

Accumulation of extracellular proteins bearing unique proline-rich motifs in intercellular spaces of the legume nodule parenchyma

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Summary. Nodulins encoding repetitive proline-rich cell wall proteins (PRPs) are induced during early interactions with rhizobia, suggesting a massive restructuring of the plant extracellular matrix during infection and nodulation. However, the proteins corresponding to these gene products have not been isolated or characterized, nor have cell wall localizations been confirmed. Posttranslational modifications, conformation, and interactions with other wall polymers are difficult to predict on the basis of only the deduced amino acid sequence of PRPs. *PsENOD2* is expressed in nodule parenchyma tissue during nodule organogenesis and encodes a protein with distinctive PRP motifs that are rich in glutamate and basic amino acids. A database search for the ENOD2 signature motifs indicates that similar proteins may have a limited phylogenetic distribution, as they are presently only known from legumes. To determine the ultrastructural location of the proteins, antibodies were raised against unique motifs from the predicted ENOD2 sequence. The antibodies recognized nodule-specific proteins in pea (*Pisum sativum*), with a major band detected at 110 kDa, representing a subset of PRPs from nodules. The protein was detected specifically in organelles of the secretory pathway and intercellular spaces in the nodule parenchyma, but it was not abundant in primary walls. Similar proteins with an analogous distribution were detected in soybean (*Glycine max*). The use of polyclonal antibodies raised against signature motifs of extracellular matrix proteins thus appears to be an effective strategy to identify and isolate specific structural proteins for functional analysis.

Keywords: ENOD2 cell wall protein; *Glycine max*; Immunolabeling; Nodulin; Peptide antibody; *Pisum sativum*.

Introduction

The legume root nodule is a nitrogen-fixing organ that develops in response to an exchange of signals between the roots of host plants and the soil bacteria, rhizobia (Limpens and Bisseling 2003). Development of the root

nodule is characterized by plant cell proliferation and differentiation and cell-specific gene expression. New cell wall synthesis occurs during cell proliferation and infection, and several nodule-enhanced genes, or plant nodulins, encode putative cell wall proteins belonging to the hydroxyproline-rich glycoprotein (HRGP) superfamily (Kieliszewski and Lamport 1994). On the basis of their predicted protein sequences, these nodulins represent three branches of the HRGP family, including putative extensins (Perlick and Puhler 1993, Arsenijevic-Maksimovic et al. 1997, Dahiya and Brewin 2000, Rathbun et al. 2002), a possible arabinogalactan protein (ENOD5) (Horvath et al. 1993, Fruhling et al. 2000), and proline-rich proteins (PRPs). Nodulins of the PRP class, including *ENOD2*, *ENOD10*, *ENOD11*, *ENOD12*, and *PRP4* have been cloned from various legumes, such as pea, soybean, *Medicago* spp., and *Sesbania rostrata* (Franssen et al. 1987, Dickstein et al. 1988, Scheres et al. 1990a, van de Wiel et al. 1990, Govers et al. 1991, Dehio and de Bruijn 1992, Pichon et al. 1992, Allison et al. 1993, Lobler and Hirsch 1993, Wilson et al. 1994, R. Chen et al. 1998, Foster et al. 2000, Karlowski et al. 2000, Journet et al. 2001). The diagnostic features of the predicted amino acid sequences of the PRP nodulins are the occurrence of pentameric (hydroxy)proline-rich repeats and the relative scarcity of serine. Furthermore, where the whole sequence is known, these nodulins have a typical signal peptide that indicates that the proteins probably enter the secretory pathway (Scheres et al. 1990a, Govers et al. 1991, Pichon et al. 1992, Lobler and Hirsch 1993, Wilson et al. 1994).

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Although the proline-rich nodulins share some sequence characteristics, they differ in predicted molecular weight, the proportion of the mature peptide made up of (hydroxy) proline-rich repeats, and the sequences of repeated motifs. Furthermore, their temporal and spatial patterns of expression suggest that they have distinct functions. For example, in pea and *Medicago* spp., *ENOD11* and *ENOD12* are induced in root hairs within hours of inoculation with the appropriate *Rhizobium* species or treatment with the corresponding chitolipooligosaccharide Nod factor, and are expressed in the prefixation zone of indeterminate nodules (Scheres et al. 1990a; Govers et al. 1991; Pichon et al. 1992; Horvath et al. 1993; Journet et al. 1994, 2001). In contrast, *ENOD2* is induced several days later, during the nodule development phase, and its expression is localized to a peripheral tissue, the nodule parenchyma, in both pea and soybean nodules (Scheres et al. 1990b, van de Wiel et al. 1990). Expression of another early nodulin, *PRP4*, is highest in the meristematic cells of *Medicago truncatula* nodule primordia (Wilson et al. 1994). *PRP4* transcript is also detected at lower levels in the nodule parenchyma, vascular bundles, and in infected tissue of the prefixation zone. Thus, the expression pattern of *PRP4* overlaps both that of *ENOD2* and that of *ENOD11* and *ENOD12*.

Despite the elegant *in situ* analyses of gene expression, proteins corresponding to these early nodulins have been neither isolated nor characterized, and little is known of their subcellular localization. From current knowledge of PRPs, it is difficult to predict the posttranslational modifications and arrangement in the cell wall of these early nodulins, only on the basis of their deduced primary amino acid sequences (Kieliszewski and Lamport 1994). Thus, questions about PRP function in nodules require identification and characterization of the mature proteins *in vitro* and *in situ*.

In previous work, we used polyclonal antibodies against soybean PRP2 to identify PRPs in pea nodules (Sherrier and VandenBosch 1994, Wycoff et al. 1998). PRP2 contains abundant repeats of the motifs ProHypValGluLys and ProHypValTyrLys, which also occur in PRP1, PRP4, and other proteins (Wilson et al. 1994, Kieliszewski and Lamport 1994, and references therein). This antibody identified a group of common and nodule-specific proteins that were found in the extracellular matrix of infection threads and intercellular spaces of the nodule parenchyma, as well as in endodermal cells and xylem elements. PRPs were found in the primary wall, secondary wall, or intercellular space, depending on the plant tissue in which they were localized. The protein localization pattern coincided with the sum tissue-specific expression patterns of known PRP-like

early nodulins and suggested important symbiotic roles for PRPs. However, this approach was limited by its inability to distinguish among proteins of the group.

The aim of the current work was to precisely determine the subcellular location, or subdomain of the plant cell wall, of ENOD2, to help define its function in nodule formation. To this end, we raised polyclonal antibodies against a peptide containing three distinctive motifs from the predicted PsENOD2 amino acid sequence. We have previously reported the use of these antibodies to monitor ENOD2 protein levels in alfalfa nodules grown under different oxygen levels (Wycoff et al. 1998). Here, we have used these antibodies to immunolocalize a nodule-specific protein epitope in peas and soybeans. The results indicate that motif-specific antibodies may be useful to investigate the location and function of specific members of the PRP family.

