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The Aberrant Cell Walls of Boron-Deficient Bean Root Nodules Have No Covalently Bound Hydroxyproline-/Proline-Rich Proteins[1]

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B-deficient bean (*Phaseolus vulgaris* L.) nodules examined by light microscopy showed dramatic anatomical changes, mainly in the parenchyma region. Western analysis of total nodule extracts examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that one 116-kD polypeptide was recognized by antibodies raised against hydroxyproline-rich glycoproteins (HRGPs) from the soybean (*Glycine max*) seed coat. A protein with a comparable molecular mass of 116 kD was purified from the cell walls of soybean root nodules. The amino acid composition of this protein is similar to the early nodulin (ENOD2) gene. Immunoprecipitation of the soybean ENOD2 in vitro translation product showed that the soybean seed coat anti-HRGP antibodies recognized this early nodulin. Furthermore, we used these antibodies to localize the ENOD2 homolog in bean nodules. Immunocytochemistry revealed that in B-deficient nodules ENOD2 was absent in the walls of the nodule parenchyma. The absence of ENOD2 in B-deficient nodules was corroborated by performing hydroxyproline assays. Northern analysis showed that ENOD2 mRNA is present in B-deficient nodules; therefore, the accumulation of ENOD2 is not affected by B deficiency, but its assembly into the cell wall is. B-deficient nodules fix much less N_2 than control nodules, probably because the nodule parenchyma is no longer an effective O_2 barrier.

B is an essential micronutrient for higher plants (Sommer and Lipman, 1926) and diatoms (Lewin, 1966); however, its mechanism of action is not yet clearly understood. Numerous studies have implicated B in the biogenesis of plant cell walls (Cohen and Lepper, 1977; Goldbach et al., 1991). Deficiency symptoms first appear in actively growing tissues, within hours in root tips and within minutes or seconds in pollen tube tips, and are characterized by cell wall abnormalities. B is localized essentially in the cell wall (Loomis and Durst, 1992; Hu and Brown, 1994), although there is some in the plasmalemma; no B could be detected in vacuoles (Martini and Thellier, 1993). In cell walls B is linked primarily with the rhamnogalacturonan II fraction of pectins (Hu and Brown, 1994; Ishi and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996). Loomis and Durst (1992) proposed that borate esters with apiose (which is preferentially present in the rhamnogalacturonan II) are responsible for cross-linking cell wall polymers and thus are necessary for cell wall stability.

In a previous report, it was established that there is a relationship between B availability and the N_2 fixation process in blue-green algae (Cyanobacteria) (Bonilla et al., 1990). In these microorganisms B deficiency induced alterations of the heterocyst envelope that might facilitate O_2 diffusion, resulting in the inhibition of nitrogenase activity (García-González et al., 1991). In addition, recent results have shown that B is also required for the legume-*Rhizobium* symbiotic process (Bolaños et al., 1994). B deficiency in pea (*Pisum sativum* L.) caused a decrease in the number of nodules and an alteration in indeterminate nodule development, leading to an inhibition of nitrogenase activity. Electron micrographs of B-deficient nodules showed dramatic cell wall changes and alterations in both peribacteroid and infection thread membranes, suggesting a role of this microelement in the stability of these structures (Bolaños et al., 1994) and in the correct establishment of the symbiosis between pea and *Rhizobium* (Bolaños et al., 1996). These data support a putative role of B as a cross-linker that stabilizes cell wall structures and/or envelopes (Loomis and Durst, 1992).

It has been established that B-deficient nodules fix less N_2 and contain abnormal cells and aberrant cell walls. We wanted to determine what might be different about these walls. Could HRGPs and PRPs be missing? Therefore, we studied the accumulation and deposition of HRGPs and

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Abbreviations: HRGP, Hyp-rich glycoprotein; PRP, Pro-rich protein.

PRPs in B-deficient bean (*Phaseolus vulgaris* L.) root nodules by western analysis, immunocytochemistry, and chemical determinations of Hyp. In root nodules the level of Hyp-containing molecules is developmentally regulated (Cassab, 1986). In the cortex (outer cortex, endodermis, vascular strands, and nodule parenchyma), Hyp is mainly localized in the wall, presumably as an HRGP-like molecule and/or nodulin (Cassab, 1986; Franssen et al., 1987; van de Wiel et al., 1990). Extensins, a family of HRGPs, are the best characterized and perhaps the most abundant structural proteins of dicotyledonous cell walls (Cassab and Varner, 1988; Showalter, 1993; Kieliszewski and Lamport, 1994).

Extensins are abundant in sclerenchyma tissue and might have a specific function in this cell type (Cassab and Varner, 1987); however, they are also commonly associated with phloem tissue and cambium cells (Showalter, 1993). PRPs represent another class of plant cell wall proteins rich in both Pro and Hyp. Pro-rich proteins are components of normal plant cell walls and some nodulins (i.e. proteins produced in response to infection by N_2-fixing bacteria). Early nodulin genes (*ENOD2* from soybean [*Glycine max*] and pea and *ENOD12* from pea) are characterized by the presence of the repeating pentapeptide sequence (Pro)-Pro-Pro-X-Y-Lys, where X and Y could be Val, Tyr, His, Asn, or Glu (Franssen et al., 1987; Scheres et al., 1990; van de Wiel et al., 1990). This repetitive pentamer also occurs in more complex extensins (Kiesliszewski and Lamport, 1994). These HRGPs and PRPs seem to play an important role in nodule morphogenesis (Cassab, 1986; van de Wiel et al., 1990; Wilson et al., 1994). To date, there is little direct evidence as to what these functions might be. Furthermore, we still do not know how each class of structural protein is self-assembled into a functional and dynamic cell wall of any cell type.

In the present paper we report that B is not only a requirement for determinate nodule development and N_2 fixation but also that in B-deficient nodules covalently bound *ENOD2* is practically nonexistent in the walls of nodule parenchyma. Our data strongly suggest that the absence of *ENOD2* in the cell wall of nodule parenchyma correlates with an irregular wall structure of this tissue that ultimately generates few *Rhizobium*-containing cells and thus fixes less N_2. Particularly, we discuss the presence of an abnormal nodule parenchyma, which contains no covalently bound *ENOD2* and could lead to a deficient O_2-diffusion barrier, which is required to protect the N_2-fixing enzyme nitrogenase. Finally, we discuss whether B might be involved in the cross-linking of *ENOD2* in the cell walls of legume nodules.

MATERIALS AND METHODS

Plant Growth and Inoculation

Bean (*Phaseolus vulgaris* L. cv Negro Jamapa) seeds were surface-sterilized with 10% (v/v) sodium hypochlorite, soaked for 1 h in sterile, distilled water, and then germinated on wet filter paper in covered stainless steel pans at 25°C, according to the method of Lara et al. (1984). After 3 d seedlings were transferred to plastic growth pots and cultivated on vermiculite with Murashige-Skoog medium without N (Murashige, 1974). Bean plants were inoculated with *Rhizobium tropici* (strain CIAT 899). Plants were grown in a greenhouse with natural light at 25°C for a 16-h day with at 20°C at night. RH was kept between 60 and 70%.

All solutions were prepared and stored in polyethylene containers previously tested to prevent release of B under sterilizing conditions according to the method of Mateo et al. (1986). For control cultures, B (as H_3BO_3) was added to a final concentration of 1.6×10^{-6} M (Fig. 1).

