Plant Physiol. (1993) 103: 719–726

Expression and Localization of Plant Protein Disulfide Isomerase

Basil S. Shorrosh[1], Jayaram Subramaniam, Karel R. Schubert, and Richard A. Dixon*

Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73402 (B.S.S., R.A.D.); and Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019 (J.S., K.R.S.)

A cDNA clone encoding a putative protein disulfide isomerase (PDI, EC 5.3.4.1) from alfalfa (*Medicago sativa* L.) was expressed in *Escherichia coli* cells, and an antiserum was raised against the expressed PDI-active protein. The antiserum recognized a protein of approximately 60 kD in extracts from alfalfa, soybean, and tobacco roots and stems. Levels of this protein remained relatively constant on exposure of alfalfa cell suspension cultures to the protein glycosylation inhibitor tunicamycin, whereas a slightly lower molecular mass form, also detected by the antiserum, was induced by this treatment. A lower molecular mass form of PDI was also observed in roots of alfalfa seedlings during the first 5 weeks after germination. PDI levels increased in developing soybean seeds up to 17 d after fertilization and then declined. Tissue print immunoblots revealed highest levels of PDI protein in the cambial tissues of soybean stems and petioles and in epidermal, subepidermal, cortical, and pith tissues of stems of alfalfa and tobacco. Immunogold electron microscopy confirmed the localization of PDI to the endoplasmic reticulum in soybean root nodules.

PDI (EC 5.3.4.1) is a luminal endomembrane protein that catalyzes the thiol/disulfide exchange reactions leading to the formation of the disulfide bonds, which stabilize the tertiary and quarternary structures of many proteins that are processed through the endomembrane system (Freedman, 1989; Noiva and Lennarz, 1992). Vertebrate PDI is a multifunctional protein: it is the β-subunit of prolyl 4-hydroxylase (Pihlajaniemi et al., 1987), exhibits dehydroascorbate reductase activity (Wells et al., 1990), is a component necessary for maintaining the catalytic activity of the bovine microsomal triglyceride transfer protein complex (Wetterau et al., 1990, 1991), and is a thyroid hormone-binding protein (Cheng et al., 1987). It has been suggested that PDI may also function as the glycosylation site-binding protein of the oligosaccharyl transferase complex (Geetha-Habib et al., 1988). However, recent studies have indicated that the enzyme binds several peptides in addition to those containing the N-glycosylation signal (Noiva et al., 1991b) and that, although depletion of PDI from microsomes results in defective co-translational formation of disulfide bonds (Bulleid and Freedman, 1988), it does not fully prevent oligosaccharyl transferase activity (Noiva et al., 1991a). PDI is essential for cell viability in yeast (Farquhar et al., 1991; LaMantia et al., 1991).

Genes encoding PDI have been cloned from vertebrates (Edman et al., 1985; Pihlajaniemi et al., 1987; Parkkonen et al., 1988) and from yeast (LaMantia et al., 1991). In contrast, little is known about plant PDI. Earlier studies have suggested that, as in animals, it may play a role in protein processing in the ER (Roden et al., 1982). We recently cloned a putative PDI (termed B2) from an alfalfa cell suspension cDNA library (Shorrosh and Dixon, 1991). The deduced amino acid sequence of B2 contained the two thioredoxin-like active sites characteristic of vertebrate and yeast PDIs (Noiva and Lennarz, 1992) and the C-terminal KDEL ER retention signal (Pelham, 1990). B2 transcripts were strongly induced in alfalfa cell suspensions by the protein glycosylation inhibitor tunicamycin (Shorrosh and Dixon, 1991). In the present paper we describe the production of a monospecific polyclonal antiserum against the alfalfa B2 protein. We have used this antiserum to confirm that B2 codes for a PDI, to study the developmental expression of B2, and to determine the cellular and subcellular localization of plant PDI.

MATERIALS AND METHODS

Plant Material

Plant tissues were collected from alfalfa (*Medicago sativa* L. cv Apollo), soybean (*Glycine max* L., U.S. Department of Agriculture germplasm PI 360965A), and tobacco (*Nicotiana tabacum* cv Xanthi) plants grown in a controlled environment chamber, the soybean plants being grown under high-irradiance conditions (Cregan and Hartwig, 1984). Soybean nodules were isolated from roots of cv Williams 82 that had been inoculated with *Bradyrhizobium japonicum* strain U.S. Department of Agriculture 31b 110 (Nitragin Co., Milwaukee, WI), and the plants were grown in a growth chamber as previously described (Polayes and Schubert, 1984). Cell suspension cultures of alfalfa cv Apollo were initiated and maintained in a modified Schenk and Hildebrandt medium as described (Dixon et al., 1981). Cells were treated with elicitor from the cell walls of *Colletotrichum lindemuthianum* at a final concentration of 60 μg of Glc equivalents mL^{-1} of culture medium or with tunicamycin at 10 μg mL^{-1} of culture medium as described previously (Shorrosh and Dixon, 1992).

[1] Present address: Michigan State University, Department of Botany and Plant Pathology, Plant Biology Building, East Lansing, MI 48824-1312.

* Corresponding author; fax 1-405-221-7380.

Abbreviations: IPTG, isopropylthio-β-galactoside; PDI, protein disulfide isomerase; pI, isoelectric point; TBS, Tris-buffered saline.

Cells were collected by filtration through Miracloth, frozen in liquid nitrogen, and stored at −80°C.

