Journal of Cell Science 109, 2539-2550 (1996)
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JCS4255

Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat

Inge Hohl[1], David G. Robinson[1], Maarten J. Chrispeels[2] and Giselbert Hinz[1,*]

[1]Pflanzenphysiologisches Institut, Universität Göttingen, Untere Kurspüle 2, D-37073 Göttingen, Germany
[2]Department of Biology, University of California, San Diego, CA 92093-0116, USA

*Author for correspondence

SUMMARY

Storage parenchyma cells of developing legume cotyledons actively transport large amounts of storage proteins to protein storage vacuoles (PSV). These proteins are synthesized on the endoplasmic reticulum and pass through the Golgi apparatus. Clathrin coated vesicles (CCV) and small electron dense vesicles found near the *trans*-Golgi network (TGN) have both been implicated in the Golgi-to-vacuole transport step. Recent findings that protein storage cells contain more than one type of vacuole have necessitated a re-examination of the role of both types of vesicles in vacuolar protein transport.

Immunoblots of highly purified CCV preparations and immunogold labelling with antibodies to the storage proteins vicilin and legumin, indicate that the dense vesicles, but not the CCV, are involved in storage protein transport in pea cotyledons. This result is supported by the finding that α-TIP, a protein characteristic of the PSV membrane, is absent from CCV. In addition, complex glycoproteins appear to be carried by CCV but are not detectable in the PSV. We suggest on the basis of these data that storage proteins and other vacuolar proteins such as acid hydrolases are not sorted by the same mechanism and are transported by different types of vesicles to different types of vacuoles.

Key words: α-TIP, Clathrin coated vesicle, Complex glycan, Pea cotyledon, Protein storage vacuole, Storage polypeptide

INTRODUCTION

The secretory system of eukaryotic cells is composed of a number of distinct membrane-bound compartments including the endoplasmic reticulum (ER), the Golgi complex and the vacuole. Transport of proteins between these compartments and from the plasma membrane is mediated by vesicles that have protein coats (Rothman, 1994). The coat proteins are part of the machinery that facilitates the fission, targeting, docking and fusion of the vesicles from the leaving compartment and with the destination compartment (Schekman and Orci, 1996). Two types of vesicles have been characterized in some detail: clathrin coated vesicles (CCV) and COP vesicles (Schmid and Damke, 1995). The COP vesicles transport proteins between the ER and the Golgi and between different Golgi cisternae. CCV are involved in the endocytosis of ligands that bind to the plasma membrane and the delivery of these ligands to early endosomes (Robinson, 1994). In mammalian cells, CCV also bud from the *trans*-Golgi network (TGN) and package newly synthesized lysosomal hydrolases bound to mannose-6-phosphate (M6P) receptors for delivery to endosomes and thence to lysosomes (Kornfeld and Mellman, 1989). Acid hydrolases that escape from this normal delivery route and become secreted can be delivered to lysosomes when they bind to M6P receptors at the plasma membrane. After internalization in CCV these receptors are then cycled back to the Golgi via the endosomes.

There is very little information about the nature of the vesicles that transport proteins between compartments of the endomembrane system in plant cells (Bassham and Raikhel, 1996). Whereas COP vesicles remain to be identified, CCV are known to be formed both at the plasma membrane (Robinson and Hillmer, 1990) and at the Golgi apparatus (Griffing, 1991). An endosome-like structure, the multivesicular body has also been described in plants (Record and Griffing, 1988). In contrast to mammals plant cells do not contain M6P receptors, and vacuolar targeting information is not contained in phosphorylated glycans but rather in peptide domains (Chrispeels and Raikhel, 1992; Neuhaus, 1996). The identification and characterization of the various components of the targeting machinery are of very topical interest in plant cell biology (Bassham and Raikhel, 1996).

