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Purification and Characterization of a Soybean Leaf Storage Glycoprotein[1]

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ABSTRACT

Removing the pods from soybean (*Glycine max* [L.] Merr. cv Wye) plants induces a change in leaf function which is characterized by a change in the leaf soluble protein pattern. The synthesis of at least four polypeptides (~27, 29, 54, and 80 kilodaltons) is enhanced, and these polypeptides accumulate to levels comprising over 50% of the soluble protein. Heat girdling the petiole also causes the accumulation of these polypeptides, suggesting that the signal for changing leaf function may be associated with inhibition of phloem transport. The 27 and 29 kilodalton polypeptides are glycosylated and have been purified to greater than 90% by $(NH_4)_2SO_4$ fractionation, concanavilin A affinity, and gel filtration chromatography. These peptides appear to comprise a single protein. Mouse antiserum has been prepared against this glycoprotein and has been used to check for cross-reactivity with seed proteins and to quantitate changes with leaf development. No cross-reactivity was observed with seed soluble proteins from several stages of development. Quantitation showed the highest content in podded plants at, and shortly following, flowering, with levels subsequently declining in conjunction with seed growth. In depodded plants, the level of glycoprotein continued to increase following flowering and accounted for 45% of the soluble leaf protein by 4 weeks after depodding.

Removing pods from soybean plants delays the loss of Chl and soluble protein associated with normal leaf senescence, but does not delay, and in fact enhances, the decline in photosynthesis (8, 12). The separation of functional senescence from changes leading to leaf death results from induction of qualitative changes in soluble protein due to pod removal (12). Several polypeptides, three in particular (~27, 29, and 80 kD), were shown to increase dramatically following pod removal, while some such as ribulose bisphosphate carboxylase (small and large subunits), were lost earlier and at a faster rate than during normal senescence.

In the previous paper, (12) several lines of evidence suggested these three accumulated polypeptides were not breakdown products of carboxylase. If this is true, then what is the function of these polypeptides and where are they located? Some evidence already exists as to their possible localization. In soybean leaves, there is a unique layer of cells, paraveinal mesophyll, located between the palisade meosphyll cells and the spongy parenchyma (2, 3). Recently, Franceschi and Giaquinta showed that this layer of cells functions in the synthesis, compartmentation, and remobilization of protein prior to and during seed filling. They also demonstrated a marked increase in the accumulation of protein within the vacuoles of these cells following pod removal. Therefore, the polypeptides that accumulate following depodding may be localized in these cells.

The purpose of this study was to further characterize these polypeptides and to purify one or more to obtain antiserum for quantitation and cellular localization work. This paper describes the purification and antiserum production to a glycoprotein apparently composed of a 27 and 29 kD polypeptide. In addition, quantitation of this protein with development and further characterization of its accumulation are presented.

MATERIALS AND METHODS

Plant Material. Soybean (*Gycine max* [L.] Merr. cv Wye [determinate]) plants were grown as previously described (12). Depodding was initiated 1 week after flowering and was repeated at weekly intervals.

Protein Purification. Trifoliate leaves were harvested from the main stem of plants which had been depodded for 4 to 5 weeks. The leaves were extracted in 20 mM Tris-HCl buffer (pH 7.6) containing 4 mM DTT and 1 mM EDTA (4 ml: 1 g of tissue) at 4°C using a Waring Blendor. The homogenate was centrifuged at 30,000*g* for 20 min, and the supernatant fractions were decanted and combined. Saturated $(NH_4)_2SO_4$ was added to the supernatant fraction to bring it to a concentration of 2.0 M and, after standing for 30 min on ice, the solution was centrifuged at 12,000*g* for 10 min. The precipitate was discarded and the supernatant solution was brought to 2.8 M $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 100 mM acetate buffer (pH 5.6) containing 0.9% NaCl. This solution was applied to a Con A-Sepharose column (1 × 5 cm) equilibrated with the resuspension buffer. The column was washed with buffer until no more protein was eluted; then 50 mM 1-*O*-methyl-α-D glucopyranoside in the same buffer was added to elute the bound protein. The fractions containing the bound protein were then applied to a Sephacryl S-200 column (5 × 105 cm) equilibrated with 0.9% NaCl in 20 mM Tris-HCl buffer (pH 7.6). One major peak (A_{280}) was obtained, and the fractions from the center of this peak were combined and concentrated for antiserum production and standardization of the quantitative radial immunodiffusion assay.

