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Characterization and Gene Expression of Nodulin Npv30 from Common Bean[1]

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We previously reported that transcripts for a 30-kD nodulin (Npv30) are very abundant in the nodule. In this paper we describe the isolation and characterization of Npv30 cDNA and genomic clones. Npv30 has the following characteristic features: (a) a putative signal sequence at the deduced amino-terminal region, (b) a proline-rich stretch at the carboxy terminus, and (c) a characteristic domain of four cysteines that resemble metal-binding sites. In *Phaseolus vulgaris* L., Npv30 is encoded by a small gene family that shares discrete sequence homologies with another small gene family in soybean. An antibody against a β-galactosidase-Npv30 fusion protein detected two proteins of 28 and 30 kD. Although Npv30 transcripts are very abundant, they encode proteins that are hardly detected in nodule fractions, suggesting that these proteins have a short half-life and/or the mRNAs are strongly regulated at the translational level. Npv30 transcripts were detected in the infected cells of the nodule by in situ hybridization experiments.

The nitrogen-fixing nodule is a specialized organ induced on the roots or stems of leguminous plants by Gram-negative soil bacteria of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium.* During the development and functioning of this organ, sets of both rhizobial and plant host genes are specifically expressed (Sánchez et al., 1991; Fisher and Long, 1992; Hirsch, 1992).

Several nodule-specific plant genes that encode proteins called nodulins are activated during rhizobial infection, nodule morphogenesis, and the establishment of the appropriate environment for nitrogen fixation (Legocki and Verma, 1980; van Kammen, 1984). According to their timing of appearance during nodule development, these genes have been classified into early and late nodulin genes (Govers et al., 1987; Nap and Bisseling, 1990). Genes encoding early nodulins are induced during rhizobial infection, growth of the infection thread, and stimulation of cortical cell divisions (Franssen et al., 1992; Kouchi and Hata, 1993). The expression of some early nodulin genes, such as *enod12,* can be induced by rhizobial lipooligosaccharides known as nodulation (Nod) factors (Pichon et al., 1992; Journet et al., 1994). However, others, such as *enod2,* cannot be induced by nodulation factors, and they seem to be under hormonal regulation (Dehio and Bruijn, 1993; Cooper and Long, 1994).

Late nodulin genes are activated in the developing and mature nodule concomitantly with the onset of nitrogen fixation activity. There are reports of late nodulin genes that appear to be activated later in nodule development. A nodule-specific protease inhibitor is expressed in senescent nodules of winged bean (Manen et al., 1991). Also, in pea a late nodulin gene, PsNOD6, is activated in the infected cells following the induction of the leghemoglobin genes (Kardailsky et al., 1993). In soybean and common bean, several late nodulin genes have been studied and the function of some of their products have been described, for example leghemoglobin (Appleby, 1984; Lee and Verma, 1984; Campos et al., 1987), the γ subunit of Gln synthetase from *Phaseolus vulgaris* (Lara et al., 1983; Padilla et al., 1987), uricase II (Bergmann et al., 1983, Sánchez et al., 1987), and Suc synthase (Thummler and Verma, 1987), among others.

A highly expressed late nodulin gene family of unknown function, the nodulin-A family, has been found in soybean (Fuller et al., 1983; Jacobs et al., 1987). The nodulin-A family comprises at least six members: Ngm-20 (Sandal et al., 1987), Ngm-23 (Mauro et al., 1985), Ngm-26b (Jacobs et al., 1987), Ngm-22/Ngm-27 (Jacobs et al., 1987; Sandal et al., 1987), Ngm-44 (Sengupta-Gopalan et al., 1986), and 15–9-A (Gottlob-McHugh and Johnson, 1991). The members of this gene family show two common characteristics: (a) two domains that are arranged in paired Cys-X_7-Cys motifs, resembling zinc-finger sequences; and (b) a putative signal peptide (Sandal et al., 1987).

In *P. vulgaris,* a group of abundant late nodule-specific transcripts that encode proteins in the 30-kD range (Npv30) was identified by in vitro translation of hybrid-released mRNA (Campos et al., 1987). Npv30 exhibits a peculiar electrofocusing pattern that appeared as a streak across the IEF gradient (pH 3–10). These results suggested that the 30-kD nodulin group could be derived from multiple related transcripts encoding polypeptides of similar molecular mass (Campos et al., 1987).