Developing legume cotyledons are an excellent system to study the delivery of proteins to the vacuole because a large proportion of the cellular protein traverses the secretory system and accumulates in protein storage vacuoles (PSV; Chrispeels, 1991; Okita and Rogers, 1996). The PSV are formed de novo during seed development (Hoh et al., 1995) and have a limiting membrane (tonoplast) with a unique aquaporin, α-TIP (Johnson et al., 1989) that is not present in the tonoplast of other vacuoles. Globulins, lectins, enzyme inhibitors and processing proteases accumulate in the PSV. Pea cotyledons contain two major types of storage proteins, vicilin and legumin, each encoded by a number of genes (Lycett et al.,

1983, 1984). These are synthesized as propolypeptides of 75 and 60 kDa (legumin) and 75, 70, 50 and 48 kDa (vicilin; Chrispeels et al., 1982), whose proteolytic processing occurs in the PSV (Chrispeels et al., 1982; for a review see Müntz, 1996). In previous experiments these propolypeptides were found to be associated with CCV-enriched fractions (Robinson et al., 1989; Harley and Beevers, 1989; Hoh et al., 1991). Moreover, a putative vacuolar transport receptor has been isolated from CCV-enriched fractions prepared from pea cotyledons. This 80 kDa protein binds specifically to the N-terminal vacuolar targeting domains of barley aleurain and sweet potato sporamin (Kirsch et al., 1994; Paris and Rogers, 1996).

The above results are very suggestive of a role for CCV in TGN vacuole transport. However, in earlier pulse-labelling experiments carried out with developing bean cotyledons, a fraction rich in dense vesicles labelled after the Golgi fraction and the radioactivity could be subsequently chased to the vacuole fraction (Chrispeels, 1983). Dense vesicles at the *trans*-face of the Golgi have been described in pea cotyledons (e.g. Craig and Goodchild, 1984; Craig, 1988; Harris et al., 1989) and in other legume cotyledons (e.g. Herman, 1994; zur Nieden et al., 1984) as well as in the endosperm of developing cereal grains (e.g. Bechtel and Gaines, 1982; Kim et al., 1988; Levanony et al., 1992). These dense vesicles resemble somewhat the dense-core vesicles that bud from the TGN in neuropeptide-secreting endocrine cells (Tooze and Tooze, 1986).

To determine whether CCV or dense vesicles carry storage proteins from the TGN to the PSV we examined the polypeptides present in highly purified CCV and used immunocytochemistry on pea cotyledon storage parenchyma cells and isolated CCV fractions. Both dense vesicles and CCV were found at the TGN in pea cotyledons, but only the dense vesicles were labelled with gold-conjugated antibodies to storage proteins. The PSV membranes stained with antibodies to the aquaporin α-TIP and these antibodies possibly stained the dense vesicles, but not the CCV. Furthermore, immunoblots of highly purified CCV fractions did not contain detectable amounts of storage proteins. Together these results lead to the conclusion that in developing cotyledons transport of vacuolar proteins to the next compartment, either the endosome or the PSV, occurs by dense vesicles. The results also indicate that the visual aggregation of storage proteins seen in electron micrographs of osmium-fixed tissue starts to occur at the periphery of the Golgi cisternae in these vesicles and not at a later stage in the transport process.

MATERIALS AND METHODS

Plant material

Pea plants (*Pisum sativum* L. var. Haubner's Exzellenz) were cultivated under greenhouse conditions as previously given (Hoh et al., 1995). Maturing seeds were removed from plants at 24 days after flowering (cotyledon size: 9 mm, 200 mg) and used fresh for both electron microscopy as well as subcellular fractionation.