Material and methods

Antibody production

A peptide corresponding to the first 21 amino acids predicted by the pPsENOD2 cDNA (Fig. 1) (van de Wiel et al. 1990) was synthesized according to established methods with tertiary butyloxycarbonyl at the Peptide Services Laboratory, Texas A&M University. The lyophilized synthetic peptide, designated PsENOD2-PEP, was dissolved in 1% acetic acid and then lyophilized again to remove volatile compounds left from the synthesis process. PsENOD2-PEP was then conjugated to bovine serum albumin (BSA) by standard protocols (Harlow and Lane 1988). The BSA-peptide conjugate was used to raise antibodies in two female New Zealand white rabbits. For the initial immunization a total of 200 µg of conjugate in Freund's complete adjuvant was delivered to each rabbit via several subcutaneous injections. The rabbits received two additional boosts, using 200 µg of peptide-BSA conjugate in Freund's incomplete adjuvant each time, at 2 and 6 weeks after the initial injections. Final bleeds were performed by cardiac puncture nine days after the final injections.

To make an affinity column for the purification of PsENOD2-PEP-specific antibodies, the same synthetic peptide was coupled to CNBr-Sepharose 4B (Sigma), according to existing protocols (Stiller and Nielsen 1983). Ammonium sulfate precipitation was used to isolate immunoglobulins G (IgGs) from the rabbit sera (Harlow and Lane 1988). The IgG precipitate was resuspended in PBS (phosphate-buffered saline, containing 140 mM NaCl

	1
PsENOD2-PEP	POHEKPOHEHTPOEYQPOHEK
pPsENOD2	PPHEKPPHEHTPPEYQPPHEKPPHEHPPPEYQ
	33
pPsENOD2	PPHEKPPHEKPSPKYQPPHEKSPPEYQPPHEK
	65
pPsENOD2	PPHENPPPVYKPPYENSPPPHVYHRPLFQAPP
	97
pPsENOD2	PVKPSRPFPGFPAFKN

Fig. 1. Amino acid sequence of the PsENOD2 synthetic peptide (PsENOD2-PEP), in comparison to the predicted amino acid sequence of the pPsENOD2 cDNA insert (van de Wiel et al. 1990). Hydroxyproline is designated by the letter O in the synthetic peptide

[except where noted], 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) and washed in an Amicon ultrafiltration cell (fitted with a YM 10 filter) to remove the ammonium sulfate and to concentrate the IgG fraction. PsENOD2-PEP-specific IgGs were isolated by affinity chromatography according to the methods of Harlow and Lane (1988), using 100 mM triethylamine buffer (pH 11.5) to elute the specific antibodies. The antibodies were again washed with PBS in the Amicon cell. Specificity of the antibody was verified by probing immunoblots of nodule proteins. Following affinity purification, antibodies from both rabbits showed the same specificity, and so the antibodies were pooled for future use. Sodium azide was added to 0.02% and the antibodies were stored at -20 °C.

Additional antibodies were obtained from other investigators for comparative localization of other cell wall components in nodules. These included the rat monoclonal antibodies MAC265, which reacts with a nodule-enhanced 100 kDa extensin-like glycoprotein (VandenBosch et al. 1989, Rathbun et al. 2002), and JIM5, which reacts with polygalacturonic acid (Knox et al. 1990), as well as a polyclonal antibody against soybean PRP2 (Marcus et al. 1991).

Protein extraction and immunoblot analysis

Peas (*Pisum sativum* L. cv. Sparkle) were inoculated with *Rhizobium leguminosarum* bv. *viciae* strain B556 and grown as previously described (Sherrier and VandenBosch 1994). Soybean (*Glycine max* L. cv. Essex) was grown and nodulated as described by Weaver et al. (1991). Nodules were harvested 21 days after inoculation (peas) or from 28-day-old plants (soybean), frozen in liquid nitrogen, and stored at -20 °C. Roots were harvested similarly from uninoculated pea plants that had been grown under the same conditions as the nodulated plants. Proteins were extracted from plant organs, prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Immobilon P membrane (Millipore), and probed with antibodies as previously described (Sherrier and VandenBosch 1994). Proteins were first extracted from tissue with a low-salt buffer containing 3 mM EDTA, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg of leupeptin per ml in 40 mM Tris-HCl at pH 8.0. The pellet was re-extracted with a high-salt buffer, consisting of 200 mM CaCl₂ added to the above buffer. Each lane was loaded with 8 µg of protein. The concentration of affinity-purified IgGs used to detect ENOD2 on immunoblots was 1–5 µg/ml.

Immunogold labeling for light and electron microscopic visualization of ENOD2 distribution

Pea and soybean nodules were fixed and embedded in LR White resin, as described previously for pea nodules (Sherrier and VandenBosch 1994). Immunogold-silver staining (IGSS) was used for light microscopic visualization of ENOD2 distribution in nodules (VandenBosch et al. 1994). Following IGSS, nodule sections were stained with 1% basic fuchsin in 1% sodium *m*-borate buffer and then photographed on an Olympus BH-2 microscope with bright-field optics.

The ultrastructural distributions of ENOD2 protein and other extracellular matrix components were demonstrated with single and double immunogold labeling by previously reported protocols (VandenBosch 1992). All secondary-antibody-gold conjugates were obtained from Amersham. After probing with PsENOD2-PEP-specific antibodies, reactive proteins were visualized using 15 nm diameter gold-labeled goat anti-rabbit IgG (Auroprobe EM GAR G15). Polygalacturonic acid was labeled with the monoclonal antibody JIM5 (Knox et al. 1990), and the high-molecular-weight extensin was labeled with MAC265 (VandenBosch et al. 1989); both of these rat monoclonal antibodies were detected with 5 nm diameter gold conjugated to goat anti-rat IgG (Auroprobe EM GARa G5). Most specimens were poststained with uranyl acetate and lead citrate before examination in a Zeiss 10C transmission electron microscope operated at 80 kV. Lead citrate staining was omitted for specimens labeled with 5 nm diameter gold.

