Soybean nodulin genes: Analysis of cDNA clones reveals several major tissue-specific sequences in nitrogen-fixing root nodules

(symbiotic nitrogen fixation/cDNA library/hybrid-selection of mRNAs/*in vitro* translation/immunoprecipitation)

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ABSTRACT Plant gene products that could play a role in the process of symbiotic nitrogen fixation in leguminous plants were detected by screening a cDNA library prepared from soybean nodule poly(A)⁺ RNA. About 13% of the 5,700 clones screened contained sequences detectable with a root cDNA probe while about 2,100 clones (37% of the library) contained sequences that were detectable only with nodule cDNA. Five unique sequence species, accounting for more than half of the 2,100 nodule-specific clones, were identified by cross-hybridization experiments. The most abundant species, represented by 860 clones, encodes the well-characterized protein, leghemoglobin (Lb). The other four species, designated NodA, NodB, NodC, and NodD, are represented by 350, 55, 61, and 6 clones, respectively. Each of these four species was found to be encoded by the plant nuclear genome at low copy number. The transcripts corresponding to the nodule-specific clones represented 12–15% (Lb), 6% (NodA), and 0.5–1.1% (NodB, NodC, and NodD) mole fraction of nodule polysomal mRNAs but could not be detected in root polysomal RNA. Hybrid-selection of nodule mRNAs by representative clones and *in vitro* translation indicated that polypeptides of *M*_r 44,000, 27,000, 24,000, and 100,000–120,000 are encoded by NodA, NodB, NodC, and NodD sequences, respectively. These polypeptides reacted with antiserum prepared against total soluble nodule proteins suggesting that the cloned sequences encode nodule-specific proteins, nodulins.

Leguminous plants are able to directly utilize nitrogen gas because of a complex symbiotic association with soil bacteria of the genus *Rhizobium* (for review, see refs. 1–3). Several events are required to achieve this association, including variety- and strain-specific recognition, invasion of the root hair, development of an infection thread, endocytosis of bacteria from the infection thread, and differentiation of bacteria into bacteroids in the host cell cytoplasm. As a result of these interactions, a highly specialized organ, the root nodule, is formed in which symbiotic nitrogen fixation occurs. Lesions in a number of bacterial genes have been shown to interrupt the development of effective nodules at various stages (4–6). On the other hand, several lines of evidence suggest that plant genes also play an active role in this process. Mutations in several legumes have been identified that render them unsusceptible to normally infective strains of *Rhizobium* or unable to form effective nodules (7–9). To determine the role of the plant in this symbiosis, our approach is to isolate the plant gene products that are synthesized specifically in mature nitrogen-fixing nodules and to use these as probes to analyze the organization and regulation of expression of the corresponding genes.

At least three types of nodule-specific proteins, termed nodulins (10, 11), may exist: (i) proteins responsible for the main-

tenance of nodule structure, (ii) enzymes necessary for the specific assimilation of reduced nitrogen, and (iii) proteins that support bacteroid function and thus facilitate nitrogen reduction. One superabundant protein of the third type, leghemoglobin (Lb), has been identified in nodules of all legumes. Other nodule-specific polypeptides have been observed as *in vitro* translation products of nodule mRNAs (11), and one *M*_r 35,000 polypeptide (nodulin-35) has been isolated (10). The latter may be a subunit of the enzyme uricase (12, 13) and would thus be a protein of the second type. Analyses of RNA:cDNA hybridization kinetics have revealed the presence of a moderately abundant nodule-specific RNA class (14, 15) that has been postulated to encode nodulins (16). To isolate these sequences for the analysis of nodulins and nodulin genes, we have constructed a soybean nodule cDNA library. In this report, we describe the isolation and characterization of four unique nodulin coding sequences.