Subcellular fractionation

CCV were isolated from pea cotyledons by centrifugal means modified after procedures given by Depta and Robinson (1986), Hoh et al. (1991) and Demmer et al. (1993). Pea cotyledons were homogenized in a medium containing 0.3 M sorbitol, 1 mM $Na_2S_2O_5$, 3 mM EDTA, 1.5% (w/v) fatty acid-free BSA, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} E-64, 0.7 μg ml^{-1} pepstatin, and 1 mM *o*-phenanthroline dissolved in buffer A (0.5 mM $MgCl_2$, 1 mM EGTA in 0.1 M MES-NaOH, pH 6.4) and the microsomal membranes removed by centrifuging at 40,000 ***g*** for 30 minutes. CCV were purified from a crude postmicrosomal fraction (40,000-133,000 ***g*** pellet) in a five-step procedure: Step 1, treatment with pancreatic RNase (4.7 Kunitz units mg $protein^{-1}$, 25 minutes at 27°C) followed by centrifugation at 8,500 ***g*** for 30 minutes. Step 2, supernatant from step 1 was loaded onto a sucrose step gradient (15 ml, 5%, w/v; 10 ml, 30%, w/v) and centrifuged at 67,000 ***g*** for 40 minutes. The 5% layer and the 5/30% interphase were removed, diluted in buffer A and pelleted (133,000 ***g*** for 70 minutes). Step 3, pellet from step 2 was resuspended in buffer A, layered onto a linear (5-35%, w/v) sucrose gradient, and centrifuged at 67,000 ***g*** for 90 minutes. Fractions corresponding to 9-19% sucrose were pooled, diluted with buffer A and again pelleted (133,000 ***g*** for 70 minutes). Step 4, pellets from step 3 were resuspended in buffer A and recentrifuged in an Eppendorf tube at 9,000 ***g*** for 10 minutes. Step 5, supernatant from step 4 was layered onto a linear (25-55%, w/v) sucrose gradient and centrifuged in a vertical rotor at 167,000 ***g*** for 2.5 hours. Fractions from 40-45% sucrose were pooled, diluted with buffer A and centrifuged at 133,000 ***g*** for 70 minutes. The resulting pellet was resuspended in buffer A and recentrifuged in an Eppendorf tube as above. The supernatant contained highly purified CCV. All centrifugations were performed at 4°C.

In order to isolate protein storage vacuoles we have employed the procedure originally described by Mäder and Chrispeels (1984) in a modified form: 3 ***g*** testa-free cotyledon (11 mm) tissue was hand-chopped with a razor blade for 10 minutes in a Petri dish with 3 ml of a medium containing 0.1 M Mops, pH 5.5, 0.6 M mannitol, 1 mM EDTA, 0.6 mg *o*-phenanthroline, 1.5 μg pepstatin, 3 μg leupeptin. After filtering through 2 layers of Miracloth (Calbiochem, Bad Soden, Germany) the homogenate was pre-centrifuged at 100 ***g*** for 1 minute. The supernatant was then layered onto 3 ml of a Ficoll solution (5%, w/v, Ficoll in homogenizing medium) and centrifuged at 200 ***g*** for 10 minutes. The resulting pellet was carefully resuspended in 5 ml homogenizing medium and recentrifuged at 400 ***g*** for 20 minutes. This procedure was repeated again, the final pellet represents an almost pure PSV fraction.

Immunocytochemistry in situ

Slices (1 mm) of cotyledon tissue were fixed, dehydrated and embedded exactly as described by Hoh et al. (1995). For single antigen localizations sections on Formvar-coated nickel grids were blocked for 30 minutes in a solution containing 3% (w/v) BSA + 0.02% (v/v) BSA-C (Aurion, Biotrend, Köln, Germany) in TBS, before challenging with the primary antibody for 1 hour. After washing (3× 10 minutes) in a solution containing 1% (w/v) BSA + 0.01% (v/v) BSA-C in TBS, the sections were exposed to a gold-conjugated secondary antibody solution for 1 hour. For double antigen localizations (legumin + vicilin) incubations were carried out sequentially with legumin antibodies (1 hour) and then with a mixture of legumin and vicilin antibodies (1 hour). Treatment with gold (5, 10 nm)-conjugated secondary antibodies was also performed in two 1 hour steps corresponding to the primary antibodies. Before poststaining the grids were washed (3× 5 minutes) in 1% (w/v) BSA + 0.01% BSA-C in TBS (2× 5 minutes) in double-distilled water, then exposed to a 1% (v/v in water) solution of glutaraldehyde for 10 minutes, and finally washed (3× 5 minutes) in water. Sections were stained for 10 seconds in an aqueous 3% (w/v) uranyl acetate solution and for 15 seconds in a 0.3% (w/v) lead citrate solution. All on-grid operations were performed at room temperature.