Enzyme Activity

Nitrogenase activity was measured by acetylene reduction as described by Dart et al. (1972). Ten plants for each determination were harvested weekly.

Light Microscopy

Nodule samples for light microscopy were fixed for 24 h at 4°C in 4% paraformaldehyde and 1% glutaraldehyde in PBS. After three changes of the same buffer, tissues were dehydrated through 10, 30, 50, 70, and 90% ethanol and three changes of 100% ethanol (30 min per step). The sections were infiltrated in Spurr's resin for 24 h. Semithin sections (2 μm) were made with a glass knife in an ultratome (Nova, LKB, Bromma, Sweden) and stained with toluidine blue for light-microscopy observations. Micrographs were taken in a Diaphot-TMD inverted microscope (Nikon). Photomicrographs were recorded on tungsten film at 160 ASA (Kodachrome, Kodak).

Chemical Analyses

Protein content was determined by the method of Bradford (1976), and Hyp was quantitated using the method of Drozdz et al. (1976).

Analysis of DEAE-Bound, Nodule-Soluble Protein Fractions

Two grams of plant nodules previously frozen in liquid N_2 was pulverized in an electric grinder. Plant powder was mixed with 2.5 volumes of cold extraction buffer (30 mM Tris-HCl, 30% Suc, and 2.5% PVP, pH 7.4). The slurry was stirred for 5 min and centrifuged (20 min at 26,900*g*, 4°C). All buffers were used ice-cold, and every fractionation step was performed at 4°C to minimize proteolytic activity. The low-ionic-strength supernatant was mixed with one-tenth volume of DEAE-Sephacel resin previously equilibrated with 30 mM Tris-HCl and 150 mM KCl, pH 7.4, according to the method of Pérez et al. (1994). The plant supernatant and the resin were mixed gently for 10 min, and then the resin was washed with 20 volumes of equilibration buffer using a Buchner funnel. The damp resin was collected and incubated for 10 min with 5 volumes of elution buffer (30 mM Tris-HCl and 500 mM KCl, pH 7.4). The slurry was filtered and the eluted proteins were precipitated with 3 volumes of acetone (prechilled at −20°C). Acetone pellets were stored at −70°C until use.

One-dimensional 10% SDS-PAGE gels were prepared according to the method of Laemmli (1970), with the modifications for minigels recommended by Hoefer Scientific Instruments (San Francisco, CA). Gels were loaded with 10 μg of protein. After electrophoresis, proteins were detected with either Coomassie brilliant blue R250 (Laemmli, 1970) or silver staining (Bio-Rad).

Western Analysis

Western analysis was performed as described previously (Towbin et al., 1979). A rabbit polyclonal antibody against soybean (*Glycine max*) seed coat extensin was used at a 1:10,000 dilution; this antibody recognizes both native and deglycosylated soybean seed coat extensin (Cassab and Varner, 1987).

Cell Wall Isolation

Fresh nodules were ground in a glass homogenizer and treated as described by Cassab et al. (1985).

Isolation of a Hyp-/Pro-Rich Cell Wall Glycoprotein from Soybean Root Nodules

The 0.2 M $CaCl_2$ cell wall extract from soybean nodules was loaded onto a CM-Sepharose CL-6B column (30 × 2.5 cm; Sigma) previously equilibrated with 0.02 M Tris-HCl, pH 8.0. The column was washed with 2 volumes of 0.02 M Tris-HCl, pH 8.0, and the attached material was eluted with a linear gradient of 0.02 to 0.5 M Tris-HCl, pH 8.0. The Hyp-containing fractions (salt concentration, 0.35 M Tris-HCl) were pooled, concentrated, and desalted with Centricon (10 kD cutoff, Amicon, Beverly, MA), and solid CsCl was added to a final density of 1.4 g mL^{-1}. Samples were centrifuged for 72 h at 250,000*g* in a rotor (SW-65Ti, Beckman). The resulting CsCl gradient was fractionated.

The Hyp-containing fractions (fraction I, mean density of 1.417 g mL^{-1}; fraction II, mean density of 1.37 g mL^{-1}) were pooled, desalted, and concentrated with Centricon (30 kD cutoff). In all fractionation procedures, the salt concentration of the samples was determined using a refractometer, the protein content was determined by UV absorption at 280 nm, and the Hyp content was determined as described above. The amino acid composition of fraction II was determined by acid hydrolysis in constantly boiling HCl (Pierce) under N_2 for 24 h at 110°C. The resulting amino acids were then analyzed.

Light-Microscopic Immunocytochemistry

Two-week-old bean nodules were fixed, dehydrated, and infiltrated as described above. Sections (2–3 μm thick) were cut using glass knives and mounted onto glass slides. Immunocytochemistry was done with the LAB-SA system histostain-SAP kit (Zymed Laboratories, San Francisco, CA) and performed according to the manufacturer's instructions with some modifications. The kit incorporated alkaline phosphatase, streptavidin, and affinity-purified antibodies into the labeled-[strept]Avidin-Biotin (LAB-SA) method, also known as streptavidin-biotin amplification. The chromogen/substrate system used was AP-red, for a signal that creates an intense red deposit around the antigen-antibody-enzyme complex in the sample. The sections were counterstained with hematoxylin. Primary antibody against soybean seed coat HRGP was diluted 1:25 and incubated for 2 h and 30 min. The biotinylated second antibody and enzyme conjugate were incubated for 1 h and 30 min instead of the 10 min recommended by the manufacturer, since this kit is recommended for frozen and paraffin sections. Micrographs were taken as above and recorded with Kodak Ektapress film at 100 ASA. Two controls were made, one using preimmune serum diluted 1:25 and the other without any serum. In both controls there was no signal observed in the cell walls of bean root nodules.

Coupled in Vitro Transcription-Translation and Immunoprecipitation

The pGmENOD2 cDNA clone encoding soybean nodulin-75 (*ENOD2*) was subcloned as an *Eco*RI/*Xho*I fragment into the plasmid vector pBluescript SK^-. The expression of the cDNA in this vector is under the control of a T7 promoter. This construction was transcribed and translated in vitro in the presence of [^{35}S]Met (40 μCi, 1175 Ci/mmol, DuPont) using the linked T7 transcription-translation system (Amersham). The transcription-translation product was immunoprecipitated with anti-HRGP antibodies from the soybean seed coat as described by Sánchez et al. (1987). The products were analyzed by autoradiography after electrophoretic separation on a 12% SDS-PAGE system.

