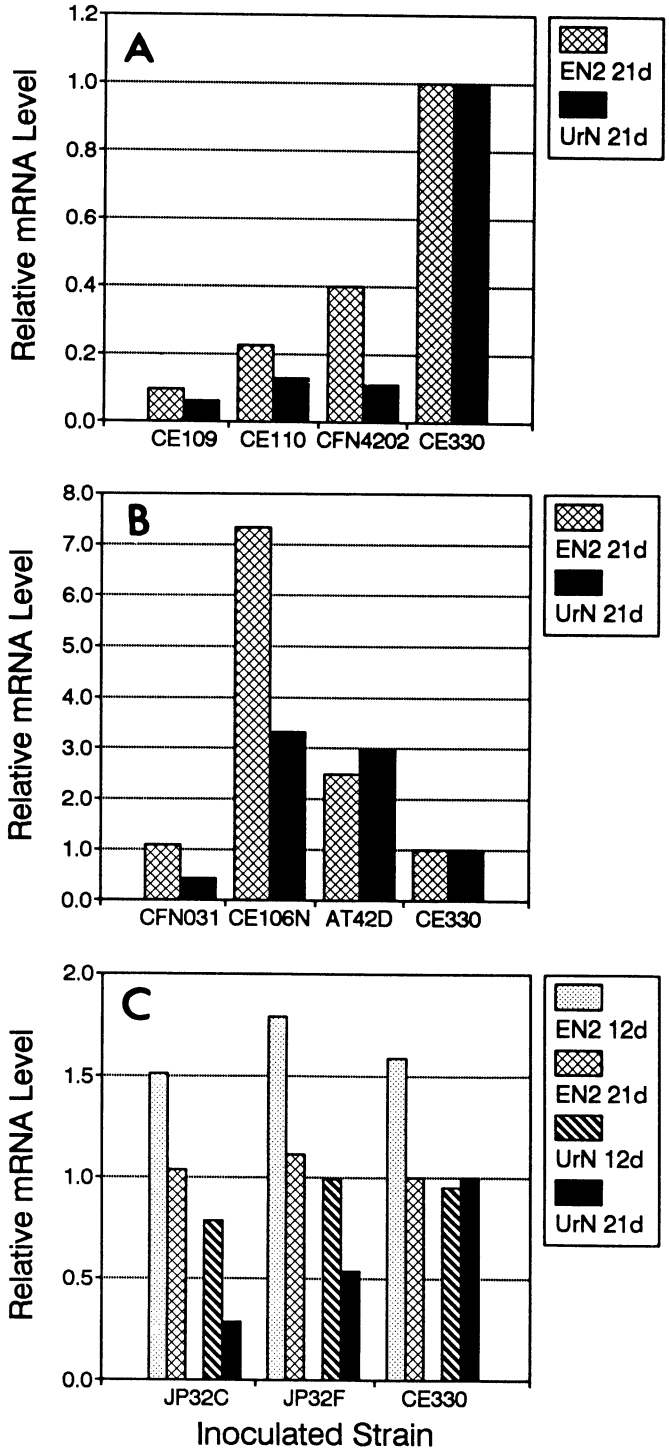

Fig. 3. Relative levels of ENOD2 (EN2) and uricase-II (UrN) mRNAs in mutant-induced nodules. **A,** Empty (group I); **B,** arrested or slow-developed (group II); and **C,** ineffective (group III) nodules. Nodulin mRNA levels were quantitated by densitometry of slot-blot hybridizations of RNA dilutions and were plotted with respect to the 21-day-old wild type (CE330) level.

Nodulin gene regulation in blocked nodulation. The expression of indicative nodulin genes in mutant-induced nodules were compared to those of wild type nodules at initial (12 days) and/or terminal (21 days) stages of development. Relative transcript levels are shown in Figures 3 and 4.

ENOD2 transcripts were found in empty nodules (Fig. 2B), but as judged by densitometry, they represent 10–40% of those from wild type nodules (Fig. 3A). This lower expression may reflect the incipient development of nodule parenchyma. The presence of uricase-II transcripts in empty nodules (Fig. 2B) was reproducibly detected. The band observed in northern blots showed the same size of uricase-II mRNA from wild type nodules (Fig 2A; Sánchez *et al.* 1987). Previous studies on soybean nodules, postulated that uricase-II is expressed only in uninfected cells that differentiate after bacterial release and peroxisome biogenesis (Nguyen *et al.* 1985; Vandenbosch and Newcomb 1986). The absence or reduction of uricase-II antigen in soybean nodules lacking or containing few intracellular bacteria has supported this idea (Stanley *et al.* 1986; Morrison and Verma 1987). On the other hand, uricase-II was immunodetected in cells of the vascular parenchyma of effective nodules (Vaughun and Stegink 1987) and, additionally, marginal uricase-II activity was found in isolated cortex cells (Kohuchi *et al.* 1988). Thus, the observed basal expression (≈10%) of uricase-II gene may be influenced by an initial differentiation of uninfected-type cells in nodule parenchyma, or by vascularization in the central zone, as observed in *P. vulgaris* empty nodules (Vandenbosch *et al.* 1985; Noel *et al.* 1986). Concerning regulatory conditions, it has been proposed that also the transport of uric acid from infected to uninfected cells is responsible for uricase-II synthesis (Nguyen *et al.* 1985). Because expression of this nodulin in common bean nodules occurs before nitrogen fixation and *de novo* ureide production (Sánchez *et al.* 1987), other factors may act as regulators of uricase-II transcription. Moreover, Larsen and Jochimsen (1986) showed that low levels of uricase-II synthesis were found in soybean callus tissue and sterile root cultures subjected to low O_2 levels, although no further evidence of oxygen control of uricase-II is available (Lyzell *et al.* 1990). *In situ* localization of uricase-II transcripts in empty nodules could further identify additional regulatory conditions.