Immunocytochemistry of CCV-fractions

Highly purified CCV were mixed with a primary fixative containing (end concentration) 2% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde, 50 mM, pH 6.8, potassium phosphate buffer and allowed

to stand for 16 hours at 4°C. In order to assist in subsequent sedimentations 2 μl of a resuspended Ponceau-stained (10 mg ml^{-1}; 10 minutes) baker's yeast pellet was added. Fixed organelles and yeast cells were pelleted by centrifuging in an Eppendorf tube at 142,000 ***g*** for 1 hour. The pellet was resuspended in 10 μl of 2% (w/v) low melting point agarose (Sigma, Deisenhofen, Germany) and gelled by standing on ice for 5 minutes. The block was washed in phosphate buffer (3× 10 minutes), removed and post-fixed for 1 hour in phosphate-buffered 0.5% (w/v) OsO_4. After washing in phosphate buffer then water (each 2× 10 minutes) the blocks were immersed in 0.1% (w/v) $NaBH_4$ for 30 minutes before dehydrating in a graded ethanol series. All of these steps were performed on ice. Embeddment in LR-White hard grade was as given by Hoh et al. (1995). On-section immunogold labelling was carried out as given above.

Antibodies and their dilutions

The antibodies used in this study were as follows (dilutions for western blotting are given in parentheses; dilutions for immunogold labelling are given in square brackets): (1) affinity-purified polyclonal antibodies raised in rabbits against *Pisum sativum* legumin (Casey, 1979) (1:4,000), [1:30]. (2) Affinity-purified polyclonal antibodies raised in rabbits against *Vicia faba* vicilin (1:5,000). (3) Affinity-purified polyclonal antibodies raised in mice against the 50 kDa vicilin polypeptide of *Vicia faba* (1:3,000), [1:200] for single immunogold labelling, [1:100] for double immunogold labelling. (4) Polyclonal antibodies raised in rabbits against the 25 kDa integral membrane protein (α-TIP) of *Phaseolus vulgaris* protein bodies (Johnson et al., 1989) (1:4,000), [1:60]. (5) Polyclonal antibodies raised against the 27 kDa integral membrane (β27) of *Beta vulgaris* storage root parenchyma vacuoles (Marty-Mazars et al., 1995) (1:5,000). (6) Polyclonal antibodies raised in rabbits against the complex glycoprotein β-fructosidase (βF_1) from *Daucus carota* cell walls (Laurière et al., 1989) (1:5,000), [1:50-100]. (7) Polyclonal antibodies raised in rabbits against the heavy chain of zucchini clathrin (Drucker et al., 1995) (1:10,000). (8) Polyclonal antibodies raised in rabbits against a fusion protein of tobacco BiP (Denecke et al., 1991) (1:5,000). Antisera (2) and (3) which also recognize pea vicilin were prepared by Dr Renate Manteuffel (Institut für Pflanzengenetik und Kulturpflanzenforschung, D-06466 Gatersleben, Germany). Secondary antibodies conjugated to 10 nm gold (α-TIP-, βF_1- and legumin-GARG), 5 nm or 12 nm gold (vicilin-GAMG) were obtained from Biocell (Cardiff, UK) and diluted 1:30 in TBS containing 1% (w/v) BSA and 0.01% (v/v) BSA-C.

Gel electrophoresis and western blotting

Polypeptides were separated on 10% SDS-PAGE. Staining of gels, electrophoretic transfer and visualization of bound antibodies were performed by standard procedures (Holstein et al., 1994).

Electron microscopy

Grids were examined in a Philips CM 10 electron microscope operating at 80 kV. For statistical analysis of Golgi parameters only micrographs in which *cis*- and *trans*-cisternae were clearly identifiable were used. Negatives with a primary magnification of ×16,000 or ×24,000 were evaluated directly using a 10-fold magnifying glass.