MATERIALS AND METHODS

Growth of Plant Tissues. Twenty-one-day nodule and 4-day control root tissues of soybean (*Glycine max*, cv. Prize) were obtained as described (17, 18) and stored under liquid nitrogen.

cDNA Synthesis and Cloning. Poly(A)⁺ RNA was isolated from total polysomes of nodules or roots as described (14, 17). Double-stranded cDNA was synthesized to nodule RNA essentially by the method of Wickens *et al.* (19), except that the second strand was synthesized at pH 8.0. First-strand reaction mixtures contained 0.5 mM dATP/dCTP/dGTP/dTTP, MeHgOH-denatured nodule poly(A)⁺ RNA at 100 µg/ml, oligo-(dT) at 100 µg/ml, and reverse transcriptase at 10 units/µg of RNA. The yield of first strand was typically 2 µg/10 µg of RNA, which was quantitatively converted to double strand in 30 min by using 10 units of DNA polymerase I per µg of input RNA. Double-stranded cDNA was treated with nuclease S1 (20) and tailed with dCTP (21). *Pst* I-cut pBR322 was tailed with dGTP and hybridized to cDNA. Transformation of *Escherichia coli* strain DH1 by the method of Hanahan as given in ref. 22 gave 3–20 × 10³ tetracycline-resistant colonies per µg of plasmid DNA.

Isolation of DNAs. Plasmid DNAs were purified on CsCl/ethidium bromide gradients (23). *Rhizobium* DNA, mtDNA from 8-day-etiolated soybean shoots, and genomic DNA from soybean embryonic axes were isolated as described (24–26).

Preparation of RNA and DNA Filters. A dot blot apparatus (Bethesda Research Laboratories) was used to bind RNAs to GeneScreen (New England Nuclear) paper in SET buffer (0.15 M NaCl/2 mM EDTA/30 mM Tris·HCl, pH 8.0). Before binding, DNAs were partially depurinated with 0.3 M HCl at room temperature for 5 min, cleaved with NaOH (27), neutralized,

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Abbreviation: Lb, leghemoglobin.

and diluted with 2 M ammonium acetate. Binding was linear up to 50 μ g for DNA and 20 μ g for RNA per 0.07-cm² area.

Hybridization and Hybrid-Selection. cDNA probes were prepared from root and nodule poly(A)⁺ RNAs as in the first-strand reaction except that dNTPs (including [α -³²P]dCTP) were used at 50 μ M. Isolated plasmid inserts were radiolabeled by nick translation. DNA-DNA hybridizations were carried out in 6-fold concentrated SET buffer/2% bovine serum albumin/0.2% polyvinylpyrrolidone/0.2% Ficoll/0.1% NaDodSO₄ containing poly(A) at 100 μ g/ml, denatured *Pst* I-cut pBR322 at 1 μ g/ml, denatured calf thymus DNA at 50 μ g/ml, and probe at 5–100 ng/ml; reactions were carried out at 65°C. RNA hybridizations were in 50% recrystallized formamide at 45°C.

For hybrid-selection, filters containing 50 μ g of purified plasmid DNA on a 0.07-cm² dot were prepared for each clone. These were incubated in 100 μ l of H buffer [0.6 M NaCl/0.1 M Pipes, pH 6.4/0.1% NaDodSO₄/1 mM EDTA/poly(A) (1 mg/ml)/50% formamide] for 4 hr at 45°C and then hybridized for 24 hr with 10 μ g of nodule poly(A)⁺ RNA in H buffer. Filters were washed 10 times at increasing stringency, starting with hybridization buffer and ending with a 55°C wash with 3 mM Pipes/20 mM NaCl and a room temperature wash with 10 mM Tris-HCl (pH 7.6). RNAs were released in 300 μ l of water containing 10 μ g of yeast tRNA by incubation for 90 sec at 100°C followed by quick-freezing in dry ice/ethanol.

Translation and Immunoprecipitation. Hybrid-selected RNAs were ethanol precipitated and then suspended in 20 μ l of rabbit reticulocyte lysate containing 200 μ Ci of [³⁵S]methionine (1 Ci = 37 G Bq) (both from Amersham) and incubated for 90 min at 30°C. Samples (2 μ l) were then boiled in SB buffer (1% NaDodSO₄/10% glycerol/1% 2-mercaptoethanol/0.1% bromophenol blue/10 mM Tris-HCl, pH 6.8) and electrophoresed on 10% acrylamide/NaDodSO₄ gels; products were visualized by fluorography. For immunoprecipitation, translation products (5 or 20 μ l) were incubated with 5 μ l of antinodule or antiroot serum in 200 μ l of 10 mM sodium borate/160 mM NaCl, pH 8.0/bovine serum albumin (1 mg/ml)/0.6% Triton X-100/10 mM methionine containing 20–100 ng of total soluble nodule proteins for 1 hr at 35°C and then at 4°C overnight. Antigen-antibody complexes were precipitated with Sepharose-protein A (Pharmacia), released in SB buffer and analyzed as above.

