AsNODc22, a Novel Nodulin Gene of Astragalus sinicus, Encodes a Protein that Localizes along the Cell Wall of Bacteria-Infected Cells in a Nodule

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A novel nodulin gene, AsNODc22, was isolated from Astragalus sinicus cv. Japan (Renge-sou) by differential screening. The transcript of AsNODc22 was nodule specific, and its size was approximately 0.8 knt. AsNODc22 encoded an unknown ORF containing a putative signal peptide and a pair of similar hepta-peptides (CSAQVSC, CSAQILSC). For immunological study, antisera against AsNODc22 protein was prepared in mice. By immunoblot analysis, the antisera detected a nodule specific band at approximately 18 kDa. Immunofluorescence microscopy was used to observe localization of the gene product in the nodule. The signals were seen along the cell wall of bacteria-infected cells, but no significant signals were seen on uninfected cells.

Key words: Astragalus sinicus (renge-sou) — Nodule — Nodulin — Rhizobium huakuii bv. renge — Symbiosis.

Astragalus sinicus (Chinese milk vetch or "Renge-sou" in Japanese) is a legume used as a green manure in rice fields in Japan and southern China. It forms indeterminate type nodules with Rhizobium huakuii (Chen et al. 1991). A. sinicus was once widely used to supply nitrogen to rice fields in Japan and is still an important source of organic nitrogen in China. The interaction between A. sinicus and R. huakuii has been studied primarily with respect to bacteria. Murooka et al. (1993a, b) characterized R. huakuii bv. renge strain B3 isolated from A. sinicus cv. Japan and established the nodulation condition in plant test tubes. Xu and Murooka (1995) identified a plasmid (pRhYM) of approximately 420 kbp, isolated from R. huakuii bv. renge B3 that is responsible for nodulation and nitrogen fixation. Hisamatsu et al. (1996) characterized a new acid exopolysaccharide and cyclic (1→2)β-glucan produced by Rhizobium huakuii.

A. sinicus is potentially valuable for use in the organic farming of rice. Molecular genetic methods are expected to improve the nitrogen fixation efficiency of A. sinicus; basic knowledge about the molecular biology of nodule formation in A. sinicus is therefore needed. Moreover, A. sinicus is small, suitable for cultivation in a laboratory, and able to regenerate adventitious shoots in cotyledon explants (Cho et al. 1995). Agrobacterium-mediated transformation methods have also been developed (our unpublished data). A. sinicus could be another good model plant for the study of molecular mechanism of nodulation in addition to Lotus japonicus (Handberg and Stougaard 1992) or Medicago species (Barker et al. 1990).

The mechanisms of symbiosis and nodule formation in legumes and other species associated with rhizobacteria have been widely studied by molecular biology (Pawlowski and Bisseling 1996). What kinds of genes are at work in the nodule formation? This fundamental problem has been studied in various plant species. A number of genes for nodule-specific proteins called nodulins have been cloned by differential hybridization (Nap and Bisseling 1988, Verma 1992) or differential display (Szczegolowski et al. 1997).

The functions of some of these nodule-specific genes have been predicted from their nucleotide sequences, although most have not been identified (Schultze et al. 1994, Cook et al. 1997). One approach is to reveal the functions of the unidentified genes through histo- and cytochemical characterization of gene expression. Sequential expression of the early nodulin genes has been detected by in situ hybridization; their gene products were thought to participate in the establishment of symbiosis (Schultz et al. 1994). For example, the mRNA of ENOD2 was detected in nodule parenchyma (van de Wiel et al. 1990). The ENOD12 gene was expressed in the distal part of the pre-fixation zone II (Scheres et al. 1990). Leghemoglobin was expressed in the distal part of the pre-fixation zone and the fixation zone (Scheres et al. 1990). Such signals can be used as markers of cell differentiation during the course of nodule formation, as well as for characterization of the gene products themselves.

Another histological and cytological approach is to study the subcellular localization of each of the nodulins. Determining the subcellular localization of the unknown nodulins will assist in the identification of their functions. However, the subcellular localization is known for only a

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few nodulins; most of these are the proline-rich proteins (Sherrier and VandenBosh 1994).

Nodule-specific structures such as infection threads or peri-bacteroid membranes (PBM) are formed in the nodule during infection by bacteria and establishment of symbiosis. Certain kinds of nodulins must be correctly transported into the appropriate organelles for the construction and maintenance of such structures. The localization of various kinds of nodulins is worth study using highly specific antibodies to enhance our understanding of sorting mechanisms.