ENOD2 motif searches

The specificity of the designed 21-amino-acid peptide shown in Fig. 1 was assessed by searching the public sequence databases for its component pentameric proline-rich repeat sequences (PPHEH, PPHEK, PPEYQ). Database searches were performed with a perl script that scans an input sequence for matches to a pattern that recognizes multiple instances of these three repeats. The following databases were scanned: the July 2003 versions of dbEST (Benson et al. 2003), the public repository of ESTs; dbGSS (Benson et al. 2003), the public database of genomic survey sequences; dbHTG (Benson et al. 2003), the collection of high-throughput genomic sequences, and Swiss-Prot/TrEMBL (SP/Tr) (Boeckmann et al. 2003), a nonredundant collection of protein sequences from all organisms. SP/Tr was searched with the pattern (P{2,3}(HEHIHEKIEYQ)_x{0,5}){2,}. This pattern matches two or more consecutive pentameric (with 2 initial prolines) or hexameric (with 3 prolines) repeats that are optionally separated by five or fewer intervening residues. An equivalent nucleotide pattern, ((CCx){2,3}(CA[CT]GA[AG](CA[CT]AA[AG])GA[AG]TA[CT]CA[AG])_x{0,15}){2,}, was used to search dbEST, dbGSS, and dbHTG. In these patterns, "x" matches any residue, "I" denotes "or", square brackets indicate a choice for a single residue, and curly braces define a range of repeat numbers to be matched.

Results

Design and production of antibodies against ENOD2

To produce antibodies expected to be specific for ENOD2, we made a synthetic peptide, PsENOD2-PEP, that corresponds to the predicted first 21 amino acids of a partial cDNA clone of *PsENOD2* from *Pisum sativum* (van de Wiel et al. 1990). This sequence was chosen because it contains the pentameric repeats ProProHisGluLys and ProProGluTyrGln that are characteristic of ENOD2 in several species of legumes (Franssen et al. 1987, Dickstein et al. 1988, van de Wiel et al. 1990, Dehio and de Bruijn 1992). Furthermore, the chosen sequence avoided the motif ProProValTyrLys, which is a minor component among predicted polypeptides of ENOD2 but is very common among other PRPs such as PRP1 and PRP2 from soybean and *M. truncatula* (Hong et al. 1987, 1990; Datta et al. 1989; Wilson and Cooper 1994), and PRP4 from *M. truncatula* (Wilson et al. 1994). Previous studies have indicated that some, but not all, prolyl residues become post-translationally hydroxylated in a predictable manner, according to the sequence context (Averyhart-Fullard et al. 1988, Kieliszewski and Lamport 1994). Therefore, in this study, hydroxyproline residues were substituted for the second proline residue contained within repeat motifs of the synthetic peptide (Fig. 1), according to known hydroxylation patterns (Cassab 1998).

ENOD2 antibodies recognize a subset of proline-rich proteins in nodules

Proteins extracted from pea tissues were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

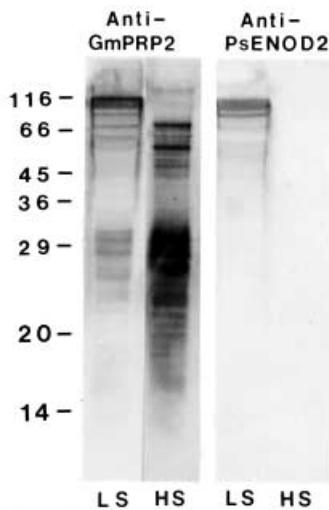


Fig. 2. Comparison of anti-pRP2 and anti-PsENOD2-PEP antibodies. Polyclonal antibodies specific for the PRP2 protein from soybean (anti-GmPRP2) or the pea ENOD2 motif (anti-ENOD2) were used as probes on immunoblots of nodule protein extracts. *LS* Low-salt extract; *HS* high-salt extract

and probed either with anti-GmPRP2 antibody, a general probe for proline-rich proteins (Marcus et al. 1991, Ye et al. 1991, Sherrier and VandenBosch 1994), or with affinity-purified anti-PsENOD2-PEP antibody. The anti-GmPRP2 antibody recognized a wide range of proteins in both low-salt and high-salt protein extracts from root nodules. The major cross-reactive proteins recognized by this antibody were high-salt soluble and had a molecular mass of approximately 30 kDa (Fig. 2). The anti-PsENOD2-PEP antibody recognized a subset of the proteins identified by anti-GmPRP2 (Fig. 2). Specifically, the major pea nodule protein recognized by the anti-PsENOD2-PEP antibody was extracted in low-salt buffer and ran at a molecular mass of approximately 100 kDa.

To evaluate organ specificity of the anti-PsENOD2-PEP antibody epitopes, pea root, nodule, leaf, stem, and flower extracts were probed with the antibody. The major band at approximately 110 kDa was detected in low-salt-extracted nodule proteins, whereas no cross-reactive proteins were detected in either low- or high-salt extracts from roots (Fig. 3). In leaf and stem extracts, a 50 kDa cross-reactive band from low-salt extracts and a 40 kDa band from high-salt extracts were detected (data not shown). No cross-reactive proteins were detected in flower tissues (data not shown).

Affinity purification and characterization of ENOD2 protein from pea nodules

The antibody against ENOD2-PEP was used to affinity-purify immunoreactive proteins from low-salt extracts of

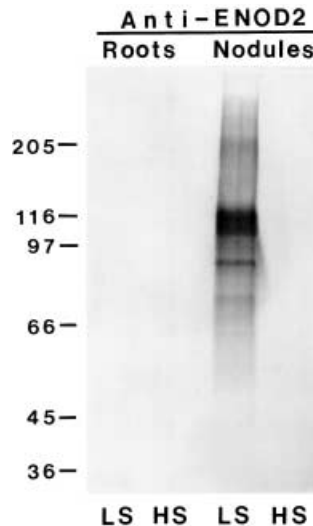


Fig. 3. Organ specificity of ENOD2 proteins. Polyclonal antibodies specific for the pea ENOD2 motif (anti-PsENOD2) were used as probes on immunoblots of uninoculated root and nodule protein extracts. *LS* Low-salt extract; *HS* high-salt extract

pea nodules (NodLS fraction). Two bands were visible after Aurodye staining of eluted proteins (Fig. 4A). The major band, which was also visible after staining with Coomassie blue (not shown), had an apparent molecular mass of approximately 110 kDa, and a second, somewhat less abundant band was visible at about 200 kDa. When NodLS proteins and immunoaffinity-purified proteins were probed with anti-ENOD2-PEP, it was evident that the two affinity-purified proteins ran at the same molecular mass as did the two proteins detected in the NodLS fraction (Fig. 4B). A lower-molecular-mass band (approx. 85 kDa), which was consistently seen on immunoblots of nodule proteins probed with anti-ENOD2-PEP, was sometimes detected in affinity-purified protein (not shown). Affinity-purified protein was found to cross-react with the more general probe anti-PRP2, suggesting that the affinity-purified proteins may be PRPs. Purified protein was also probed with the monoclonal antibody MAC265, which reacts with a high-molecular-mass extensin-like glycoprotein, in the extracellular matrix of pea nodules (Rathbun et al. 2002, VandenBosch et al. 1989). Although this monoclonal antibody recognizes pea nodule proteins of a molecular mass similar to ENOD2-related proteins, it did not cross-react with affinity-purified proteins (Fig. 4B).