Npv-30 mRNA was only observed in nodules from groups II and III (Fig. 4), indicating that the initial induction of this transcript requires the release of bacteria, similar to Lb and other late nodulins (Fuller *et al.* 1984; Verma *et al.* 1988; Nap and Bisseling 1990).

In group II nodules, three patterns of nodulin expression were found (Figs. 3B and 4A). 1) In CFN031-induced nodules, the terminal ENOD2 mRNA accumulation was unaffected by the arrest of development. However, the blockage of bacterial proliferation may parallel limited expression and degradation of late nodulin mRNAs in the central tissue. 2) In the nodules formed by "leaky" infection phenotype of CE106N, ENOD2 and uricase-II, transcripts were accumulated more than three times of the corresponding wild type levels, while Lb was present at 50%

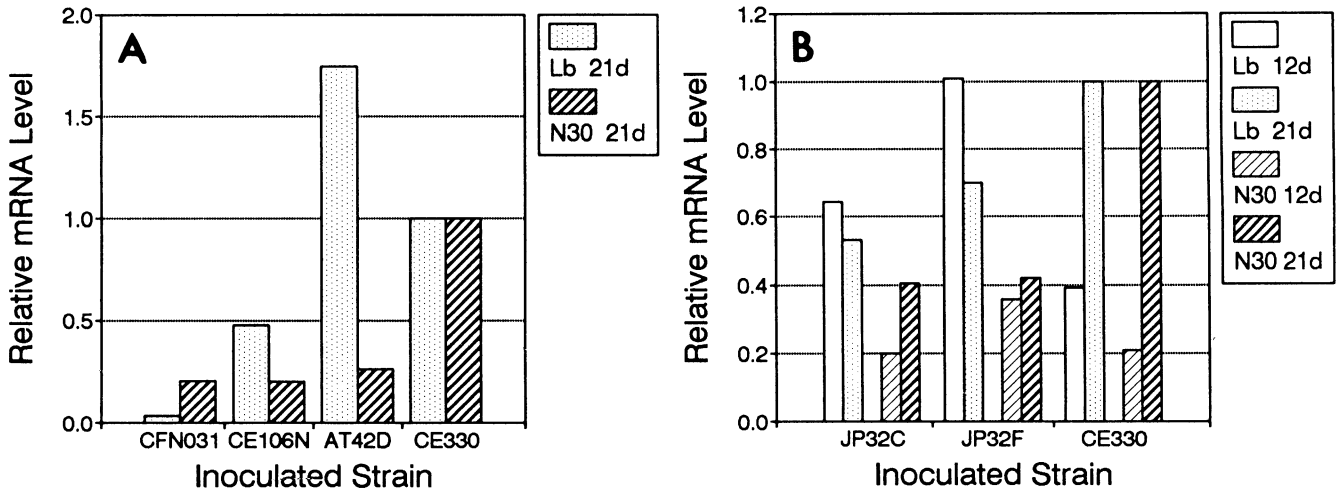

Fig. 4. Relative levels of leghemoglobin (Lb) and nodulin-30 (N30) mRNAs in mutant-induced nodules. **A,** arrested or slow-developed (group II), and **B,** ineffective (group III) nodules. Nodulin mRNA levels were quantitated by densitometry of slot-blot hybridization of RNA dilutions, and were plotted with respect to the 21-day-old wild type (CE330) level.

and Npv-30 at 20%; these were similar to the initial level found in CE330 nodules (not shown) 3) In nodules induced by AT42D, an accumulation of ENOD2, uricase-II, and also of Lb mRNA (2.5, 2.9, and 1.7 times, respectively) were detected. Npv-30 mRNA was accumulated at a very slow rate being almost undetectable at 12 days (less than 0.5%; data not shown). Thus, in slowly developed nodules, uncoupled accumulation of the early and late indicative nodulins suggests changes in dosage of cell types, and also that Npv-30 and Lb gene expression rely on different regulatory conditions (Fig. 4A).

In group III nodules. the temporal ENOD2 profile resembles that of the effective nodules (Fig. 3C). The other nodulins were expressed at similar or higher levels at 12 days, suggesting that blockages in initial nodule differentiation have not occurred. Further into 21 days, transcript levels decreased (uricase-II, Lb) or increased somewhat (Npv-30), but in all cases below the corresponding wild type amounts (Figs. 3C and 4B). It is likely that the subsequent accumulation of late nodulins, requires conditions derived from an active nitrogen fixation status (Fuller *et al.* 1984; Sánchez *et al.* 1991). This behavior is similar to the one observed for nodule GS from common bean (Cock *et al.* 1990).

We conclude from these results that the expression of uricase-II in empty nodules from *P. vulgaris* suggests the existence of regulatory conditions prior to bacterial release. Blockages in nodule development show that at least two events (nodule induction and bacterial release) are decisive for the initial induction of these nodulins. Nevertheless, the coordinated and accurate regulation may demand other steps involving multiple signals as revealed by data derived from arrested, slow-developed, and ineffective nodules. The effects of blocked development on nodulin transcript accumulation in determinate nodules could be explained in part by the characteristic mode of growth and differentiation of infected cells (Sprent 1989; Nap and Bisseling 1990). Morphological studies of nodulin expression of mutant nodules are in progress.

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