The aim of our research is to isolate novel nodule genes from A. sinicus and to characterize these genes in order to understand the mechanisms of nodule formation. In this study, a novel nodule gene was cloned from A. sinicus and found to encode an ORF with a putative signal peptide at the N-terminus. Subcellular localization of the gene product was studied by high resolution immuno-fluorescence microscopy.

**Materials and Methods**

**Plant materials**—Seeds of A. sinicus cv. Japan (supplied from Takayama Seed Co. Ltd., Kyoto, Japan) were surface-sterilized and grown in a dish for a week (Murooka et al. 1993a). After inoculation with R. huakuii bv. renge B3, the plants were grown for three to four weeks in nitrogen-free (NFR) medium as described by Murooka et al. (1993a). Harvested nodules, roots, and leaves were frozen in liquid nitrogen and kept at −80°C until used for isolation of nucleic acids or proteins.

**Isolation of DNAs and RNA**—DNA was isolated and purified from R. huakuii bv. renge B3 by the method of Saito and Miura (1963). Plant genomic DNA was isolated by the method of Liu et al. (1995). Plant RNA was isolated by the method of Shirzadegan et al. (1991).

**Construction of cDNA library and screening of nodule-specific clones**—A cDNA library was constructed using reverse-transcribed poly(A)+ RNA from 3-week-old nodules ligated into a lambda-gt10 vector (Time-Saver cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden) and Giga-pack Gold Packaging Kit (Stratagene, La Jolla, CA)) according to the manufacturers' instructions. Oligo(dT) primers and EcoRI/NorI adapters were used in the construction of the library. AMV reverse transcriptase (Takara Shuzo Co. Ltd., Japan) was used to synthesize 32P-labeled cDNA probes from 3-week-old nodule RNA and uninfected root RNA. The probes were used to screen phase plaques of the library for nodule-specific genes. The phage DNA of the positive clones was cut with EcoRI, and the inserts were cloned into the EcoRI site of the pBluescriptK81+ vector (Stratagene). A clone which contained an 0.85 kb insert was named pASc22. Both DNA strands of the pASc22 insert were sequenced using a Cy5 Autoread Sequencing Kit and ALFread automated DNA sequencer (Pharmacia Biotech).

**Southern blot hybridization**—Genomic DNA of A. sinicus and R. huakuii bv. renge B3 was digested with restriction endonucleases according to the manufacturer's instructions (Takara Shuzo). 10 µg of DNA was electrophoresed in each lane of a 1.0% agarose gel in 1×TAE buffer (Sambrook et al. 1989), and blotted onto Hybond-N membrane (Amersham International plc, Buckinghamshire, U.K.). A FITC (fluorescein isothiocyanate)-labeled probe of the EcoRI insert of pASc22 was prepared using a Gene Image Kit (Amersham International) and hybridized to the blotted membrane at 60°C according to the manufacturer's instructions. The hybridized membrane was washed with a final wash of 0.2× SSC (20× SSC is 1.5 M NaCl and 0.5 M trisodium citrate, pH 7.2) and 0.1% (w/v) SDS at 60°C. The hybridized probe was detected by exposure onto Kodak X-Omat AR film (Kodak, Rochester, NY) according to the instructions.

**Northern blotting analysis**—Northern blotting analysis was performed using 20 µg of total RNA from the nodules, roots, and leaves. The RNA was size-separated on a 10% agarose gel in 10 mM phosphate buffer pH 7.0, treated with NaOH, and transferred onto Hybond-N nylon membrane. A 32P-labeled probe of the EcoRI insert of pASc22 was prepared using a Megaprime DNA labeling system (Amersham International) as described by the manufacturer. The membrane was hybridized with the probe in 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 0.1% (w/v) SDS, 50 µg ml−1 denatured DNA, and 1× Denhardt's at 45°C for 12 h. The blots were washed at high stringency and exposed to X-ray film under an intensifying screen at −80°C. The filter was reprobed with a cDNA clone of pea β-tubulin (gift from Dr. Naoki Sato) as a control.