Localization of ENOD2-related proteins in pea and soybean nodules

In describing pea and soybean nodule anatomy, we use previously established terminology for the zones of develop-

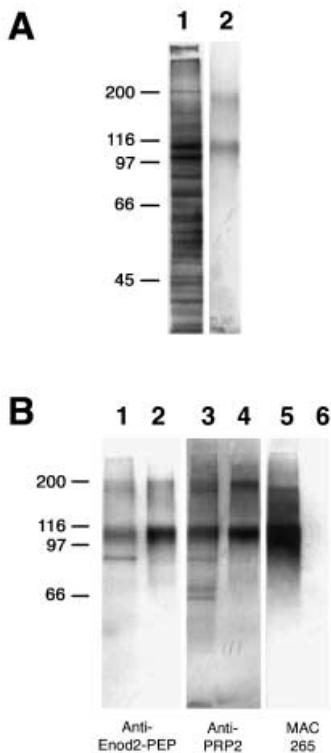


Fig. 4A, B. Characterization of affinity-purified ENOD2-related proteins. **A** Aurodye staining of total protein in low-salt extracts of pea nodules (1) and ENOD2-related proteins purified by immunoaffinity chromatography (2). **B** Immunoreactivity of nodule proteins and affinity-purified protein. 1, 3, and 5 Lanes loaded with pea nodule proteins extracted under low salt conditions; 2, 4, and 6 lanes containing affinity-purified protein. 1 and 2 Lanes probed with anti-PsENOD2-PEP; 3 and 4 lanes with anti-GmPRP2; 5 and 6 lanes with the monoclonal antibody MAC265, which identifies a nodule-enhanced extensin-like glycoprotein

ment in indeterminate nodules and for nodule tissues outside the central infected zone (Mylona et al. 1995).

Light microscopic localization of ENOD2 protein in pea nodules revealed that ENOD2 was deposited in the intercellular spaces of the nodule parenchyma (Fig. 5). This specific localization to the nodule parenchyma was continuous throughout the nodules, from the mature base of the nodule to the prefixation zone–meristem boundary. Label was completely absent from all other tissues, including meristematic cells (not shown), and the infected cells and uninfected interstitial cells in the nitrogen fixation zone (Fig. 5B). Whereas parenchyma intercellular spaces were clearly labeled in the prefixation zone, no ENOD2 could be detected within the infection threads or elsewhere in adjacent newly infected cells. Vascular tissue running through the nodule parenchyma also lacked detectable label (Fig. 5C).

Ultrastructural examination of immunogold-labeled pea nodule sections showed that ENOD2 did not become incorporated into the cellulosic portion of the cell wall (Fig. 6). In the prefixation zone, immunoreactive protein

was found in the extracellular matrix at three-way junctions between cells prior to, and concomitant with, the formation of intercellular spaces. Double labeling of ENOD2 protein and polygalacturonan demonstrated that ENOD2 partitioned into a nonfibrillar phase of the wall outside the expanded middle lamella (Fig. 6A). The area of heaviest ENOD2 labeling consisted of a moderately electron-dense, amorphous material. In the older nodule tissues in the nodule fixation zone, ENOD2 was an abundant component of the extracellular matrix, in intercellular spaces of the nodule parenchyma, and adjacent to the vascular and nodule endodermis tissues (Fig. 6B). In this zone, immunolabel was also frequently found over material between the plasma membrane and the wall (Fig. 6B). Double labeling of sections with anti-PsENOD2-PEP and MAC265 indicated that ENOD2 and the MAC265-reactive glycoprotein extensin were localized in the same area of the extracellular matrix, in the nodule parenchyma, and adjacent to the nodule endodermis (Fig. 6C). In the nodule parenchyma and endodermal tissues, label was found over endoplasmic reticulum (not shown) and Golgi bodies (Fig. 6D). Preincubation of the primary antibody with ENOD2-PEP–BSA conjugate before immunolabeling virtually eliminated deposition of gold particles on pea nodule sections (Fig. 6E). Likewise, omission of primary antibodies resulted in a lack of label on sections (not shown).

The antibody against ENOD2-PEP was also used to identify similar proteins in soybean, a determinate nodule-forming legume. The motifs ProHypHisGluLys and ProHypGluTyrGln, against which the antibody was made, are expected to be abundant in the soybean ENOD2 gene product, and the motif ProHypHisGluAsn also occurs at least once, on the basis of the predicted amino acid sequence (Franssen et al. 1987). This suggests that antibodies against ENOD2-PEP should be highly reactive against the corresponding soybean protein. On immunoblots, multiple immunoreactive bands were detected in low-salt extracts of soybean (*Glycine max* L.) nodules, with molecular masses ranging from approximately 70 kDa to well over 200 kDa (Fig. 7). The major band, at about 200 kDa, migrated more slowly than the major immunoreactive bands found in pea extracts. After immunolabeling of soybean nodule sections with anti-ENOD2-PEP, label was detectable over intercellular spaces adjacent to nodule parenchyma and nodule endodermis cells (Fig. 8A). Labeled sections showed a clear demarcation between the nodule parenchyma and the unlabeled adjacent boundary layer, an uninfected tissue that separates the nodule parenchyma from the outermost infected cells (Fig. 8B). The secondary cell walls of the sclerified nodule endodermis and all cells in the vascular bundles were also unlabeled (Fig. 8B).

