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Targeting and Release of Phytohemagglutinin from the Roots of Bean Seedlings[1]

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Phytohemagglutinin (PHA), an abundant vacuolar seed protein of the common bean (*Phaseolus vulgaris*), is a tetramer of two homologous polypeptides, PHA-E and PHA-L. The roots of bean seedlings release into the culture medium a cross-reacting lectin that is most closely related to PHA-E. Reverse-transcriptase polymerase chain reaction with root mRNA as template was used to identify PHA transcripts in the roots of bean seedlings. Roots were found to contain mRNA for PHA-E but not for PHA-L. Indirect immunocytochemical detection with colloidal gold and antibodies to deglycosylated PHA showed that in the meristem of the primary root, PHA accumulates in vacuoles. However, in elongated root cells PHA was found only in the cell walls, indicating targeting to an alternate location. These results are discussed in relation to the various mechanisms that may account for the release of a normally vacuolar protein by roots.

The seeds of the Leguminosae contain well-characterized lectins, sugar-binding proteins found in many organisms that are encoded by a large family of homologous genes (Sharon and Lis, 1990). It is likely that these lectins help defend the plant against predators and pests (Chrispeels and Raikhel, 1991). Evidence from a number of laboratories indicates that the roots of legumes release lectins into the rhizosphere and that these root lectins are either very similar to or identical with the well-characterized seed lectins (Gietl and Ziegler, 1979; Gade et al., 1981; Diaz et al., 1986; Vodkin and Raikhel, 1986). Because of their ability to discriminate between different saccharides, lectins are well-suited to play a role in recognition processes. Bohlool and Schmidt (1974) suggested that lectins released by roots may present sites on the root surface that interact specifically with saccharides on the surface of *Rhizobium* cells.

Seeds of the common bean (*Phaseolus vulgaris*) synthesize the lectin PHA, a glycoprotein with subunits of M_r 32,000 to 34,000. In bean seeds, where PHA is quite abundant and accounts for 5 to 10% of the seed protein, there are two homologous lectin polypeptides, PHA-E and PHA-L, with different sugar specificities and biological properties. PHA-E agglutinates red blood cells, whereas PHA-L agglutinates lymphocytes and is a mitogen in mammalian cells. The two polypeptides are synthesized simultaneously, and in the seed they form hetero- as well as homotetramers. PHA-like proteins are also present in much smaller amounts in the vegetative organs of bean plants (Borrebaeck, 1984). Hamblin and Kent (1973) observed that red blood cells could be agglutinated by *Rhizobium leguminosarum* bv *phaseoli* previously incubated with PHA purified from seeds. This early experiment led to the formulation of the "lectin recognition hypothesis." Recent experiments (Marino and Boiardi, 1992) indicate that such lectin-treated rhizobia have a greatly enhanced infectivity so that many more infection threads are formed in the roots.

Legume lectins such as PHA are vacuolar proteins that accumulate primarily in the protein storage vacuoles of the cotyledons. In beans, the embryonic axis also synthesizes PHA, and in the axis, the protein also accumulates in vacuoles (Greenwood et al., 1984). This raises the important question of how vacuolar lectins or lectin-like proteins synthesized in root cells reach the outside surface of the root. Several mechanisms could account for the appearance of extracellular PHA: (a) Root PHA is encoded by a different gene and lacks vacuolar targeting information. (b) Root PHA is encoded by the same gene as seed PHA, but in roots the vacuolar targeting information in PHA is not recognized or is poorly recognized, allowing PHA to be secreted. Such alternate targeting could occur in all cells or in a subset of cells. (c) In roots, PHA first goes to the vacuole but is then secreted, following an as-yet-undescribed pathway.

In this paper, we present evidence that bean seedlings synthesize and release authentic PHA-E into the surrounding medium and that this PHA-E has the same amino acid sequence as seed PHA-E. This PHA accumulates in vacuoles of root cells in the meristem of the primary root, indicating correct targeting. However, in elongated cells, PHA is found only in the cell wall, indicating poor recognition of the targeting signal. We suggest that this cell-wall PHA is the source of the PHA in the culture medium.

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Abbreviations: endoglycosidase-H, endo-β-*N*-acetylglucosaminidase-H; PHA, phytohemagglutinin.

MATERIALS AND METHODS

Plant Material

Phaseolus vulgaris L. cv Greensleeves seeds were sterilized by washing with 70% ethanol for 1 min and 1% $HgCl_2$ for 2 min, followed by extensive washing with sterile water. Sterile seeds were plated on 1% agar plates containing 1× Gamborg's salts and vitamins, 1% Suc, pH 5.7. Plates were placed in the light for 3 weeks in a vertical position to facilitate the growth of long roots.

