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Short Communication

Paraveinal Mesophyll of Soybean Leaves in Relation to Assimilate Transfer and Compartmentation[1]

III. IMMUNOHISTOCHEMICAL LOCALIZATION OF SPECIFIC GLYCOPEPTIDES IN THE VACUOLE AFTER DEPODDING

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ABSTRACT

Immunohistochemical staining was used to determine the cellular distribution of two glycosylated polypeptides (molecular weights of 27 and 29 kilodaltons) which are normally present at low levels in soybean (*Glycine max* var 'Wye') leaves but which markedly accumulate after depodding. These polypeptides, which comprise a substantial portion of the total leaf soluble protein of depodded plants, were exclusively located in the vacuoles of paraveinal mesophyll and associated bundle sheath cells. These results support the unique role of the soybean leaf paraveinal mesophyll in the transport and spatial compartmentation of nitrogen reserves in relation to seed filling.

Soybean leaves have a specialized cell layer called the PVM[4] which mediates the transfer of assimilates from the palisade and spongy mesophyll to the phloem (1, 2). We have extensively characterized the PVM and have demonstrated its unique role in the synthesis and compartmentation of leaf nitrogen reserves prior to and during seed filling (2, 3). Ultrastructural, cytochemical, and microautoradiographic studies showed that a dictyosome-derived glycoprotein is transferred to and accumulated within the vacuoles of the PVM prior to flowering and that this glycoprotein is remobilized from the leaf during the early stages of seed filling (3). Moreover, a decrease in sink demand caused by depodding caused a marked accumulation of this, as yet uncharacterized, glycoprotein within the PVM, suggesting its remobilization may play a role in the temporal allocation of nitrogen to the seeds.

Other studies from this department (6) have demonstrated that the loss of the major soluble leaf protein, ribulose bisphosphate carboxylase, following depodding of soybeans is accompanied by the appearance of three major polypeptides of unknown cellular location, two of which (27 and 29 kD) were glycosylated. It was of interest to determine whether the glycoprotein(s) which was found to accumulate selectively in the PVM by Franceschi and Giaquinta (2, 3) includes the 27 and 29 kD glycoproteins that Wittenbach (6) found to increase following depodding.

This study uses antibodies prepared against purified 27 and 29 kD glycoproteins to determine their cellular location by immunohistochemical techniques. Our findings conclusively show that these glycoproteins are exclusively localized in the vacuoles of the paraveinal mesophyll.

MATERIALS AND METHODS

Soybean plants (*Glycine max* var 'Wye') were grown under controlled conditions in environmental chambers as described previously (4). Samples for microscopy were collected from leaves of plants 3 to 4 weeks after continual depodding and were fixed in FAA (20 ml 37% v/v formaldehyde, 95 ml 100% ethanol, 95 ml glacial acetic acid, 75 ml H_2O) overnight at 4°C. Samples were dehydrated with ethanol, infiltrated with xylene, and then embedded in Paraplast medium (Fisher Scientific). Fifteen-μm-thick sections were mounted on gelatin coated slides, deparaffinized in xylene, and rehydrated prior to the immunohistochemical localization of the 27 and 29 kD polypeptides.

The purification of the glycosylated polypeptides from depodded soybean plant leaves and production of the mouse antiserum to these polypeptides is described elsewhere (7). Leaf sections are treated with antibody produced in mice and then reacted with a sheep anti-mouse antibody which is conjugated to peroxidase. In the presence of H_2O_2, the peroxidase catalyzes the oxidation of DAB to form an insoluble, colored product. The following protocol was used for the immunohistochemistry (all steps were performed at 21°C): (a) 5-min rinse in PBS (Gibco Laboratories, NY); (b) 2-h incubation in mouse antiserum (approx. 1 mg IgG/ml in PBS); (c) 10-min rinse in PBS; (d) 2-h incubation in peroxidase-conjugated sheep anti-mouse IgG (1:10 dilution of stock solution obtained from Cappel Laboratories, West Chester, PA.); (e) 10-min rinse in PBS; (f) 5-min rinse in distilled H_2O; (g) 2-h incubation in 0.05% (w/v) DAB (Sigma) in 0.001% (v/v) H_2O_2 in the dark (DAB solution was changed every 30 min); (h) distilled H_2O rinse; (i) dehydrate with ethanol, infiltrate with xylene, and mount with Permount medium (Fisher Scientific).