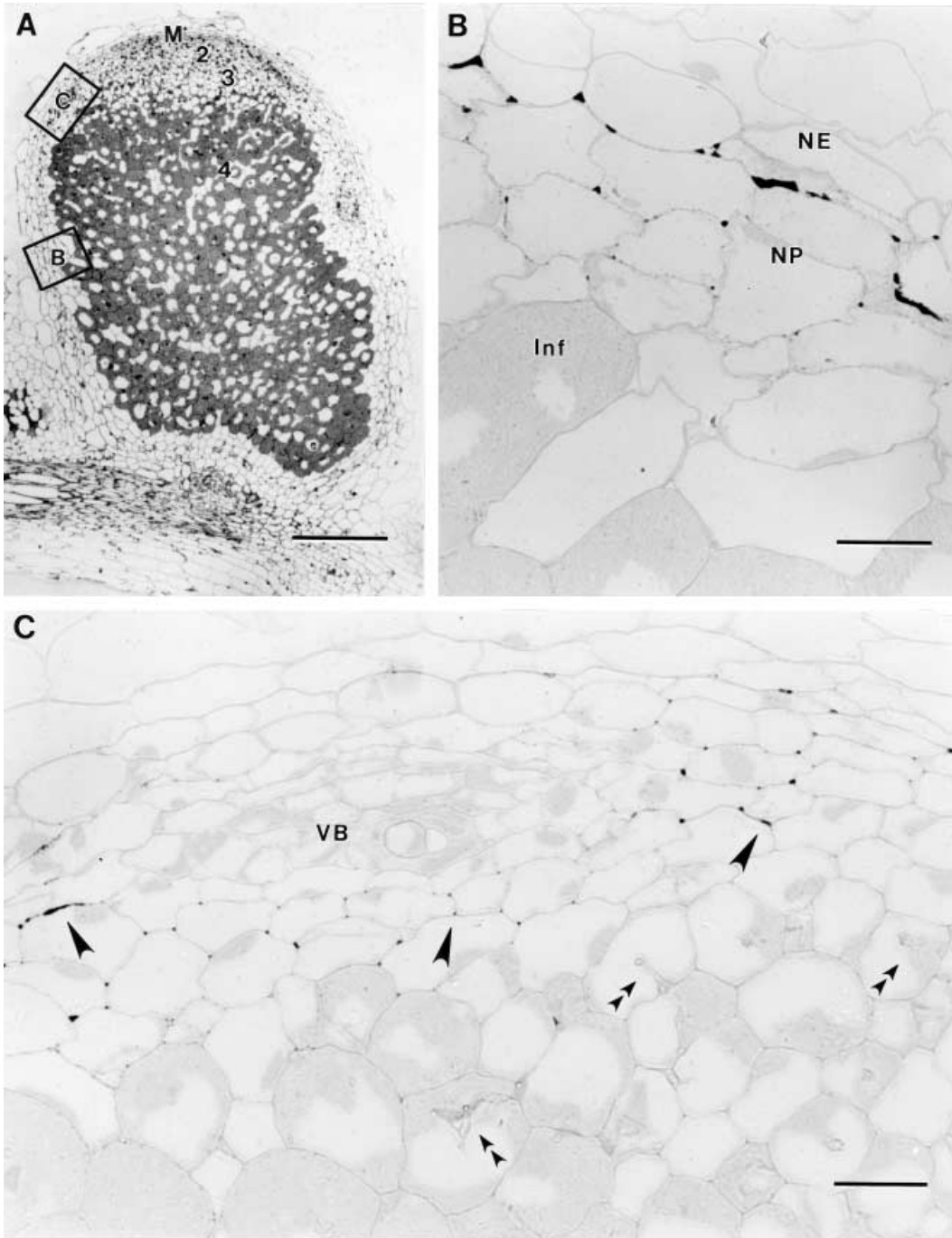


Fig. 5A–C. Light microscopic localization of ENOD2 in sections of pea nodules. **A** Low-magnification view of a longitudinal section of a pea nodule indicating the different developmental zones. Boxes denote areas depicted in panels B and C. Section was stained with azur II and methylene blue. Bar: 250 μm . **B** and **C** Higher magnification views of a sequential section after immunogold-silver staining and counterstaining with basic fuchsin. **B** Localization of ENOD2 in the intercellular spaces of the nodule parenchyma adjacent to the nitrogen fixation zone. Label appears as dense black deposits between cells. No label is present in the infected cells or in the uninfected interstitial cells. Bar: 25 μm . **C** Localization of ENOD2 in the intercellular spaces (large arrowheads) of the nodule parenchyma adjacent to the prefixation zone. No label is visible within the vascular bundle or infection threads (small double arrowheads). Bar: 25 μm . *M* Nodule meristem; 2 prefixation zone; 3 interzone; 4 nitrogen fixation zone; *Inf* infected cell; *NE* nodule endodermis; *NP* nodule parenchyma; *VB* vascular bundle

