

# Transport of Storage Proteins to Protein Storage Vacuoles Is Mediated by Large Precursor-Accumulating Vesicles

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**Novel vesicles that accumulate large amounts of proprotein precursors of storage proteins were purified from maturing pumpkin seeds. These vesicles were designated precursor-accumulating (PAC) vesicles and had diameters of 200 to 400 nm. They contained an electron-dense core of storage proteins surrounded by an electron-translucent layer, and some vesicles also contained small vesicle-like structures. Immunocytochemical analysis revealed numerous electron-dense aggregates of storage proteins within the endoplasmic reticulum. It is likely that these aggregates develop into the electron-dense cores of the PAC vesicles and then leave the endoplasmic reticulum. Immunocytochemical analysis also showed that complex glycans are associated with the peripheral region of PAC vesicles but not the electron-dense cores, indicating that Golgi-derived glycoproteins are incorporated into the PAC vesicles. These results suggest that the unique PAC vesicles might mediate a transport pathway for insoluble aggregates of storage proteins directly to protein storage vacuoles.**

## INTRODUCTION

Higher plants have two types of vacuoles. One type, protein storage vacuoles, develops mainly in storage organs, such as seeds, and the other type, lytic (vegetative) vacuoles, which contain various lytic enzymes, develops in the vegetative organs. Both types of vacuoles, however, are found in the same cells of barley roots (Okita and Rogers, 1996; Paris et al., 1996) and of maturing pea cotyledons (Robinson et al., 1995). In these cells, vacuolar proteins synthesized on the rough endoplasmic reticulum (RER) are sorted and delivered to their respective vacuoles. Thus, different targeting machineries for each type of vacuole must be involved in the protein transport in these cells.

Kirsch et al. (1994) demonstrated that the clathrin-coated vesicles contain a potential receptor for a precursor of barley aleurain, which is a thiol proteinase. This implicated these vesicles in the transport of the proteinase precursor to lytic vacuoles. On the other hand, clathrin-coated vesicles isolated from maturing pea cotyledons contain no seed protein precursors (Hohl et al., 1996). Thus, the clathrin-coated vesicles may mediate the transport of lytic vacuolar proteins but not the transport of seed proteins to protein storage vacuoles. This raises the question of how seed proteins are sorted and delivered to protein storage vacuoles in the cells of maturing seeds.

Most storage proteins, including globulins and some prolamins, have been shown to be transported to protein storage vacuoles via the Golgi apparatus (Chrispeels, 1985; Shotwell and Larkins, 1988). Chrispeels (1983) reported the presence of electron-dense vesicles close to the Golgi stacks in maturing bean cotyledons. Recently, Hohl et al. (1996) demonstrated immunocytochemically in maturing pea cotyledons that electron-dense vesicles with a diameter of ~100 nm associated with any *cis*, medial, and *trans* cisternae of the Golgi apparatus contain storage proteins. These results suggest that the storage proteins are transported to protein storage vacuoles via the Golgi-derived dense vesicles. However, a Golgi-independent pathway for storage proteins has also been reported. Levanony et al. (1992) reported that wheat prolamins are transported to the protein storage vacuoles without any contribution by the Golgi apparatus. However, the mechanism responsible for such Golgi-independent transport has not been characterized or determined.

Cell fractionation of pulse-chase—labeled maturing seeds of pumpkin and castor bean has demonstrated that both proglobulin (Hara-Nishimura et al., 1985; Fukasawa et al., 1988) and pro2S albumin (Hara-Nishimura et al., 1993a) are transported from the RER to the protein storage vacuoles via the vesicles having a density of 1.24 g/cm<sup>3</sup>. At the protein storage vacuoles, the precursors are converted to their mature forms (Hara-Nishimura and Nishimura, 1987). This

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suggests that the vesicles with a density of  $1.24 \text{ g/cm}^3$  mediate the transport of major storage proteins to protein storage vacuoles in maturing seeds of pumpkin and castor bean. However, the origin of these vesicles remains unknown. The ultrastructure of these vesicles, which includes an electron-dense core surrounded by electron-translucent structures, is distinct from the ultrastructures of the clathrin-coated vesicles (Robinson, 1996) and Golgi-derived vesicles (Hohl et al., 1996) in pea cotyledons.

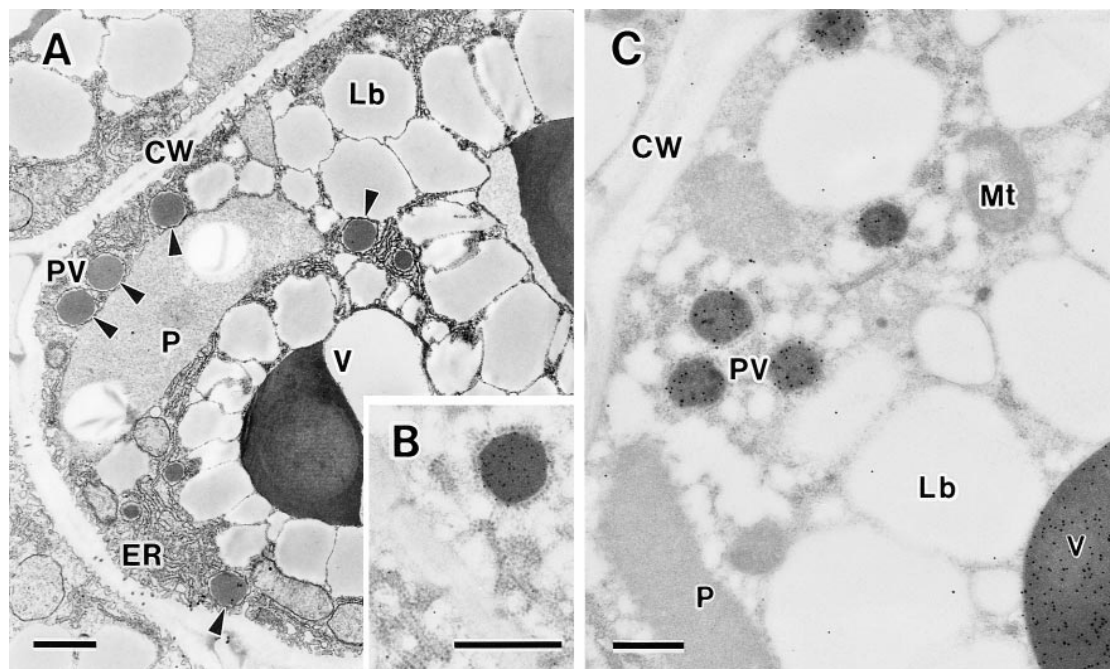
Immunoelectron microscopy showed that electron-dense aggregates of storage proteins are present within the RER. This suggests that the vesicles with a density of  $1.24 \text{ g/cm}^3$  might be derived directly from the protein aggregates in the RER. If this is true, then it is unclear how seed proteins with complex glycans, such as ricin of castor bean, are transported to the protein storage vacuoles, because complex glycans would not be expected to originate in the RER. In this study, we prepared biochemically and electron microscopically pure vesicles from maturing pumpkin seeds and characterized them to answer the question of how seed protein precursors are accumulated in the vesicles to be delivered to protein storage vacuoles. We show here a unique

transport pathway provided by the vesicles that we have designated as precursor-accumulating (PAC) vesicles.