Procedures for protein extraction and SDS gel electrophoresis (6) and tissue protein staining with mercuric bromophenol blue (2) have been described previously.

After electrophoresis, protein was transferred from the gels to

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[4] Abbreviations: PVM, paraveinal mesophyll; DAB, diaminobenzidine; PBS, phosphate-buffered saline, 10 mM, pH 7.3; IgG, immunoglobulin G; MBPB, mercuric bromophenol blue.

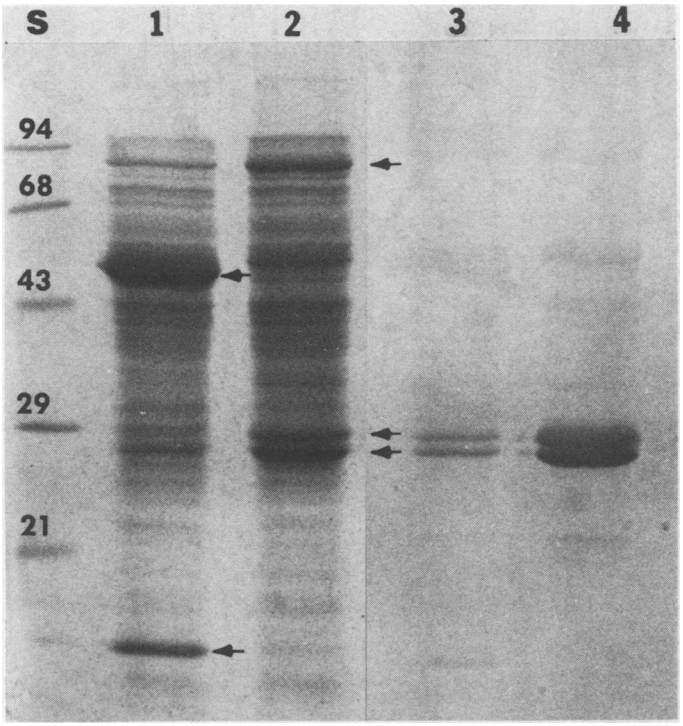

FIG. 1. Polypeptide profiles and peroxidase-conjugated antibody staining of soluble protein in soybean leaves. Lanes 1 and 3 are from podded leaves, while lanes 2 and 4 are from leaves depodded for 4 weeks after flowering. Each lane has a total of 40 μg protein. Lanes 1 and 2 were stained for protein with Coomassie blue, and lanes 3 and 4 were stained for peroxidase activity following immunological detection by peroxidase-conjugated antibodies. S is mol wt standards (kD). Arrows show the large (55 kD) and small (13 kD) subunits of RuBPCase in lane 1, and in lane 2 the 27, 29, and 80 kD proteins.

nitrocellulose paper according to the procedure of Towbin *et al.* (5). The transfer was made using a constant current of 220 mamp for 16 h. Following transfer, the blot was either stained for protein or used for immunological detection of the 27 and 27 kD polypeptides. Blots were stained for protein with Coomassie blue (0.1% w/v in 40% v/v methanol/10% v/v acetic acid) and destained with 90% methanol/2% acetic acid. Immunological detection using peroxidase-conjugated antibody followed the methods of Towbin *et al.* (5) except that 3% BSA was used to saturate additional protein binding sites in the paper and 0.2% BSA was used in subsequent steps. Also, DAB was substituted for *o*-dianisidine in the color reaction.

RESULTS AND DISCUSSION

Depodding soybean plants has been shown previously (6) to delay the normal loss of total leaf soluble protein associated with senescence. Depodding does, however, cause marked qualitative changes in polypeptide profiles of the leaf protein. Figure 1, lane 1 shows the polypeptide profile of soluble protein from soybean leaves with pods 4 weeks after flowering. Following Coomassie blue staining, the large and small subunits of ribulose bisphosphate carboxylase, 55 and 13 kD (arrows), are the most prominent bands. Leaves of depodded plants are almost totally devoid of ribulose bisphosphate carboxylase for 4 weeks after flowering (Fig. 1, lane 2). Even though these major proteins are lost, the level of total soluble protein remains high because several polypeptides, three in particular (27, 29, and 80 kD, arrows) increase in amounts sufficient to offset the loss of carboxylase protein. The role of these three major polypeptides is unknown.