Northern Analysis

Total RNA was extracted from 5 g of 2-week-old bean root nodules with 2 volumes of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol. One volume of phenol-chloroform was added and mixed for 15 min. The suspension was centrifuged at 10,000*g* for 4 min at 4°C. Phenol-chloroform was added until the interphase was clear. RNA was precipitated with one-tenth volume of 3 M sodium acetate, pH 4.8, and 2 volumes of cold ethanol for 15 min at 4°C. The pellet was washed with cold 70% (v/v) ethanol and dried under a vacuum. It was then resuspended with water at the initial volume. RNA was precipitated with 1 volume of 4 M LiCl at 4°C for 12 h and was then centrifuged at 14,000*g* for 5 min at 4°C. The pelleted RNA was washed with 70% (v/v) ethanol and resuspended in 300 μL of water. RNA was extracted with 1 volume of phenol-chloroform and precipitated with one-tenth volume of 3 M sodium acetate, pH 4.8, and 2 volumes of cold ethanol for 60 min at −20°C. RNA was pelleted by centrifugation at 14,000*g* for 20 min at 4°C. The pelleted RNA was washed with cold 70% (v/v) ethanol and dried under a vacuum. Finally, RNA was resuspended in water. After denaturation in 40% formamide and subsequent separation on 1.5% agarose gels containing 15% formamide, the gels were blotted in 20× SSC onto Hybond N membranes (Amersham). The *Hin*dIII/*Pst*I cDNA insert of

clone pGmENOD2 was used to prepare a radioactive probe by random primer. Hybridization and washes were performed at high stringency.

Slot-Blot Analysis

Serial dilutions of total RNA from root nodules were slotted onto nylon filters and hybridized to extensin (pTom 5.10) and actin cDNA clone (Plact-6). Hybridization and washes were performed at high stringency.

Reproducibility

Data in the figures are mean values ± SE from four independent experiments.

RESULTS

Bean plants grown under B deprivation showed a significant reduction in growth, mainly in root development, compared with control plants (Fig. 1). Both nodule number and fresh weight (50% less after 2 weeks of treatment) were reduced in B-deficient plants (Fig. 2A). B starvation resulted in more than 50% inhibition of nitrogenase activity, detected as acetylene reduction, after 3 weeks of treatment and about 60% inhibition after 4 weeks (Fig. 2B).

Light-microscopy examination of B-deficient nodule thin sections (Fig. 3, B and D) showed dramatic changes and alterations in cell size and structure compared with control nodules (Fig. 3, A and C) after 2 weeks of treatment. In B-deficient nodules, the anatomy of the peripheral, uninfected tissues (which includes nodule outer cortex, endodermis, nodule parenchyma, and vascular bundles) was severely affected. The cell walls in the nodule parenchyma were wrinkled and contained expanded intercellular spaces (Fig. 3, B and D). Within the peripheral tissues, the endodermal cells with their characteristic thick and lignified secondary cell walls could not be distinguished (Fig. 3, B and D) as in control nodules (Fig. 3, A and C).

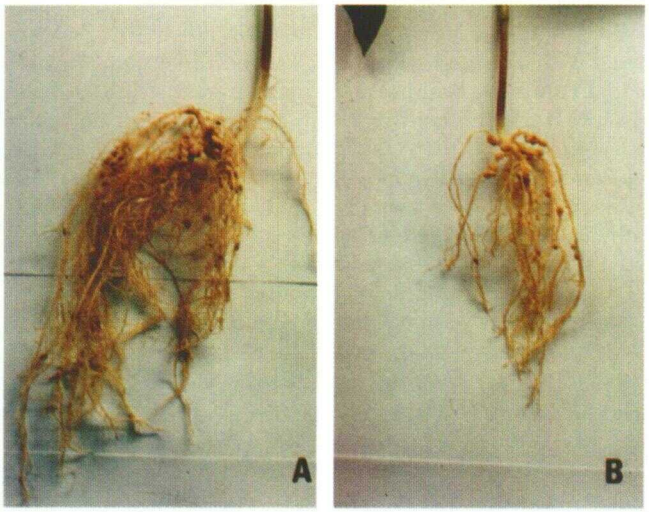

Figure 1. Differences between roots of bean plants grown in the presence of B (A) and under B deficiency (B).

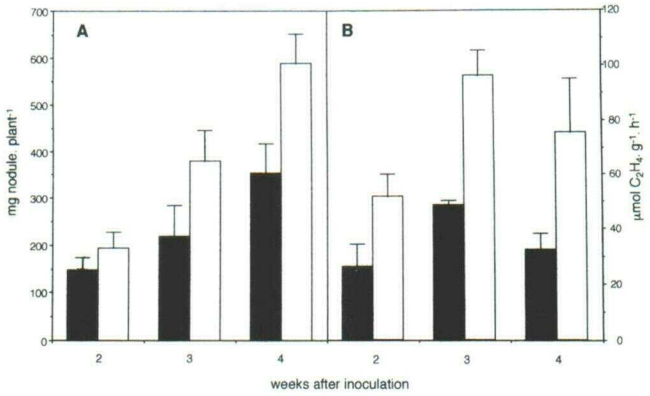

Figure 2. Effect of B deprivation on fresh weight (A) and nitrogenase activity expressed as acetylene reduction (B) of bean root nodules at different developmental times. White bars, Controls; black bars, B-deficient plants.

Furthermore, in B-deficient nodules, a distal-proximal gradient (from the root axis) of cell size developed in the central tissue (Fig. 3B), which is surrounded by several layers of small cells with heavily stained nuclei.

On the proximal side of the central tissue, uninfected cells formed a boundary layer, where amyloplasts filled with starch accumulation were clearly visible, and a few *Rhizobium*-containing cells could be observed (Fig. 3B). Moreover, cells in the central tissue were of smaller size and irregularly shape. On the distal side of the central tissue, clusters of small cytoplasm-rich cells could be observed (Fig. 3B, arrow). These cells seemed to be mitotically active, because their cell walls, in contrast to the central part, were not deformed (Fig. 3B). A higher magnification of the severely damaged peripheral tissues in B-deficient nodules showed the presence of expanded intercellular spaces in the nodule parenchyma (Fig. 3D, arrows) compared with control nodules (Fig. 3C). Vascular bundles are apparently well developed in B-deficient nodules (data not shown), so this deficiency does not influence the formation of these complex tissues.

Because cell walls frequently looked disturbed under B deficiency, we decided to investigate the presence of HRGPs and PRPs, normal nodule wall components (Cassab, 1986; Scheres et al., 1990; van de Wiel et al., 1990; Benhamou et al., 1991). We studied the pattern of accumulation of HRGPs and PRPs using western analysis in bean root nodules grown in the presence or absence of B. To visualize as many nodule proteins as possible and to eliminate polysaccharides that interfere with the electrophoretic analysis, B-deficient and control nodules were extracted in a low-ionic-strength buffer and the extracts were chromatographed by DEAE-batch adsorption (see "Materials and Methods"). These total protein extracts were prepared from 2- and 3-week-old B-deficient and control nodules and subjected to SDS-PAGE (Fig. 4A) for protein analysis.