Production of Antiserum. The purified protein was emulsified in complete Freund's adjuvant. Mice (C57/BL6) were immunized initially by footpad injection with 50 μg protein/mouse. Subsequent boosts after 2 and 3 weeks were done intraperitoneally using 10 and 5 μg protein, respectively, plus 1 mg of Alhydrogel/mouse. The mice were then bled 1 week later by retroorbital puncture to obtain antiserum. Thereafter, the mice were given boosts of 1 to 1.5 μg protein every 2 to 3 weeks and were bled 1 week after each boost for collection of antiserum.

Immunodiffusion and Immunoelectrophoresis Techniques. An-

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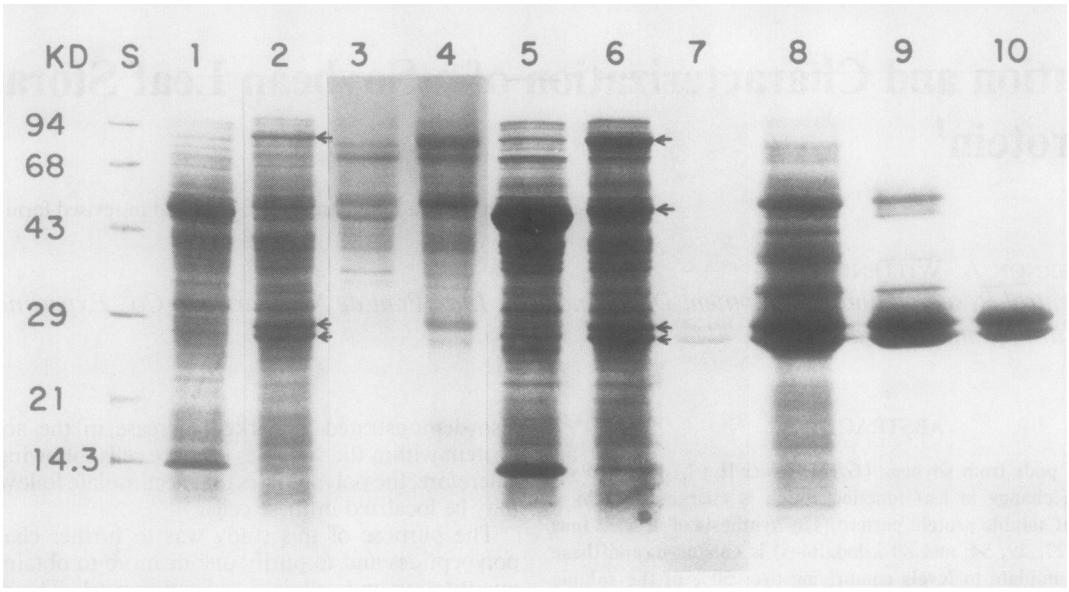

FIG. 1. Polypeptide profiles from SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2, soluble protein from leaves of podded (1) and depodded (2) plants 5 weeks after flowering. Lanes 3 and 4, [^{35}S]methionine-labeled polypeptides following a 2-h pulse to leaves from podded (3) and depodded (4) plants 4 weeks after flowering. Lanes 5 and 6, soluble protein from nongirdled (5) and girdled (6) leaves 4 weeks after flowering (3 weeks after heat girdling). Lane 7, soluble protein of lane 2 sample after staining with periodic acid-Schiff reagent. Lane 8, 2.0 to 2.8 M $(NH_4)_2SO_4$ fraction of soluble proteins from depodded leaves 5 weeks after flowering. Lane 9, polypeptides from the $(NH_4)_2SO_4$ fraction that are bound to a Con A-Sepharose column and subsequently eluted by methyl glucopyranoside. Lane 10, Con A-purified protein after passing through a Sephacryl S 200 column. Lane S is the protein standards with their respective mol wt. Lanes 1–7 represent 40 μg protein each, while lanes 8, 9, and 10 represent 85, 45, and 20 μg, respectively.