Figure 1, lanes 3 and 4, show the polypeptide profiles from the same leaves of these podded and depodded plants after staining with peroxidase-conjugated antiserum prepared to the previously purified 27 and 29 kD proteins. It is clear that staining is highly specific for the 27 and 29 kD polypeptides since essentially no reaction product is associated with the numerous other polypeptides which appear in the Coomassie blue-stained gels (lanes 1 and 2). It is clear that the 27 and 29 kD proteins are normally present in soybean plants and that depodding results in their marked accumulation through either an increase in their synthesis and/or a decrease in their degradation. 'Normal' mouse serum did not cross react with the 27 and 29 kD glycoproteins in an Ouchterlony assay, thus eliminating the possibility of pseudo-binding.

Because of this selective staining we were able to use this peroxidase-conjugated antibody procedure to determine the cellular location of these proteins. Depodded plants were used in the immunohistochemical studies because (a) these plants have a much greater amount of protein than the podded controls, and (b) previous ultrastructural studies have shown that depodding only affected the amount, not the cellular distribution of the protein found in the PVM (3).

Figure 2A shows a cross section of a depodded soybean leaf stained with mercuric bromophenol blue, which is a general protein stain. Although staining is evident in all the leaf cells, as would be expected with a general protein stain, it is clear that the PVM and associated bundle sheath cells contain the greatest intensity of protein staining. When a similar unstained leaf cross section is treated by the immunohistochemical procedure for the localization of the 27 and 29 kD glycoproteins, the specific reaction product is found only in the PVM and associated bundle sheath (Fig. 2, B and C). Unlike the general protein staining in Figure 2A, no staining is evident in any other cell type. Sections treated with mouse antibody to the glycosylated polypeptides but not with subsequent peroxidase-conjugated sheep anti-mouse IgG, showed no reaction product (data not shown), thus demonstrating that endogenous peroxidase activity was not responsible for reaction product formation. When the sections were incubated with sheep IgG, but not with mouse antibody, no reaction product was formed (Fig. 2D), ruling out nonspecific adsorption of the peroxidase-conjugated sheep anti-mouse IgG to the sections.

The subcellular compartmentation of the glycoproteins as determined by antibody-mediated stain could be assessed at the light microscope level. Figure 2, E and F, are of the same section in the region of the PVM as viewed with bright field (stain found only in PVM and bundle sheath) and phase contrast optics, respectively. It is evident from this comparison that the antibody-linked peroxidase reaction product, and hence the glycosylated polypeptides, are localized solely within the vacuoles of both the PVM and bundle sheath cells. This is particularly evident in the phase contrast micrograph where the vacuole, cytoplasm, and cell wall can be clearly distinguished (Fig. 2F, arrows).

CONCLUSION

We have previously shown that the highly specialized PVM of soybean leaves plays a novel role in the compartmentation of nitrogen reserves in relation to seed filling (2, 3). This study further characterizes the glycoprotein which accumulates in this cell type in response to alterations in sink demand. Immunohistochemistry was used to demonstrate clearly that 27 and 29 kD glycosylated polypeptides, which accumulate in soybean leaves following depodding, are exclusively localized in the vacuoles of the PVM and associated bundle sheath. Inasmuch as other polypeptides increase following depodding (*e.g.* 80 kD, Fig. 1), it is possible that PVM represents a synthesis and storage compartment for more than one related or unrelated protein. It is interesting to note that the 27 and 29 kD polypeptides which are present in the PVM of leaves in both podded and depodded plants do not show cross reactivity (Wittenbach, personal observations) with soybean storage seed