In 2-week-old B-deficient nodules there are apparently four proteins, in contrast to control nodules, which show several polypeptides. Only one high-molecular-mass polypeptide (116 kD) was stained with anti-HRGP soybean

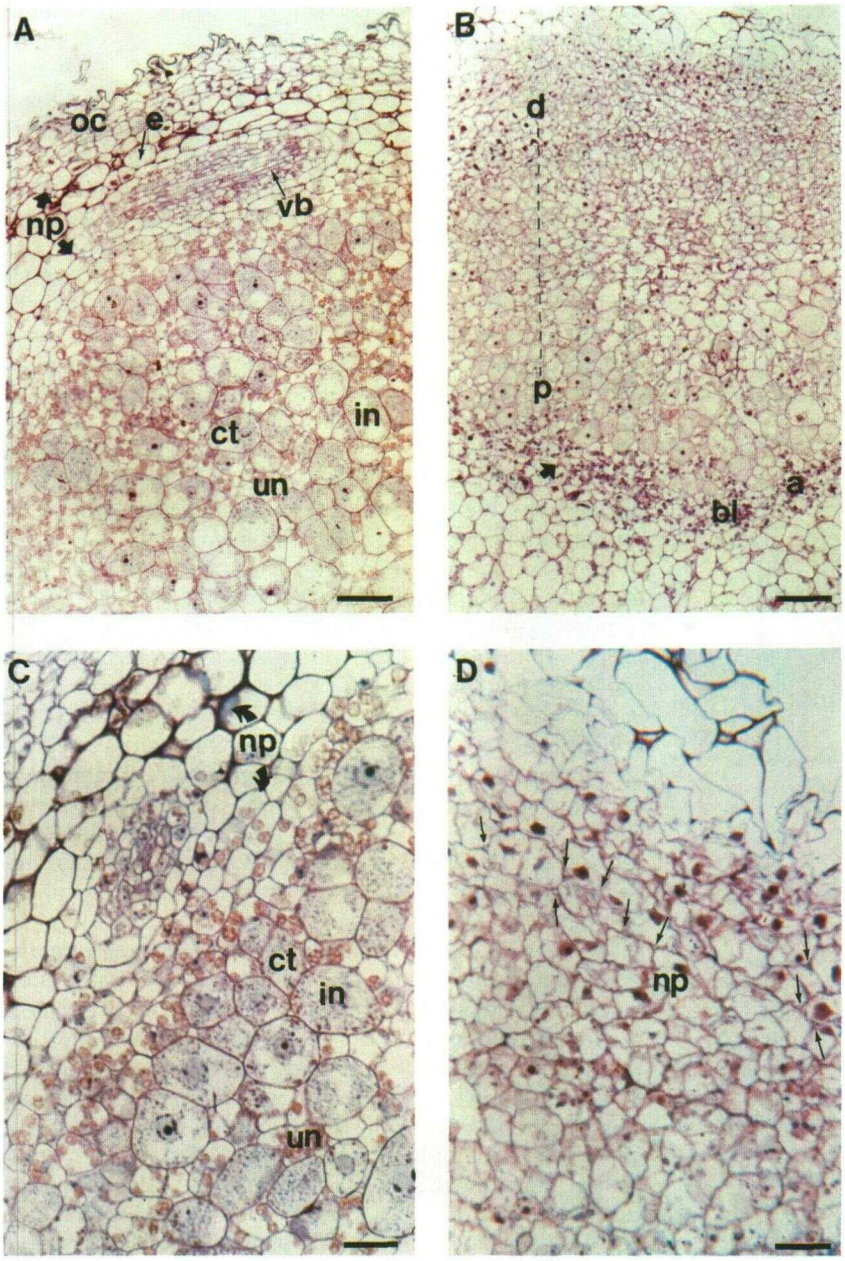

Figure 3. Bright-field micrographs of semithin longitudinal sections of 2-week-old bean root nodules. A and C, Control nodules; B and D, B-deficient nodules. A, Control nodule showing the outer cortex (oc), vascular bundles (vb), endodermis (e), nodule parenchyma (np), and central tissue (ct) with infected (in) and uninfected (un) cells. Bar = 36 μm. B, B-deficient nodule showing a proximal-distal gradient of cell size (d- - - -p), and a boundary layer (bl) of uninfected cells with amyloplasts (a) in the proximal side of the central tissue. Note the absence of endodermis, the abnormal nodule parenchyma, and the small clusters of mitotically active cells with undeformed cell walls (arrow). Bar = 36 μm. C, Detail of a section of control nodule showing the nodule parenchyma, which contains relatively few and small intercellular spaces (np). Bar = 18 μm. D, Detail of a section of B-deficient nodule showing the fewer cell layers at the outer cortex and the irregular cell walls in the nodule parenchyma (np), particularly the presence of expanded intercellular spaces (arrows). Bar = 11 μm.

seed coat antibodies in both B-deficient and control nodules (Fig. 4B). This polypeptide was present in 2-week-old B-deficient nodules at higher levels than in control nodules but decreased in 3-week-old control and B-deficient nodules (Fig. 4, A and B). The 116-kD protein was temporarily stained with Coomassie blue (only for 10 min) and was usually labeled at both sides of the lane either by Coomassie blue or by anti-HRGP antibodies (Fig. 4A).

High-concentration salt extracts (0.2 M $CaCl_2$) and boiling SDS total protein extracts from 2-week-old B-deficient bean nodules were also subjected to western analysis with anti-HRGP antibodies. In both cases, only one polypeptide band of 116 kD was stained with these antibodies (Fig. 4D). The polypeptide band stained with anti-soybean seed coat HRGP antibodies in bean nodules had the same molecular mass and staining pattern as a purified protein from soybean nodule cell wall extracts (Fig. 4C). Western analysis of this purified cell wall protein examined by 7% SDS-PAGE also stained only one polypeptide of 116 kD with soybean seed coat anti-HRGP antibodies (Fig. 4D). The amino acid composition of this 116-kD cell wall protein was very similar to the one derived from the cDNA of *ENOD2* from soybean (Franssen et al., 1987; Table I). Therefore, we were probably analyzing the accumulation of an early nodulin that belongs to the PRP family instead of studying the presence of HRGP-like molecules.

These data indicate that in the 116-kD protein about 21% of the Pro residues were hydroxylated (Table I), as is the case with soybean PRPs (Averyhart-Fullard et al., 1988; Datta et al., 1989; Kleis-San Francisco and Tierney, 1990;