Isolation of Secreted Proteins from Roots

Seedlings were removed from the plates and inserted into plastic tube caps with slits so that the roots could grow through the slit. In this set-up the roots hang below the cap, whereas the leaves and cotyledons remain above the cap. Roots were immersed in 15 mL of water containing a mixture of protease inhibitors (1 μg mL^{-1} leupeptin, aprotinin, and pepstatin A) in a Corning (Corning, NY) tube with the cap screwed down. Water from the incubation was isolated at specified time points and either lyophilized or concentrated with Centricon concentrators (Amicon, Danvers, MA).

Immunodetection

For immunoblotting, protein samples that had been boiled for 2 to 3 min were fractionated by SDS-PAGE and transferred to nitrocellulose, and the proteins were detected using a rabbit antiserum. Goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) was used as the secondary antibody. The antisera against PHA and binding protein have been described (Sturm et al., 1988; Höfte and Chrispeels, 1992).

Treatment with Endoglycosidase-H

Endoglycosidase-H (Boehringer Mannheim) digestion of glycoproteins was carried out at 37°C for 24 h in 100 mM sodium acetate (pH 5.8) with 10 milliunits of enzyme.

Affinity Purification of Lectins

One gram of 5- to 7-d-old lateral roots or two rehydrated bean seeds were ground in an ice-cold mortar and pestle in 5 mL of tumbling buffer (50 mM Tris-HCl [pH 6.8], 150 mM NaCl, 0.1% Triton X-100, 18 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% β-mercaptoethanol). Debris was sedimented with a 10,000*g* spin for 10 min. Supernatants were added to 8 mL of tumbling buffer and 2 mL of Sepharose-linked porcine thyroglobulin was added to each sample. After tumbling overnight at 4°C, sample and beads were poured through a disposable minicolumn and sequentially washed with 10 mL of tumbling buffer, 10 mL of tumbling buffer without Triton X-100, and 10 mL of tumbling buffer without Triton X-100 and containing 1 M NaCl. Bound lectin was eluted with four changes of 0.5 mL of elution buffer (0.2 M Gly [pH 3.2] 0.5 M NaCl). The elution buffer was changed to PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 1.8 mM KH_2PO_4) using Centricon microconcentrators (Amicon).

RNA Isolation and PCR

Roots and cotyledons were ground in liquid nitrogen and then extracted in a 3:2 ratio of extraction buffer (0.1 M NaCl, 0.01 M Tris-HCl [pH 7.5], 1 mM EDTA, 1% SDS) to phenol/chloroform. After centrifugation, aqueous supernatants were subject to a series of precipitations beginning with 2.5 volumes of 100% ethanol, then an equal volume of 4 M LiCl, and finally 2.5 volumes of 100% ethanol. The final pellets were resuspended in diethylpyrocarbonate-treated water.

cDNA was transcribed from 1 μg of total RNA using 200 units of Molony murine leukemia virus reverse transcriptase (BRL) in the provided buffer, 200 μM deoxynucleoside triphosphates, and 20 units of RNasin (Promega) in 20 μL at 37°C for 30 min. The PCR primers were based on the conserved amino-terminal sequence Met-Ala-Ser-Ser-(Lys/Asn) of PHA-L and the carboxy-terminal sequence Leu-Asn-Gln-Ile-Leu of PHA-E. The C-terminal primer is called P1 and has the following sequence: 5′-GGCTCGAGTCTAGAGGATTTGGTTG-3′. The N-terminal primer is called P2 and its sequence is the following: 5′-CCATCGATAGATGGCTTCCTCCAA(C/G)T-3′. Thirty micromoles of each primer were added to the cDNA, and PCR was carried out in 100 μL of the following mixture: 1× Vent polymerase buffer (New England Biolabs) [10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100], 200 μM each deoxynucleoside triphosphate, and 2 units of Vent polymerase (New England Biolabs). Conditions for PCR were 95°C for 2 min, 40 cycles of 55°C for 2 min, 72°C for 2 min, 94°C for 1 min. The resulting PCR products were cloned directly into pCRII (Invitrogen Corp., San Diego, CA) and sequenced with Sequenase dideoxy chain-termination method (United States Biochemical).

RNase Protection

RNase protection assays were done with the RPA II kit from Ambion (Austin, TX). Briefly, a [^{32}P]UTP-labeled RNA probe was made from a linearized plasmid transcribed with either SP6 RNA polymerase or T7 RNA polymerase. Probes were gel-purified after transcription and 5 × 10^4 counts of probe were hybridized at 42°C overnight with the following RNA samples: 200 pg of sense transcript, 2 μg of total yeast RNA, 1 μg of cotyledon RNA, 10 μg of lateral root RNA, and 10 μg of whole-root RNA. RNase digestion was done with a 1:70 dilution of the RNase A and RNase T1 mixture for 1 h at 37°C. After precipitation, resuspended samples were loaded onto a 5% acrylamide/urea gel and subjected to electrophoresis for 1 h at 200 V. The gel was exposed overnight at −60°C.