**Preparation of antibody against AsnODC22 protein**—The pASc22 insert was initially subcloned in-frame into pGEX-4T-3 (Pharmacia Biotech) to express the full-length ORF. To improve the product yield, part of the ORF (amino acids 19–147) was amplified by PCR (pGEX-4T-3 containing the ORF as a template, and 5'-GGCATGGATCCTCAATCTTTGCTAAA-3' and 5'-GTTGTGCCATTGGCTGAGGCA-3' as primers). The PCR product was digested with BamHI and PstI and then subcloned in-frame into pGEX-4T-3 again. The GST-AsnODC22 fusion protein was expressed in E. coli strain BL21 and purified according to the manufacturer's instructions. The purified fusion protein was cleaved with thrombin and separated by 15% SDS-PAGE following the method of Laemmli (1970). The target peptide was electroluted from the gel. The recovered peptide was injected into mice for the preparation of antisera (Harlow and Lane 1988).

**Immunoblot analysis**—Frozen samples (20 mg) were ground in liquid nitrogen, resuspended in 60 µl of 2× sample buffer (50 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue), and boiled at 100°C for 5 min. After a brief centrifugation, the proteins in 10 µl of supernatant were size-separated by SDS-PAGE on a 15% resolving gel and blotted onto PVDF membrane (Immobilon, Millipore corporation, MA). The filter was reacted with the primary antibody (anti-AsnODC22 protein) at a dilution of 1: 250 for 2 h and then incubated with alkaline phosphatase-conjugated anti-mouse IgG antibody (Boehringer Mannheim GmbH, Germany) at a dilution of 1: 2,500 according to the manufacturer's instructions.

Detection was accomplished using nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP).

**Immunofluorescence microscopy**—Three-week-old nodules of A. sinicus were fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4 for 12 h, embedded in Technovit 7100 resin (Hermaus Kulzer GmbH, Wehrheim, Germany), and cut into thin sections (0.5–2 µm) as previously described (Kuroiwa et al. 1990, Fujie et al. 1993, 1994). The sections were attached to a coverslip coated with 3-amino-propyltriethoxysilane and glutaraldehyde (Fujie et al. 1995), washed briefly in PBS, and blocked with 1% BSA in PBS. Primary antibody (anti-AsnODC22 protein antiserum) was added to the block-
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ing solution at a dilution of 1:200 and incubated for 2 h. The sections were washed twice in PBS for 15 min with gentle agitation at room temperature, incubated with FITC-conjugated anti-mouse IgG antibody (TAGO Inc., Burlingame, CA) in PBS at a dilution of 1:100 for 30 min at room temperature, washed twice in PBS for 5 min at room temperature, stained with 4',6-diamidino-2-phenylindole (DAPI) as described previously (Fujie et al., 1993), and observed under an epi-fluorescence microscope (BX-2 system, Olympus, Japan) equipped with appropriate filters. When preimmun serum was used as primary antibody, no significant signal was observed. Photographs were taken using Ektachrome 400 film (Kodak).

Results

Screening of nodule specific cDNA clone and its DNA sequence—To obtain cDNA clones involved in nodule development in *A. sinicus*, a cDNA library from *A. sinicus* nodules was constructed, and about 1×10^7 plaques were differentially screened with nodule and root mRNA probes. We identified more than 100 nodule-specific or nodule-enhanced clones and classified them into 11 groups based on cross hybridization. It was confirmed that these clones were derived from *A. sinicus* by genomic Southern hybridization (unpublished data).

Partial nucleotide sequences of the clones were determined, and homology searches were performed. One of the clones (pASC22) did not have significant homology to any previously isolated genes and appeared to be a novel nodulin gene. Therefore, the entire nucleotide sequence of the clone was determined (DDBJ accession number AB009450). The clone encoded an ORF consisting of 147 amino acids and was designated as AsNODc22. The deduced amino acid sequence of the ORF was used for a homology search of the sequences reported to the DNA Data Bank of Japan, but no significant homology was found. The ORF contains a leucine-rich hydrophobic region of 24 amino acids at the N-terminus, an N-linked glycosylation signal, and one pair of similar hepta-peptides, CSQVSC and CSQVSC (Fig. 1). The hydrophobic region seems to

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Fig. 1 DNA sequence of AsNODc22. Nucleotide and deduced amino acid sequences of AsNODc22 cDNA. AsNODc22 contained an ORF of 147 amino acids. The ORF has a hydrophobic region of 24 amino acids, a putative signal peptide at the N-terminus (bold letters), and two similar hepta-peptide sequences (boxed regions). An N-linked glycosylation signal is underlined by a dotted line. A putative polyadenylation signal is underlined by a simple line.
function as a signal peptide, and this protein appears to be sorted into the cell membrane or vacuole.