RESULTS

Highly purified CCV fractions do not contain storage proteins

To improve the separation of CCV from other vesicles we have combined and improved upon features of the isolation protocols of Demmer et al. (1993) and Hoh et al. (1991): the linear D_2O-Ficoll gradient (Hoh et al., 1991) was replaced by a rate zonal centrifugation on sucrose (Demmer et al., 1993; step 3 in Materials and Methods), and additionally two low speed clearing centrifugations (in steps 4 and 5) were included. An immunoblot of protein samples taken at each of the five steps of the purification scheme, separated by SDS-PAGE and developed with antisera against clathrin heavy chain, the cargo proteins vicilin and legumin, the ER marker BiP is shown in Fig. 1a-e. Clathrin is present in all fractions (Fig. 1a); the ER marker BiP disappears after the second purification step (Fig. 1b). The vicilin 75 and 70 kDa precursor (Fig. 1d) also are almost completely lost after the third step, but the 50 kDa vicilin precursor (Fig. 1c) and the legumin precursors (Fig. 1e) persist until the penultimate step (Fig. 1, lane 4). Processed forms of legumin (40 and 20 kDa) were absent from all CCV-containing fractions.

The fraction obtained after step 5 was fixed, embedded and used for immunocytochemistry with antibodies against vicilin (Fig. 2a) and legumin (Fig. 2b). We obtained an extremely low level of labelling and gold particles were not associated with any vesicle type or structural element in particular. This is consistent with the absence of labelling on the immunoblot (Fig. 1, lane 5). We conclude therefore that highly purified CCV do not contain cargo storage proteins that are in transit to the PSV in developing cotyledons.

Storage proteins exit from the Golgi in dense vesicles

Cotyledon storage parenchyma cells contain Golgi apparatus profiles consisting of four to five cisternae and associated vesicles (Fig. 3a). These vesicles can be either electron dense or electron translucent. When sections are simultaneously incubated with antibodies to legumin and vicilin the different sized gold particles linked to secondary antibodies are found over the dense vesicles (Fig. 3a, arrows) but not over the translucent vesicles or over the CCV (arrowheads). The same information is obtained with single antibody incubations (Fig. 3b,c,d).

To determine the *cis*/*trans* distribution of dense vesicles we marked their presence on 75 micrographs and scored those vesicles that showed a distinct cisternal connection. We found equal proportions of dense vesicles associated with the *cis*, medial and *trans* cisternae and with the TGN (Table 1). Those

Table 1. Position of DV in the Golgi apparatus of developing pea cotyledons and their immunogold labelling with storage protein antisera

Cisternae*	*Cis*	Median	*Trans*	TGN	Number of micrographs evaluated
(a) DV†	21	20	19	24	75
(b) DV labelled with antisera against:					
Legumin	6	6	8	13	17
Vicilin	5	4	5	5	16
Legumin and vicilin	2	2	4	3	9

*Only the outermost cisternae were designated *cis* or *trans*. All the cisternae lying between were termed median; *cis* and *trans* poles were distinguished on the basis of cisternal and intercisternal widths (Robinson and Kristen, 1982).

†Only those DV which showed a distinctly clear cisternal connection were counted.