## RESULTS

### Ultrastructural Analysis and Isolation of a Novel Transport Vesicle for Storage Proteins

Previously, we demonstrated that vesicles with a density of  $1.24 \text{ g/cm}^3$  mediate the transport of proprotein precursors of two major storage proteins, namely, proglobulin and pro2S albumin, from the RER to protein storage vacuoles in maturing seeds of pumpkin (Hara-Nishimura et al., 1985, 1993a) and castor bean (Fukasawa et al., 1988). To characterize these vesicles, we performed an ultrastructural analysis of pumpkin cotyledons at the middle stage of seed maturation. Electron microscopy of the cotyledons revealed numerous electron-dense vesicles  $\sim 300$  to  $400 \text{ nm}$  in diameter in the cells, as indicated by arrowheads in Figure 1A. These vesicles are much larger than the clathrin-coated vesicles



**Figure 1.** Electron Microscopy of Pumpkin Cotyledons at the Middle Stage of Seed Maturation.

(A) Numerous PAC vesicles (PV, arrowheads)  $\sim 300$  to  $400 \text{ nm}$  in diameter are visible in the cell. PAC vesicles contain an electron-dense core that is surrounded by an electron-translucent layer.

(B) An electron-dense vesicle surrounded by ribosomes can be seen close to the ER in the cell. Immunoelectron microscopy with 2S albumin-specific antibodies shows that the vesicle was also labeled with gold particles.

(C) Immunoelectron microscopy with 11S globulin-specific antibodies shows that gold particles are distributed on the electron-dense area of PAC vesicles (PV). Gold particles are also visible on a protein storage vacuole. The labeling density of the vesicles is similar to that of the protein storage vacuoles. CW, cell wall; ER, endoplasmic reticulum; Lb, lipid body; Mt, mitochondrion; P, plastid; V, protein storage vacuole. Bars in (A) to (C) =  $500 \text{ nm}$ .

(Robinson, 1996), with diameters of 50 to 70 nm, and Golgi-derived dense vesicles, with diameters of  $\sim 100$  nm (Hohl et al., 1996). The vesicles were frequently observed close to the well-developed RER, and some were surrounded by ribosomes in the cotyledon cells at the middle stage of seed maturation (Figure 1B).

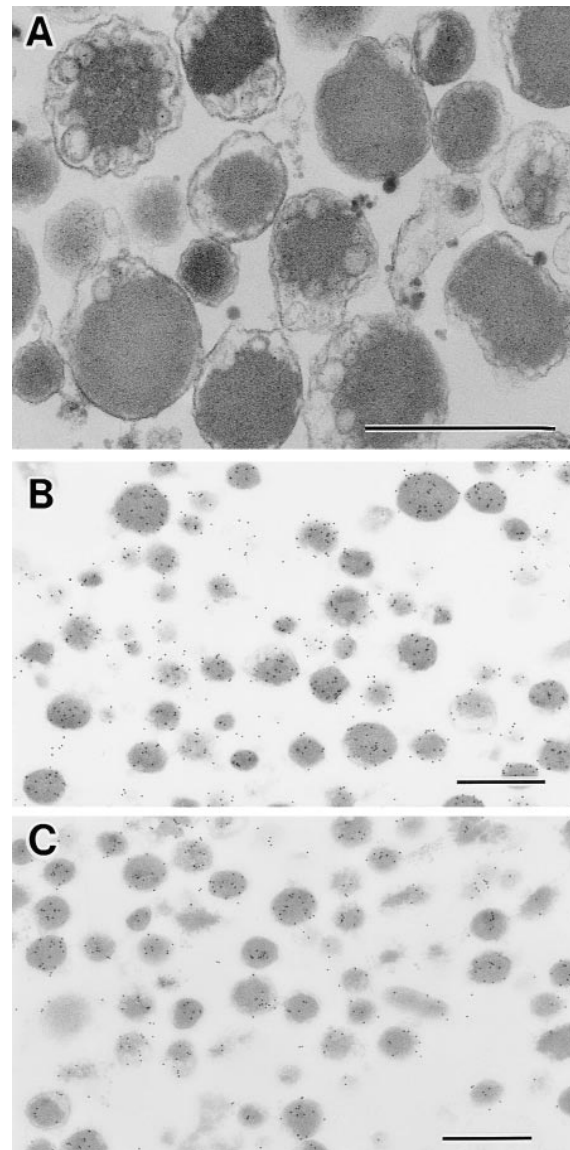
Electron microscopy after immunogold labeling with 11S globulin-specific antibodies revealed gold particles on the vesicles as well as on the protein storage vacuoles (Figure 1C), which contain large amounts of 11S globulin, the most abundant storage protein. Immunocytochemistry with 2S albumin-specific antibodies gave a similar result (Hara-Nishimura et al., 1993a). The gold particles were distributed uniformly on the electron-dense area of the vesicles, and the labeling density of the vesicles was similar to that of the protein storage vacuoles (Figure 1C).

To characterize the vesicles in greater detail, we isolated them from maturing pumpkin cotyledons by centrifugation on a self-generated Percoll gradient. The vesicles formed one band in the Percoll gradient. Further centrifugation of the isolated vesicles on a sucrose density gradient revealed a density of  $1.24 \text{ g/cm}^3$ . This indicated that the purified vesicles are identical to those found in the previous pulse-chase experiments with the maturing seeds of pumpkin (Hara-Nishimura et al., 1985, 1993a) and castor bean (Fukasawa et al., 1988).

Figure 2A shows electron microscopy of the isolated vesicles. Microscopy revealed that the vesicle fraction was not contaminated with any other organelles or any microsomal membranes. The vesicles had the same diameter as those observed in the cells. They had a characteristic structure, with an electron-dense core surrounded by an electron-translucent layer. Some vesicles contained small electron-translucent vesicle-like structures, and the content of the vesicle-like structures was observed to be higher in the vesicles of the younger cotyledons (data not shown). Immunoelectron microscopy of the isolated vesicles with specific antibodies raised against either 11S globulin or 2S albumin revealed that electron-dense cores of all the vesicles were labeled uniformly with gold particles, as shown in Figures 2B and 2C. This result indicates clearly that both 11S globulin and 2S albumin are localized in the electron-dense core of the vesicles.

### PAC Vesicles Are Packed with a Significant Amount of Proprotein Precursors of Storage Proteins

Our previous study showed that maturation of storage proteins occurs in protein storage vacuoles by the action of a vacuolar processing enzyme (Hara-Nishimura and Nishimura, 1987; Hara-Nishimura et al., 1993a). Therefore, if it is true that the vesicles with a density of  $1.24 \text{ g/cm}^3$  function as the transport vesicles for storage proteins to the protein storage vacuoles, then the vesicles should contain proprotein precursors of storage proteins but not mature proteins.