Here we show that Npv30 is indeed encoded by a gene family similar to the well-characterized nodulin-A gene

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family of soybean described by Jacobs et al. (1987). We were able to detect nodulin Npv30 with antisera against β-galactosidase-Npv30 in soluble nodule fractions, and by using in situ hybridization, we concluded that the Npv30 transcripts were expressed in the infected cells of the nodule.

MATERIALS AND METHODS

Plant Material

Phaseolus vulgaris L. cv Negro Jamapa seeds were obtained from Pronase (México City, México). Seeds were germinated for 2 d, transferred to pots, inoculated with *Rhizobium tropici* strain CIAT 899 (Martínez-Romero et al., 1991), and grown in the greenhouse as previously described by Lara et al. (1984). Nodules were harvested 21 d after inoculation. Uninfected bean roots were obtained and collected 6 d after sowing. Both tissues were harvested and frozen immediately in liquid nitrogen and stored at −70°C.

Isolation of Plant DNA and Southern Blot Analysis

Genomic bean DNA was isolated from leaves by the cetyltrimethylammonium bromide method described by Doyle and Doyle (1990). Approximately 5 μg of DNA were digested, subjected to electrophoresis on 0.8% agarose gel, and transferred to nylon filters. The blot was hybridized at 42°C to random-primed probes in 50% formamide according to the method of Sambrook et al. (1989). The blots were washed three times for 15 min each in 0.1× SSC, 1% SDS at 65°C.

Northern Dot-Blot Analysis

Total RNA from 8-d-old roots and 21-d-old nodules was prepared by the "hot-phenol" method, following the procedure of de Vries et al. (1991). Serial dilutions of root and nodule RNA were dotted onto nylon filters and hybridized to leghemoglobin and Npv30 cDNAs. Hybridization and washes were done at a high stringency as previously described by Sambrook et al. (1989). After hybridization of northern dot blots, the filters were autoradiographed and the intensity of spots was measured with a Bio Image System (Millipore) using Visage software.

DNA Sequencing

Appropriate DNA restriction fragments of cDNA and genomic Npv30 clones were subcloned into the pKS^+ and pSK^+ vectors (Stratagene) and sequenced using the Sequenase kit (United States Biochemical) following the instructions of the manufacturer. Sequence data were analyzed using the software package of the University of Wisconsin Genetics Computer Group.

Production of Npv30 Fused to β-Galactosidase Protein in *Escherichia coli*

A gene fusion between the 3′ terminus of *lacZ* and part of the Npv30 cDNA clone was made using pUR289 as expression vector (Rhüter and Müller-Hill, 1983; Sambrook et al., 1989). A 594-bp *Rsa*I-*Rsa*I fragment of the pNF311 clone coding for the last 159 amino acids of the carboxy terminus (see "Results") was subcloned into the *Sma*I site of pKS^+ (pN308 clone). A *Bam*HI-*Hin*dIII fragment of this clone was introduced into the pUR289 plasmid to produce the pSYC30 plasmid. *E. coli* JM109 was transformed with pSYC30 and a fusion protein was induced as recommended by Sambrook et al. (1989). The β-galactosidase-Npv30 fusion protein was purified by affinity chromatography using a *p*-aminobenzyl-1-thio-β-D-galactopyranoside agarose column (Das, 1990). Antibodies against this fusion protein were produced in a rabbit by standard methods.

Protein Extraction

Total proteins from 8-d-old roots and 21-d-old nodules were extracted by grinding 1 g of frozen tissue and homogenized in 2 mL of 100 mM Tris-HCl, pH 6.8, 5% (w/v) SDS, 5 mM 2-mercaptoethanol. The homogenate was boiled for 5 min and centrifuged at $(1 \times 10^4)g$ for 5 min. The supernatant is referred to as the "total fraction." Freshly extracted roots and nodules were used to prepare soluble protein fractions. One gram of tissue was gently ground in 100 mM Tris-HCl, pH 8.0, 0.5 M Suc, and 5% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at $(1 \times 10^4)g$ for 15 min and the supernatant was considered to be the "soluble fraction."