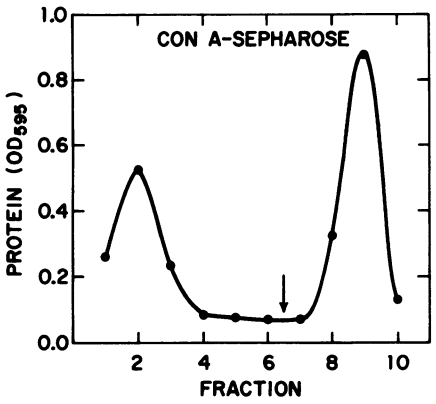

FIG. 2. Protein elution pattern from the Con A-Sepharose column following application of the 2.0 to 2.8 M $(NH_4)_2SO_4$ fraction in 100 mM acetate buffer (pH 5.6). The arrow represents the time of addition of 50 mM methyl glucopyranosides.

tigen-antibody precipitation patterns were observed using the double diffusion and immunoelectrophoretic techniques described by Ouchterlony and Nilsson (9). Quantitation of the glycoprotein was performed using the Mancini radial immunodiffusion technique (7) with 1-mm-thick, 1% agar gels containing 0.4% antiserum. Leaf and seed samples (200–500 mg) were extracted in 4 ml Tris buffer (pH 7.6) containing 4 mM DTT and 1 mM EDTA. Extracts were centrifuged at 30,000*g* for 20 min, and serial dilutions of the supernatant fractions were made and used for quantitation. Aliquots of 10 μl were added to wells 3 mm in diameter, and the plates were incubated in a humid chamber at 25°C for 30 h. The area of the precipitation halos for the extracts and glycoprotein standards were determined after Coomassie blue staining, and the quantity of glycoprotein was determined from a reference curve based on the response of the purified protein standards.

Gel Electrophoresis and Protein Transfer. Procedures for sample preparation and SDS-polyacrylamide gel electrophoresis were described earlier (12). Glycosylated polypeptides were detected in the gels using periodic acid-Schiff stain following the procedure of Segrest and Jackson (10). Labeled polypeptides were detected by autoradiography. Gels were stained and dried as normal and then autoradiographed using Cronex x-ray film and enhancing screen.

Proteins were transferred from gels to nitrocellulose paper following electrophoresis according to the procedure of Towbin *et al.* (11). Blots were either stained with Coomassie blue or used for immunological detection using peroxidase-conjugated antibody (5, 11).

Labeling Proteins. The seventh trifoliate was used for the labeling studies. The trifoliate leaf and stem were removed from the plant by cutting under water, and the stem of this explant was then placed in a vial of water on the growth room bench. One of the branch veins near the middle of the center leaflet was cut and trimmed under water near the main vein so that a 50-μl capillary tube would fit over it. The capillary tube was sealed over the vein using Petrolatum, and the leaf section was allowed to take up water for 0.5 h to check the system. Uptake was enhanced by increasing the air flow across the leaf with a small fan. Then 150 to 200 μCi of [^{35}S]methionine (500–600 Ci/mmol) was applied to the capillary. After uptake of the label (15–30 min), water was again added to the capillary as necessary and labeling was allowed to continue for 2 h. The leaf section was then extracted in 20 mM Tris buffer (pH 7.6), and gel samples were prepared as described earlier (12).

RESULTS AND DISCUSSION

Depodding soybean plants or explants results in the marked accumulation of several polypeptides (Fig. 1, lanes 1 and 2, arrows). These polypeptides are not breakdown products but are