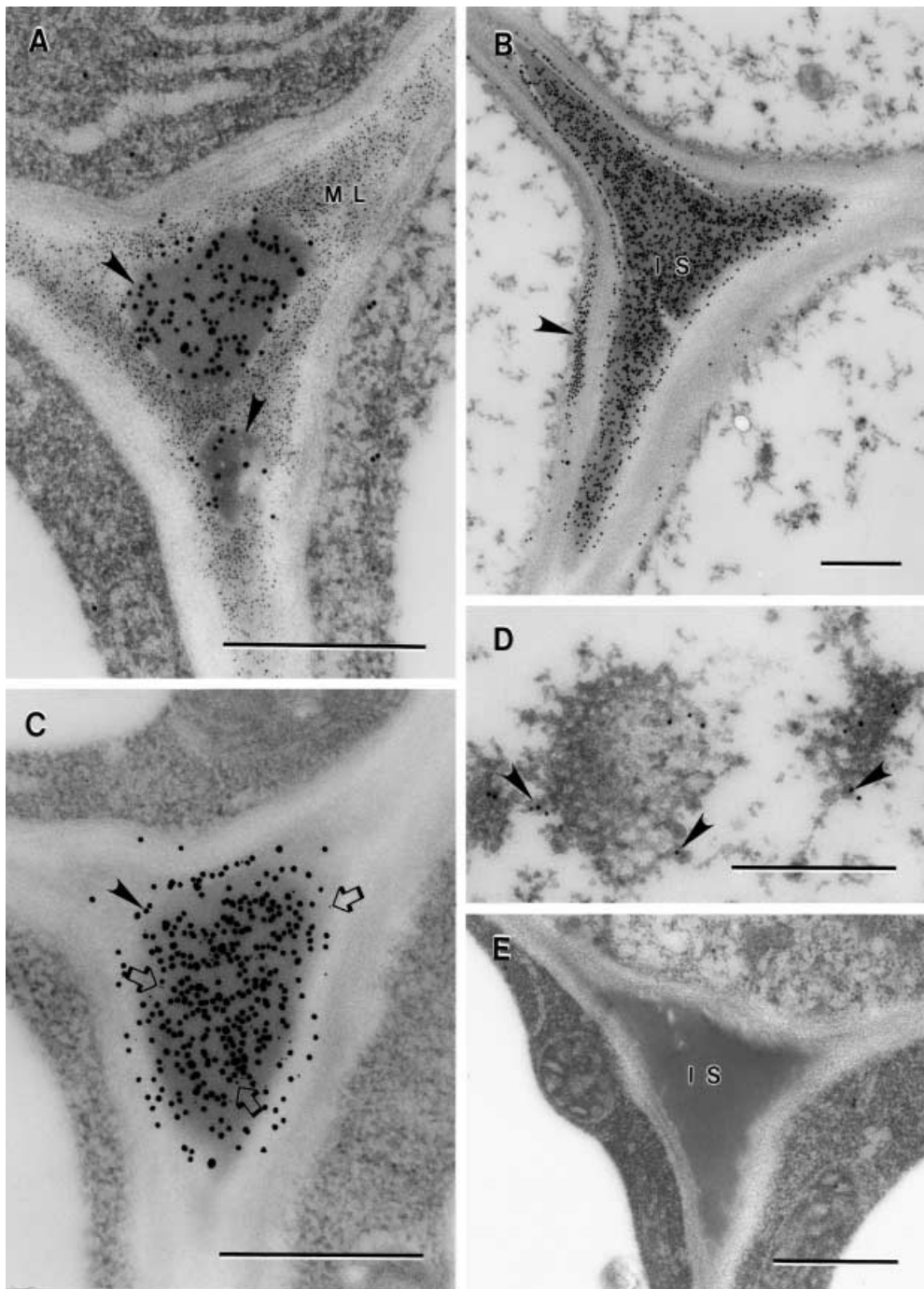


Fig. 6A–E. Ultrastructural localization of ENOD2 in pea nodules. Bars: 0.5 μm . **A** Localization of ENOD2 and polygalacturonan in a three-way junction between nodule parenchyma cells in the prefixation zone. The distribution of ENOD2 is marked by 15 nm diameter gold particles that occur chiefly over the amorphous matrix (arrowheads) between the cells. The middle lamella surrounds accumulations of ENOD2, as seen by the 5 nm diameter gold particles that label polygalacturonan. **B** Localization of ENOD2 in an intercellular space between three nodule parenchyma cells in the nitrogen fixation zone. Labeled protein also occurs between the plasma membrane and the cell wall (arrowhead). **C** Colocalization of ENOD2 (15 nm gold particles, e.g., at arrowhead) and MAC265-reactive glycoprotein (5 nm gold particles, e.g., arrows) in an intercellular space. The cells depicted are at the junction of the nodule parenchyma and vascular endodermis in the nitrogen fixation zone. **D** Face and oblique views of Golgi bodies in a nodule parenchyma cell. Gold particles, marking the presence of ENOD2, are often seen in association with Golgi cisternae and buds or associated vesicles (arrowheads). **E** Nonimmune control labeling of an intercellular space between the nodule parenchyma and vascular endodermis. Primary antibody against ENOD2 was preadsorbed with excess ENOD2-PEP-BSA conjugate before labeling of section. *IS* Intercellular space; *ML* middle lamella

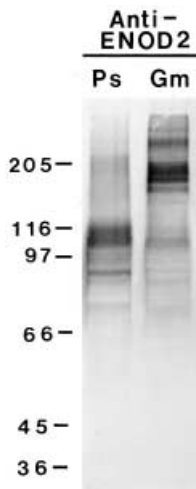


Fig. 7. Immunoblot detection of ENOD2-related proteins in low-salt extracts from pea and soybean nodules. *Ps* Immunoreactive pea proteins; *Gm* immunoreactive soybean proteins

Occurrence of ENOD2 signature motifs in known angiosperm protein sequences

The plant protein and nucleotide sequences deposited in public databases constitute an important resource for investigating the distribution of protein motifs that was not available when the ENOD2 antibodies were produced. To verify that the amino acid motifs used for ENOD2 antibody production were clearly diagnostic of ENOD2, database searches were designed to identify related motifs in similar proteins. Exhaustive searches for the pentameric motifs PPHEH, PPHEK, and PPEYQ identified 12 sequences from Swiss-Prot/TrEMBL, 6 sequences from dbEST, and no sequences from dbGSS or dbHTG. All 18 hit sequences were from legumes. The majority of the sequences were known to have a nodule-specific accumulation pattern and are likely homologs of *ENOD2*. The exceptions came from two nonnodulating legumes, *Cladrastis lutea* and *Maackia amurensis*, where *ENOD2*-like transcripts were detected in stems, roots, and flowers (Foster et al. 2000). The 18 sequence accessions and their motif distribution counts are shown in Table 1.

Discussion

ENOD2 is a proline-rich protein (PRP) that is induced during nodule differentiation in the interaction between a diverse group of legumes and their nitrogen-fixing symbionts of the bacterial family Rhizobiaceae. Although the expression of *ENOD2* has been well characterized, its function is unknown and the ultrastructural location was previously undetermined. In this study, we examined the

occurrence of ENOD2 within pea and soybean root nodules. In the past, PRPs were shown to be present in the extracellular matrix of the nodule parenchyma, in the infection thread matrix, and in the secondary wall of xylem in root nodules (Sherrier and VandenBosch 1994). However, the location of ENOD2 could not be specifically determined because no probes specific for this protein were available.

In this study, an ENOD2-specific antibody probe was generated against a synthetic peptide derived from the predicted amino acid sequence of the *ENOD2* gene from garden pea. Members of the PRP class of cell wall proteins typically share sequences among their proline-rich repeats. The most highly conserved tandem repeats are variants of the pentamer PPVX(K/T), where X is often Y, H, or E (Fowler et al. 1999). ENOD2 proteins are distinct from other PRPs in that they contain the repeat motifs PPHEH/K and PPEYQ, and relatively less valine and tyrosine. These ENOD2 signature motifs were used to design a synthetic antigen. After affinity purification, the antibody specifically recognized a subset of proteins recognized by the more generic PRP2 antibody probe.

As the synthetic peptide antigen was derived from a motif common among predicted ENOD2 sequences from several legume species, the antibody was used to probe protein extracts and tissues from pea and soybean root nodules, as examples of indeterminate and determinate nodules, respectively. On Western blots, the major reactive band from pea root nodules was approximately 110 kDa. Cross-reactive proteins ranging from 70 kDa to over 200 kDa were also detected in extracts from soybean nodules with the ENOD2-specific antibody. This diversity in molecular mass may be due to differential glycosylation or strong interactions with another component of the extracellular matrix. No immunoreactive protein was detectable in roots, consistent with predictions from Northern blot analysis of pea and soybean transcripts (van de Wiel et al. 1990). Immunologically related proteins of different molecular masses were also found in low- and high-salt extracts from stems but were not detectable in flowers.