**Figure 2.** Ultrastructures of Isolated PAC Vesicles and Immunolocalization of Storage Proteins in the Vesicles.

(A) Highly purified PAC vesicles from pumpkin cotyledons at the middle stage of seed maturation after centrifugation on a self-generated Percoll gradient. PAC vesicles contain electron-dense cores that are surrounded by an electron-translucent layer and/or small electron-translucent vesicles. The PAC vesicle fraction was not contaminated with other cellular components.

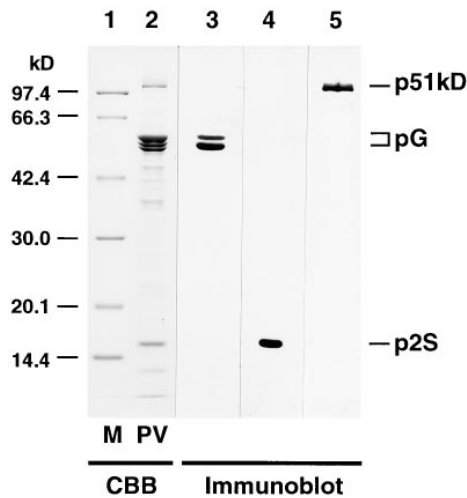
(B) Immunoelectron microscopy of isolated PAC vesicles with 11S globulin-specific antibodies. Electron-dense cores of all of the vesicles are labeled uniformly with gold particles.

(C) Immunoelectron microscopy of vesicles with 2S albumin-specific antibodies. Electron-dense cores of all the vesicles are uniformly labeled with gold particles.

Bars in (A) to (C) = 500 nm.

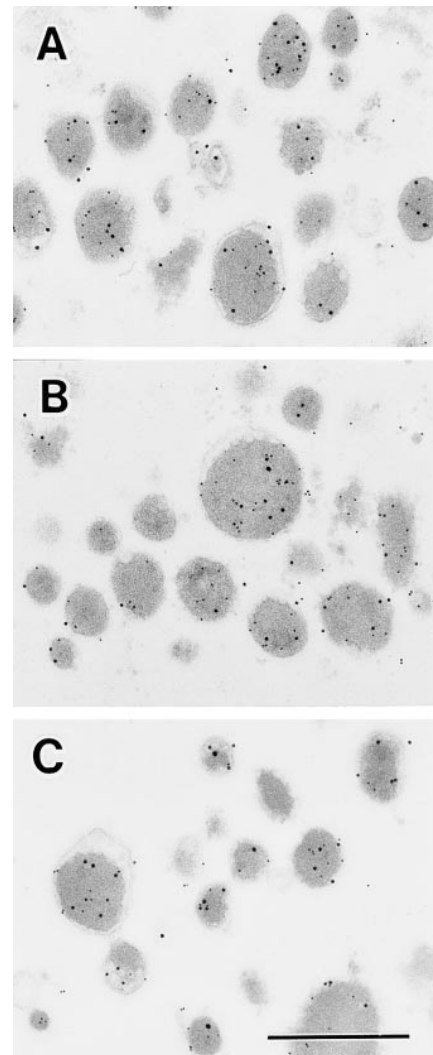
To examine the protein composition of the vesicles, we subjected the pure vesicle preparation to SDS-PAGE and then either to staining with Coomassie blue or to immunoblotting with specific antibodies against each of three major storage proteins. Figure 3 shows that the vesicles accumulated considerable amounts of three proteins that stained with Coomassie blue (lane 2). The immunoblot showed that these proteins corresponded to proprotein precursors of 11S globulin (lane 3), 2S albumin (lane 4), and the 51-kD protein (lane 5), respectively. No mature forms of these proteins were detected in the vesicles, indicating that the vesicle fraction was not contaminated by protein storage vacuoles. Thus, the vesicles accumulate proprotein precursors of all the major storage proteins and transport them to protein storage vacuoles. Accordingly, the vesicles were designated PAC vesicles.

The results indicate that the major storage proteins are transported to protein storage vacuoles via PAC vesicles. This raised the question of whether each PAC vesicle contains all three precursors that are to be transported or whether there are PAC vesicles that are specific to each storage protein. To answer these questions, we performed double immunogold labeling of the isolated vesicles by using protein A conjugated with gold particles 10 and 15 nm in



**Figure 3.** Accumulation of the Proprotein Precursors of All Major Storage Proteins of Pumpkin in PAC Vesicles.

Isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie blue (CBB; lanes 1 and 2) or immunoblot analysis (lanes 3 to 5). Lane 1 (M) contains molecular mass markers (labeled in kilodaltons at left); lane 2 (PV), isolated PAC vesicles; and lanes 3 to 5, immunoblots with antibodies raised against the specified protein. Lane 3 contains 11S globulin; lane 4, 2S albumin; and lane 5, the 51-kD protein. Labeling at right identifies the major protein components of PAC vesicles, which are all proprotein precursors to storage proteins: pro51-kD protein (p51kD), proglobulin (pG), and pro2S albumin (p2S).



**Figure 4.** Double Immunogold Labeling of the Isolated PAC Vesicles by Using Protein A Conjugated with Gold Particles of Different Sizes.

(A) Immunolocalization of proglobulin (15-nm gold) and pro2S albumin (10-nm gold).  
 (B) Immunolocalization of proglobulin (15-nm gold) and pro51-kD protein (10-nm gold).  
 (C) Immunolocalization of pro2S albumin (15-nm gold) and pro51-kD protein (10-nm gold).  
 Bar in (C) = 500 nm for (A) to (C).

diameter. Double immunogold labeling using specific antibodies raised against various pairs of storage proteins resulted in gold particles of both 10 and 15 nm in each PAC vesicle, as shown in Figures 4A to 4C. This finding strongly suggests that each PAC vesicle mediates the transport of all three major storage proteins of pumpkin.

### The Electron-Dense Core of a PAC Vesicle Originates from a Storage Protein Aggregate That Is Formed within the ER

The next issue to be resolved was the origin of the PAC vesicles. Are they derived from the ER or the Golgi apparatus? Most storage proteins are actively synthesized in the cells of pumpkin cotyledons at the middle stage of seed maturation (Hara-Nishimura et al., 1987; Inoue et al., 1995a). Ultrastructural analysis of the cells of maturing pumpkin cotyledons at this stage revealed that Golgi stacks are less in these cells, in contrast to the extensive development of the RER, as shown in Figures 1 and 5.