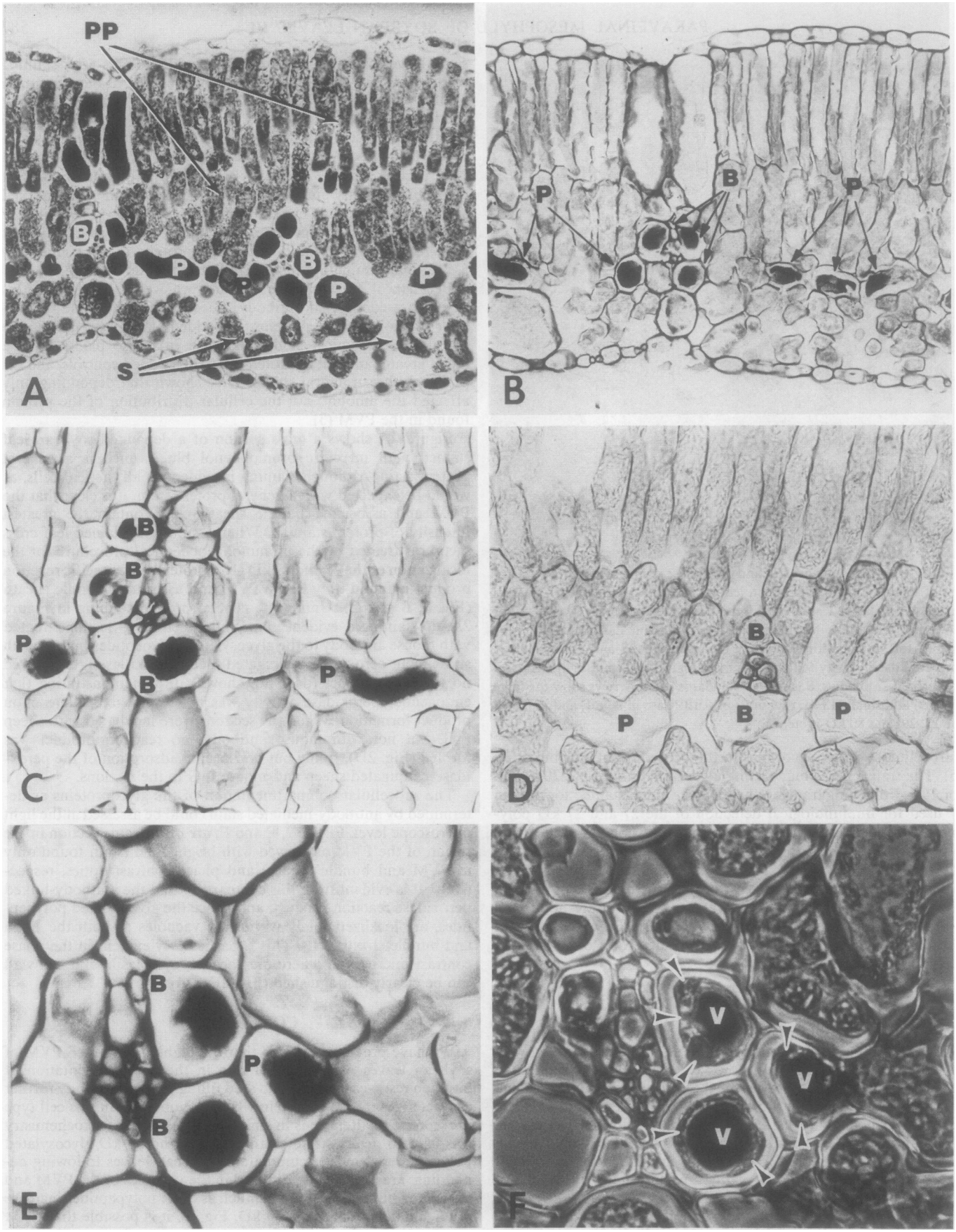

FIG. 2. Immunohistochemical localization of specific glycoproteins in soybean leaves. Bundle sheath (B), PVM (P), palisade parenchyma (PP), spongy mesophyll (S). A, Section stained for protein with MBPB. The PVM and bundle sheath show the greatest stain intensity (× 90). B, Antibody-mediated localization of the 27 and 29 kD glycosylated polypeptides. Only the PVM and bundle sheath show positive reaction for antibody binding (× 90). C, Higher magnification showing localization of the antibody-linked peroxidase reaction product (× 180). D, Control sections not exposed to mouse antiserum. No endogenous peroxidase activity or nonspecific adsorption of peroxidase-conjugated sheep anti-mouse IgG to the sections can be detected (× 180). E, High magnification of antibody-bound peroxidase activity reaction product in PVM and bundle sheath cells as seen with bright field microscopy. Cytoplasm of adjacent cells is not apparent (× 340). F, Same field of view as 'E,' seen with phase contrast microscopy. The cytoplasm of the PVM and bundle sheath is clearly delineated (arrowheads). It is evident that antigenicity is restricted primarily to the vacuole (V) (× 340).

protein (some of which are also glycosylated). Unlike the other leaf cell types, the PVM appears to express the increased synthesis of these glycoproteins and thus the PVM may be a unique system to study the spatial and temporal aspects of gene expression.

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