RESULTS

Construction and Screening of a Nodule cDNA Library. Double-stranded cDNA was prepared from poly(A)⁺ RNA of 21-day nodules and cloned into the *Pst* I site of pBR322. From some 12,000 tetracycline-resistant colonies obtained in several transformations, 5,700 ampicillin-sensitive clones were isolated. Plasmid DNAs isolated from 25 clones picked at random

all contained DNA inserts; however, *Pst* I restriction sites were re-created with only about 90% efficiency. To identify nodule-specific clones, radiolabeled cDNA probes synthesized from root and nodule poly(A)⁺ RNAs were hybridized to replica copies of colonies. About 13% of the clones hybridized well to the root probe and were set aside, while 2,100 other clones (37% of the library) hybridized to the nodule probe only.

To determine which clones contained unique nodule-specific sequences, DNAs isolated from the 25 randomly selected clones were dot blotted onto multiple GeneScreen filters and hybridized to root and nodule cDNA probes. DNAs from 13 clones that hybridized to the nodule cDNA probe only were then probed with a Lb genomic clone, Ch4GmLb11 (28). Five clones hybridized and one of these (clone 23) has been proven to contain Lb coding sequences by restriction enzyme and sequence analysis (data not shown). The insert DNAs from six nonleghemoglobin nodule-specific clones and from clone 23 were then isolated on polyacrylamide gels. The sizes of the inserts are given, together with other data, in Table 1. The inserts were nick-translated and separately hybridized to the plasmid DNA dot blots. Clones 24, 45, and 60 appeared to be unique species while clones 14 (not given in Table 1), 15, and 25 cross-hybridized to each other. The unique-sequence species represented by these clones are designated NodA (clones 14, 15, and 25), NodB (clone 45), NodC (clone 60), and NodD (clone 24).

Relative Abundance of NodA, NodB, NodC, NodD, and Lb RNAs. Probes prepared from isolated insert DNAs were hybridized to replica copies of the 2,100 nodule-specific clones. Clones 14 (which has a 180-base-pair insert), 15, and 25 hybridized to 250, 359, and 325 clones, respectively, that overlapped extensively. Each of the other probes hybridized to unique sets of clones: clone 23 to 860, clone 60 to 61, clone 45 to 55, and clone 24 to 6 clones. The mole fraction of mRNA represented by each species can be estimated from these cloning frequencies assuming that all RNAs were cloned with equal efficiency. These values are given in Table 1.

Quantitation of the relative concentrations of the cloned RNA species was carried out by RNA dot-blot hybridization. Total polysomal RNAs isolated from root and nodule tissues were immobilized on blots and hybridized to individual clone inserts at approximately equal probe DNA concentrations (0.4 pmol of double-strand/ml). Under these conditions, the fraction of probe hybridizing to each spot should be proportional to the amount of homologous sequence present (30). The plot of the fraction of probe hybridized versus the amount of RNA was linear for up to 20 μ g of RNA (Fig. 1). The relative concentration of each species was determined from the slope of the line for it. These values (Table 1) are comparable with those determined on the basis of cloning frequency except for clone 24, which may have been cloned with low efficiency. No hybridization to root RNA

Table 1. Characterization of soybean nodulin clones

Sequence	Clone no.	Insert size, bp	Fraction of library represented, %	RNA, relative mole fraction	Size of genomic <i>Eco</i> RI fragment(s), kb	Translation product, $M_r \times 10^{-3}$
Lb	23	510	15	1.00 (15)	0.6–11.5*	(16) [†]
NodA	15	700	6.3	0.60 (9.0)	7.0	44
	25	900	5.7	0.40 (6.0)	7.0	44
NodB	45	370	1.0	0.07 (1.1)	5.8	27
NodC	60	450	1.1	0.04 (0.6)	11.6	24
NodD	24	600	0.1	0.04 (0.6)	2.9, 3.4	100–120