Protein Extraction

Alfalfa suspension culture cells (75 g) for preparation of PDI activity were homogenized on ice in 150 mL of 0.1 M sodium phosphate (pH 7.5), 0.1 M NaCl, 5 mM EDTA, 0.5% (w/v) Triton X-100, 1 mM benzamidine, 0.1 mM PMSF. The homogenate was sonicated 10 times and incubated on ice for 2.5 h, and cell debris was pelleted by centrifugation at 13,000*g* for 30 min. Solid ammonium sulfate was added slowly to the supernatant to bring the final concentration to 90% saturation, the mixture was stirred overnight at 4°C, and protein was collected by centrifugation at 15,000*g* for 30 min and resuspended in 10 mL of 0.1 M sodium phosphate (pH 7.5). The same extraction conditions, including ammonium sulfate precipitation, were used, on a small scale (1 g of tissue), for extracts for western blot analysis.

Proteins expressed in *Escherichia coli* SB221 cells were extracted as described previously (Shorrosh and Dixon, 1991). Protein content was determined by the Bradford (1976) assay using BSA as a standard.

Production of Anti-(Alfalfa B2) Serum

Alfalfa B2 was expressed in *E. coli* SB221 cells as described previously (Shorrosh and Dixon, 1991). The expressed protein was resolved by electrophoresis in an 8% preparative SDS-PAGE gel (Laemmli, 1970). The protein band encoded by the B2 clone was localized by staining with Coomassie brilliant blue, and regions from 12 gel lanes were excised, cut into small fragments, destained to completion with 50% (v/v) isopropanol/3% (w/v) SDS overnight, rinsed with water, vacuum dried, ground in liquid nitrogen, and finally resuspended in PBS buffer. Antiserum was obtained by immunizing a female New Zealand White rabbit. The primary immunization contained approximately 30 μg of B2 protein in 2.7 mL of complete Freund's adjuvant, injected subcutaneously along the back at nine separate sites (300 μL per site). Booster injections containing B2 from six excised gel lanes (approximately 15 μg) in incomplete Freund's adjuvant were given at 4 and 6 weeks after the primary injections. The serum was stored at −20°C.

Immobilization of B2 Antibodies

Antibody immobilization was performed essentially as described by Harlow and Lane (1988). Protein-A beads (0.5 g) were swollen in 2 mL of PBS buffer (pH 7.0) and then mixed with 1 mL of anti-(B2) serum for 1 h at room temperature. The beads were washed twice in 10 volumes of 0.2 M sodium borate (pH 9.0) and resuspended in 30 mL of 0.2 M sodium borate (pH 9.0). Solid methylpimelimidate was then added to a final concentration of 20 mM. After the beads were mixed for 30 min at room temperature, they were washed once in 0.2 M ethanolamine (pH 8.0), incubated in the same buffer for 2 h with gentle mixing, washed, and resuspended in PBS containing 0.01% (w/v) merthiolate. Immobilized antibody was stored at 4°C.

Immunoprecipitation and Assay of PDI Activity

Alfalfa cell extracts (425 μg protein) were incubated overnight at 4°C with gentle mixing in 200 μL of 0.1 M sodium phosphate buffer (pH 7.5) with the addition of either 0.5 mL of bed volume of protein-A beads coupled to anti-(B2) antibodies or 0.5 of mL bed volume of uncoupled protein-A beads. After the extract was centrifuged, 90 μL of the supernatant was mixed with 110 μL of a solution containing 0.18 M sodium phosphate (pH 7.5), 9 mM EDTA, 0.56 μg μL^{-1} of GSH, 1 μCi of ^{125}I-insulin (monoiodinated on A14, 371 μCi μg^{-1}, NEN), and 0.9 μg μL^{-1} of unlabeled insulin and incubated for 1 h at 37°C. At the end of the incubation, 300 μL of 2.5% (w/v) BSA and 500 μL of 15% (w/v) TCA were added to enzyme reactions and controls and mixed, and the mixtures were incubated on ice for 10 min. The samples were centrifuged at 15,300*g* for 15 min at 4°C. Radioactivity in an aliquot (850 μL) of the supernatant was determined by γ counting. Background activity was determined with protein extract incubated at 4°C overnight, treated with 0.1 M DTT for 20 min at 37°C, and then boiled for 20 min. This background is due to PDI-independent degradation/solubilization of ^{125}I-insulin and was subtracted from the data presented.

The assay for PDI activity of B2 expressed in *E. coli* SB221 cells was performed as described previously (Shorrosh and Dixon, 1991).

Electrophoresis and Blotting Procedures

Proteins from plant tissues were separated by SDS-PAGE according to standard procedures (Laemmli, 1970). Resolved proteins were transferred electrophoretically to nitrocellulose for 1 h at 100 V in 25 mM Tris, 192 mM Gly, 20% (v/v) methanol (pH 8.3) and reversibly stained with Ponceau S. IEF gels were run as previously described (Robertson et al., 1987), rinsed with water, and then incubated in 100 mM Tris (pH 7.0), 1% (w/v) SDS, 10% (v/v) glycerol for 30 min. Proteins were transferred from IEF gels to nitrocellulose in a solution prepared by mixing 84 mg of $NaHCO_3$ with 318 mg of Na_2CO_3 in 800 mL of water and 200 mL of methanol. Transfer was for 2 h at 300 mA, and blots were reversibly stained with Ponceau S. Tissue printing of plant sections onto nitrocellulose was as described elsewhere (Ye and Varner, 1991). Blots were blocked in TBS (10 mM Tris, 0.9% [w/v] NaCl), 2.5% (w/v) BSA, 0.02% (w/v) NaN_3. B2 protein was detected with immune serum (1:10,000 dilution) in TBST (TBS plus 0.05% [v/v] Tween 20), 1% (w/v) BSA, 0.02% (w/v) NaN_3, and visualized with alkaline phosphatase-conjugated anti-(rabbit immunoglobulin G) (Promega) according to the manufacturer's instructions.