Electron-Microscope Immunocytochemistry

Small pieces of *P. vulgaris* primary and lateral roots from 5-d-old seedlings were fixed in 2% paraformaldehyde, 0.8% glutaraldehyde in 10 mM sodium phosphate buffer for 2 h.

Postfixation, dehydration, and embedding of the root tissue were done according to Schroeder et al. (1993). The immunocytochemical procedure was similar to that described by Bednarek and Raikhel (1991) with some modifications. The sections were first probed either with the preimmune serum or with the anti-PHA serum and then either with goat anti-rabbit IgG, followed by protein A coupled to 15-nm colloidal gold, or directly with protein A-colloidal gold (EY Laboratories, San Mateo, CA).

RESULTS

Bean Roots Release a PHA-Like Protein into the Culture Medium

There are several reports that roots of seedlings release lectins into the culture medium (Gietl and Ziegler, 1979; Gade et al., 1981, and others subsequently). We confirmed these observations for the common bean by incubating the roots of 5-d-old seedlings in water containing protease inhibitors and measuring the appearance of protein that cross-reacts with a serum specific for deglycosylated PHA (PHA-E and PHA-L). Care was taken to keep the cotyledons well out of the water during seedling growth. After 24 h, the water surrounding the roots was concentrated 75-fold and the proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 1A). The results show the presence in the water, as well as in the root extract, of a single PHA-cross-reacting polypeptide with a M_r similar to that of seed PHA. A semiquantitative estimation suggests that after 24 h, the water contained about 1 to 2% as much of this protein as the roots.

Because the embryonic axis of beans contains PHA-E and PHA-L synthesized during embryogeny (Greenwood et al., 1984), the primary root of a bean seedling can be expected to contain these polypeptides. The root system of a 5-d-old seedling was divided into three parts: the tip of the primary root, the primary root without tip, and the lateral roots. Extracts of all three parts were subjected to SDS-PAGE, and an immunoblot (Fig. 1B) shows that all regions contain the PHA-cross-reactive protein. Thus, newly formed cells (lateral roots) also contain PHA, suggesting that this PHA did not come from the embryo.

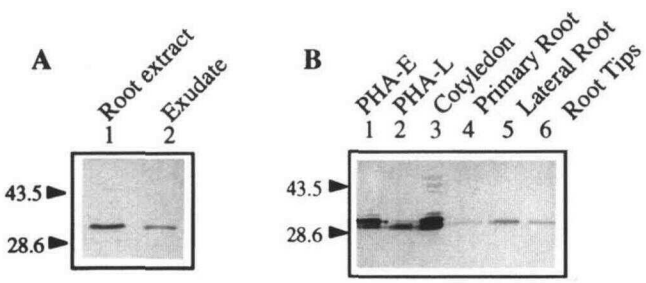

Figure 1. A, PHA-related protein is released from bean roots. Five-day-old bean seedling roots were incubated in 15 mL of water. After 24 h, the water was frozen and lyophilized. The lyophilized protein was resuspended into 200 μL; 25 μL was loaded on an SDS-PAGE gel. It is compared to 30 μg of protein of a root extract. B, PHA-related protein in different parts of bean roots. Thirty micrograms of protein from extracts of each fraction were loaded onto an SDS-PAGE gel. The immunoblot was probed with anti-PHA antibody. Lanes 1 and 2, Purified PHA-E and PHA-L from red kidney bean; lane 3, cotyledon extract; lane 4, primary root; lane 5, lateral root (3-cm-long tip portions); lane 6, root tip (2-cm-long tips).

PHA is a lectin that binds specifically to the complex glycan of porcine thyroglobulin; this glycoprotein was used for the affinity purification of PHA. PHA can be eluted from a thyroglobulin-Sepharose column by lowering the pH to 3.2 (Felsted et al., 1975). When extracts of cotyledons or lateral roots were passed over such a column and the bound protein eluted, cotyledon PHA, as well as the cross-reacting root protein, bound to the column and was eluted under the same conditions (Fig. 2A), suggesting that the root protein is a lectin with carbohydrate-binding ability. The root lectin in Figure 2A, lane 4, appears to run as a doublet. Such doublets are often seen when lectins are separated by SDS-PAGE and are thought to result from carboxy-terminal or glycoprotein processing of the polypeptide in the vacuole (Vitale et al., 1984). Note that a single band is predominantly seen in root exudates and extracts (Fig. 1A, lanes 1 and 2, and Fig. 1B, lanes 4–6).

The Root Lectin Is More Closely Related to PHA-E than to PHA-L

Seed PHA is a tetrameric glycoprotein, and the PHA-E and PHA-L subunits carry two Asn-linked glycans, one attached to Asn^{12} and the other attached to Asn^{60}. When the polypeptides are first synthesized in the ER, both glycans are typical high-Man glycans, but as the protein moves through the Golgi apparatus, one of the two high-Man glycans is modified to a complex glycan (Sturm and Chrispeels, 1986). These two types of glycans can be distinguished on the basis of their sensitivity to endoglycosidase-H (only high-Man glycans are cleaved from the protein by this hydrolase) and reactivity with a serum that reacts specifically with complex glycans (Laurière et al., 1989).