Western Blot Detection

Proteins were separated by 15% SDS-PAGE as described by Laemmli (1970). Protein gel blotting on Immobilon (Millipore) was carried out as described by Towbin et al. (1979). The immunodetection was performed using secondary antibodies conjugated with alkaline phosphatase (Blake et al., 1984). The preimmune serum did not recognize either Npv30 in a nodule extract or pure β-galactosidase. At a 1:5000 dilution, the antiserum recognized, in the same blot, Npv30 in a nodule extract and β-galactosidase as one of the molecular weight markers.

In Situ Hybridization

Twenty-one-day-old nodules were fixed, dehydrated, and embedded into paraffin according to procedures described by van de Wiel et al. (1990). Nodule sections were hybridized with ^{35}S-UTP-labeled antisense or sense RNA probes (100–1500 Ci/mmol) made from the pN308 plasmid under the conditions reported by Scheres et al. (1990). Sections were stained with 0.025% toluidine blue after 1 week of exposure at 4°C and photographed with a microscope equipped with dark-field and epipolarization optics.

RESULTS

Isolation of the Npv30 cDNA and Gene

A nodule cDNA library of *P. vulgaris* L. Negro Jamapa made in pBR322 (Campos et al., 1987) was screened using the 700-bp *Pst*I insert of pNF-30–1 (Campos et al., 1987). Attempts to isolate a full-length Npv30 cDNA clone in this

library were unsuccessful. A partial 1-kb cDNA clone from Npv30 (pN311) was isolated, sequenced, and used for further analysis. The genomic λ123 clone was isolated from a *P. vulgaris* L. Saxa library (Clontech, Palo Alto, CA) as previously described (Carsolio et al., 1994). The restriction map of the genomic λ123 clone, showing that the *npv*30–1 gene is contained within a 7.2-kb *Sal*I genomic fragment, is presented in Figure 1. The 2.1-kb *Sal*I-*Hin*dIII fragment from λ123 that hybridized to a 5′-specific probe from pN311 was subcloned into the vector pKS$^+$, giving rise to the plasmid pGN338 (Fig. 1). This 2.1-kb *Sal*I-*Hin*dIII fragment was completely sequenced. The analysis of the sequence revealed the presence of a putative promoter, an exon (exon 1) containing most of the coding region, and part of an intron, as compared with the sequence of pN311 clone. The sequence analysis of the control region of this gene has been reported by Carsolio et al. (1994). To complete the sequence of the intron and the other exon (exon 2), we constructed the plasmid pGN339 (Fig. 1) by introducing the 7.2-kb *Sal*I fragment into pKS$^+$. The sequencing reactions were primed with specific oligonucleotides.

Sequence Analysis

The nucleotide sequences of the partial-length cDNA (pN311) and genomic subclones are shown in Figure 2. The nucleotide sequence of Npv30 cDNA includes a 663-bp open reading frame, a 175-bp 3′ untranslated region containing a putative polyadenylation signal, and a poly(A$^+$) tail (Fig. 2).

The deduced protein sequence of the genomic clone (referred to as Npv30–1) corresponds to a polypeptide of 220 amino acid residues and a molecular mass of 23.6 kD. Hydropathy analysis determined by the method of Kyte and Doolittle (1982) (Fig. 3) predicted that the amino terminus of Npv30–1 is highly hydrophobic, and sequence analysis of this region suggested a potential cleavage site for the putative signal peptide (Fig. 2) (von Heijne, 1986).

The deduced amino acid sequence analysis revealed high homology to discrete regions of the previously mentioned late nodulin-A gene family from soybean (Fig. 4) (Jacobs et al., 1987; Sandal et al., 1987). Both the bean gene and the soybean gene family exhibited conserved regions that could encode for putative signal peptides and the two domains arranged in paired Cys-X_7-Cys motifs, resembling zinc-finger sequences or metal-binding domains (Sandal et al., 1987). The highest scores of identity and similarity of the amino acid sequence deduced from the genomic clone was 53.71% identity and 71.42% similarity to Ngm-20 and 47% identity and 65.51% similarity to Ngm-22/Ngm-27.