Specimen Preparation for EM

Soybean root nodules (16–21 d old) were fixed in 3% (w/v) glutaraldehyde in 50 mM Sorensen's phosphate buffer (pH 7.2) for 1.5 h at room temperature and washed in the same buffer for 1 h with at least three changes. The fixed nodules were dehydrated through a series of ethanol concentrations (10, 30, 50, 70, 90, and 100%) and then embedded in LR White acrylic resin at 50 to 55°C for 24 to 36 h.

Immunolabeling

Immunolabeling of ultrathin sections was carried out as described by Van den Bosch and Newcomb (1986). Sections were poststained with 2% (w/v) aqueous uranyl acetate for 5 to 10 min before observation in a JEOL 2000 FX electron microscope operated at 100 kV.

RESULTS

The B2 cDNA was cloned into the expression vector pSE380, and the mouse ERp59 PDI sequence in the expression vector T19 was obtained from Dr. Michael Green (St. Louis University Medical Center). The encoded proteins were expressed in *E. coli* cells. *E. coli* lysates were assayed for PDI activity, measured as release of ^{125}I-labeled insulin A-chain in the presence of GSH (Shorrosh and Dixon, 1991). Lysates from cells induced with IPTG and expressing B2 or ERp59 contained approximately equal specific activities of PDI (Fig. 1A). The induced activities were in each case associated with the presence of new proteins of approximately 60 kD detected by SDS-PAGE analysis of the *E. coli* lysates (data not shown). *E. coli* cells harboring B2 exhibited a higher basal (non-IPTG-induced) apparent PDI activity than did cells harboring ERp59; this could have been due to use of the two different expression vectors. Immobilized antibodies raised against B2 protein purified from *E. coli* lysates immunoprecipitated approximately 65% of the apparent PDI activity present in alfalfa suspension cell extracts (Fig. 1B); the remaining activity is probably not due to the action of PDI. These data further support the conclusion that B2 encodes alfalfa PDI.

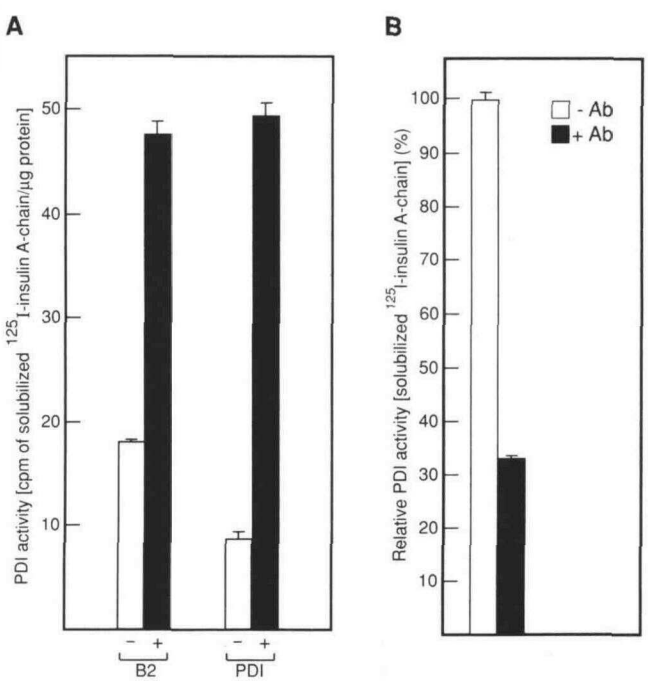

Figure 1. PDI activity determined by the GSH:insulin transhydrogenase assay. A, Activity in lysates of uninduced (−) or IPTG-induced (+) *E. coli* cells harboring the expression vector pSE380 containing the alfalfa B2 sequence or T19 harboring the mouse ERp59 (PDI) sequence. B, PDI activity in extracts from alfalfa suspension cells after treatment with anti-(B2) serum linked to protein-A beads (+Ab) or protein-A beads alone (−Ab). Error bars represent the spread of values from duplicate determinations.

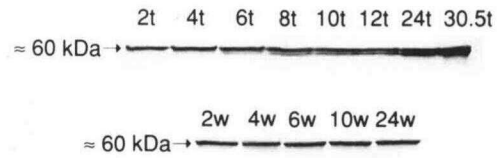

Figure 2. Western blot analysis of PDI levels in alfalfa cell suspension cultures. Total protein (25 µg) was isolated from suspension cultures treated with tunicamycin (t) at 10 µg mL^{-1} or with an equal amount of water (w) for the times (h) indicated. Blots of SDS-PAGE gels were probed with anti-(B2) serum. The arrow marks the migration of a standard molecular mass marker.