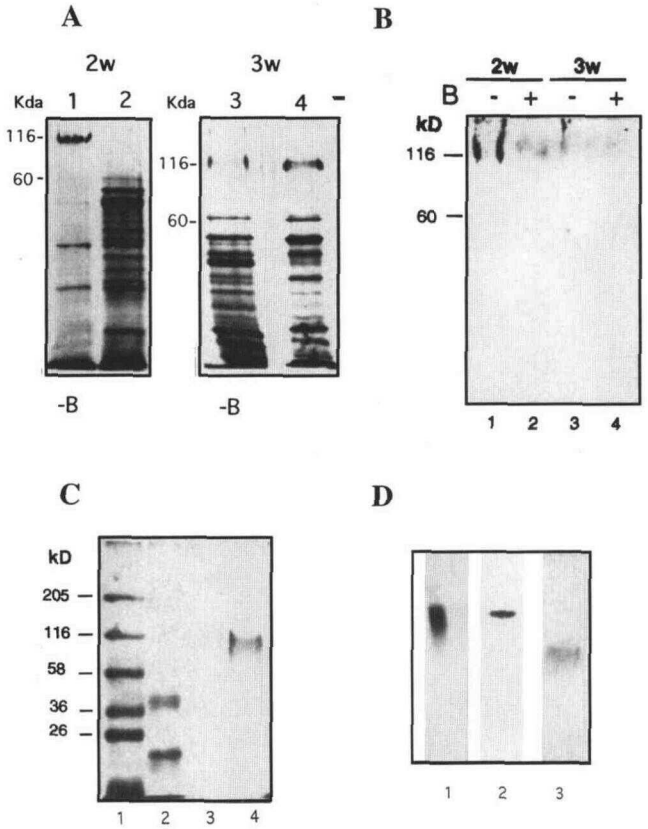

Figure 4. Western analysis of total proteins from bean nodules grown in the presence or absence of B with anti-HRGP antibodies. A, Polypeptide pattern of low-ionic-strength nodule extract from both normal and B-deficient bean plants. Total proteins were separated by SDS-PAGE on a 10% acrylamide gel and visualized by Coomassie blue staining. Lanes 1 and 3, B-deficient nodule proteins; lanes 2 and 4, control nodule proteins. B, HRGP-PRPs detected by reaction with anti-HRGP antibodies from soybean seed coat. Lanes 1 and 3, B-deficient nodule proteins; lanes 2 and 4, control nodule proteins. Time of nodule harvesting is indicated at the top of each lane: 2w, 2 weeks old; 3w, 3 weeks old. Immune complexes were detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies. C, 7% SDS-PAGE of purified soybean nodule cell wall protein visualized by silver staining. Lane 4 contains approximately 0.2 μg of Hyp and corresponds to a fraction isolated from a CsCl gradient peak of δ 1.39 mg mL^{-1}. Note that this polypeptide has the same molecular mass as the one visualized by reaction with anti-HRGP antibodies in bean extracts. Lanes 1 and 2 contain wide-range and high-molecular-mass markers. No sample was loaded in lane 3. Molecular mass markers are given at left in kilodaltons. D, Western analysis of different protein samples from B-deficient bean nodules and control soybean nodules with anti-HRGP antibodies. Lane 1, High-concentration salt extract of 2-week-old B-deficient bean nodules; lane 2, boiling SDS total protein extract from 2-week-old B-deficient bean nodules. (Note that in both cases the anti-HRGP antibodies recognize only one polypeptide.) Lane 3, Purified soybean cell wall protein (0.05 μg of Hyp). In all three lanes, the molecular mass of the decorated protein band is 116 kD.

Lindstrom and Vodkin, 1991). However, comparison of the amino acid composition of *ENOD2* from soybean and pea (*Pisum sativum*) nodules with PRPs from seedlings showed that they differ in that they have fewer Val and Tyr residues and have a higher His and Glx (Glu or Gln) content (Hong et al., 1987; Kleis-San Francisco and Tierney, 1990).

The amino acid composition of other *ENOD* genes that code for PRPs, such as *ENOD12* and *MtPRP4*, is also shown for comparison with *ENOD2* (Scheres et al., 1990; Wilson et al., 1994). Both *ENOD12* and *MtPRP4* have higher Lys and Val contents than *ENOD2*. The amino acid composition of the HRGP from soybean seed coats is also listed for comparison with nodule PRPs (Table I). Both HRGP and *ENOD2* are similar since the levels of Val, Lys, and Tyr are comparable; however, in the seed coat HRGP, the ratio of Hyp to Pro and the content of Ser are higher, and the level of Glx is lower than in *ENOD2*. The slower migration of the nodule cell wall protein in SDS-PAGE gels, i.e. 116 versus 45 kD for the predicted translation product of *ENOD2* cDNA clone and versus 75 kD from the in vitro translation product of *ENOD2* cDNA clone (Franssen et al., 1987), is presumably due to the high Pro content of the protein, to glycosylation, and/or to the high ionic charge.

To determine whether anti-HRGP from soybean seed coat antibodies recognizes *ENOD2* in bean nodules, in vitro transcription-translation and immunoprecipitation of the cDNA clone pGmENOD2 (Franssen et al., 1987), which codes for the soybean *ENOD2* gene, was performed. As shown in Figure 5 (lane 3), anti-HRGP soybean seed coat antibodies immunoprecipitate the in vitro-synthesized recombinant *ENOD2*. The *Eco*RI/*Xho*I cDNA fragment of *ENOD2* subcloned in pBluescript SK^- plasmid vector was 726 bp; therefore the predicted size of the in vitro transcribed and translated product was 26.6 kD, which is similar to the observed molecular mass of the recombinant polypeptide.

To investigate the cellular distribution of putative *ENOD2* in bean nodules grown under B deprivation, immunocytochemistry by light microscopy was performed. Sectioned 2-week-old nodule tissue of B-deficient and control plants was incubated with anti-soybean seed coat HRGP antibodies. As shown in Figure 6A, a strong signal was observed within the cell walls of control nodules, primarily in the nodule parenchyma. There was, however, no labeling in the walls of vascular bundle and outer cortex cells (Fig. 6A). In contrast, in B-deficient nodules, no labeling was detected in the cell walls of B-deficient nodule parenchyma cells, even though we used a very concentrated dilution of the antibody (see "Materials and Methods"; Fig. 6B). This result indicates that, in the absence of B, putative *ENOD2* is not covalently bound in cell walls of nodules, even though it accumulates in total cell extracts at higher levels than in control nodules (Fig. 4B).

The fact that *ENOD2* did not accumulate in the cell walls of B-deficient nodules (Fig. 6B) suggests that there are higher levels of this protein in total cell extracts than in control nodules. Furthermore, the antibody also decorates vesicle-like structures in nodule parenchyma and uninfected cells of both control and B-deficient nodules (Fig. 4, A and B). These aggregates are also positive to Amido black and periodic acid-Schiff staining for proteins and glycoproteins or carbohydrates (data not shown) and were detected in developing soybean seed coat palisade cells (Cassab and Varner, 1987). Therefore, the major difference

Table I. *Amino acid composition of several Hyp-/Pro-rich cell wall proteins and early nodulins*

Amino Acid	Sb Nodule Proteins[a]	SbENOD2[b]	PsENOD2[c]	SbENOD12[d]	MtPRP4[e]	30 kD[f]	SbPRP1[g]	HRGP[h]
				mol %				
Hyp	21.0					16.8		36.2
Pro	20.7	40.8[i]	37.5[i]	26.7[i]	33.7	19.0	39.6[i]	9.9
Lys	7.4	8.4	8.0	15.1	18.4	14.8	19.6	10.8
Val	2.7	3.2	2.7	7.0	15.8	14.7	16.1	2.5
Tyr	4.1	6.7	6.2	3.5	4.6	7.4	16.1	8.5
Ser	1.0	1.0	3.6	2.3	0.9	1.4	0.4	8.2
His	8.8	8.4	11.6	12.8	6.6	0.4	0	8.8
Glu	17.6[j]	14.2	11.6	8.1	9.8	9.8[j]	3.9	2.4
Gln		3.6	3.6	5.8	0.4		0	
All others	6.3	4.2	14.3	18.8	9.7	16	4.2	16.2

[a] Determined from protein composition of the nodule cell wall protein. [b] Determined from cDNA nucleotide sequence (Franssen et al., 1987). [c] Determined from cDNA nucleotide sequence (van de Wiel et al., 1990). [d] Determined from cDNA nucleotide sequence (Scheres et al., 1990). [e] Determined from genomic clone nucleotide sequence (Wilson et al., 1994). [f] Determined from protein composition of the purified 30-kD hook protein (Kleis-San Francisco and Tierney, 1990). [g] Determined from genomic clone nucleotide sequence (Hong et al., 1987). [h] Determined from protein composition of the purified HRGP from soybean seed coats (Cassab et al., 1985). [i] Hyp is a posttranslational modification of Pro and cannot be distinguished from nucleotide sequence. [j] Glu/Gln cannot be distinguished after hydrolysis of protein.

observed between B-deficient and control nodules was the absence of staining of nodule parenchyma cell walls in B-deficient nodules. Control root nodule sections incubated with preimmune serum or no serum were not labeled (data not shown).