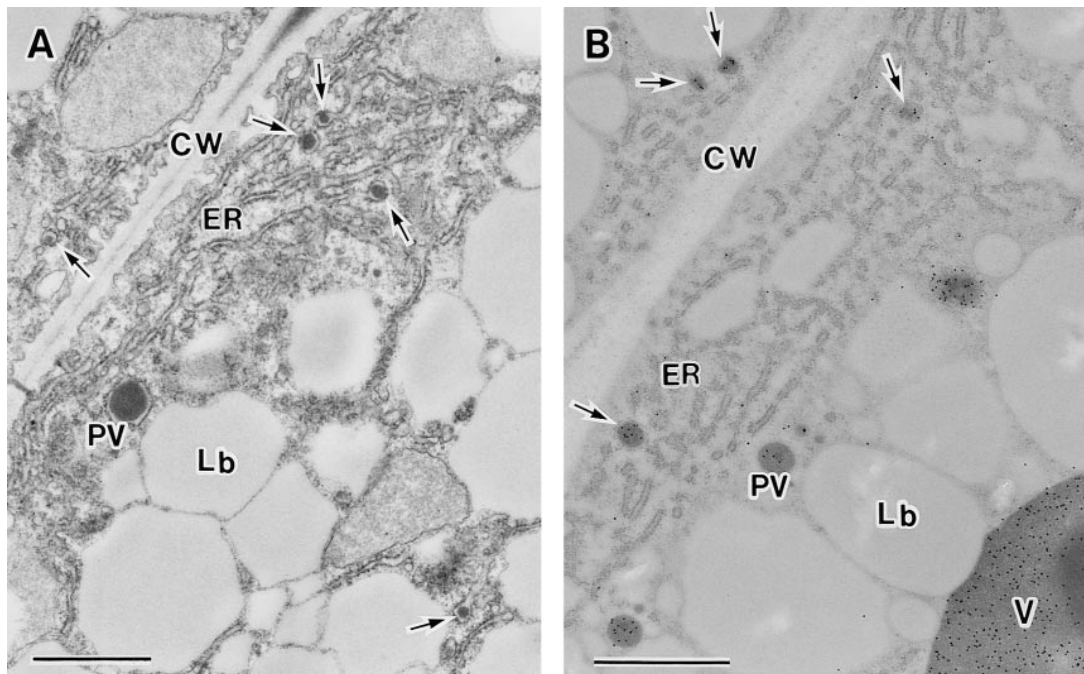
Electron microscopy of pumpkin cotyledons at the middle stage revealed numerous electron-dense aggregates within the RER, as indicated by arrows in Figure 5A. Immunocytochemical analysis with 2S albumin-specific antibodies demonstrated that the ER aggregates contained 2S albumin, as shown in Figure 5B. Similar immunogold labeling of the ER aggregates with 11S globulin-specific antibodies was observed (data not shown), indicating that these ER aggregates are composed of both pro2S albumin and proglubulin. This result indicates that the proprotein precursors,

which are synthesized and translocated into the luminal side of the ER, form core aggregates. The diameters of the ER aggregates ranged from 100 to 300 nm, suggesting that the aggregates might increase in size by incorporating newly synthesized storage proteins in the lumen of the RER.

Most aggregates were not surrounded directly by the ER membrane. There was an electron-translucent space between each aggregate and the ER membrane, as indicated by arrows in Figures 5A and 5B. The structure of the aggregates is analogous to that of the PAC vesicles, although the ER aggregates were smaller than the PAC vesicles. It seems possible that the aggregates might bud out of the ER to be transformed into PAC vesicles.

### Golgi-Derived Glycoproteins Are Localized in the Peripheral Region of PAC Vesicles

The next question is whether the PAC vesicles are involved in a transport of storage proteins with complex glycans to protein storage vacuoles. Such glycoproteins must be passed through the Golgi apparatus so that their oligosaccharide chains can be modified. However, the major storage



**Figure 5.** Electron Microscopy of Maturing Pumpkin Cotyledons Showing Numerous Electron-Dense Aggregates within the ER.

**(A)** Electron-dense core aggregates (arrows) formed within the ER in cells at the middle stage of seed maturation. The electron-translucent space between the aggregate and the ER membrane is loaded with ribosomes. PAC vesicles (PV) are also visible.

**(B)** Immunoelectron microscopy with 2S albumin-specific antibodies. The aggregates within the ER (arrows) are labeled with gold particles. PAC vesicles (PV) and a protein storage vacuole (V) are also visible.

CW, cell wall; Lb, lipid body. Bars in **(A)** and **(B)** = 500 nm.

proteins of pumpkin, namely, 11S globulin and 2S albumin, are not glycosylated. Recently, we isolated a cDNA of the third major storage protein of pumpkin, the 51-kD protein. The cDNA encodes the 100-kD precursor with no possible N-linked glycosylation site (data not shown). Thus, it is not necessary for these pumpkin storage proteins to pass through the Golgi apparatus.

In contrast to pumpkin seeds, castor bean seeds contain glycoproteins, ricin, and *Ricinus communis* agglutinin, with complex glycans. Previously, we reported that 11S globulin of the maturing endosperm of castor bean, like pumpkin storage proteins, was transported from the ER to protein storage vacuoles via the vesicles with a density of 1.24 g/cm<sup>3</sup> (Fukasawa et al., 1988). We also observed vesicles identical to pumpkin PAC vesicles in the endosperm cells of castor bean at the middle stage of seed maturation (Hiraiwa et al., 1993). Except for the glycoproteins, the compositions of soluble proteins and membrane proteins of castor bean protein storage vacuoles are similar to those of pumpkin (Inoue et al., 1995a, 1995b; Hiraiwa et al., 1997a). Therefore, we used the endosperm of maturing castor bean seeds to study the transport pathway of glycoproteins.

We prepared complex glycan-specific antibodies that recognized mainly ricin and *R. communis* agglutinin (data not shown) and used them for immunocytochemical analysis. Immunoelectron microscopy revealed the presence of gold particles on the peripheral region of PAC vesicles but not on the electron-dense area of PAC vesicles, as shown in Figures 6A and 6B. Gold particles were also distributed on the periphery of the protein deposits in a protein storage vacuole, a cell wall, and a Golgi stack. This result suggests that Golgi-derived glycoproteins might be incorporated into the mature PAC vesicles after the vesicles are released from the ER.