Anti-(alfalfa B2) serum recognized a single protein band of approximately 60 kD on western blot analysis of proteins extracted from control alfalfa cell suspension cultures treated with water (Fig. 2). Levels of this protein remained relatively constant for up to 24 h after exposure of cells to tunicamycin. Tunicamycin treatment induced the appearance of a slightly lower molecular mass protein, which was first apparent approximately 6 to 8 h after treatment with the protein glycosylation inhibitor. Western blotting of IEF gels of soluble protein extracts of alfalfa cells from cultures 24 h after exposure to water or tunicamycin indicated the presence of a single band of pI 4.65 (Fig. 3B). This band was also observed in lysates of *E. coli* cells expressing B2 (Fig. 3A). This protein and a slightly lower pI component were detected on western blots of IEF separations of alfalfa microsomal proteins isolated from control suspension cultures or cultures exposed to elicitor from *C. lindemuthianum* (Fig. 3C), a treatment that

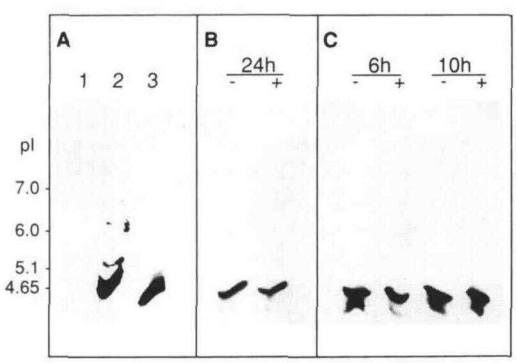

Figure 3. Western blot analysis of alfalfa cell suspension proteins resolved by IEF and probed with anti-(B2) serum. A, Total protein (25 µg/lane) from IPTG-treated *E. coli* SB221 cells harboring either the pSE380 vector alone (lane 1) or the B2 sequence in pSE380 (lane 2). Lane 3 shows total protein from an alfalfa cell suspension. B, Total protein (25 µg) from alfalfa cell suspension cultures treated with (+) or without (−) tunicamycin (10 µg mL^{-1}) for 24 h. C, Microsomal protein (25 µg) from alfalfa cell suspension cultures treated with (+) or without (−) *C. lindemuthianum* cell wall elicitor at a final concentration of 60 µg of Glc equivalents mL^{-1} of culture medium. The pIs of IEF standard proteins are indicated.

strongly induces prolyl 4-hydroxylase activity. The PDI doublet seen in tunicamycin-treated material is unlikely to be the result of the induction of a new charge isoform of the enzyme in view of the single band observed on western blots of IEF gel separations of proteins from tunicamycin-treated cells. Therefore, it is most likely a nonglycosylated form of the enzyme.

Western blot analyses were performed to investigate the developmental regulation of PDI levels in alfalfa, soybean, and tobacco (Fig. 4). The anti-(B2) serum detected a single band of approximately 60 kD in total protein extracts from alfalfa roots and soybean roots and stems. A single band of slightly lower molecular mass was detected in tobacco root and stem extracts (Fig. 4A). Leaves, roots, and root nodules were harvested from alfalfa plants at various stages of development. PDI was constitutively expressed in leaves, roots, and nodules. PDI levels in leaves appeared low at all stages of development, and the protein was of slightly higher molecular mass than in roots and nodules (Fig. 4B). The absolute level in leaves compared to other tissues is probably an underestimate in view of the equal protein loadings and the high level of Rubisco in leaves. A lower molecular mass form of the enzyme was detected in roots of seedlings during the first 5 weeks of development; this may be a degradation product, because it was not seen in extracts from freshly harvested roots that had not been stored at −80°C and, therefore, had not undergone freezing and thawing.

PDI may be necessary for processing of storage proteins in developing seed endosperm (Roden et al., 1982). PDI levels relative to total protein were greatest in developing soybean seeds at approximately 17 DAF and then gradually declined (Fig. 4C).

Tissue print immunoblots of alfalfa stem sections appeared to reveal the presence of PDI in epidermal, subepidermal, vascular, and cortical cells (Fig. 5, A–C), although the resolution was not good because the alfalfa stems were soft and did not blot well. Better resolution was seen on tissue print blots of soybean stems, in which most PDI was localized in the cambial region, with no apparent signal in epidermal cells (Fig. 5, D–F). Expression in the vascular cambium was also clearly seen in tissue prints of soybean petioles (Fig. 5 G-I). In tobacco stems, equally strong staining for PDI was observed in cambial, epidermal, and subepidermal regions (Fig. 5, J–L). PDI was present throughout the endosperm in developing soybean seeds (data not shown).

To investigate the subcellular localization of plant PDI, we performed EM of immunogold-labeled sections of active nitrogen-fixing soybean nodules, a source material rich in ER. In preliminary experiments, nodule extracts from which bacteroids had been separated by differential centrifugation were shown to posses a single protein band of approximately 60 kD recognized by the anti-(alfalfa B2) serum on western blots. Bacteroids contained a number of cross-reacting proteins, the major one being of approximately 100 kD; they did not contain a cross-reacting protein of similar molecular mass to plant PDI (data not shown). Gold particles were localized exclusively to the ER and the bacteroids in cross-sections of nodules developed with anti-(B2) serum (Fig. 6, A and B). The Golgi apparatus (Fig. 6A) and plasma membrane (data not shown) were not labeled. Labeling of bacteroids occurred in the central region, possibly associated with chromosomal material. No signal was observed with preimmune serum (Fig. 6C).