The comparison of the inferred amino acid sequences between the cDNA and genomic clones (Fig. 2) showed that the cDNA encoded a larger protein with 19 additional amino acids and more Pro residues at the carboxy terminus. Other base changes and mismatches between the clones can also be observed in Figure 2.

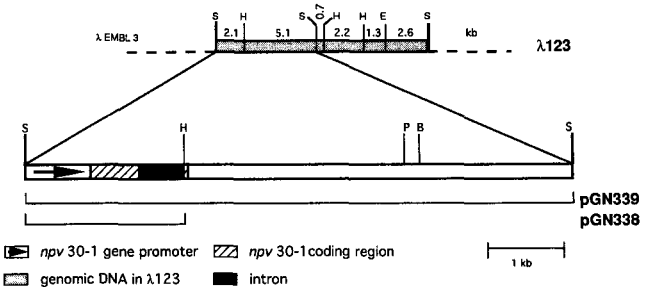

Figure 1. Restriction map of the genomic region of *P. vulgaris* present in the λ123 clone is displayed. The regions of *npv30*–1 are diagrammed as it is shown in the figure. DNA fragments contained in the two derived plasmids, pGN338 and pGN339, used to determine the sequence of *npv30*–1 are indicated below the restriction map. The restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; S, *Sal*I.

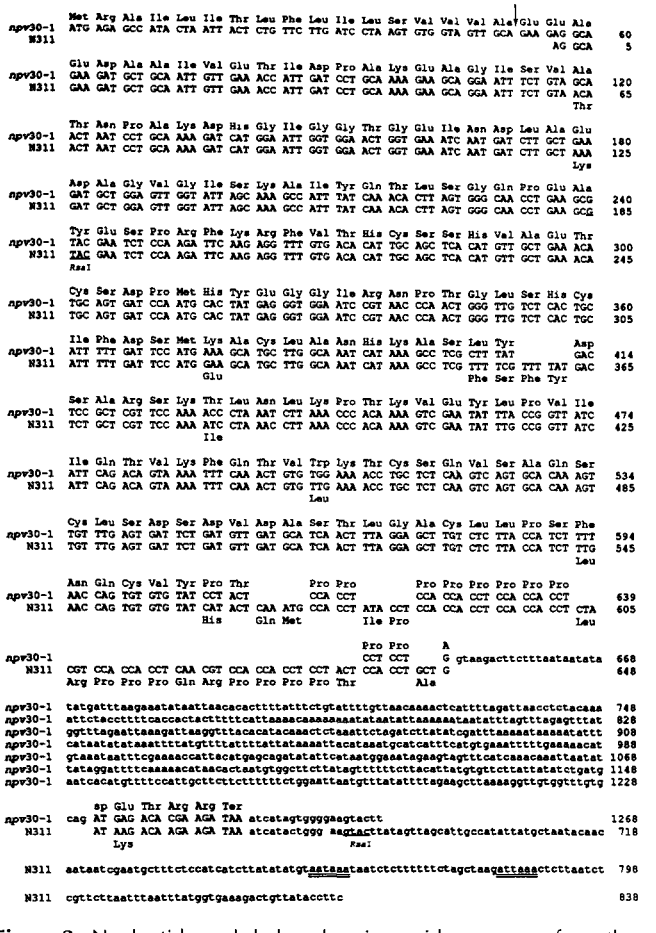

Figure 2. Nucleotide and deduced amino acid sequences from the cDNA (pn311) and genomic (*npv30*–1) clones. Two potential polyadenylation signals (double underlined) located in the cDNA and internal *Rsa*I restriction sites are also indicated. The arrow indicates the potential cleavage site of the putative signal peptide.