ENOD2 transcripts have been detected in alfalfa pseudonodules formed after treatment of roots with auxin transport inhibitors (Hirsch et al. 1989), in empty alfalfa nodules induced by rhizobial mutants or *Agrobacterium tumefaciens* encoding rhizobial *nod* genes (Hirsch et al. 1985, Franssen et al. 1987, Dickstein et al. 1988), and in alfalfa roots infected with Nod⁻ rhizobia carrying a transzeatin secretion gene (Cooper and Long 1994). In addition, an *ENOD2*-like gene is expressed in roots, flowers, and stems of two nonnodulating woody legumes (Foster

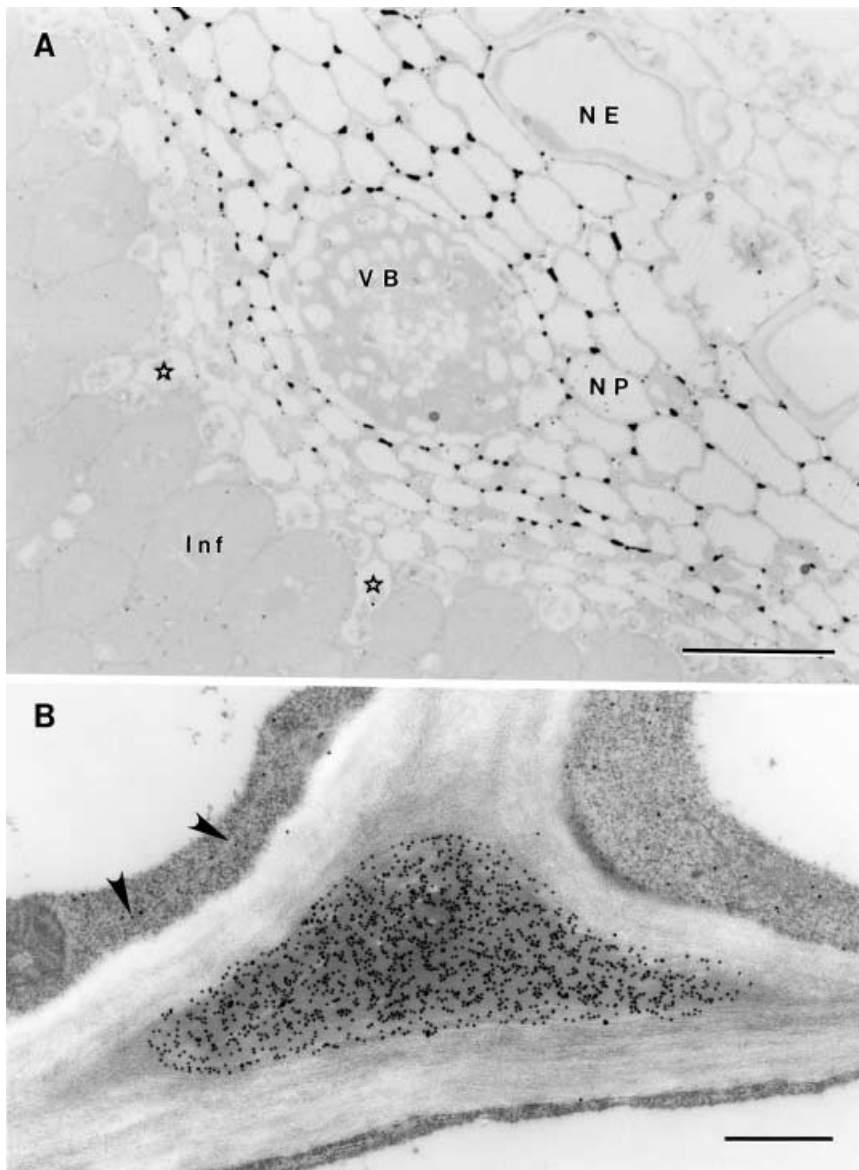


Fig. 8A, B. Detection of ENOD2 in soybean nodule parenchyma. **A** Light microscopic view of ENOD2 immunolabeling. Silver-enhanced gold label is visible as opaque black deposits over intercellular spaces adjacent to nodule parenchyma (*NP*) and nodule endodermal (*NE*) cells. Infected tissues (*Inf*) and the vascular bundle (*VB*) are unlabeled. Stars indicate junctions between rays of uninfected interstitial cells with the unlabeled border zone. Bar: 50 μ m. **B** Electron microscopic view of ENOD2 immunolabeling in an intercellular space between three nodule parenchyma cells. Arrowheads indicate intracellular label over endomembranes in the nodule parenchyma. Bar: 0.5 μ m

et al. 2000). These data suggest that ENOD2 may be important for restructuring the plant extracellular matrix during plant organ development.

Bonilla and co-workers (1997) suggested that ENOD2 assembly into the wall of developing nodules was dependent on boron. Boron binds rhamnogalacturonan II within the plant extracellular matrix (Hu and Brown 1994) and may be critical for the maintenance of cell wall integrity (Loomis and Durst 1992). Using general HRGP antibodies, Bonilla and co-workers (1997) showed that a protein identified as ENOD2 was present in the parenchyma walls and tissue extracts of normal bean nodules. In contrast, ENOD2 was present in tissue extracts, but absent from walls, of boron-deficient bean nodules in which cells of the uninfected

nodule periphery demonstrated aberrant development and altered cellular morphology. Therefore, the altered targeting of ENOD2 during boron deficiency may be a consequence of abnormal cellular differentiation or changes in the physical and biochemical nature of the extracellular matrix. To understand the possible interactions of ENOD2 with other cellular constituents, it is critical to know the submural distribution of ENOD2 and its spatial relationship to other cell wall components within developing nodule tissues. However, the methodology utilized in this study was limited in resolution, and the extracellular distribution of the protein could not be determined precisely.

In our study, the anti-PsENOD2-PEP antibody strongly recognized protein in the expanded intracellular spaces of the

Table 1. Distribution of ENOD2 repeat motifs in sequences from dbEST and Swiss-Prot/TrEMBL

Species ^a	Accession	Reference(s)	Nr. of appearances of motif					
			PPHEK	PPPHEK	PPHEH	PPPHEH	PPEYQ	PPPEYQ
Cl ^b	Q9FUR6 ^c	Foster et al. 2000	1	11	0	0	0	0
Gm	P08297 ^c	Franssen et al. 1987, 1990	15	2	0	0	0	8
Ll	P93237 ^c	Karlowski et al. 1997	8	24	0	0	0	0
Ll	Q06841 ^c	Szczyglowski and Legocki 1990	5	11	0	0	0	0
Lj	AV407549 ^d		3	0	0	0	0	1
Lj	BI419058 ^d		3	0	0	0	0	0
Lj	CB828470 ^d		3	0	0	0	0	0
Lj	CB829392 ^d		3	0	0	0	0	0
Ma ^b	O49151 ^c	Foster et al. 1998	3	15	0	0	0	6
Ma	Q9FUR5 ^c	Foster et al. 2000	3	8	0	0	0	4
Ms	P11728 ^c	Dickstein et al. 1988	5	0	1	0	0	3
Mt	BE998393 ^d		2	0	1	0	0	2
Mt	BQ165835 ^d		1	0	1	0	0	3
Ps	P16329 ^c	van de Wiel et al. 1990	5	0	2	0	2	1
Sj	Q9FUR7 ^c	Foster et al. 2000	0	6	0	0	0	7
Sr	Q41402 ^c	Dehio and de Bruijn 1992	17	0	0	0	0	7
Sr	Q41404 ^c	Strittmatter et al. 1989	3	0	0	0	0	1
Vf	Q9S9A7 ^c	Perlick and Puhler 1993	0	1	2	0	0	0