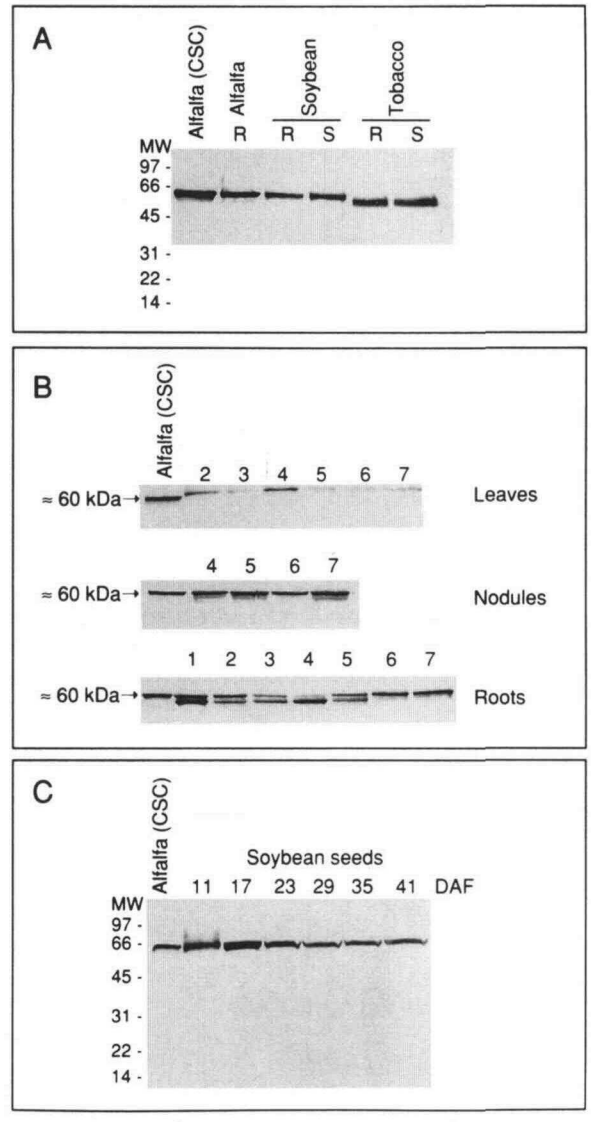

Figure 4. Western blot analysis of PDI levels in developing tissues of alfalfa, soybean, and tobacco. Total proteins (25 μg/lane) were isolated from plant tissues, resolved by 8.5% SDS-PAGE, blotted, and probed with anti-(B2) serum. A, Alfalfa, soybean, tobacco roots (R), and stems (S). B, Alfalfa leaves, roots, and nodules at the indicated developmental stages (in weeks). C, Soybean seeds at the indicated developmental stages, in DAF. Total protein from an alfalfa cell suspension culture (CSC) was included on all gels as a control. The positions of standard protein molecular mass markers are indicated.

DISCUSSION

The alfalfa B2 cDNA was originally identified as a putative PDI on the basis of sequence similarity to mammalian PDI and expression in *E. coli* (Shorrosh and Dixon, 1991). The specific activity of PDI expressed from the B2 clone was