To determine if root PHA contains high-Man and/or complex glycans, affinity-purified PHA was treated with endoglycosidase-H and subjected to electrophoresis on an SDS gel, and an immunoblot was prepared with the serum that specifically recognizes complex Asn-linked glycans. A comparison of the mobility of cotyledon PHA (always consisting of the two polypeptides PHA-E and PHA-L) before and after treatment with endoglycosidase-H shows that the enzyme treatment causes a shift in the molecular mass of about 2000 D (Fig. 2B, compare lanes 1 and 2), confirming the removal of a single high-Man chain. Recognition by this serum is indicative of the presence of a complex glycan chain. A shift of the same magnitude in the electrophoretic mobility is observed with root PHA (Fig. 2B, compare lanes 3 and 4). These results show that endoglycosidase-H-treated root PHA is still recognized by the serum, indicating that it has at least one complex glycan. The change in M_r after endoglycosidase-H treatment indicates that the protein also has one high-Man glycan. The similarity between root PHA and cotyledon PHA-E and PHA-L shows that the former, like the latter, has one of each type of glycan.

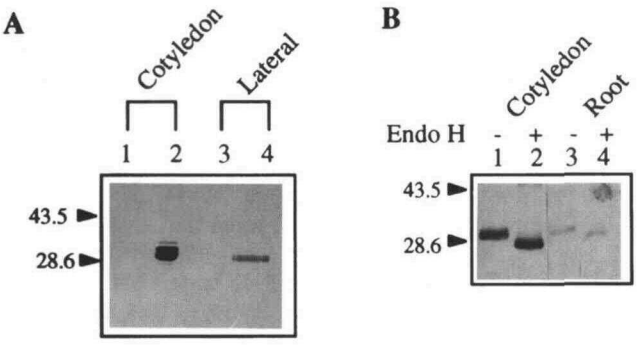

Figure 2. Affinity purification of the PHA-like protein in roots. A, Cotyledon and lateral root extracts from 5- to 7-d-old roots were incubated with thyroglobulin-linked Sepharose. The gel was washed and the bound protein was eluted. Lanes were loaded for equivalent volumes of bound (lanes 2 and 4) and unbound (lanes 1 and 3) fractions. The immunoblot was probed with anti-PHA. B, Root lectin has high Man and complex glycans. The thyroglobulin-purified lectin from root and cotyledon was incubated with endoglycosidase-H (Endo H) or with buffer only as the control. The immunoblot was probed with an antibody that recognizes the Xyl residue on plant complex glycans.

To determine if the root PHA may be more closely related to PHA-E or to PHA-L, we used two different immune sera: one made against a mixture of the two deglycosylated polypeptides and one made against deglycosylated PHA-L. When blots were probed with these two sera, we found that lateral root PHA was recognized by the serum made against the mixture of polypeptides (Fig. 3A, lane 4) but not by the serum against PHA-L (Fig. 3B, lane 4). This indicates that the root PHA is more closely related to PHA-E. However, no serum specific for PHA-E was available to confirm this suggestion.

The Root Lectin from *P. vulgaris* cv Greensleeves Is PHA-E-Like

To establish the identity of the root lectin unambiguously, we isolated and characterized the root lectin cDNA using PCR. Initial high-stringency northern blot analysis using the cDNA of seed PHA-L as a probe revealed that a PHA-related RNA transcript is expressed more abundantly in the lateral roots than in the primary root (data not shown). These data indicated that reverse-transcriptase PCR with lateral root RNA as the substrate would be a feasible approach for isolating PHA clones. The nucleotide sequences for the amino and carboxy termini of the PHA gene family that includes arcelin and α-amylase inhibitor are well conserved. These sequences are useful templates for PCR primers because they border the entire reading frame of PHA-related proteins, and they have allowed us to isolate PHA, arcelin, and α-amylase inhibitor cDNAs (Mirkov et al., 1995). Using these sequences as primers in two separate PCR procedures, we obtained products of the same molecular weight (about 820 bp) from both lateral root RNA and cotyledon RNA (Fig. 4A). These products hybridized with a PHA probe on a Southern blot of the PCR products (data not shown). Restriction enzyme digests and sequencing of the cloned PCR products indicated that PHA-E was recovered from both cotyledon and root RNA. At the nucleotide level, the root clone of this cultivar (Greensleeves) is 98% similar to PHA-E and 90% similar to PHA-L from cv Tendergreen (Hoffman and Donaldson, 1985). A multiple alignment of the derived amino acid sequences of the root PCR product with the cotyledon PCR product from *P. vulgaris* cv Greensleeves (Fig. 4B) reveals only two amino acid differences between the PHA-E proteins. In cv Greensleeves the residues Ser^{220} and Phe^{266} found in cotyledon PHA-E have been changed to Asn^{220} and Leu^{266} in root PHA-E. There is also one difference (not shown) between Greensleeves and Tendergreen cotyledon PHA-E at amino acids 88 to 90, where Thr-Phe-Ala (Greensleeves) becomes Ala-Ser-Pro (Tendergreen). Thus, there are small differences between the cotyledonary forms of PHA-E in different bean cultivars. Therefore, it appears that the PHA in the roots is authentic PHA-E.