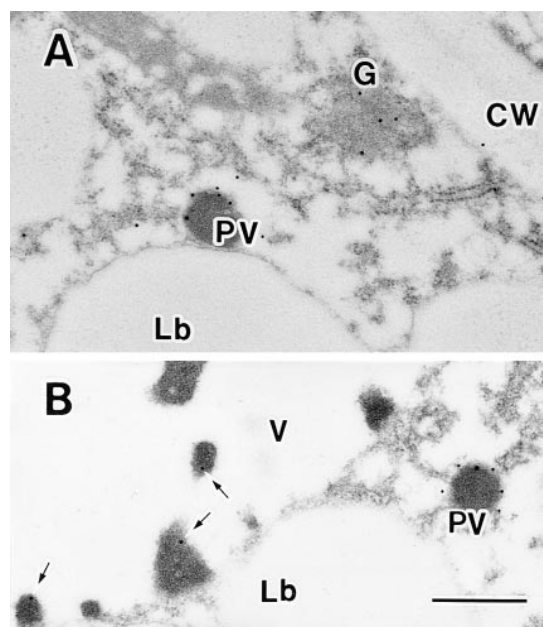
#### An ER-Resident Protein BiP Is Trapped in the ER Aggregates and Is Transported to Protein Storage Vacuoles via PAC Vesicles

To confirm that the storage protein cores of PAC vesicles are derived directly from the ER, we measured the content of BiP, an ER-resident molecular chaperone, in the vesicles. If BiP were detected in the vesicles, it would suggest that BiP is trapped in the protein aggregates of ER and then transported to the vesicles, as discussed by Levanony et al. (1992). To compare the BiP content in vesicles with that in the ER, the ER was prepared from maturing pumpkin cotyledons by sucrose density gradient centrifugation. The specific activity of NADH-cytochrome *c* reductase, an ER marker enzyme, was 104 milliunits per mg of protein in the ER preparation. In contrast, no activity was detected in the isolated PAC vesicles by using this assay, indicating that the specific activity in the vesicles was estimated to be <0.3 milliunits per mg of protein. Thus, the isolated vesicles were barely contaminated with the ER. The ER and PAC vesicles (15  $\mu$ g of protein each) were subjected to SDS-PAGE on a 5 to

20% gradient polyacrylamide gel with subsequent Coomassie blue staining. The protein composition of the PAC vesicles was different from that of the ER, as shown in Figure 7A (lanes 1 and 2).

To examine the presence of BiP in the PAC vesicles, ER (0.4  $\mu$ g of protein) and vesicles (40  $\mu$ g of protein) were subjected to SDS-PAGE and subsequent immunoblotting with BiP-specific antibodies. The intensity of the BiP signal in the PAC vesicles was equal to that in the ER, as shown in Figure 7A (lanes 4 and 5). BiP content in the PAC vesicles, on the basis of the amount of protein, was  $\sim$ 1% of that in the ER. This result indicates that BiP is present at a significant level in the vesicles. This is supported by the results of the immunogold labeling analysis of vesicles with BiP-specific antibodies, as shown in Figure 7B. These results support the possibility that the electron-dense cores of the PAC vesicles might have been derived directly from the aggregates in the ER.

BiP found in PAC vesicles could be transported to protein storage vacuoles together with proprotein precursors of storage proteins. Further analysis was performed to deter-



**Figure 6.** Immunoelectron Microscopy of Castor Bean Endosperm at the Middle Stage of Seed Maturation with Complex Glycan-Specific Antibodies.

(A) Gold particles are visible at the peripheral region of the PAC vesicles (PV) but not inside the area of the PAC vesicles. A Golgi stack (G) and cell wall (CW) are also labeled with gold particles.

(B) Gold particles are also visible on the peripheral region of PAC vesicles (PV) as well as on the peripheral region of storage protein deposits in a protein storage vacuole (V, arrows).

Lb, lipid body. Bar in (B) = 500 nm for (A) and (B).

mine how much BiP accumulated in protein bodies of dry seeds. Protein bodies have been shown to be directly converted from protein storage vacuoles at the late stage of seed maturation (Hara-Nishimura et al., 1987). We isolated protein bodies from dry pumpkin seeds by using glycerol, as described previously (Hara-Nishimura et al., 1982). The specific activity of NADH-cytochrome *c* reductase in the whole homogenate of the tissues was 0.29 milliunits per mg of protein, whereas the activity in the protein bodies was only 0.0028 milliunits per mg of protein. Thus, contamination of the protein bodies with the ER was estimated to be <1%.

Each 200  $\mu$ g of protein of the whole homogenate and the protein bodies was subjected to SDS-PAGE and then to staining with Coomassie blue or immunoblot analysis with BiP-specific antibodies. Approximately 20% of the total amount of BiP in the whole homogenate was detected in the protein bodies, as shown in Figure 8A (lanes 5 and 6). A significant amount of BiP accumulated in the protein bodies. To examine the suborganellar localization of BiP, the protein bodies were separated further into a soluble fraction and an insoluble crystalloid that was composed of 11S globulin. Figure 8A shows that most of the BiP was located in the soluble matrix (lane 7) of the protein bodies but not in the crystalloid (lane 8). Immunocytochemical labeling of maturing pumpkin cotyledons with BiP-specific antibodies resulted in labeling of protein storage vacuoles with gold particles, as shown in Figures 8B and 8C. This result supports the proposed association of BiP with the protein storage vacuoles. Taken together, these results suggest that BiP is trapped in the ER aggregates and is transported to protein storage vacuoles via PAC vesicles in the cells of maturing pumpkin cotyledons.

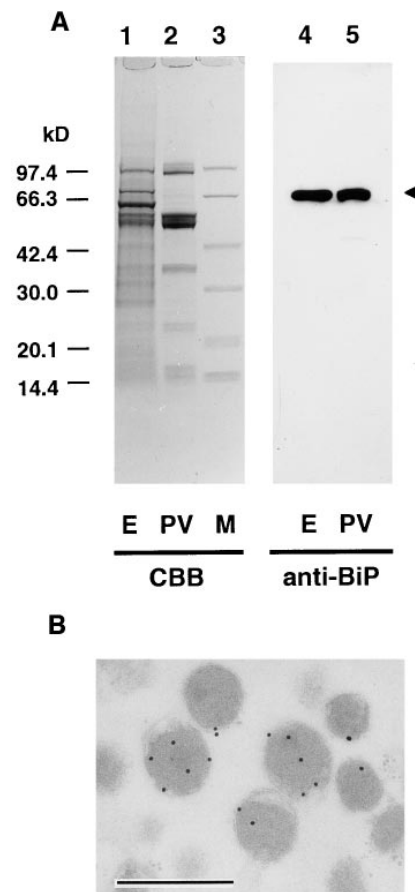
## DISCUSSION

### Formation of Core Aggregates of Storage Protein Precursors within the ER

Proprotein precursors of storage proteins are synthesized on the RER and translocated into the luminal side of the RER. There, they might form core aggregates, similar to those shown in Figures 5A and 5B. Active synthesis of storage proteins might facilitate formation of protein aggregates within the ER. We previously demonstrated that trimeric oxidized proglobulin molecules are produced within the ER (Hara-Nishimura et al., 1985; Hara-Nishimura, 1987) and that the increased accumulation of storage proteins parallels the increase in the amount of BiP during seed maturation of pumpkin (Hatano et al., 1997). It seems likely that BiP might play some role in the formation of the aggregates in the ER of pumpkin cotyledons. Li et al. (1993) showed that BiP retains prolamins, alcohol-soluble storage proteins, in the lumen of the ER by facilitating their folding and assembly into protein bodies of rice. In the case of rice (Okita and Rogers, 1996) and maize (Larkins and Hurkman, 1978), prolamins ag-

gregates that are formed within the ER are converted directly to protein bodies but are not transported to protein storage vacuoles.