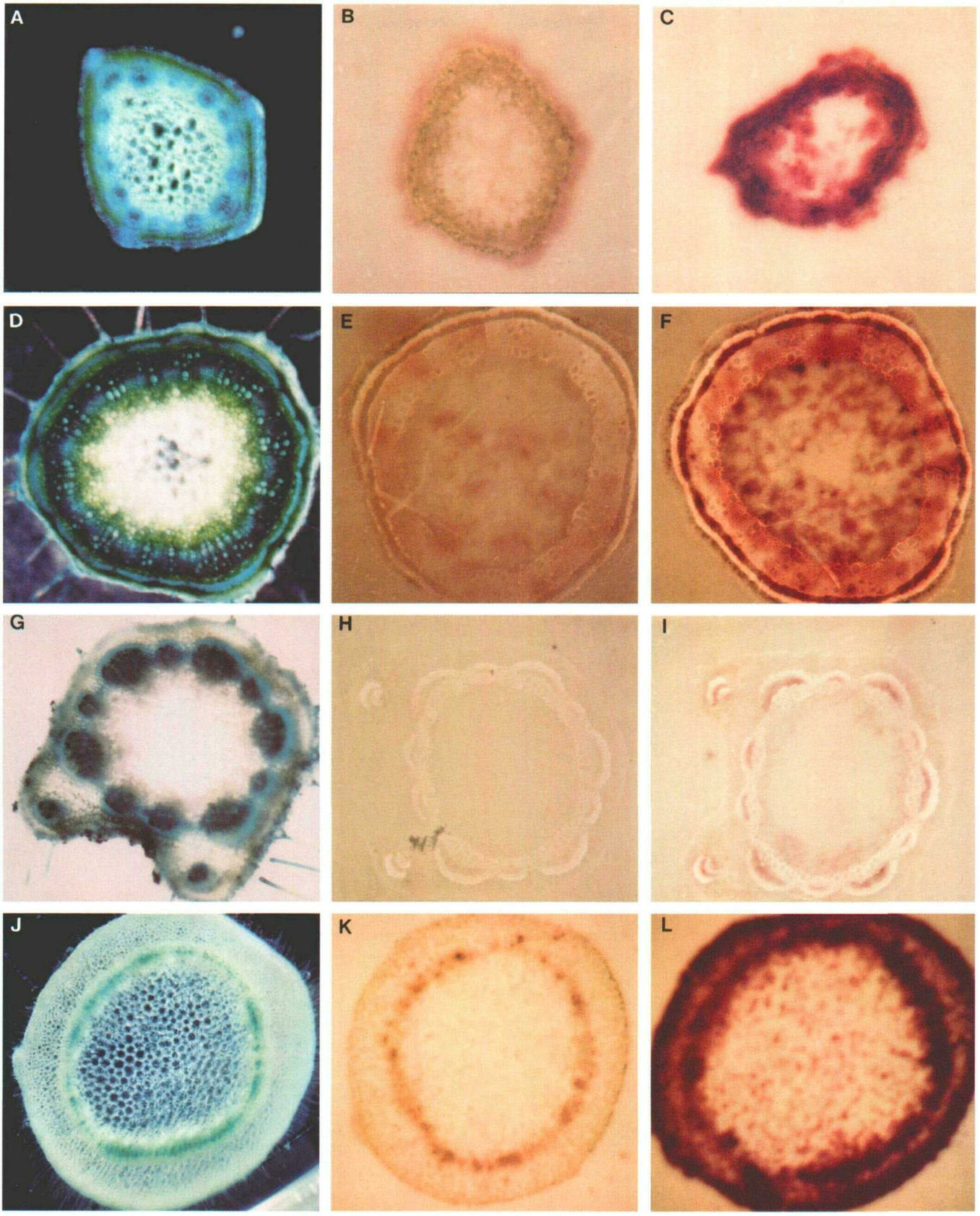

Figure 5. Localization of PDI in tissue print immunoblots. Sections were stained with toluidine blue (A, D, G, J), preimmune serum (B, E, H, K), or anti-(alfalfa B2) serum (C, F, I, L). Sections are from alfalfa stems (A–C), soybean stems (D–F), soybean petioles (G–I), and tobacco stems (J–L).

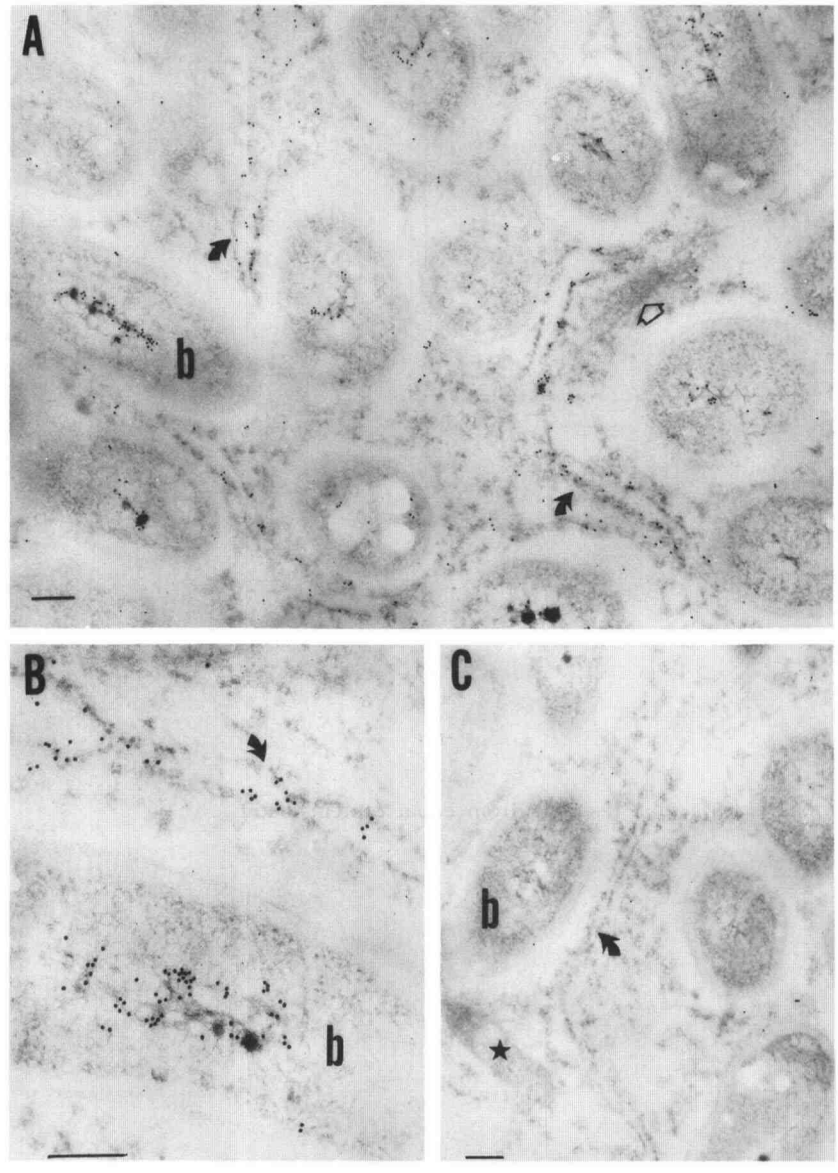

Figure 6. Electron micrographs showing immunogold labeling of PDI in soybean root nodule tissue embedded in LR White acrylic resin. A and B, Sections labeled with anti-(B2) serum followed by protein-A gold. Note localization of PDI in the ER (curved arrows) and the bacteroids (b). No labeling was observed on Golgi (large arrow) or other structures. C, Control treated with preimmune serum and protein-A gold. Note the lack of label in ER (arrow) and the bacteroids, as well as mitochondria (*). Bar = 200 nm.

similar to that from the mouse ERp59 PDI. The antiserum raised against the B2 protein expressed in *E. coli* immunoprecipitated PDI activity from crude alfalfa cell extracts. Other plant proteins, e.g. thioredoxin and G1, a 40-kD protein related to the mammalian endomembrane protein ERp72 (Shorrosh and Dixon, 1992), contain the same catalytic sites as PDI and may exhibit PDI activity, at least in vitro. This may explain the basal PDI activity remaining after immunoprecipitation of alfalfa cell extracts. The antiserum did not inhibit PDI activity in solution, suggesting that the antibodies do not recognize the thioredoxin-like active sites, which are the only conserved sequences between B2 and G1. The anti-(B2) serum recognized a single band (occasionally a doublet) on western blots of alfalfa cell proteins. The pI of the recognized band was identical with that calculated from the deduced amino acid sequence of B2, whereas the molecular mass determined by SDS-PAGE was approximately 5 kD higher than the calculated value. This could be the result of unusual protein conformation or possibly glycosylation.