Genomic Blot Analysis

To estimate the number of Npv30 genes in common bean, Southern analysis was performed. The 1-kb *Pst*I insert from pN311 was used to probe blots of *P. vulgaris* genomic DNA digested with several enzymes (Fig. 5). At high stringency (0.1× SSC, 65°C), the probe hybridized to

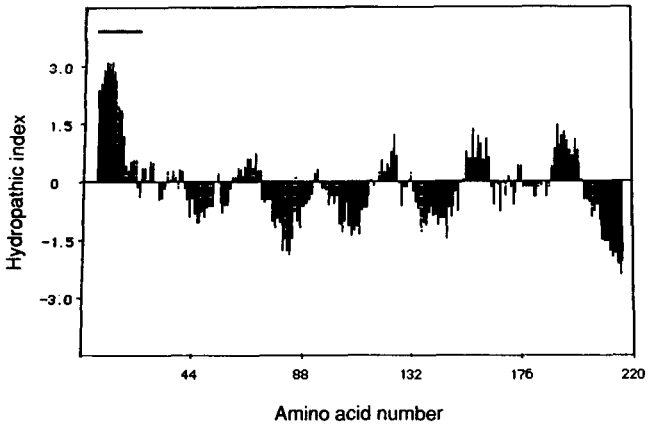

Figure 3. Hydropathic profile of the deduced Npv30–1 amino acid sequence determined by the method of Kyte and Doolittle (1982) with a window of 11 residues. The horizontal bar above indicates the region of the putative signal peptide.

at least three discrete DNA bands. These data together with the alignment of the genomic and the Npv30 cDNA sequences suggest that Npv30 is encoded by a small gene family.

Northern Dot-Blot Analysis

To quantify Npv30 mRNA in the nodule, dot blots of total RNA from 21-d-old nodules of common bean were probed at high stringency with both Npv30 and leghemoglobin cDNAs (Campos et al., 1987). The lengths of both probes were similar (about 600 bp). Densitometric evaluation of the autoradiographs showed that Npv30 transcripts are about 3-fold more abundant than leghemoglobin transcripts (Fig. 6).

Detection of Npv30 in the Nodule

A *lacZ*-Npv30 gene fusion was constructed by subcloning an internal pN311 *Rsa*I-*Rsa*I fragment into pUR289 (see "Materials and Methods"). Such a fusion protein lacked the 81 amino acid residues from the amino terminus of Npv30. An antiserum was raised against the β-galactosidase-Npv30 fusion protein. The antiserum reacted with total and soluble nodule fractions. The antibody detected two bands, of approximately 30 and 28 kD. No signal was obtained with leaf (data not shown) or root extract (Fig. 7).

Localization of the Npv30 Transcripts in the Nodule

The localization of the Npv30 transcripts in nodules was analyzed by in situ hybridization. Sections of bean nodules from 21-d-old plants were hybridized with ^{35}S-UTP-labeled RNA probes transcribed from the pN308 insert (sense and antisense). In Figure 8, only the hybridization with the Npv30 antisense probe is presented. Npv30 transcripts accumulated to high levels in the infected cells, whereas no hybridization was detected in the uninfected cells of the nodule central zone (Fig. 8, B and D).

DISCUSSION

We previously described a group of abundant nodule-specific transcripts in *P. vulgaris* that produce proteins in the 30-kD range when translated in vitro (Npv30). We were also able to isolate a cDNA clone that hybrid selected an mRNA of the Npv30 group (Campos et al., 1987).

In this study, we have continued the characterization of Npv30. Although the cDNA and genomic clones were isolated from different cultivars, the nucleotide sequences indicated that the Npv30 cDNA was derived from a gene different from the *npv30*-1 genomic clone. Nevertheless, the comparison of these clones showed that they were highly homologous (95% identical). These data, together with additional results, such as (a) the electrofocusing pattern of in vitro translated products from hybrid-selected mRNA by pNF-30–1 clone (Campos et al., 1987); (b) Southern blot analysis (Fig. 5); and (c) the partial sequence of another cDNA clone different from N311 (data not shown), suggest that Npv30 is encoded by a gene family.

Analysis of the deduced amino acid sequence revealed that the Npv30 had homology with a highly transcribed nodulin-A family from soybean (Gottlob-McHugh and Johnson, 1991; Richter et al., 1991). Like all members of this soybean family, Npv30 had two regions that contained Cys-X_7-Cys motifs. Cys residues in Npv30 may be involved in forming inter- or intramolecular disulfide bonds. Although it has been reported that proteins with regularly spaced Cys residues may be metal-binding proteins (Berg, 1990), searches of protein data bases did not show homology between Npv30 and any metal-binding protein so far reported. The conservation of these domains in both common bean and soybean suggests a similar function for these nodulins.