The origin and expression of AsNODc22—Genomic Southern hybridization studies confirmed that AsNODc22 was encoded in the *A. sinicus* genome. There appear to be one or two homologues (Fig. 2A). Expression studies of AsNODc22 confirmed its nodule-specificity. Total RNA was isolated from 3-week-old nodules, uninfected roots, and mature leaves, and the expression of AsNODc22 was determined by northern blot hybridization. The signal was observed only in the nodule lane at approximately 0.8 knt. AsNODc22 is certainly a nodule-specific gene, and the 844 bp insert of pASC22 appears to contain a nearly full length transcript (Fig. 2B).

The accumulation of AsNODc22 protein—For immunological studies of the AsNODc22 protein, a glutathione S-transferase (GST) fusion protein of AsNODc22 was prepared in *E. coli*, and anti-AsNODc22 antisera were raised in mice. The antisera detected a band at 18 kDa in the lane for 3-week-old nodules, but corresponding bands were not detected in the lanes for roots or mature leaves (Fig. 3). No significant signal was detected with preimmune sera at a dilution of 1 : 100 (data not shown). This result demonstrates that the AsNODc22 protein is nodule-specific and that the antisera used in this study is highly specific for the protein.

Localization of AsNODc22 protein in the nodule—Subcellular localization of the AsNODc22 protein was observed by immunofluorescence microscopy of anti-AsNODc22 protein antisera-treated sections embedded in Technovit 7100 resin. The nodule of *A. sinicus* is an indeterminate type (Fig. 4A). The shapes of the cell nuclei, organelle nucleoids, and bacteria nucleoids were made visible by staining the sections with DAPI. In meristems or in the infection zone, the immature cells were small. After infection, the cells grew rapidly larger. In the course of cell development, the size of the nuclei also became larger. This may be due to endoduplication of DNA in the cell nuclei. The mature bacteria-infected cells contained a large vacuole, and the cell nuclei were located along the tonoplast.

When sections were stained using anti-AsNODc22 protein antiserum, the signals were observed along the cell walls of the bacteria-infected cells (Fig. 4B). At low magnification, the signals were seen along the walls of mature cells that contained large numbers of bacteroids. The signal was not observed in meristematic cells, nodule parenchyma, cortex, or other uninfected cells. At high magnification observation around the infection zone, the signals were observed prior to the development of the bacteroids and enlargement of the host cell (Fig. 4C, D).

![Fig. 2 Genomic Southern hybridization and northern hybridization of AsNODc22.](image)

![Fig. 3 Immunoblot analysis of AsNODc22 protein.](image)
Fig. 4 Localization of AsNODc22 protein. Three-week-old nodules were embedded in Technovit 7100 resin and cut into thin longitudinal sections. The sections were stained with DAPI for DNA (A, C, E) and anti-AsNODc22 protein antiserum followed by FITC-conjugated secondary antibody for immunofluorescence microscopy (B, D, F). (A) A longitudinal section of the nodule of A. sinicus. Cell nuclei of A. sinicus are seen as small bright spots. A nodule meristem is seen on the left of the section. Cytoplasm of the infected cells is bluish because the nucleoids of R. huakuii bv. renge B3 were stained with DAPI. (B) The AsNODc22 protein-specific signals are seen as ring-shapes in this section. (C, D) Higher magnification near the meristem. Signals are barely visible in uninfected cells. The signals became visible just after infection and increased in intensity during the course of differentiation. (E, F) Higher magnification of the mature cells in the fixation zone. Individual nucleoids of R. huakuii bv. renge B3 were observed in the cytoplasm of the infected cells. Signals are visible along the cell walls of the infected cells. No significant signals were observed in the nodule parenchyma. Only autofluorescence of the cell wall was observed with excitation by UV or blue light in the endodermis (lower left corner of the picture). Asterisks, infected cells; large arrows, nuclei of infected cells; small arrowheads, nodule parenchyma; large arrow heads, nuclei of uninfected cells (other than nodule parenchyma); small arrow, cell wall of infected cells. Scale bars are 100 µm (A, B) and 50 µm (C, D, E, F).
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This zone may be equivalent to the prefixation zone (zone II) or the inter zone (zone II-III; Vasse et al. 1990, Yang et al. 1991). The signals were not seen in endodermis or nodule parenchyma even at higher magnification (Fig. 4E, F).