PHA-E mRNA Is Present in Roots, but mRNA for PHA-L Is Not Found There

Although reverse-transcriptase PCR was used to isolate the root lectin cDNA, it is possible that the root lectin PCR product is derived from contaminating DNA. To be certain that the root lectin gene is transcribed in this tissue, we did an RNase protection assay. In these experiments, a radiolabeled minus-sense transcript was annealed with a plus-sense transcript or with total RNA and then digested with a mixture of RNaseA and RNaseT1. Transcripts that hybridize to the labeled minus strand will be protected from RNase digestion and appear as a band with a molecular weight similar to that of the labeled transcript on an autoradiograph after electrophoresis. We used a 3′ antisense probe from the root lectin cDNA (Fig. 5A). The full-length probe is shown in lane 1. Lane 2 shows the control hybridization, demonstrating that the RNase digestion is complete. In lane 3, hybridization of root PHA to a seed PHA-L transcript results in a lower-molecular-weight, fragmented

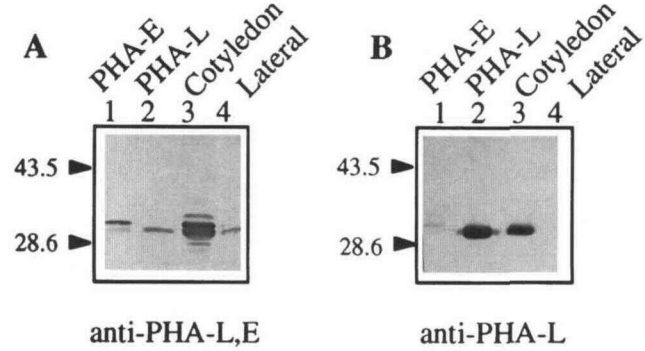

Figure 3. The root lectin is immunologically more related to PHA-E than to PHA-L. An equal amount of protein from both cotyledon and lateral root extracts was loaded in lanes 3 and 4, respectively, of both immunoblots. PHA-E and PHA-L were purified from red kidney bean. A, The blot was probed with an antibody that recognizes both PHA-E and PHA-L. B, The blot was probed with an antibody raised against the PHA-L protein only. Note that the PHA-L antiserum does not detect the root lectin.

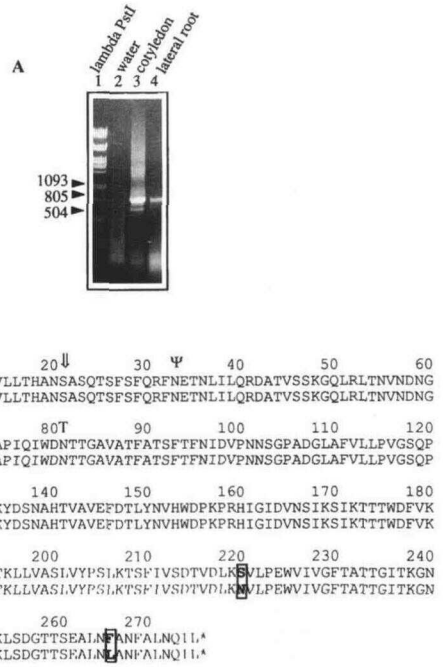

Figure 4. Products of the PCR of cotyledon and root cDNA. A, One microgram of cotyledon RNA (lane 3) and 5 μg of lateral root RNA (lane 4) were transcribed to cDNA and used in a PCR reaction with PHA carboxy- and amino-terminal-specific primers. Lane 1 contains λ *Pst*I molecular weight markers. Water was included as a contamination control (lane 2). The lower-molecular-weight product in lane 3 is α-amylase inhibitor, a protein related to PHA. B, Alignment of the amino acid sequences of PHA-E from the root and cotyledon of cv Greensleeves. The differing amino acid residues are boxed.

band pattern due to the partial identity of the transcripts. When the probe was hybridized to the seed PHA-E and root PHA sense transcripts, (lanes 4 and 5, respectively), a single transcript was protected. Lanes 6 and 7 show that the root transcript probe protects RNA in lateral roots as well as in whole roots; note that the protected fragment is enriched in the lateral roots. The full-length band, as well as the band pattern of lane 3, are both present in the cotyledon sample (lane 8), indicative of the presence of both PHA-E and PHA-L. We conclude that root PHA-E mRNA is indeed present in roots.