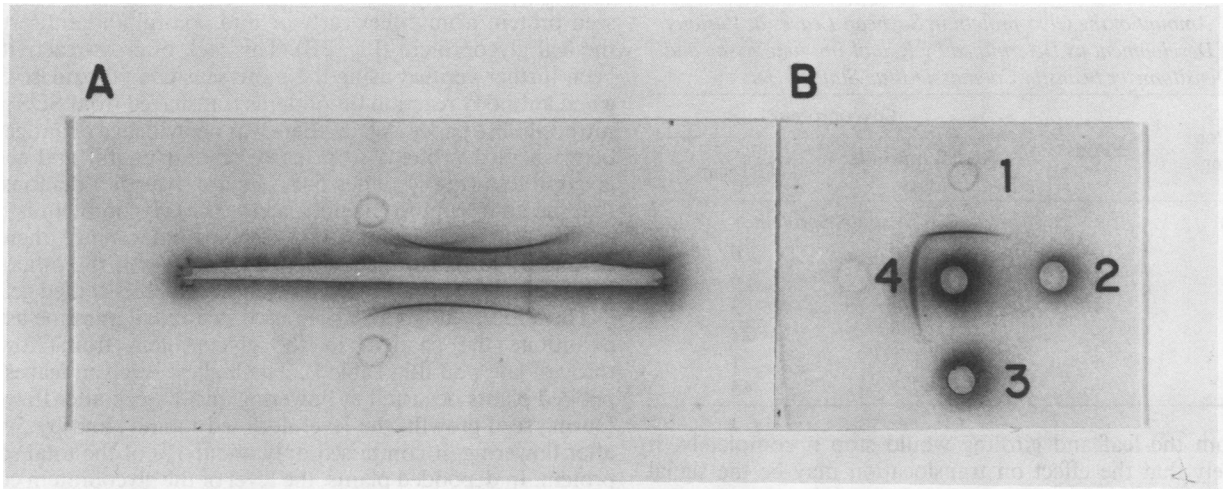

FIG. 3. A, Immunoelectrophoresis (left to right) of the purified glycoprotein (4 μg in 10 μl) followed by double diffusion with the center trough containing 50 μl of antiserum. B, Double diffusion assay with the center well containing 10 μl antiserum and wells 1 to 4 containing 2 μg purified glycoprotein (1), 40 μg soluble protein from seeds 2 weeks (2) and 5 weeks (3) after flowering, and 4 μg soluble protein from depodded leaves 5 weeks after flowering (4).