Relative mole fraction of RNA values are taken from the slopes of the hybridization vs. amount of RNA plots (Fig. 1) after normalization to a value of 1.0 for leghemoglobin. Numbers in parentheses represent normalization to a value of 15% for leghemoglobin. M_r values for translation products are means of at least five determinations except for NodD, which was determined once. bp, Base pairs; kb, kilobase pair(s).

* Several bands; see ref. 28.

[†] Leghemoglobin, not observed in this analysis because soybean leghemoglobins do not contain methionine (see ref. 29).

or to *Rhizobium* or mtDNAs could be detected (e.g., see *Inset* to Fig. 1). In control experiments (not shown) in which either 1 or 0.1 genome equivalent of clone 23 DNA was added to these DNA spots, hybridization of clone 23 probe was clearly visible.

The Nodule-Specific Clones Encode Nodulins. To determine whether the four nodule-specific sequences encode translatable mRNAs, RNAs isolated by hybrid-selection (31) were translated in rabbit reticulocyte lysate containing [35 S]-methionine. Clones 45 and 60 selected mRNAs encoding polypeptides of M_r 27,000 and 24,000, respectively (Fig. 2). Clones 14 (not shown), 15, and 25 each select a mRNA encoding a M_r 44,000 polypeptide. In addition to this major product, clone 25 appears to select mRNAs encoding smaller polypeptides. In five separate experiments, the appearance of smaller polypeptides was variable, indicating that they may be due to peptide or mRNA degradation.

The translation products of the various hybrid-selections were allowed to react with antiserum against root or nodule proteins to determine whether the polypeptides identified in Fig. 2 were specifically present in nodules. The products of clones 60 (NodC), 45 (NodB), 15 (NodA), and 25 (NodA) reacted specifically with the antinodule serum (Fig. 3a and c) while reaction with the antiroot serum was insignificant. In addition, the results show that the NodD sequence is homologous to a mRNA encoding a polypeptide of M_r 100,000–120,000 (Fig. 3b). Limited amounts

precluded testing the reaction of the NodD product with the antiroot serum.

Translation products of total nodule poly(A)⁺ mRNAs were also allowed to react with antinodule and antiroot sera (Fig. 3c). These reactions indicated two polypeptides (M_r , 22,000 and 29,500), which may be more abundant in root tissue. Several polypeptides (M_r , 120,000, 44,000, 41,000, 36,000, 27,500, 27,000, 24,000, and 20,000) reacted with nodule but not (or poorly) with root antiserum. Lb does not appear in the translation products of total nodule mRNA because soybean Lbs lack methionine (29). The M_r 44,000, 27,000, and 24,000 polypeptides may correspond to NodA, NodB, and NodC species (see also Fig. 2), respectively. Several minor bands are observed in the (overexposed) antinodule lane (N) for clone 60 (Fig. 3c). These may be due to some homology of clone 60 with other RNAs. However, the fact that clone 60 hybridizes to a single genomic DNA *Eco*RI fragment (see below) makes this unlikely. It is also possible that this clone contains long G-C tails that weakly select a subset of mRNAs. Whatever the reason, polypeptides of M_r 45,000, 41,000, 30,000, and 20,000 react specifically with the antinodule serum. Thus, in addition to the nodulins encoded by NodA, NodB, NodC, and NodD sequences, the data in Fig. 3 identify six to eight other polypeptides that may be nodulins.