PDI protein levels were not induced by fungal elicitor in alfalfa suspension cells, consistent with previous observations at the transcript level (Shorrosh and Dixon, 1991). Thus, if PDI serves as a β-subunit for prolyl 4-hydroxylase in plants, the induction of the activity of this enzyme by elicitor (Bolwell et al., 1985; B. Shorrosh, unpublished results) is most likely the result of an increase in the levels or activity of the catalytic α-subunits.

The increase in PDI levels in cells exposed to tunicamycin is probably initiated by the build up of underglycosylated or incorrectly processed proteins in the ER, as observed in relation to induction of the so-called Glc-regulated proteins of mammalian cells (Lee, 1987). Tunicamycin likewise induces transcripts encoding B2 and G1 in alfalfa cells (Shorrosh and Dixon, 1991, 1992), and a plant homolog of the

immunoglobulin-binding protein (BiP) in maize cells (Fontes et al., 1991). It is possible that the lower molecular mass immunoreactive band seen on western blot analysis of proteins from tunicamycin-treated cells is a non- or underglycosylated form of PDI; B2 contains a single putative N-glycosylation site at amino acid 278 (Shorrosh and Dixon, 1991). Because IEF blot analysis of extracts from tunicamycin-treated cells did not reveal the presence of charge isoforms of PDI at the level of resolution obtained, it is unlikely that the lower molecular mass band is the product of a second PDI gene in alfalfa (Shorrosh and Dixon, 1991).

PDI protein was expressed constitutively in alfalfa roots, root nodules, leaves, and stem tissue. The protein in alfalfa leaves was of slightly higher molecular mass than from other alfalfa tissues. Both root and nodule tissues contained a lower molecular mass form of PDI, which may or may not be identical with that seen in tunicamycin-treated cells. Tobacco PDI was of slightly lower electrophoretic mobility than the enzyme from the legumes soybean and alfalfa.

PDI activity appears in wheat endosperm from 10 to 50 d postanthesis (Roden et al., 1982), consistent with a requirement for PDI activity for the formation of disulfide bonds during storage protein synthesis. PDI levels in developing soybean seeds appeared maximal at approximately 17 DAF. This coincides with the early stages of accumulation of storage protein (glycinin and β-conglycinin) transcripts (Walling et al., 1986). The apparent decline in PDI after 17 d is probably artifactual, resulting from equal protein loadings from tissues that produce increasing levels of storage proteins.

The cellular localization of PDI in plant vegetative tissues, as assessed by tissue print immunoblot analysis, varied somewhat depending on the species. In alfalfa and tobacco stems, considerable signal was observed in the epidermal and subepidermal regions, whereas in soybean stem sections these same regions stained only weakly. This may be due to the fact that the soybean tissues were more mature, showing extensive secondary thickening in the stem. In this respect, PDI in more mature alfalfa stems was not found in epidermal and subepidermal tissues, being localized exclusively in the cambial zone (data not shown). Likewise, in soybean stems and petioles, PDI was localized predominantly to the cells of the cambial zone and its immediate phloem derivatives. PDI was also present in stem pith from all species examined. The level of PDI may reflect the metabolic activity of particular cell types in relation to production of proteins that require processing in the ER. Cambial cells are highly active in production of new cell wall material. Wall-bound Hyp-rich glycoproteins and their corresponding transcripts have recently been shown, by tissue print analysis, to be localized primarily in cambial tissues of soybean stems and petioles (Ye and Varner, 1991). PDI may serve a dual role in Pro hydroxylation and glycosylation of Hyp-rich glycoproteins.

Because the alfalfa B2 PDI contains a KDEL C-terminal ER retention sequence (Pelham, 1990), it would be predicted that the protein would be localized in the ER. However, rat PDI is exported from the ER to the plasma membrane in exocrine pancreatic cells (Yoshimori et al., 1990), and the maize auxin-binding protein exhibits plasma membrane localization in spite of the possession of a C-terminal KDEL tetrapeptide (Klambt, 1990; Jones and Herman, 1993). Our data confirm exclusive localization of PDI to the endomembrane system in soybean nodule tissue, a rich source of ER. Immunogold labeling of bacteroids is due to the presence of a possible chromosomal protein of high molecular mass from the *Rhizobium* that is antigenically related to plant PDI. Localization of PDI to plant endomembranes has been previously suggested based upon results of subcellular fractionation studies (Roden et al., 1982).

Taken together, our results confirm that the alfalfa B2 clone encodes a PDI, the expression and localization of which are consistent with current hypotheses of PDI function. The highly specific plant PDI antibodies should be of value in future studies of protein processing and will help in assessing the role of PDI in the plant prolyl 4-hydroxylase reaction.

ACKNOWLEDGMENTS

We wish to thank Scott Russell for the use of the Oklahoma State University EM facility, Gregory Strout for sectioning and technical assistance, Jack Blount for assistance with production of antiserum in rabbits, Frank Coker for propagating plant material, Michael Green for a gift of the mouse ERp59 cDNA, Robert Gonzales and Scott White for critical reading of the manuscript, and Scotty McGill for preparation of the manuscript.

Received April 23, 1993; accepted July 14, 1993.
Copyright Clearance Center: 0032-0889/93/103/0719/08.