Another intriguing feature is the presence of a Pro-rich carboxy terminus in Npv30 and some members of the soybean family, such as Ngm-20 and 15–9-A (Sandal et al., 1987; Gottlob-Mchugh and Johnson, 1991). It is interesting

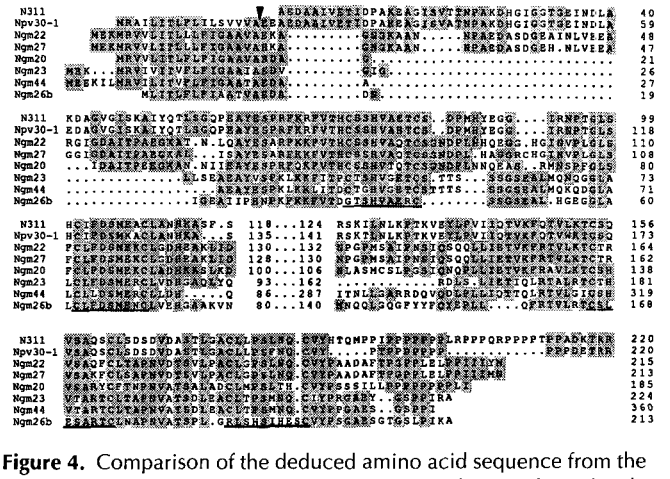

Figure 4. Comparison of the deduced amino acid sequence from the cDNA (pN311) and genomic (*npv30*–1) clones of Npv30 bean family with members of soybean nodulin-A family: Ngm-44, Ngm-27, Ngm-26b, Ngm-23, Ngm-22, and Ngm-20. The shaded areas show the conserved regions. The arrowhead indicates the potential cleavage site of the putative signal peptide.

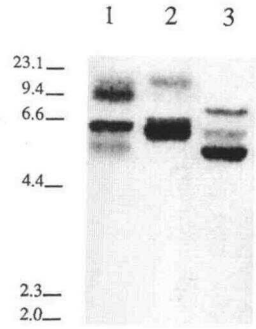

Figure 5. Genomic DNA Southern blot analysis of *P. vulgaris* L. Negro Jamapa. The DNA was digested with *Bgl*II (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3). The blot was hybridized to the *Pst*I insert of the pN311 cDNA clone. DNA markers are indicated in kb.

to note that pN311 cDNA encodes for eight additional Pro residues at the carboxy terminus as compared to the *npv30*-1 clone. These Pro residues could be involved in creating an adequate environment for protein-protein interaction (Yu et al., 1994). Also, the possibility of a loose interaction of these proteins with cell wall proteins through the Pros (Hyps?) cannot be discarded (Showalter, 1993).

The observation that *npv30*-1 and the Ngm-20, Ngm-22 (Sandal et al., 1987), and Ngm-23 genes (Mauro et al., 1985) are interrupted by introns at equivalent positions suggests that the bean *npv30*-1 and soybean nodulin-A family have evolved from a common ancestor.

The fact that the *npv30*-1 gene, as well as all members of the nodulin-A soybean gene family, encodes proteins with a putative signal peptide suggests that these proteins may be associated with membranes or secreted from the cell, possibly to the peribacteroid space. Although the Npv30 proteins were detected in the soluble fraction (Fig. 7), different members of the nodulin-A gene family have different cellular localizations: some have been detected in the cytosol (Ngm-44, Ngm-22), whereas others can be located both in the cytosol and in the peribacteroid membrane (Ngm-26). Some others have been found to be associated with the peribacteroid membrane (Ngm-20, Ngm-23) (Jacobs et al., 1987; Richter et al., 1991). This indicates that it is necessary to carry out a more detailed and careful analysis of the subcellular localization of the Npv30 protein.

Although pN311 cDNA is a partial clone, we might predict that both the genomic *npv30*-1 and pN311 encode for proteins of similar size at the amino terminus (Fig. 2). Nevertheless, pN311 should encode a protein of about 2 kD larger than the Npv30-1 protein, because the former has 19 additional amino acids (Fig. 2).