Nodulin Coding Sequences Hybridize to Unique Soybean Genomic Fragments. Southern blots of *Eco*RI-cleaved soybean and *Rhizobium* DNAs were hybridized to nick-translated insert DNAs from clones 15, 25, 24, 45, and 60. These clones hybridized to single *Eco*RI fragments in the soybean genome, except clone 24, which hybridized to two fragments (Fig. 4). The sizes of these fragments are indicated in Table 1. Clones 15 and 25 hybridized to the same size *Eco*RI fragment, further sug-

FIG. 1. Determination of relative RNA concentrations of cloned sequences. Soybean total polysomal RNA from nodule and root and DNAs of *Rhizobium* and soybean mitochondria were fixed to filters and hybridized to various probes (▲, clone 23; ●, clone 15; ■, clone 25; ○, clone 45; □, clone 60; △, clone 24). (*Inset*) Example of dot-blot hybridization. (*Right*) Positions at which nucleic acids were fixed to the filter. (*Left*) Autoradiograph depicting hybridization of clone 15. Spots: row A (top to bottom), 0, 1, 5, 10, 20, and 40 μ g of nodule RNA, 5 μ g of *Rhizobium* DNA; row B (top to bottom), 0, 1, 5, 10, 20, and 40 μ g of root RNA, 0.3 μ g of soybean mtDNA. The fraction of probe hybridizing to nodule RNA spots was determined by scintillation counting and these values are plotted versus μ g of RNA in the spot. The relative concentrations of the various species, determined from the slopes of the lines, are 3.0, clone 23; 1.8, clone 15; 1.2, clone 25; 0.22, clone 45; 0.11 (clones 60 and 24) $\times 10^{-5}$ per μ g of nodule RNA. Values for slopes of root RNA hybridizations were insignificant.

FIG. 2. Translation of hybrid-selected mRNAs. Plasmid DNAs from clones 60, 45, 25, and 15 were immobilized on dot blots and hybridized to poly(A)⁺ RNA from total polysomes of nodules. Hybridized RNA was released, translated in a rabbit reticulocyte lysate containing [35 S]-methionine, and visualized on NaDodSO₄ gels by fluorography. Arrowheads indicate major products corresponding to the indicated clones. Control translations were poly(A)⁺ RNA of total polysomes from root (Rt) and nodule (Nd), RNA selected by pBR322 (pBR), and background (-RNA). M_r markers are $\times 10^{-3}$.

FIG. 3. Immunoprecipitation of translation products of hybrid-selected RNAs. (a) Translation products of clones 15, 25, and 45 (Fig. 2) were allowed to react with antisera prepared against total soluble proteins of root (R) or nodule (N), precipitated with Sepharose-protein A, and electrophoresed. (c) Translation products of clone 60, total nodule poly(A)⁺ RNAs (Total), and RNA selected by pBR322 (pBR) (Fig. 2) were processed similarly. (b) Immunoprecipitation by nodule antiserum of the translation product of RNA selected by clone 24 (N) and background (B). Filled arrowheads indicate products that react specifically with antinodule serum. Products indicated by dots in lane 60 (N) are discussed in the text. Open arrowheads indicate products that react preferentially with antiroot serum. M_r markers are indicated as numbers ($\times 10^{-3}$) on the left (a and b) and as dots on the right (c).

gesting that they represent the same gene. All hybridization intensities were low, indicating that none of these fragments are highly reiterated within the genome. No hybridization to *Rhizobium* DNA was detected with any of the probes, even though several-hundred-fold more genome equivalents were used as compared with soybean DNA. These results, together with those in Fig. 1, confirm the nuclear origin of the NodA, NodB, NodC, and NodD sequences and suggest that they may be encoded by low-copy genes. Furthermore, genomic sequences have been isolated by using cDNA clones 15, 25, 24, and 60 as probes and their restriction maps are consistent with the data shown in Fig. 4 (unpublished data).

DISCUSSION

The nodule cDNA library appears to properly reflect the composition of soybean nodule poly(A)⁺ RNA. About 50% of the clones hybridize to root (13%) or nodule (37%) cDNA probes and most of the clones detectable with the root probe also hybridize to nodule cDNA. Since all clones appear to contain inserts, those that do not hybridize to cDNA probes probably correspond to low-abundance RNA sequences. Extrapolation of our previous data (14) indicates that we are detecting species that are present at more than about 50 molecules per cell. Of the nodule RNAs in this abundance range (>50 molecules per cell), about one-fourth are also present in root (15), which corresponds to the fraction of clones found to hybridize to root

cDNA. Furthermore, a moderately abundant class of nodule-specific RNAs exists containing an estimated 20–40 species, present at about 2,400 molecules per cell, that are presumed to encode nodulins (15, 16). These species should be represented in the 2,100 nodule-specific clones. In fact, the abundances of NodB, NodC, and NodD sequences are approximately in this range.