To determine whether the absence of putative *ENOD2* in the cell walls of nodule parenchyma of B-deficient nodules was due to a deficient cross-linking of HRGPs and PRPs into the wall, we quantitated the levels of Hyp in total cell walls. In Table II, the ratio of Hyp to dry weight in total cell walls of B-deficient and control nodules is shown. This ratio decreased to about 5-fold less in B-deficient compared with control nodules at both developmental times analyzed. Therefore, B-deficient nodule cell walls have much less covalently bound HRGPs and PRPs.

Finally, we analyzed the transcript levels of *ENOD2* and extensin in 2-week-old B-deficient and control nodules by RNA gel- and slot-blot hybridizations (Figs. 7 and 8). Lower levels of *ENOD2* RNA were observed in B-deficient nodules compared with control nodules (Fig. 7). Similarly, transcript levels of extensin declined in B-deficient nodules compared with control nodules (Fig. 8). With both treatments, actin levels were measured, but we could not detect any significant difference in the level of this transcript. Therefore, in root nodules B deficiency affects the expression pattern of both *ENOD2* and extensin mRNA. Extensin has been shown to accumulate mainly in the walls of infected cells and in peribacteroid membranes surrounding groups of bacteroids in bean nodules (Benhamou et al., 1991). The fact that B-deficient nodules contain a reduced number of infected cells and, consequently, less peribacteroid membrane (Fig. 3B) may contribute to the diminished extensin transcript levels. On the other hand, because the morphology of the nodule parenchyma is markedly altered in B-starvation conditions, the accumulation of *ENOD2* mRNA may also be negatively affected.

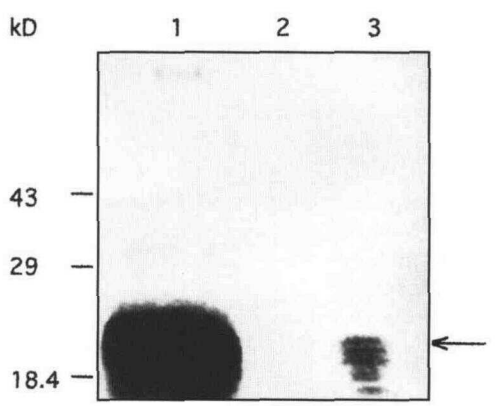

Figure 5. Identification of the product encoded by cDNA pGmENOD2 as *ENOD2* with anti-HRGP soybean seed coat antibodies. pGmENOD2 cDNA was cloned in pBluescript SK$^-$ plasmid, transcribed, and translated in vitro using a linked T7 transcription-translation system (see "Materials and Methods"). The protein products were immunoprecipitated with anti-HRGP soybean seed coat antibodies. Lane 1, Autoradiogram of the ^{35}S-labeled in vitro-translated products; lane 2, immunoprecipitation of the ^{35}S-labeled in vitro translated products using normal serum; lane 3, immunoprecipitation of the ^{35}S-labeled in vitro-translated products using anti-HRGP soybean seed coat antibodies. Molecular mass markers are given at left in kilodaltons.

DISCUSSION

The major focus of our study was to contribute to the understanding of the mechanism of action of B by studying the accumulation of the cell wall components HRGPs and PRPs in B-deficient root nodules. In previous papers, B was shown to be essential for indeterminate nodule development in pea (Bolaños et al., 1994, 1996). In pea nodules, the absence of B resulted in dramatic changes in cell walls in both peribacteroid and infection thread membranes. To extend these studies to other leguminous plants, its role in determinate nodules was also established. Indeterminate nodules are characterized by a persistent nodule meristem

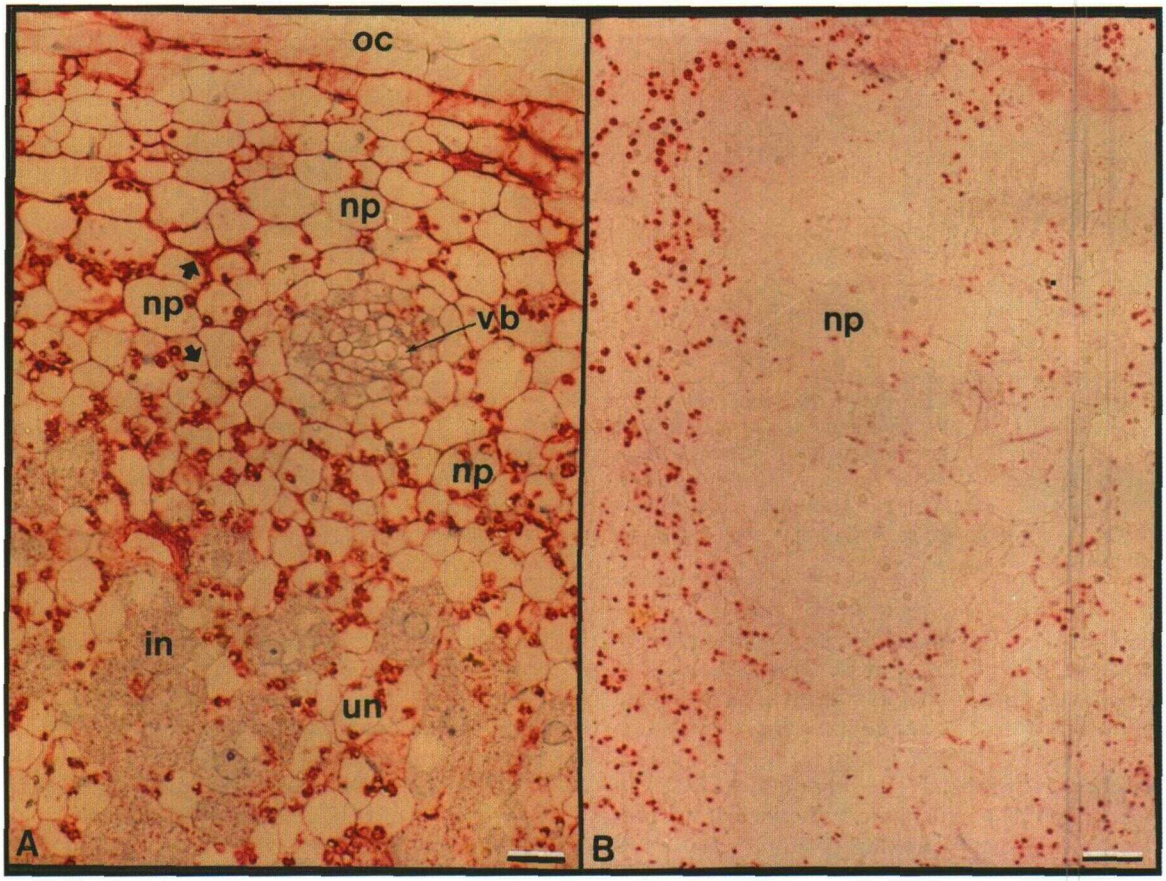

Figure 6. Immunostreptavidin-biotin staining of *ENOD2* in 2-week-old bean nodules grown with and without B. A, Control nodules. B, B-deficient nodules. Anti-HRGP antibodies intensely stain the cell walls of the nodule parenchyma of control nodules red but not B-deficient nodules. The tissue sections were counterstained with hematoxylin. oc, Outer cortex; vb, vascular bundle; np, nodule parenchyma; un, uninfected cells; and in, infected cells. Bars = 8 μm.