We used a 360-bp 3′ transcript of PHA-L as the minus-sense probe to determine if PHA-L-related transcripts are also present in roots (Fig. 5B). The full-length probe is shown in lane 1. After hybridization with yeast RNA and digestion with RNase, there is no signal, indicating that the RNase digestion is complete (lane 2). In lane 3, a PHA-L sense transcript is protected from digestion after hybridization with the probe. The presence of lower-molecular-weight bands in lanes 4 and 5 shows that the PHA-L minus-strand transcript has only partial identity with PHA-E. There is no signal in lanes 6 and 7, which contain 10 μg of lateral root RNA and whole-root RNA, respectively. Even after 3 d of exposure, a signal in these lanes did not appear. RNase treatment of cotyledon RNA (0.5 μg) hybridized with the PHA-L sense transcript results in a band of the same size as the sense transcript itself (lane 8). The lower-molecular-weight band pattern is most likely derived from partial hybridization to PHA-E. This is the PHA-E-specific pattern seen in lanes 4 and 5, which contain PHA-E sense transcripts. Multiple bands in lanes with one transcript are probably due to probe degradation and/or interprobe hybridization. That a PHA-L-related transcript from lateral roots is not protected from RNase digestion is further confirmation that a PHA-L-related mRNA is not present in this tissue.

PHA Is Located in Vacuoles as Well as in the Cell Wall

We used immunocytochemistry to localize root PHA at the subcellular level in the primary and lateral roots of bean seedlings. The thin sections were first probed either with a preimmune serum or with the anti-PHA serum (serum to deglycosylated PHA-E plus PHA-L) and then

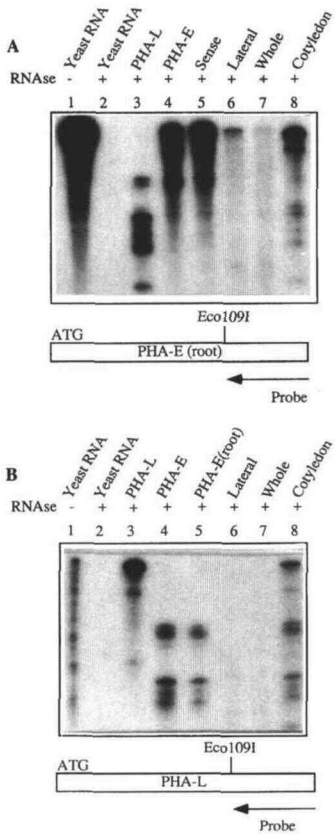

Figure 5. A, Presence of PHA-E transcripts in root tissue. The probe for this assay is an antisense transcript from the 3′ end of the root PHA clone cleaved at an *Eco*1091 site illustrated below the autoradiograph. The amount of probe used in all samples was 5×10^4 cpm. The probe was hybridized with the following RNA samples and only lane 1 was not digested with RNase: lane 1, yeast RNA; lane 2, yeast RNA; lane 3, sense RNA transcript from PHA-L clone; lane 4, sense RNA transcript from PHA-E clone; lane 5, sense RNA transcript from root PHA clone; lane 6, 10 μg of lateral root RNA; lane 7, 10 μg of total root RNA; lane 8, 1.0 μg of cotyledon RNA. B, PHA-L transcripts are not present in roots. The probe is an antisense transcript from the 3′ end of a PHA-L clone cleaved at an *Eco*1091 site as illustrated below the autoradiograph. The amount of probe used in all samples was 5×10^4 cpm. The RNA samples are the same as in A.

with goat anti-rabbit IgG coupled to colloidal gold. On the sections of bean roots, except for the occasional stray gold particle, we observed no staining with the preimmune serum (Fig. 6, A and C). With the anti-PHA serum, we found gold particles over the vacuolar content of the meristematic cells of the primary and lateral root tips (Fig. 6B) and over the cell walls of the cells in the zones of elongation and differentiation in both the primary and lateral roots (Fig. 6, D and E). This cell-wall labeling, although light, was clearly above background and was specific for the walls. In the cells that showed labeling of the cell wall, we observed no vacuolar labeling (Fig. 6, D and E). Since cross-reacting proteins other than PHA are not present in root extracts, we presume that the cell-wall labeling represents cell-wall-localized PHA.

DISCUSSION

Several published studies show that the roots of legumes contain low levels of lectin and/or proteins that cross-react with lectin antibodies and that these proteins are released into the environment. However, there are very few detailed molecular and cellular studies of this phenomenon. The lectin-recognition hypothesis postulates the involvement of these lectins in the specific interaction between *Rhizobium* and legumes, but there is no information on how those normally vacuolar proteins achieve this extracellular location. Recent immunochemical evidence supports the idea that, at least in roots, lectins may be present in the cell walls as well as in vacuoles (Sherrier and VandenBosch, 1994). To investigate how roots may release lectins, we characterized the lectin released by bean roots and examined its intracellular location.