When the antibody directed at the β-galactosidase-Npv30 fusion protein from *E. coli* was used against total and soluble nodule bean fractions, two proteins of 30 and 28 kD were detected by western blot analysis. These proteins may be the unprocessed protein and the matured protein after the removal of the signal peptide, or alternatively, the antibody may detect pN311 and *npv30*-1 products and/or other members of the Npv30 family.

There is a discrepancy between molecular weight of the derived amino acid sequence and the molecular weight of the immunodetected proteins in the nodule. This phenomenon has already been observed for Ngm-22, Ngm-23 (Jacobs et al., 1987; Richter et al., 1991), and Ngm-24 (Katinakis and Verma, 1985).

In spite of the fact that Npv30 mRNAs are very abundant, it was difficult to immunodetect these proteins in total or soluble nodule fractions. It is worth mentioning that we have been unable (Campos et al., 1987; F. Campos and F. Sánchez, unpublished results) to detect abundant proteins in the 20- to 30-kD range in soluble, peribacteroidal, or total nodule fractions. On the contrary, we can detect mRNAs less abundant than Npv30 that encode abundant proteins such as leghemoglobin (20% of nodule protein), uricase II (2% of nodule protein), and Gln synthetase (2% of nodule protein) by using one- or two-dimensional polyacrylamide gels that are silver stained or stained with Coomassie blue. This suggests that the Npv30 pro-

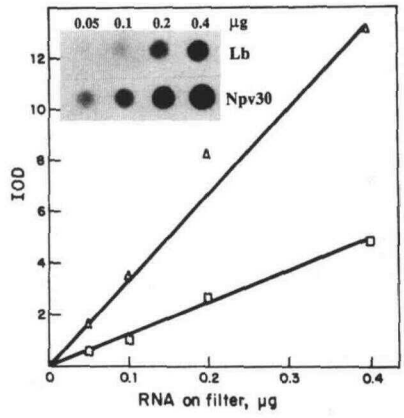

Figure 6. Determination of relative concentration of Npv30 transcripts compared to leghemoglobin (Lb) mRNAs. Serial dilutions of total RNA from 21-d-old nodules were fixed to filters and hybridized to the pN311 *Pst*I insert (△) and the pNF-Lb01 *Pst*I insert (□). Autoradiography of dot-blot hybridization is shown in the inset. The mRNA levels were quantified using an analytical imaging instrument and plotted versus µg of RNA. IOD, Integral optical density.

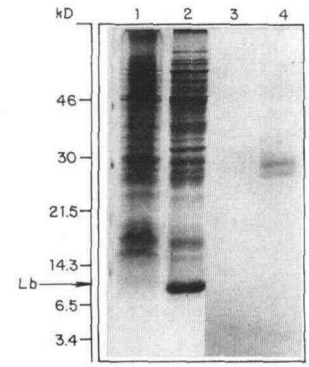

Figure 7. Western immunoblotting analysis of total 8-d-old uninfected root fraction (lanes 1 and 3) and total 21-d-old nodule fraction (lanes 2 and 4). Lanes 1 and 2 were stained with Coomassie blue, and lanes 3 and 4 were immunoblotted with antibodies to β-galactosidase-Npv30 fusion protein. Molecular mass markers are shown in kD on the left margin. The arrow indicates the position where leghemoglobin (Lb) migrated.