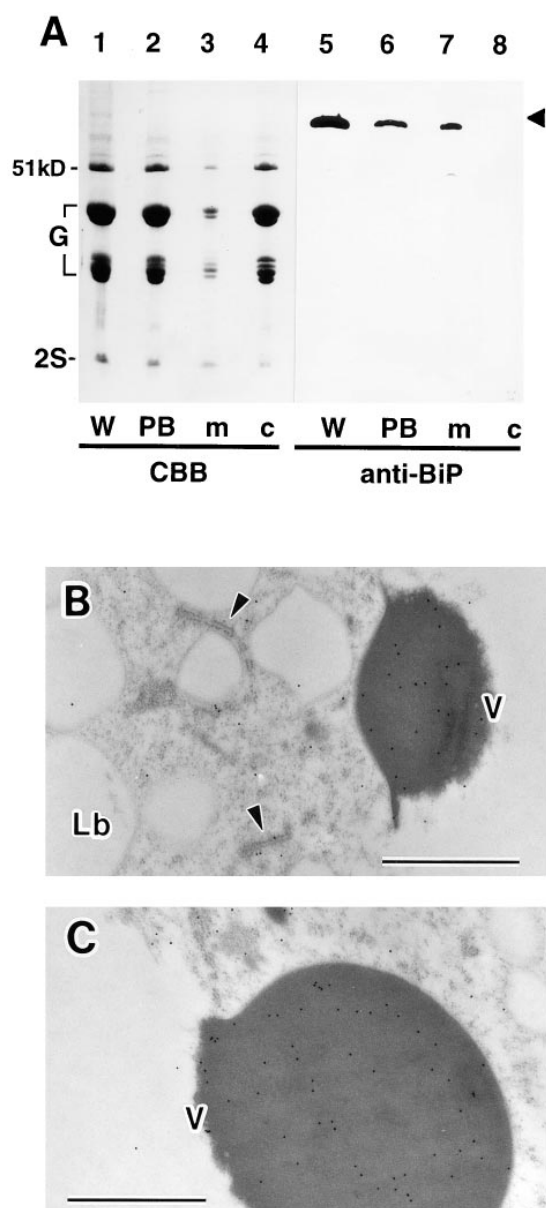
The electron-dense aggregate structures surrounded by a ribosome-studded membrane also were observed in maturing pea cotyledons (Craig, 1988). The ER-derived aggregates of pea should be transported to the protein storage vacuoles. However, the mechanism of transport is unknown.



**Figure 7.** Association of an ER-Resident Molecular Chaperone, BiP, with PAC Vesicles.

**(A)** ER and PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie blue (CBB; lanes 1 and 2) or immunoblot analysis with pumpkin BiP-specific antibodies (anti-BiP; lanes 4 and 5). Lane 1 (E) contains the ER protein (15  $\mu$ g of protein); lane 2 (PV), PAC vesicles (15  $\mu$ g of protein); lane 3 (M), molecular mass markers (labeled in kilodaltons at left); lane 4 (E), ER protein (0.4  $\mu$ g of protein); and lane 5 (PV), PAC vesicles (40  $\mu$ g of protein). Arrowhead indicates BiP.

**(B)** Immunogold labeling of isolated PAC vesicles with BiP-specific antibodies shows localization of BiP in PAC vesicles. ER and PAC vesicles were prepared from pumpkin cotyledons at the middle stage of seed maturation. Bar = 500 nm.



**Figure 8.** Association of BiP with Both Protein Bodies from Dry Pumpkin Seeds and Protein Storage Vacuoles in Maturing Pumpkin Seeds.

**(A)** Protein bodies and the suborganellar fractions were subjected to SDS-PAGE and subsequent staining with Coomassie blue (CBB; lanes 1 to 4) or immunoblot analysis with pumpkin BiP-specific antibodies (anti-BiP; lanes 5 to 8). Lane 1 (W) contains whole protein (0.2 mg of protein) of dry seeds; lane 2 (PB), whole protein (0.2 mg of protein) of protein bodies; and lanes 3 (m) and 4 (c), suborganellar fractions of matrix and crystalloids of 11S globulin, respectively, prepared from protein bodies (0.2 mg of protein) by centrifugation. Lanes 5 to 8 show the results of the immunoblot analysis with BiP-specific antibodies (anti-BiP) of the same fractions as given for lanes 1 to 4. The arrowhead indicates BiP. G indicates 11S globulin subunits; 2S, 2S albumin; and 51kD, the 51-kD protein.

Levanony et al. (1992) reported that the wheat prolamin aggregates forming in the ER might be transported to the vacuoles, bypassing the Golgi apparatus, via protein bodies that are surrounded by electron-translucent vesicles. These protein bodies could be structurally analogous to the PAC vesicles in pumpkin, even though the protein components are different in each case: the wheat aggregates are composed of insoluble prolamin, and the pumpkin aggregates are composed of proglubulin and pro2S albumin. It seems likely that the PAC vesicles are targeted to protein storage vacuoles by a mechanism similar to that for the wheat protein bodies. These results suggest that plant cells might have a common machinery for the delivery of protein aggregates produced within the ER to the vacuoles.

#### PAC Vesicle-Mediated Transport of Storage Proteins to Protein Storage Vacuoles

Robinson and Hinz (1996) reported that a type of pea protein body, PB<sub>3</sub>, has a density of 1.19 to 1.22 g/cm<sup>3</sup> and might contain a proprotein precursor of legumin. They also reported that PB<sub>3</sub> is formed at the late stage of pea seed maturation and is derived from the cisterna, whose origin is unclear. These characteristics of pea PB<sub>3</sub> are similar to those of PAC vesicles. PAC vesicles, like pea PB<sub>3</sub>, are observed more frequently in the cotyledon cells at the later stage of pumpkin seed maturation (data not shown). The increase in the number of PAC vesicles might be correlated with the disappearance of Golgi stacks in the cell from the middle to late stages of seed maturation.

PAC vesicle-mediated transport of aggregates implies the existence of a Golgi-independent pathway for storage proteins in maturing pumpkin cotyledons. This is supported by our previous results with maturing pumpkin cotyledons treated with the carboxylic ionophore monensin (Hayashi et al., 1988). Monensin has been reported to inhibit Golgi-dependent transport of  $\alpha$ -amylases in the rice scutellum cells (Mitsui et al., 1985) and in the barley aleurone cells (Heupke and Robinson, 1985). However, monensin did not inhibit the transport of proglubulin into protein storage vacuoles at the middle stage of pumpkin seed maturation (Hayashi et al., 1988). This result indicates that at this stage, proglubulin synthesized on the RER is transported to the vacuoles, bypassing the Golgi apparatus. The majority of storage proteins in pumpkin are not glycosylated and need not pass through the Golgi apparatus. A pathway bypassing the Golgi apparatus is presumed to have evolved for the efficient

**(B)** and **(C)** Immunoelectron microscopy of pumpkin cotyledons at the middle stage of seed maturation with anti-BiP antibodies shows localization of BiP together with storage proteins in protein storage vacuoles (V). Lb, lipid body; arrowheads, endoplasmic reticulum. Bars = 1  $\mu$ m.



transport of unglycosylated proteins in maturing seeds that synthesize a large amount of storage proteins. On the other hand, glycoproteins of castor bean seeds that pass through the Golgi apparatus can be incorporated into the mature PAC vesicles and then transported to protein storage vacuoles together with the other storage proteins (Figure 6). Further analysis of the sorting of storage proteins with or without complex glycans will give us a detailed mechanism of the vacuolar targeting machineries in maturing seeds.