A superabundant RNA class composing about 18% by mass of nodule mRNA has been interpreted to be Lb RNA (14). However, the present data suggest that the Lb RNA/NodA RNA ratio (mol/mol) is about 2:1. It is therefore likely that previous experiments could not distinguish these two species by hybridization kinetics. Thus, instead of being composed entirely of Lb RNA, the superabundant RNA class probably contains both NodA and Lb sequences. Despite the surprising abundance of the NodA RNA, its translation product (the M_r 44,000 polypeptide) does not appear to accumulate in the soluble cytoplasmic fraction of the nodule (10). Furthermore, this product does not react with antiserum against nodulin-35, the second most abundant protein in this fraction. It is of obvious importance to determine the function of this nodulin.

Assuming that the NodA and Lb RNAs represent a total of 110,000 molecules per cell as previously estimated for the superabundant RNA class (14), then the molar ratios derived from our hybridization experiments imply mRNA abundancies of about 73,000 molecules per cell (Lb), 37,000 molecules per cell (NodA), and 2,900–5,100 molecules per cell (NodB, NodC, and NodD).

FIG. 4. Southern blot hybridization of soybean genomic and *Rhizobium* DNAs to individual nodulin clones. *Rhizobium* DNA (Rh; 5 μ g) and soybean genomic DNA (S; 10 μ g) were cleaved with *Eco*RI, electrophoresed on adjacent lanes of a 1% agarose gel, and blotted. Strips were hybridized to the probes indicated on the top. M_r markers are $\times 10^{-3}$.

Since soybean nodules contain about 611,000 molecules of total polysomal poly(A)⁺ RNAs per cell (14), the above values correspond to mole fractions of 12% (Lb), 6% (NodA), and 0.5–0.8% (NodB, NodC, and NodD), in good agreement with the values determined by cloning frequencies (15%, 6%, and 0.1–1.1%, respectively).

Specific immunoprecipitation of translation products of total nodule poly(A)⁺ RNAs with root vs. nodule antisera has shown that several discrete nodule-specific polypeptides of M_r 20,000–120,000 exist. Moreover, we have shown that the NodA, NodB, NodC, and NodD sequences specifically encode polypeptides of M_r 44,000, 27,000, 24,000, and 100,000–120,000, respectively, that react with nodule antiserum. In keeping with previous nomenclature, we refer to these nodule-specific polypeptides as nodulin-44, nodulin-27, nodulin-24, and nodulin-100. Earlier studies identified, in addition to nodulin-35 (10), several small (M_r <20,000) nodule-specific *in vitro* translation products (11). Since these small polypeptides were reactive with nodule-specific antiserum, they may have contained antigenic determinants of the larger nodulins. They are therefore likely to have arisen due to degradation of RNA or “early quitters” in the wheat germ translation system or to proteolysis (large amounts of total root protein were used in the preparation of nodule-specific antiserum). In fact, larger nodule-specific polypeptides were observed on two-dimensional gels of total translation products and on one-dimensional gels of immunoprecipitated products (11).

Since nodule-specific RNAs are induced in parallel to Lb RNAs and since a number of mutations in *Rhizobium* have been shown to affect the expression of nodulins and nodulin mRNAs (11, 16, 32), we believe that these nodulins play an important role in symbiotic nitrogen fixation. In contrast to the Lb genes, which represent a polymorphic family of closely related sequences (28, 33), the four nodulin sequences identified in this study appear to be represented by unique low-copy genes. The availability of unique nodule-specific clones and the identification of the polypeptides they encode should enable us to study the regulation of these genes, to determine the amino acid sequences of the proteins they encode, and to purify individual nodulins to determine their functions with regard to the role of the legume in symbiotic nitrogen fixation.

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