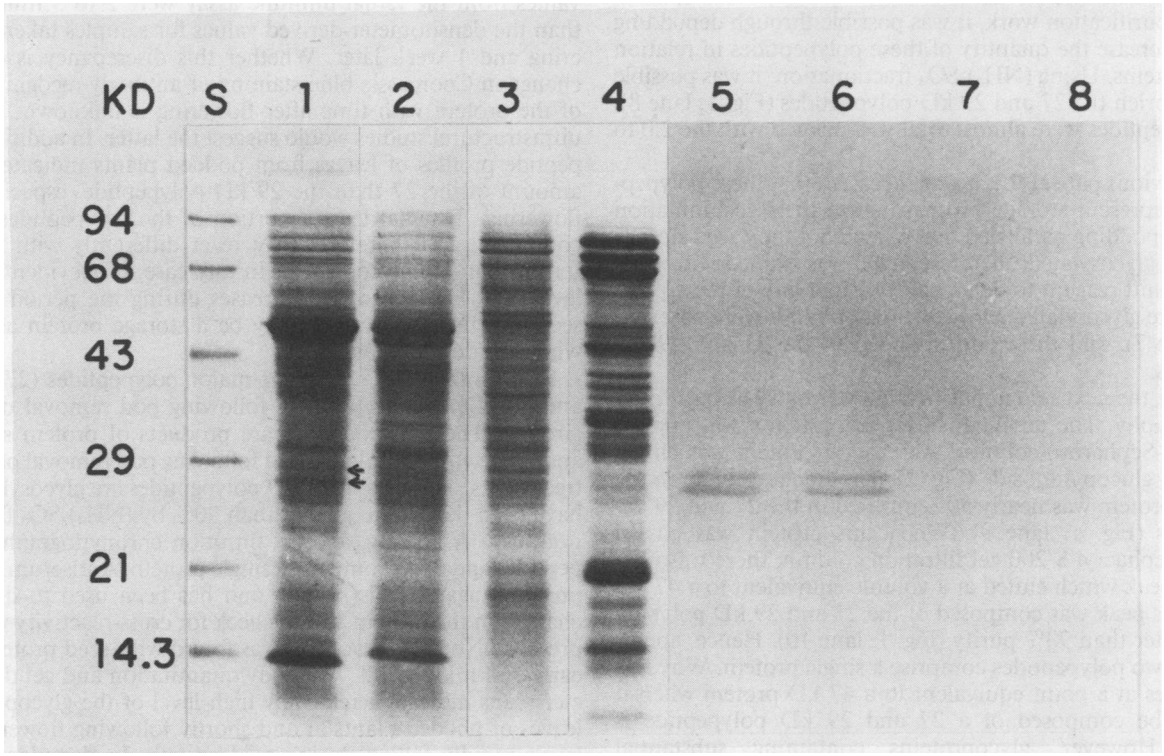

FIG. 4. Polypeptide profiles of soluble protein (40 μg/lane) from leaves of podded plants 1 and 4 weeks after flowering (lanes 1 and 2) and from seeds 2 weeks (lane 3) and 5 weeks after flowering (lane 4). Lanes 5 to 8 are the same as lanes 1 to 4 except the proteins have been transferred to nitrocellulose paper and stained for peroxidase activity following immunological detection with peroxidase-conjugated antibody. Lane S is the protein standards with their respective mol wt.

normal products of protein synthesis (Fig. 1, lanes 3 and 4). Following depodding, there is an increase in [^{35}S]methionine incorporation into at least four polypeptides, the three denoted by arrows (27, 29, and 80 kD) plus a polypeptide running just slightly behind the large subunit of ribulose bisphosphate carboxylase. It was originally thought that this latter band was an altered large subunit of carboxylase, even though it was demonstrated to lack antibody recognition sites (12). However, when the autoradiograph was compared with the Coomassie blue-stained gel, it was evident that the band was a distinct polypeptide. The 27 and 29 kD polypeptides accumulate in nearly identical amounts even though their induced rate of [^{35}S]methionine incorporation appears quite different. This may be due to a difference in turnover rate or sulfur content. Although there appear to be differences in other polypeptides, it is evident from these major changes that pod removal significantly alters the protein composition of soybean leaves.

These changes in protein composition can also be induced by heat-girdling the petiole, thereby blocking phloem translocation (Fig. 1, lanes 5 and 6, arrows). In fact, the polypeptide changes occur faster following girdling than depodding. Since pod removal in this determinate variety would greatly reduce translo-

Table I. *Amount of the Glycoprotein in Soybean Leaves at Various Stages of Development as Determined by Radial Immune Assay and Densitometer Scans of Coomassie Blue-Stained Gels.*

Time after Flowering	±Pods	Glycoprotein	
		Radial immune assay	Gel scan
weeks		*% soluble protein*	
0	+	15	6
1	+	15	6
3	+	7	3
3	−	29	22
5	+	1	1
5	−	47	45

cation from the leaf and girdling would stop it completely, it seems likely that the effect on translocation may be the signal responsible for initiating the changes in protein. Whether the effect on protein synthesis is mediated by a hormone is unknown at this time.

From the Coomassie blue-stained gels, it appears that the four major polypeptides accumulate to levels representing at least 50% of the soluble protein in leaves from depodded plants. Hence, for purification work, it was possible through depodding to greatly increase the quantity of these polypeptides in relation to other proteins. Using $(NH_4)_2SO_4$ fractionation, it was possible to further enrich the 27 and 29 kD polypeptides (Fig. 1, lane 8). These polypeptides were almost totally associated with the 2.0 to 2.8 M cut.

In the previous paper (12), it was suggested that these polypeptides may represent storage proteins, since their accumulation following depodding paralleled that of starch. Many seed storage proteins are glycosylated; therefore, a gel was stained with periodic acid-Schiff reagent to determine whether any of these polypeptides were glycosylated. Only two major bands were observed (Fig. 1, lane 7), and these corresponded to the 27 and 29 kD polypeptides.

Therefore, the next step in purification was to try using affinity chromatography. The desalted $(NH_4)_2SO_4$ fraction was applied to a Con A-Sepharose column, and bound protein was eluted with methyl glucopyranoside (Fig. 2). The fraction representing the bound protein was nearly 90% purified in the 27 and 29 kD polypeptides (Fig. 1, lane 9). When this protein was passed through a Sephacryl S 200 gel filtration column, there was only one major peak which eluted at a volume equivalent to a 47 kD protein. This peak was composed of the 27 and 29 kD polypeptides at greater than 90% purity (Fig. 1, lane 10). Hence, apparently these two polypeptides comprise a single protein. Why this protein elutes at a point equivalent to a 47 kD protein when it appears to be composed of a 27 and 29 kD polypeptide is unknown. However, glycoproteins containing substantial amounts of carbohydrate are known to act in an anomalous manner during both gel filtration and gel electrophoresis.