### Multiple Transport Pathways for Vacuolar Proteins to Protein Storage Vacuoles

Elucidation of these machineries for the targeting of proteins to protein storage vacuoles requires biochemical analyses of isolated vesicles. Recently, we found two homologous proteins, PV72 and PV82, in the membrane fraction of isolated PAC vesicles and showed that PV72 and PV82 bind to peptides derived from pumpkin pro2S albumin (Shimada et al., 1997). Both PV72 and PV82 are type I integral membrane proteins with epidermal growth factor-like motifs and homologs of a potential vacuolar targeting receptor in pea, BP-80 (Paris et al., 1997), and an epidermal growth factor receptor-like protein in Arabidopsis, AtELP (Ahmed et al., 1997). Further investigations of the PV72 and PV82 proteins should help us to clarify the molecular mechanisms responsible for sorting within the cisternae of ER and/or Golgi apparatus.

Most proprotein precursors of storage proteins are converted into their respective mature forms by the action of the vacuolar processing enzyme after arrival at protein storage vacuoles (Hara-Nishimura et al., 1993a, 1995). We have shown that protein storage vacuoles contain the vacuolar processing enzyme (Hara-Nishimura et al., 1993b, 1995; Shimada et al., 1994a) as well as an aspartic proteinase (Hiraiwa et al., 1997a). This raises the question of how such hydrolytic enzymes are incorporated into protein storage vacuoles. We previously reported that the precursor of the vacuolar processing enzyme is localized in PAC vesicles in maturing castor bean endosperm (Hiraiwa et al., 1993) and is converted self-catalytically to an active enzyme after incorporation into the vacuoles (Hiraiwa et al., 1997b). However, we could not detect the aspartic proteinase in PAC vesicles. To clarify the multiple transport pathway to protein storage vacuoles, the transport mechanism of these hydrolytic enzymes to the vacuoles should be established.

### Targeting of PAC Vesicles to Protein Storage Vacuoles

The final step in the transport of a storage protein is the incorporation of the proprotein into a protein storage vacuole. There are two possible modes of incorporation: one is autophagy by the vacuoles, and the other is membrane fusion between PAC vesicles and the vacuoles. Autophagic engulfment by protein storage vacuoles has been observed in ma-

turing seeds of pumpkin (data not shown) and pea (Robinson et al., 1995) and in seedlings of mung bean (Van der Wilden et al., 1980). Autophagy should be accompanied by the incorporation and breakdown of membrane proteins of protein storage vacuoles (Maeshima et al., 1994; Inoue et al., 1995a; Strzalka et al., 1995). If such autophagy were to mediate the incorporation of PAC vesicles into the protein storage vacuoles, some of the tonoplast should remain inside the vacuoles. Detailed immunocytochemical studies about the tonoplast should answer the question of whether autophagic engulfment is involved in the incorporation of storage proteins into the protein storage vacuoles.

Alternatively, the incorporation of PAC vesicles into protein storage vacuoles could occur by membrane fusion. In yeast, distinct members of the Ras superfamily of small GTP binding proteins (Pryer et al., 1992) and members of the syntaxin family (Becherer et al., 1996) facilitate the targeting of vesicles to the appropriate membranes. We reported previously that two small GTP binding proteins are associated with the membrane of PAC vesicles (Shimada et al., 1994b). Recently, two syntaxin homologs have been shown to be localized in specific compartments in Arabidopsis: AtPEP12 is localized on a post-Golgi compartment (da Silva Conceição et al., 1997), and aVAM3 is localized on the contact sites between small vacuoles and large vacuoles (Sato et al., 1997). Thus, machinery analogous to that described in yeast could mediate the fusion of PAC vesicles with protein storage vacuoles. Further analysis is required to elucidate the involvement of members of these families in the transport of the storage proteins to protein storage vacuoles.

## METHODS

### Plant Materials

Pumpkin (*Cucurbita* sp cv Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubuyo Seed Co. (Nagoya, Japan). The seeds were used directly for the isolation of protein bodies (protein storage vacuoles in dry seeds) and also were planted in the field during the summers of 1995 and 1996. The cotyledons of maturing seeds, freshly harvested 22 to 28 days after anthesis, were used for some of the experiments.

Castor bean (*Ricinus communis*) seeds were grown in a greenhouse during the summer of 1996. The maturing seeds were freshly harvested 25 to 30 days after anthesis, and the endosperm was used.

### Isolation of Precursor-Accumulating Vesicles from Maturing Pumpkin Cotyledons

Precursor-accumulating (PAC) vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation by a method described previously (Hara-Nishimura et al., 1991; Shimada et al., 1994b). The cotyledons (10 to 20 g) were homogenized in a solution (7 mL/g fresh weight of cotyledons) of 20 mM sodium pyrophosphate, pH 7.5, 1 mM EDTA, and 0.3 M mannitol with an ice-chilled

mortar and pestle, and the homogenate was filtered through cheese-cloth. The filtrate was centrifuged at 3000g for 15 min, and the supernatant was centrifuged again at 8000g for 20 min at 4°C. The resulting supernatant was used to isolate microsomes, as described below. The pellet was suspended in 1 mL of 10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, and 0.3 M mannitol. The suspension was layered on a solution of 28% Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden) in 10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, and 0.3 M mannitol on a cushion of 2 mL of 90% Percoll. Centrifugation was at 40,000g for 30 min at 4°C. The vesicle fraction was centrifuged again in a self-generated Percoll gradient. The resulting vesicle fraction was washed in the above-described Hepes-KOH buffer. The isolated PAC vesicles were subjected to either immunoelectron microscopy or immunoblot analysis.

#### Isolation of Microsomes from Maturing Pumpkin Cotyledons

Microsomes were also isolated from pumpkin cotyledons at the middle stage of seed maturation. An extract of cotyledons, prepared as described above, was centrifuged at 8000g and then at 100,000g for 30 min at 4°C. The pellet was suspended in 2 mL of 0.15 M Tricine-KOH, pH 6.8, 1 mM EDTA, and 13% (w/w) sucrose, and the suspension was layered on a step gradient of sucrose, which consisted of 50% (w/w) sucrose, 35% (w/w) sucrose, and 20% (w/w) sucrose. Centrifugation was at 21,000 rpm for 1 hr at 4°C in an SW 28 rotor (Beckman Instruments Ltd., Tokyo, Japan). Most microsomes formed a band between 20 and 35% (w/w) sucrose. The microsomal fraction was collected and diluted with the above-described Tricine-KOH buffer, and centrifugation was at 150,000g for 30 min at 4°C. The isolated microsomes were subjected to SDS-PAGE and then to an immunoblot analysis.