LITERATURE CITED

Bolwell GP, Robbins MP, Dixon RA (1985) Metabolic changes in elicitor-treated bean cells. Enzymic responses in relation to rapid changes in cell wall composition. Eur J Biochem **148:** 571–578

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem **72:** 248–254

Bulleid NJ, Freedman RB (1988) Defective co-translational formation of disulphide bonds in protein disulphide isomerase deficient microsomes. Nature **335:** 649–651

Cheng S-Y, Gong Q-H, Parkison C, Robinson EA, Apella E, Merlino GT, Pastan I (1987) The nucleotide sequence of human cellular thyroid hormone binding protein present in endoplasmic reticulum. J Biol Chem **262:** 11221–11227

Cregan PB, Hartwig EE (1984) Characterization of flowering response to photoperiod in diverse soybean genotypes. Crop Sci **24:** 659–662

Dixon RA, Dey PM, Whitehead IM (1981) Dose responses for *Colletotrichum lindemuthianum* elicitor-mediated enzyme induction in French bean cell suspension cultures. Planta **151:** 272–280

Edman JC, Ellis L, Blacher RW, Roth RA, Rutter WJ (1985) Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. Nature **317:** 267–270

Farquhar T, Honey N, Murant SJ, Bossier P, Schultz L, Montgomery D, Ellis RW, Freedman RB, Tuite MF (1991) Protein disulfide isomerase is essential for viability of *Saccharomyces cerevisiae*. Gene **108:** 81–89

Fontes EBP, Shank B, Wrobel RL, Moose SP, OBrian GR, Wurtzel ET, Boston RS (1991) Characterization of an immunoglobulin binding protein homolog in the maize *fluory-2* endosperm mutant. Plant Cell **3:** 483–496

Freedman RB (1989) Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell **57:** 1069–1072

Geetha-Habib M, Noiva R, Kaplan HA, Lennarz WJ (1988) Glycosylation site binding protein, a component of oligosaccharyl transferase, is highly similar to three other 57 kD luminal proteins of the ER. Cell **54:** 1053–1060

Harlow E, Lane D (1988) Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 522

Jones AM, Herman EM (1993) KDEL-containing auxin-binding protein is secreted to the plasma membrane and cell wall. Plant Physiol **101:** 595–606

Klambt D (1990) A view about the function of auxin-binding proteins at plasma membranes. Plant Mol Biol **14:** 1045–1050

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

LaMantia M, Miura T, Tachikawa H, Kaplan HA, Lennarz WJ, Mizunaga T (1991) Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. Proc Natl Acad Sci USA **88:** 4453–4457

Lee AS (1987) Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends Biochem Sci **12:** 20–23

Noiva R, Kaplan HA, Lennarz WJ (1991a) Glycosylation site binding protien is not required for N-linked glycoprotein synthesis. Proc Natl Acad Sci USA **88:** 1986–1990

Noiva R, Kimura M, Roos J, Lennarz WJ (1991b) Peptide binding by protein disulfide isomerase, a resident protein of the endoplasmic reticulum. J Biol Chem **266:** 19645–19649

Noiva R, Lennarz WJ (1992) Protein disulfide isomerase. A multifunctional protein resident in the lumen of the endoplasmic reticulum. J Biol Chem **267:** 3553–3556

Parkkonen T, Kivirikko KI, Pihlajaniemi T (1988) Molecular cloning of a multifunctional chicken protein acting as the prolyl 4-hydroxylase β-subunit, protein disulfide isomerase and a cellular thyroid-hormone-binding protein. Biochem J **256:** 1005–1011

Pelham HRB (1990) The retention signal for soluble proteins of the endoplasmic reticulum. Trends Biochem Sci **15:** 483–486

Pihlajaniemi T, Helaakoski T, Tasanen, K, Myllyla R, Huhtala ML, Koivu JG, Kivirikko KI (1987) Molecular cloning of the beta-subunit of human prolyl 4-hydroxylase. This subunit and protein disulphide isomerase are products of the same gene. EMBO J **6:** 643–649

Polayes DA, Schubert KR (1984) Purine synthesis and catabolism in soybean seedlings. The biogenesis of useides. Plant Physiol **75:** 1104–1110

Robertson EF, Dannelly HK, Malloy PJ, Reeves HC (1987) Rapid isoelectric focusing in a vertical polyacrylamide minigel system. Anal Biochem **167:** 290–294

Roden LT, Miflin BJ, Freedman RB (1982) Protein disulfide-isomerase is located in the endoplasmic reticulum of developing wheat endosperm. FEBS Lett **138:** 121–124

Shorrosh BS, Dixon RA (1991) Molecular cloning of a putative plant endomembrane protein resembling vertebrate protein disulfide-isomerase and phosphatidylinositol-specific phospholipase C. Proc Natl Acad Sci USA **88:** 10941–10945

Shorrosh BS, Dixon RA (1992) Molecular characterization and expression of an alfalfa protein with sequence similarity to mammalian ERp72, a glucose-regulated endoplasmic reticulum protein containing active site sequences of protein disulphide isomerase. Plant J **2:** 51–58

Van den Bosch KA, Newcomb EH (1986) Immunogold localization of nudule specific uricase in developing soybean root nodules. Planta **167:** 425–436

Walling L, Drews GN, Goldberg RB (1986) Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci USA **83:** 2123–2127

Wells WW, Xu DP, Yang Y, Rocque PA (1990) Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. J Biol Chem **265:** 15361–15364

Wetterau JR, Combs KA, McLean LR, Spinner SN, Aggerbeck LP (1991) Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. Biochemistry **30:** 9728–9735

Wetterau JR, Combs KA, Spinner SN, Joiner BG (1990) Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. J Biol Chem **265:** 9800–9807

Ye Z-H, Varner JE (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell **3:** 23–37

Yoshimori T, Semba T, Takemoto H, Akagi S, Yamamoto A, Tashiro Y (1990) Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. J Biol Chem **265:** 15984–15990