Antiserum to this purified protein was raised in mice. This antiserum gave one precipitation line with the purified protein and with a crude leaf extract in the Ouchterlony double diffusion assay (Fig. 3B), indicating only one antigen. To further check whether there was indeed only one major antigen, the purified protein was subjected to electrophoresis prior to the double diffusion assay. Again only one major line of precipitation was evident (Fig. 3A), further supporting the concept of a single glycoprotein composed of two polypeptides.

Inasmuch as soybean seeds contain glycoproteins (1, 6), it seemed likely that the leaf glycoprotein might be identical with one of the seed proteins. However, gel patterns did not show any bands corresponding to the 27 and 29 kD polypeptides (Fig. 4, lanes 1–4). In addition, no cross-reactivity was evident between seed protein from either early or mid pod-fill and antiserum to the leaf glycoprotein (Fig. 3B). This lack of cross-reactivity has been further verified using the more sensitive peroxidase-conjugated antibody reaction on proteins transferred from SDS gels to nitrocellulose paper. Again, there was no evidence of antigenicity between seed proteins from early or mid podfill and the leaf glycoprotein (Fig. 4, lanes 5–8). Figure 4 further demonstrates that the antiserum to the purified (>90%) glycoprotein is highly specific for the 27 and 29 kD polypeptides, since there was essentially no reaction product associated with the other polypeptide bands appearing in the Coomassie blue-stained gel.

The antiserum has also been used in a radial immune assay to quantitate the changes in the glycoprotein from flowering through late pod fill (Table I). The highest levels in leaves from podded plants occurred at flowering and 1 week after flowering. During seed growth, the level declined rapidly, and by 5 weeks after flowering, it comprised only about 1% of the total soluble protein. In depodded plants, the level of the glycoprotein continued to rise so that by 5 weeks after flowering (4 weeks after depodding) it comprised over 45% of the total soluble protein. In comparing these values with those calculated from densitometer scans of gels (Table I), there appeared to be a good correlation for the values from 3 and 5 weeks after flowering. However, the values from the radial immune assay were 2 to 3 times higher than the densitometer-derived values for samples taken at flowering and 1 week later. Whether this discrepancy is due to a change in Coomassie blue staining or antibody recognition sites of the protein with time after flowering is unknown; however, ultrastructural studies would suggest the latter. In addition, polypeptide profiles of leaves from podded plants indicate a larger amount of the 27 than the 29 kD polypeptide, especially near flowering. Thus, at least a portion of the polypeptides are not combined, and therefore, may react differently with the antiserum than the glycoprotein. In any case, it is evident that the level of the glycoprotein decreases during the period of rapid seed growth, suggesting it may be a storage protein associated with seed development.

In summary, there are four major polypeptides (27, 29, 54, and 80 kD) that accumulate following pod removal or petiole girdling. These polypeptides are products of protein synthesis, and their synthesis is enhanced following pod removal or girdling treatments. The 27 and 29 kD polypeptides are glycosylated and have been purified to greater than 90% by $(NH_4)_2SO_4$ fractionation, Con A affinity, and gel filtration chromatography. These peptides appear to comprise a single protein. Antiserum has been prepared against this protein and has been used to quantitate changes in the protein and to check for cross-reactivity with seed proteins. No cross-reactivity was found with seed proteins from early and midpod fill. Antibody quantitation and gel densitometer scans indicate a relatively high level of the glycoprotein in leaves of podded plants at and shortly following flowering with levels rapidly falling during seed growth. In depodded plants, levels continued to rise following pod removal with levels attaining values of 45% of the total soluble protein by 4 weeks after depodding.

In collaboration with V. Franceschi and R. Giaquinta (5), we have also used the antiserum to determine the localization of this protein in depodded soybean leaf tissue. Immunohistochemical staining, using the peroxidase-conjugated antibody reaction, was used to establish the localization. The results clearly showed that the glycoprotein was exclusively localized in the vacuoles of the paraveinal mesophyll and associated bundle sheath cells. Together, these results establish a unique role for the soybean leaf paraveinal mesophyll cells in the transport and compartmentation of a glycoprotein apparently functioning as a storage form of N for seed development.

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