#### Isolation of Protein Bodies and Suborganelle Fractionation

Protein bodies were prepared from 5 g of dry pumpkin seeds by using a nonaqueous isolation method with 100% glycerol, as described previously (Hara-Nishimura et al., 1982). Both light microscopic examination and assays of marker enzymes indicated that the protein bodies were intact and were not contaminated with other cell organelles or cytoplasmic components. The protein bodies were lysed in 1 mL of a hypotonic buffer solution of 10 mM Tris-HCl, pH 7.5, and the lysate was centrifuged at 100,000g for 20 min at 4°C to separate it into a soluble matrix fraction and an insoluble crystalloid that was composed of the major seed protein 11S globulin. Aliquots of both the matrix and crystalloid fractions that had been prepared from protein bodies were equivalent to 200 µg of protein and were subjected to SDS-PAGE and then to immunoblot analysis with BiP-specific antibodies.

#### Preparation of Specific Antisera

The 51-kD protein is one of the major proteins in pumpkin seeds. To prepare polyclonal antibodies, we purified the 51-kD protein from the protein bodies that had been isolated as described above. Isolated protein bodies were burst in 10 mM Tris-Mes, pH 6.5, sonicated, and then centrifuged at 250g for 15 min to remove insoluble crystalloids as a pellet. A solution of NaCl was added to the supernatant to a final concentration of 1 M. During centrifugation at 100,000g for 1 hr, the 51-kD protein was sedimented. The pellet was subjected to SDS-

PAGE with subsequent staining with Coomassie Brilliant Blue R 250. The band corresponding to the 51-kD protein was cut out from the gel and injected subcutaneously with complete Freund's adjuvant (Freund, 1951) into a rabbit. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. One week after the second booster injection, blood was drawn and the antiserum was prepared.

Complex glycan-specific antibodies were purified from polyclonal antibodies raised against  $\beta$ -xylosidase of sycamore (*Acer pseudo-platanus*) that had been raised previously (Tezuka et al., 1993). The  $\beta$ -xylosidase contains xylose-containing N-linked oligosaccharides (Tezuka et al., 1993). To separate complex glycan-specific antibodies, we applied 5 mL of  $\beta$ -xylosidase-specific antiserum to an affinity column of horseradish peroxidase-conjugated Sepharose 4B (Pharmacia LKB Biotechnology). After thoroughly washing the column, complex glycan-specific antibodies were eluted with glycine-HCl, pH 2.0. The affinity-purified antibodies recognized ricin, a major glycoprotein of castor bean (data not shown).

Previously prepared specific polyclonal antibodies specific to BiP (Hatano et al., 1997), 11S globulin (Hara and Matsubara, 1980), or pro2S albumin (Hara-Nishimura et al., 1993a) were also used for the experiments.

#### SDS-PAGE and Immunoblot Analysis

Both the PAC vesicles and the microsomes were subjected to SDS-PAGE on either a precast gradient gel (5 to 20% polyacrylamide; Atto Corporation, Tokyo, Japan) or a 12.5% polyacrylamide gel, and the protein body fractions were subjected to SDS-PAGE on a 12.5% polyacrylamide gel. Immunoblot analysis was performed essentially as described previously (Inoue et al., 1995a). The separated proteins on gels were stained with Coomassie blue or were transferred electrophoretically to a GVHP membrane (0.22 µm; Nihon Millipore Ltd., Tokyo, Japan). The membrane blot was incubated with antibodies for 1 hr. Dilutions of specific antisera were as follows: anti-11S globulin (1:2000), anti-2S albumin (1:2000), anti-51-kD protein (1:500), and anti-BiP (1:2000 for the experimental results shown in Figure 7 and 1:5000 for those shown in Figure 8). Horseradish peroxidase-conjugated antibodies raised in goat against rabbit IgG (Cappel, West Chester, PA) were diluted 10,000-fold and used as second antibodies. Immunodetection was performed with an enhanced chemiluminescence kit (Amersham Japan, Tokyo), according to the manufacturer's directions.

#### Ultrastructural Analysis and Immunogold Labeling

The pumpkin cotyledons and the castor bean endosperm at the middle stage of seed maturation were vacuum infiltrated for 1 hr with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer, pH 7.4. The tissues were then cut into slices <1 mm thick with a razor blade and treated for another 2 hr with freshly prepared fixative. The isolated PAC vesicles were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 0.3 M mannitol, 1 mM EDTA, and 10 mM Hepes-KOH, pH 7.2, at 4°C for 1 hr. Procedures for ultrastructural studies were essentially the same as those described previously (Hara-Nishimura et al., 1993a), except that the material was postfixated with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 2 hr.

Immunogold labeling procedures were essentially the same as those described previously (Nishimura et al., 1993), except for the

use of the appropriate antibodies raised against 11S globulin, 2S albumin, the 51-kD protein, BiP, and complex glycans. Postfixation was omitted for immunoelectron microscopy. The samples were dehydrated in a graded dimethylformamide series at  $-20^{\circ}\text{C}$  and embedded in London Resin White (London Resin Co. Ltd., Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp at  $-20^{\circ}\text{C}$  for 24 hr. Ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome (Leica, Heidelberg, Germany) and mounted on uncoated nickel grids. The sections were treated with blocking solution (1% BSA in PBS) for 1 hr at room temperature and then incubated overnight with antibodies raised against 11S globulin (diluted 1:5000), 2S albumin (1:2500), the 51-kD protein (1:250), BiP (1:100), and complex glycans (1:50) in blocking solution at  $4^{\circ}\text{C}$ . After washing with PBS, sections were incubated for 30 min at room temperature with a solution of protein A-gold (10 or 15 nm; Amersham Japan) that had been diluted 1:30 in the blocking solution. The sections were washed with distilled water and then were stained with 4% uranyl acetate and lead citrate. After staining, all sections were examined with a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan) at 80 kV.

#### Assay of a Marker Enzyme and Quantitation of Protein

The activity of NADH-cytochrome *c* reductase was measured as described previously (Hara-Nishimura et al., 1985). The reaction mixture consisted of 0.5 mL of 25 mM Hepes-KOH, pH 8.5, 0.05 mM NADH, 1 mM antimycin, 2.5 mM  $\text{NaN}_3$ , 0.05% bovine cytochrome *c*, and the sample solution. The reaction was started by the addition of NADH; the increase in absorbance at 550 nm was then measured. The NADH-independent increase in absorbance at 550 nm was measured as a control. The concentration of protein was determined by using a protein assay kit (Bio-Rad) with  $\gamma$ -globulin as the standard.

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