^a Cl, *Cladrastis lutea*; Gm, *Glycine max*; Ll, *Lupinus luteus*; Lj, *Lotus japonicus*; Ma, *Maackia amurensis*; Ms, *Medicago sativa*; Mt, *Medicago truncatula*; Ps, *Pisum sativum*; Sj, *Styphnolobium japonicum*; Sr, *Sesbania rostrata*; Vf, *Vicia faba*

^b *Cladrastis lutea* and *Maackia amurensis* are nonnodulating legumes

^c Sequence from Swiss-Prot/TrEMBL

^d Sequence from dbEST

uninfected nodule parenchyma in both pea and soybean, but did not bind to the fibrillar portion of the plant cell wall. Neither cell walls of infected cells nor infection threads contained the ENOD2 epitope. These results are consistent with *ENOD2* message localization in the nodule parenchyma, with the prediction that ENOD2 is a secreted protein (Scheres et al. 1990a, Govers et al. 1991, Pichon et al. 1992, Lobler and Hirsch 1993, Wilson et al. 1994), and with a cell wall localization which had been observed by light microscopy (Wycoff et al. 1998). To date, little is known about how proteins are targeted to subdomains within the plant cell wall. It is clear, however, that different domains of the wall have specialized functions.

Gases and liquids generally move readily through the plant apoplast, though in root nodules their passage from the rhizosphere into the nodule interior is occluded at the nodule parenchyma (Hunt and Layzell 1993, Jacobsen et al. 1998). The intercellular spaces of nodule parenchyma have previously been shown to contain an extensin-like glycoprotein

that bears an antigen recognizable by the monoclonal antibody MAC265, and that may be involved in the establishment and maintenance of the peripheral O₂ diffusion barrier (VandenBosch et al. 1989, Wycoff et al. 1998, Rathbun et al. 2002). In this study, we have shown that ENOD2 and an extensin bearing the MAC265 epitope have similar molecular weights and colocalize within the nodule parenchyma but are distinct proteins. The ENOD2 proteins isolated by immunoaffinity purification do not bear the MAC265 epitope characteristic of the nodule extensin, also suggesting that these two proteins do not become covalently cross-linked to one another. The MAC265 glycoprotein increases in abundance during times of induced O₂ stress, while ENOD2 quantities remain unchanged (Wycoff et al. 1998). ENOD2 may still contribute to the O₂ barrier, however, by occluding the intercellular space or by regulating the extent of cross-linking of the root nodule extensin.

PRPs are known to function in plant defense or in cellular development (Cassab and Varner 1988), are

expressed in a cell- or tissue-specific manner, and are induced after tissue wounding (J. Chen and Varner 1985, Tierney et al. 1988, Hong et al. 1987, van de Wiel et al. 1990, Ye et al. 1991, Santino et al. 1997, Fowler et al. 1999, Bernhardt and Tierney 2000). Extensins and some PRPs can become insolubilized within the cell wall during development or as a response to biotic or abiotic stress. This process is thought to be carried out by peroxide-mediated cross-linking involving tyrosine residues (Bradley et al. 1992, Brisson et al. 1994, Frueauf et al. 2000). In nodules, cross-linking of the extracellular matrix in the nodule parenchyma could serve to protect the plant from the spread of infection, either from external pathogens or from the internalized symbionts. However, ENOD2 proteins are relatively tyrosine poor, suggesting that they may not be candidates for oxidative cross-linking. Consistent with this prediction is the analysis of peptide sequences from chymotryptic digests of insolubilized cell walls in *M. truncatula* nodules which found that no PRPs were detectable among cross-linked nodule proteins (Frueauf et al. 2000). Interestingly, those authors did identify several extensin-related peptides in the insoluble wall fraction of nodules (Frueauf et al. 2000), including one that is characteristic of the MAC265-reactive extensins in *P. sativum* and MtN12, an ortholog in *M. truncatula* (Gamas et al. 1996, Rathbun et al. 2002). Wisniewski et al. (2000) have independently found that nodule extensin can become insolubilized in a peroxide-mediated process.

Given the amino acid content and the strict tissue distribution of ENOD2 within nodules, this protein may interact with other components in the intercellular spaces of the nodule parenchyma, conferring distinctive characteristics specifically to this tissue. The positively charged lysine and histidine residues may facilitate interaction of ENOD2 with negatively charged side chains of pectins. Here, we have shown that de-esterified pectin moieties line the intercellular spaces where ENOD2 accumulates, making such an interaction possible, perhaps promoting a “thickening” or “gelling” of the pectin at the interface. In addition, the abundance and placement of glutamate residues in ENOD2 suggests that ENOD2 polypeptides may interact with themselves or adjacent polypeptides via ionic interactions at alternating positively and negatively charged residues. These intra- and intermolecular interactions may enhance the ability of the plant to prevent infection by additional microbes, regulate gas diffusion, and delimit the extent of rhizobial cellular invasion.

The availability of extensive expressed sequence tags and genomic sequence data from angiosperms provides an out-

standing resource for investigating protein diversity in key model systems and crops and is an expanding resource for understanding the evolution of gene families in diverse taxa. Among legumes, sequences from *G. max*, *M. truncatula*, and *Lotus japonicus* are especially well represented in public databases. Well after the immunodetection of ENOD2 reported here was completed, we conducted a database search among legume proteins that are currently available, to ascertain whether the motifs that had been used for antibody production are indeed specific to ENOD2. The specificity of these signature motifs was verified and, surprisingly, an exhaustive search only retrieved protein and nucleotide sequences from the legume family (Fabaceae). Using a similar computational approach, Graham et al. (2004) identified additional PRP repeat motifs that were common in legumes but unknown from other taxa. These include PPVEK and PPVYK, the latter is the motif thought to be involved in peroxidase-mediated cross-linking of PRPs (Frueauf et al. 2000).

To date, PRPs have been studied most extensively in legumes. Although it is possible that the apparent legume specificity of ENOD2 signature motifs and other PRP motifs is due to the underrepresentation of many angiosperm taxa in large-scale sequencing projects, it appears likely that PRPs are highly variable among vascular plants. PRPs containing distinctive repeat motifs have been described in *Arabidopsis thaliana* (Fowler et al. 1999, Bernhardt and Tierney 2000) and rice (Akiyama and Pillai 2003). It is not yet clear whether these novel repeat motifs have diverged substantially from a common evolutionary origin or whether they represent convergence of unrelated genes. Whatever the case, the diversification of the PRP gene superfamily represents a rich opportunity for investigating the tailoring of protein structures to specialized cell wall functions across diverse plant taxa.

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