(Brewin, 1991). The persistence of a meristem causes indeterminate nodules to be elongated because new cells are constantly being infected, and cell division continues at the distal end of the nodule. In contrast, determinate nodules are spherical. Cell division ceases early during nodule development, and the final form of the nodules results from cell enlargement rather than cell division (Hirsch, 1992). Assuming that B is mainly required in meristematic cells (Raven, 1980) we could hypothesize that it may not be required in determinate nodules due to their lower rate of cell division later in development. However, with the data presented here, it is readily evident that B is also essential for determinate nodule development.

Table II. *Ratio of Hyp to dry weight from isolated cell walls of B-deficient and control nodules at different developmental times*

Values are the means of four different experiments.

Treatment	Hyp Content	
	2 weeks postinoculation	3 weeks postinoculation
	μg mg^{-1} dry wt	
B-deficient	0.09	0.12
Control	0.47	0.73

Under conditions of B deficiency, bean plants showed a significant reduction in growth. We also found that root elongation decreased in B-deficient plants, in particular the growth of lateral roots (Fig. 1B). The earliest symptom of B deficiency in the intact plant is the arrest of root elongation (Cohen and Lepper, 1977). We also observed that the organization of nodule tissues was dramatically affected in B-deficient plants. Between 1 and 2 weeks after inoculation, a distal-proximal gradient (from the root axis) in cell size could be distinguished in the central tissue of bean nodules grown under B-deprivation conditions (Fig. 3B). It is inter-

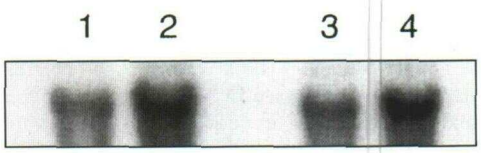

Figure 7. Gel-blot analysis of mRNA levels of *ENOD2* in 2-week-old B-deficient and control root nodules. Total RNA was separated by gel electrophoresis, transferred to a filter, and hybridized with pGm-ENOD2 cDNA probe as described in "Materials and Methods." Lanes 1 and 2, RNA from B-deficient nodules; lanes 3 and 4, RNA from control nodules; lanes 1 and 3, 5 μg of RNA per lane; and lanes 2 and 4, 10 μg of RNA per lane.

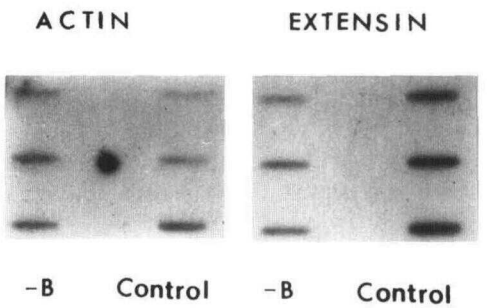

Figure 8. Slot-blot hybridization analysis of RNA levels of extensin and actin in 2-week-old bean nodules grown in the presence or absence of B. Total RNA was transferred to a filter and hybridized with extensin (pTom 5.10) and actin cDNA probes (Plact-6). The slots (from top to bottom) contain 5, 10, and 15 μg of total RNA. Left, Hybridization was with the actin probe; right, hybridization was with the extensin probe.

esting to note that in these nodules, the number of cell layers in the outer cortex diminished, the endodermis never developed, and the nodule parenchyma contained irregular cells with abnormal cell walls (Fig. 3, B and D). Furthermore, the nodule parenchyma in B-deficient nodules contains expanded intercellular spaces that are absent in control nodules (Fig. 3, D and C). In this regard, alteration and loss of structural integrity in cell walls have been reported frequently as an early symptom during B deficiency (Kouchi and Kumazawa, 1976; Hirsch and Torrey, 1980; Parr and Loughman, 1983; Loomis and Durst, 1992).

Extensins are a family of HRGPs found in the cell walls of higher plants (Cassab and Varner, 1988; Showalter, 1993). It has been reported that in soybean root nodules the level of Hyp-containing molecules is developmentally regulated (Cassab, 1986). Hyp accumulates in early nodulation and is found later in development in large amounts in the central zone and cortex. In the nodule cortex, Hyp is mainly localized in the wall, presumably as HRGPs and PRPs, but in the central zone it is present in the soluble fraction, largely as arabinogalactan proteins, a class of HRGPs (Cassab and Varner, 1988). However, HRGPs have also been found to accumulate mainly in the walls of infected cells and in peribacteroid membranes surrounding groups of bacteroids (Benhamou et al., 1991). The anti-HRGP antibodies used in this work recognize equally effectively both glycosylated and deglycosylated HRGP from the soybean seed coat; however, its cross-reactivity against glycosylated and deglycosylated carrot HRGP is just 12 and 6%, respectively (Cassab and Varner, 1987).

We have also shown that these antibodies immunoprecipitate the transcription and translation product of the *SbENOD2* cDNA clone (Fig. 5). Therefore, we presume that the anti-HRGP antibodies may recognize the sequence Pro-Pro-Pro-Val-Tyr, which is found twice in *SbENOD2* (Franssen et al., 1987) and can also be present in some extensins such as the cDNA for SbHRGP-3 from soybean hypocotyl (Hong et al., 1994). Moreover, since we do not have sequence data from the soybean seed coat HRGP, we could also speculate that this glycoprotein may contain sequences similar to the sugar beet extensin 15-mer core: Hyp-Hyp-[Val-His-Glu-Tyr-Pro]-Hyp-Hyp (Li et al., 1990). This sequence is very similar to the repetitive motif Pro-Pro-[His-Glu-Lys-Pro]-Pro-Pro present in *SbENOD2* (Franssen et al., 1987).

However, PRPs appear to differ from HRGPs in that (a) they contain equal amounts of Pro and Hyp (Table I), (b) antibodies and nucleic acid probes specific for PRPs do not cross-react with extensins (Datta et al., 1989; Marcus et al., 1991), and (c) they are poorly glycosylated (Kieliszewski and Lamport, 1994). Nevertheless, *ENOD2* must be slightly glycosylated, since its average density (1.37 g mL^{-1}) is higher than that of nonglycosylated proteins (1.33 gm L^{-1}). On the other hand, the fact that anti-soybean seed coat HRGP antibodies probably cross-react with *ENOD2* from nodules may indicate that this PRP also belongs to the extensin family of cell wall proteins (Li et al., 1990; Kieliszewski and Lamport, 1994). Moreover, anti-glycosylated and deglycosylated carrot extensin antibodies did not stain the 116-kD bean nodule protein (data not shown) but did stain two proteins of approximately 33 and 30 kD. Furthermore, anti-PRP2 antibodies stained about seven polypeptides in bean nodule extracts (data not shown), and therefore we obtained a pattern similar to the one reported for pea nodules by Sherrier and VandenBosch (1994).