Characterization of the Lectin

The lectin present in and released by seedling roots was characterized with immunochemical techniques, by its binding to an affinity matrix, and by sequencing of the cDNA obtained by PCR from root mRNA. All the evidence indicates that the lectin released by bean roots is authentic PHA-E. The nucleotide sequence of the PCR product yields a derived amino acid sequence that differs by only two amino acids from the sequence of seed PHA-E. Considering that there is a difference of three amino acids in PHA-L of the cultivars Tendergreen and Greensleeves, we propose that the PHA released by the roots is authentic PHA-L. As well as having the same amino acid sequence as seed PHA-E, root PHA has the same affinity for the glycans of porcine thyroglobulin, and the same Asn-linked glycans (one high-Man and one complex) as PHA-E from seeds.

We do not know whether transcripts from the same gene are utilized for synthesis of root PHA and PHA-E in the roots as in the seeds. Genes encoding PHA and PHA-like proteins are found on two different linkage groups in the bean genome (Nodari et al., 1993). Expression of seed PHA-E in the roots could be the result of a weak promoter element that allows this strong seed promoter to be active in roots. Our results are similar to the recent findings of Hoedemaker et al. (1994), who used the same method (reverse-transcriptase PCR) to demonstrate that the lectin present in the roots of *Pisum sativum* is identical to the pea seed lectin.

Subcellular Location of Root PHA-E

The immunocytochemical results obtained with antibodies to PHA clearly support the vacuolar location of PHA in roots of bean seedlings. Thus, the vacuolar targeting information present in PHA can be properly interpreted when the protein is synthesized in roots. In addition, PHA is also found in the cell walls in the zone of elongation. Similarly, when VandenBosch et al. (1994) studied the distribution of lectin in nodulated peanut roots, they observed labeling of the vacuoles in the nodule cortex parenchyma (uninfected cells), within the symbiosomes of the infected cells, and over the walls of the nodule parenchyma cells. Similar observations were made by Law and Van Tonder (1992). This dual location suggests that dual targeting may occur in legumes and that a certain proportion of protein that is destined for the vacuole may be diverted to the cell wall. It is interesting that we observed either labeling of the vacuoles (in the meristem) or labeling of the cell walls (in the zone of elongation and above). It is possible that in these large, vacuolated cells of the elongation zone, PHA was targeted to the vacuoles as well as to the cell wall but that the vacuolar PHA was degraded by vacuolar proteases.

Narváez-Vásquez et al. (1993) used immunocytochemistry to examine the location of proteinase inhibitors synthesized in transgenic tomato plants from chimeric genes regulated by the cauliflower mosaic virus 35S promoter. Like PHA, these proteinase inhibitors are vacuolar proteins, and in the root cells of transgenic plants they were found in the vacuoles as well as in the cell walls. Monensin increased the amount of proteinase inhibitor in the cell walls, in accordance with the known function of this drug in redirecting proteins in the secretory system away from the vacuoles and toward the extracellular space (Craig and Goodchild, 1984). When proteins are overexpressed, it is always possible that the cellular sorting system is being saturated and that the appearance of a vacuolar protein in the cell wall is caused by overloading (Rothman et al., 1986; Stevens et al., 1986). However, that is unlikely to be the case in bean roots, where PHA is not an abundant protein.

What Is the Pathway for PHA Release from Roots?

The data presented here are consistent with the interpretation that in root cells, especially in the region distal to the meristem, there is either dual targeting or complete alternate targeting of vacuolar PHA and that this results in the secretion of PHA into the cell wall and the subsequent release of lectin from the roots. This interpretation is entirely consistent with the results of Narváez-Vásquez et al. (1993), who examined transgenic tomato plants expressing vacuolar proteinase inhibitor and found the protein to be present in the cell walls, and with the immunocytochemical studies of Law and Van Tonder (1992) and VandenBosch et al. (1994) on peanut roots and nodules. Of the four possibilities raised in the introduction, the explanation that we