The cells embedded in the resin sometimes contain cracks between the cell wall and cytoplasm due to plasmolysis during fixation or embedding. When infected cells containing such cracks were stained using anti-*AsNODc22* protein antiserum, the signals were seen along the cell wall, but no significant signals were observed on the edge of the cytoplasm (Fig. 5). This suggests that *AsNODc22* protein localizes in or on the cell wall.

**Discussion**

It is likely that the signal peptide of the ORF functions as a sorting signal for localization to the cell wall and that the *AsNODc22* protein functions in or on the cell wall. In this study, it was observed that the *AsNODc22* protein localizes in the cell wall. This is the first observation of nodulin localization to the cell wall, other than that of the proline-rich proteins. ENOD2 is a proline-rich protein. The transcript of the ENOD2 was found in nodule parenchyma and is thought to localize in cell walls due to its proline-rich sequence and signal peptide at the N-terminus (van de Wiel et al. 1990). The transcript of ENOD2 was also found in the nodule parenchyma and uninfected cells surrounding the symbiotic region of both effective and ineffective nodules (Allen et al. 1991). Another study using polyclonal antibodies raised against PRP2 (soybean proline-rich protein) detected proline-rich proteins localizing along the cell wall in the nodule parenchyma, in the infection thread matrix, and on the surface of the bacteroids (Sherrier and VandenBosch 1994). In their study, a major signal was found on the cell wall of the nodule parenchyma, and no significant signal was observed on the cell wall of the bacteria-infected cells. In contrast, significant signals of the *AsNODc22* protein were found along the cell wall of the infected cells but not on the nodule parenchyma. These contrasts show that the *AsNODc22* protein and proline-rich proteins work at different places within the nodule.

The function of the *AsNODc22* protein is still unknown. The accumulation of *AsNODc22* protein, however, implies a dynamic change in the cell wall components or extracellular matrix during nodule development, in parallel with the proline-rich proteins (Sherrier and VandenBosch 1994). It is suggested that ENOD2 creates an O₂ barrier in the nodule parenchyma (van de Wiel et al. 1990). *AsNODc22* protein may also work as a secondary O₂ barrier around the infected cells comparable to that seen in the nodule parenchyma. Advanced studies using techniques such as immuno-electronmicroscopy may provide important information about the functions of these gene products.

The molecular mass of the *AsNODc22* product deduced from the nucleotide sequence is 15.6 kDa. The apparent molecular mass (18 kDa) estimated by SDS-PAGE was somewhat larger than the deduced value. The ORF, however, seems to correspond to the true *AsNODc22* product, since no other possible translation initiation site was found upstream of the ATG at nucleotide 173 (Fig. 1). Moreover, the cDNA length (0.86 kbp) corresponded to the transcript's size, as revealed by northern blot analysis (0.8 kbp). Thus, the difference in the molecular mass may be due to an apparent decrease in migration rate caused by modification (i.e., glycosylation) of the peptide. The glycosylation signal near the N-terminus is a candidate for modification.

Another valuable observation is the accumulation of *AsNODc22* protein at a rather early stage during nodule development. Sequential expression of some early nodulin genes (including ENOD12, ENOD40, or ENOD2 (reviewed by Schultz et al. 1994)) has already been used as a marker...
of nodule development. It is desirable to compare the mRNA localization of AsNODc22 to that of the other well-characterized early nodulin genes by in situ hybridization. Unfortunately, however, we have not succeeded in localizing the signal of the mRNA by in situ hybridization using ordinary methods (data not shown). Detecting the AsNODc22 protein by immunofluorescence microscopy is much simpler than detecting gene expression by in situ hybridization and could serve as a useful marker for differentiation in the nodule.

We thank Dr. N. Sato, Saitama University, for providing the pea tubulin clone. This work was supported in part by a grant from the Nissan Science Foundation to M.F. and by Grant-in-Aid (no. 09740615) from the Ministry of Education, Science, Sports and Culture of Japan to M.F.

References


(Rceived January 16, 1998; Accepted May 27, 1998)