Immunocytochemical analysis showed that B might be involved in cell wall assembly of structural proteins, since its absence possibly prevented *ENOD2* deposition, primarily into the walls of nodule parenchyma (Fig. 6B). On the other hand, it is very likely that in the immunostained section of B-deficient nodules (Fig. 6B) soluble *ENOD2* is not detected because of its glycoprotein nature, and thus it is poorly fixed. We are currently trying to purify *ENOD2* from B-deficient nodules to determine whether the polypeptide that is incubated with fixatives loses its cross-reactivity with anti-HRGP antibodies.

The expression of *ENOD2* was localized within the nodule parenchyma of soybean and pea nodules by in situ hybridization (van de Wiel et al., 1990). The nodule parenchyma appears to be an important tissue in the *Rhizobium*-legume symbiosis, since it forms a barrier to the diffusion of gaseous O_2, as demonstrated by O_2 microelectrode measurements (Tjepkema and Yocum, 1974; Witty et al., 1986). It has been suggested that the *ENOD2* protein contributes to the diffusion barrier by modifying cell walls (van de Wiel et al., 1990). The occlusion of the small intercellular spaces by extracellular matrix proteins and water would theoretically provide 104 times the resistance to gas diffusion as a continuous airway (Witty et al., 1986).

PRPs have been localized in the intercellular spaces of pea nodule parenchyma using antibodies against PRP2 from soybean cells in culture (Sherrier and VandenBosch, 1994); nevertheless, PRPs have also been localized in several nodule cell types, such as vascular bundles, xylem tracheary elements, vascular endodermis, and the infection thread matrix (Sherrier and VandenBosch, 1994). Anti-HRGP antibodies from the soybean seed coat stained only the cell walls of nodule parenchyma and endodermis of bean (Fig. 6A) and soybean nodules (data not shown); therefore, these antibodies did not recognize the same set of PRPs as the PRP2 antibodies. Furthermore, in B-deficient nodules the lack of *ENOD2* deposition in cell walls of

nodule parenchyma may be related to the decrease in their capability to fix N_2. In the nodule parenchyma, *ENOD2* may contribute to their occlusion, perhaps by functioning as a gel plug or an adhesive. In fact, there is a strong sequence similarity between PRPs and an adhesive protein from mussels (Kieliszewski and Lamport, 1994).

Since we could not immunolocalize *ENOD2* in the cell walls of nodule parenchyma from B-deficient nodules, we also studied the level of Hyp total nodule cell walls in these and control nodules (Table II). The ratio of Hyp to cell wall dry weight was 5-fold less in B-deficient nodules compared with control nodules at both developmental times studied. This seems to indicate that, even though Hyp-containing proteins can be extracted from cell walls of B-deficient nodules (Fig. 4A), the level of these proteins that are cross-linked into the wall is relatively low compared with control nodules. These data suggest that HRGPs and PRPs may be synthesized at a normal level, but assembly and cross-linking into the cell wall matrix cannot be achieved in the absence of B. Although indirect immunological evidence (Marcus et al., 1991; Sherrier and VandenBosch, 1994) argues for PRPs cross-linking in muro, currently there is no direct evidence for this (Kieliszewski and Lamport, 1994). However, there is probably an enzyme highly specific for PRPs that cross-links them to create a polymer lattice that forms an "ultrafilter" that regulates cell wall porosity (van de Wiel et al., 1990).

The endodermis, the cells of which become sclerified in the cortex, never develops in B-deficient nodules (Fig. 3B), suggesting a possible change in the composition of secondary walls, primarily lignin. It has been suggested that there is a concomitant decrease in lignin synthesis in B-deficient tissue (Skok, 1958). However, there is little evidence demonstrating a decrease in lignification under B deprivation (Lovatt, 1984). In this study we were able to detect a possible change in the lignification of cortical cells of B-deficient nodules by observing negative staining with phloroglucinol in nodule cross-sections (data not shown), a test that defines lignification (Jensen, 1962).

The RNA levels of both *ENOD2* and extensin were influenced under B-starvation conditions. B deficiency resulted in lower *ENOD2* and extensin RNA levels compared with nodules grown in normal conditions (Figs. 7 and 8). On the one hand, the decrease in *ENOD2* RNA levels in B-deficient root nodules could be due to the altered morphology of the nodule parenchyma. On the other hand, the reduction in extensin RNA levels could be due to the decrease in the number of infected cells in B-deficient nodules. In both cases, the decline of transcript levels may be due to the loss of a positive regulatory component such as a transcriptional activator or to the acquisition of a negative regulatory component. Suppressors of HRGP gene expression, which are pectic fragments solubilized from plant cell walls by fungal endopolygalacturonase, have been described previously (Boudart et al., 1995).

It was demonstrated by using antisense gene technology that tobacco plants tolerate large variations in total Hyp concentration and soluble extensin content without apparent effects on their phenotype and cell wall structure (Memelink et al., 1993). The absence of a phenotype could be due to the fact that only one cell wall component (extensin) was manipulated and other wall components may have compensated for its absence. The absence of B brings about a clear phenotype of cell wall morphology. Therefore, the effect observed on nodule cell wall structure and distribution of HRGPs and PRPs caused by B deprivation provides a good experimental system for studying cell wall assembly.

Studies of localization and interaction with other cell wall components such as B should provide insights into the rules of self-assembly of HRGPs and PRPs, for example, in the assembly of extracellular matrix components into a functioning cell wall. Therefore, B might regulate not only cell wall structure but also polymer accumulation, secretion, and bridging between hydroxyl groups of sugar polymers, since borate forms cyclic diesters with appropriate diols or polyols (Loomis and Durst, 1992).

The localization of B in plant cell walls may indicate that B has a structural role in the cell wall matrix or, alternatively, that B is required for the synthesis of new cell wall material (Martini and Thellier, 1993; Hu and Brown, 1994). Several groups have proposed that B has a primary role in the biosynthesis of the cell wall. However, a consistent effect of B deficiency on new cell wall synthesis has not been shown, even in tissue that exhibited marked alteration in cell wall ultrastructure (Slack and Whittington, 1964). Skok (1958) suggested that since B is not a reutilizable micronutrient an available supply is required by plants at all times, with the function of B being related to the formation of structural units or "building blocks" in the wall. Loomis and Durst (1992) proposed a cell wall cross-linking role for B. Recently, B was found to be present as a B-rhamnogalacturonan II complex within plant cell walls (Ishi and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996). Our data suggest that B also has a role in the assembly of some wall protein components.

We hypothesize that under B deficiency the borate-rhamnogalacturonan II complex exhibits decreased availability and thus cannot "capture" *ENOD2* into the cell wall matrix of root nodules. We also suspect that once *ENOD2* is in the cell wall compartment and cannot covalently bind to the wall it is degraded, since salt-extracted cell wall proteins from B-deficient nodules subjected to cationic gel electrophoresis for protein-blot analysis show several proteolytic products (data not shown). However, definitive confirmation of this hypothesis involves the isolation and characterization of *ENOD2* from B-deficient nodules, as well as the examination of borate-rhamnogalacturonan II levels in B-deficient nodules. These experiments are in progress. In conclusion, experimental creation of B deficiency provides a novel approach to studying plant cell wall assembly during nodule development and demonstrates that the presence of *ENOD2* and B is essential in cell walls of nodule parenchyma for normal nodule anatomy and function.

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