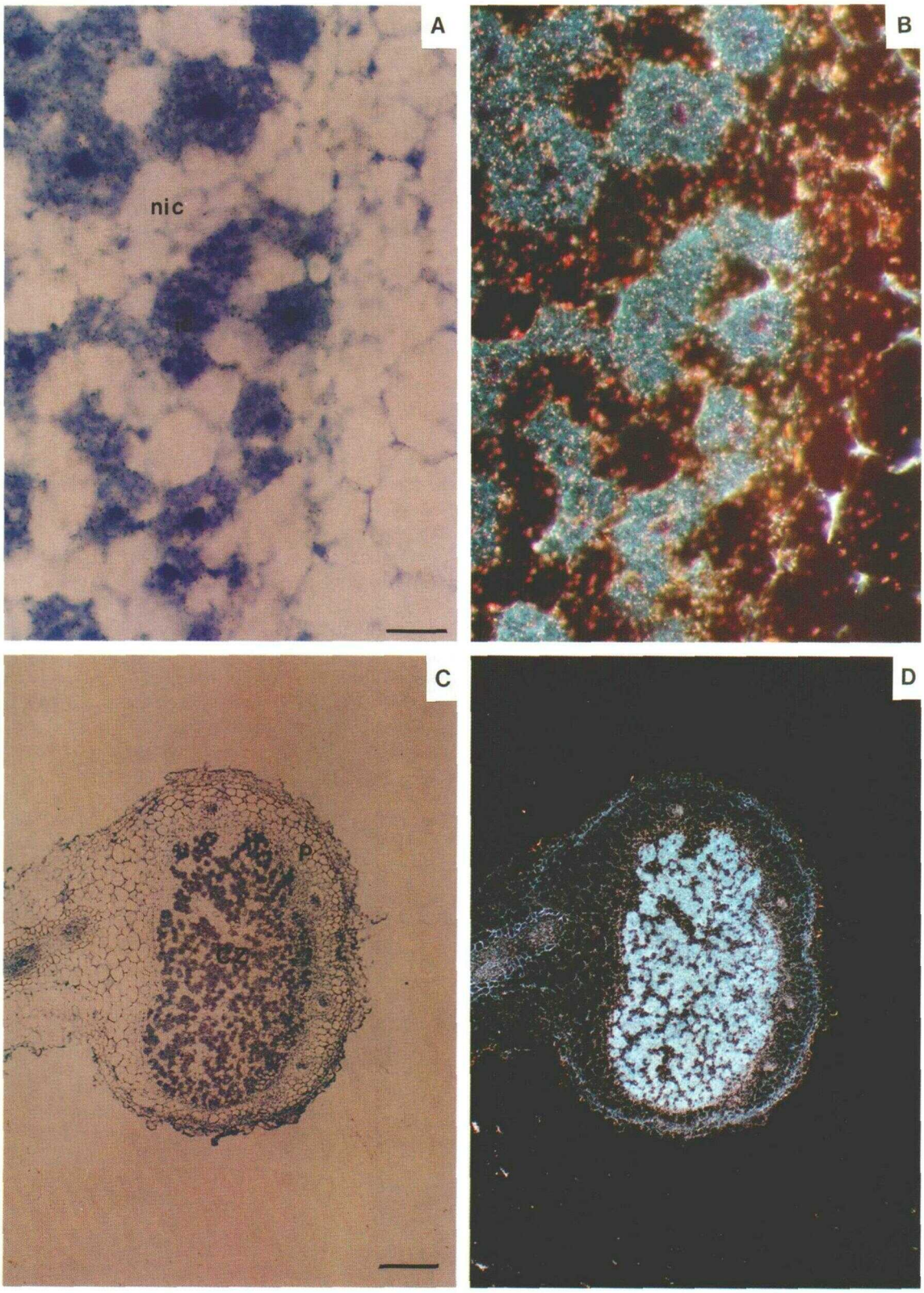

Figure 8. In situ localization of Npv30 transcripts in cross-sections of 21-d-old nodules of *P. vulgaris*. A and C are bright-field micrographs and B and D are dark-field micrographs in which silver grains are visible as white dots. The Npv30 cDNA described here was used to make a ^{35}S-UTP-labeled antisense probe. A and B, Central tissue; bar = 25 μm. C and D, Bar = 500 μm. NIC, Noninfected cell; IC, infected cell; CZ, central zone; P, nodule parenchyma.

teins could have a short half-life and/or that the mRNAs are strongly regulated at the translational level.

From the in situ hybridization light micrographs, we conclude that Npv30 transcripts accumulate in the infected cells of the central tissue (Fig. 8). These results and the finding that the expression of a chimeric *npv30*-1 promoter-GUS gene was restricted to the infected cells in *Lotus corniculatus* transgenic nodules (Carsolio et al., 1994) support the conclusion that Npv30 expression occurs exclusively in the infected cells of the bean nodule.

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The EMBL accession numbers for the sequences reported in this article are Z30345 for the cDNA pN311 and Z30347 for the genomic clone *npv30*-1.

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