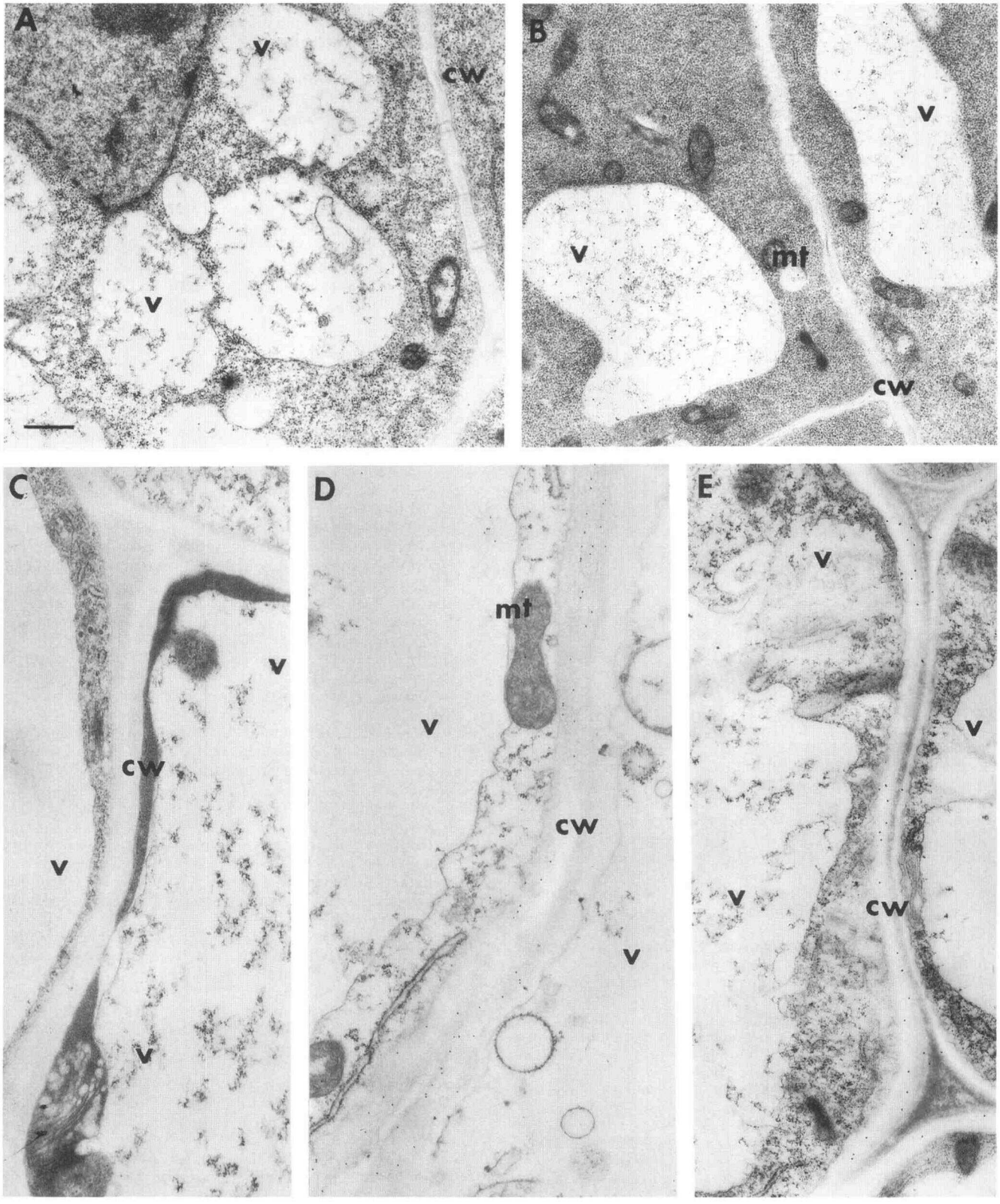

Figure 6. Immunogold labeling of sections of root cells from *P. vulgaris*. A, Lateral root tip cells from a 5-d-old root section labeled with preimmune serum. B, Lateral root tip section labeled with the anti-PHA antibody. C, Section of upper portion of the primary root cells labeled with preimmune serum. D, Section of upper portion of the primary root cells labeled with the anti-PHA antibody. E, Section of upper portion of the lateral root cells labeled with the anti-PHA antibody. Bar = 0.5 μm; 17,000×. cw, Cell wall; mt, mitochondria; v, vacuole.

favor for the release of lectins from roots is the lack of recognition by the elongating cells of the targeting information present in PHA. Root PHA has the same amino acid sequence as seed PHA-E, and the targeting information present in the protein clearly allows it to be directed to the vacuoles of meristematic cells of the primary root. In elongating and differentiating cells, root PHA is found only in cell walls. Note that only one band appears on immunoblots of root extracts and exudates, as opposed to the two bands in cotyledon extracts that arise presumably due to vacuolar proteolytic processing (Vitale et al., 1984). We feel that the single band is highly suggestive of PHA secretion to the cell wall, bypassing the vacuole. However, we cannot exclude the possibility that in these cells PHA is also targeted to the vacuoles and is then completely broken down by vacuolar proteases. Perhaps root cells secrete a variety of vacuolar proteins through alternate targeting. One then wonders if the rhizobia that respond to the signals coming from the roots of certain species of legumes also evolved the capacity to bind to the lectins that are being secreted by the roots as a result of the cell-wall targeting of these seed vacuolar proteins.

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