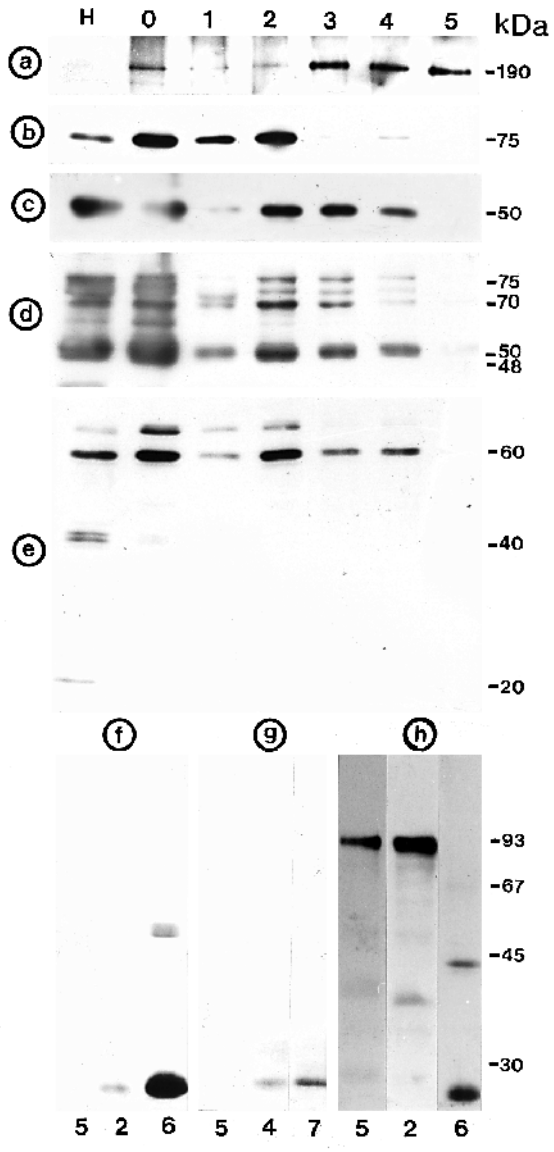

Fig. 1. Western blotting of subcellular fractions from developing pea cotyledons with antibodies against (a) clathrin heavy chain; (b) BiP; (c) 50 kDa vicilin polypeptide; (d) total vicilin pro-polypeptides; (e) legumin; (f) α-TIP; (g) γ-TIP (β27); (h) βF-complex glycan. Fractions are as follows: lane H, homogenate; lane 0, crude postmicrosomal (40,000-130,000 ***g***) pellet; lane 1, supernatant from purification step 1; lane 2, pellet from purification step 2; lane 3, pellet from purification step 3; lane 4, supernatant from purification step 4; lane 5, supernatant from purification step 5 (highly purified CCV); lane 6, PSV; lane 7, LV enriched fraction from a sucrose density gradient (see Hoh et al., 1995). Identical amounts of protein (20 μg) were applied to each lane. For details of the individual steps in the purification of CCV see Materials and Methods. The immunoreactive polypeptides around 55 kDa in f, lane 6, represent aggregates of α-TIP. This is a common feature for membrane integral proteins in SDS-PAGE (Qi et al., 1995).

at the TGN are often seen with CCV immediately adjacent (Figs 3 and 5a). Antibodies to both legumin and vicilin label the dense vesicles associated with any of the Golgi cisternae. We have not observed a preferential staining of *cis*-dense vesicles with legumin or *trans*-dense vesicles with vicilin antibodies as did Harris et al. (1989). It it likely, therefore, that these two major storage proteins travel together through the Golgi stack.

Aquaporins typical of protein storage and lytic vacuoles are not present in CCV

The limiting membrane of PSV contains the aquaporin α-TIP that is specific to seeds (Johnson et al., 1989), and antibodies to this protein react with a 27 kDa polypeptide on an immunoblot of purified PSV (Fig. 1f, lane 6). Although a trace of α-TIP is detectable in a partially purified CCV fraction (Fig. 1f, lane 2), α-TIP antibodies do not bind to any polypeptide in highly purified CCV (Fig. 1f, lane 5). The absence of α-TIP from CCV is confirmed by immunocytochemical observations on intact tissue. The antibodies against α-TIP label the PSV (Fig. 4a), but do not label the CCV (Fig. 4b). Whether the dense vesicles are specifically labelled is not clear. There are never more than two gold particles per vesicle and many of the dense vesicles were unlabelled. The labelling of the dense vesicles might possibly be background labelling. Thus while immunocytochemistry clearly supports a role for the dense vesicles in storage protein transport, the evidence in favour of them transporting α-TIP as well is equivocal.

Lytic vacuoles (LV) also possess an aquaporin (γ-TIP) which is similar but immunologically distinguishable from α-TIP (Höfte et al., 1992). β27 antiserum, which recognizes the tonoplast of LV but not of PSV in pea cotyledons (Hoh et al., 1995) also failed to cross-react with polypeptides in highly purified CCV (Fig. 1g, lane 5). However, in contrast to the immunoblot results with α-TIP antibodies, γ-TIP antigen was still detectable in CCV fractions up till purification step 4 (Fig. 1g, lane 4).

CCV and the protein storage vacuoles contain different glycoproteins

The transport of many secretory proteins is accompanied by the cotranslational attachment in the ER of high-mannose glycans and the conversion of many of these glycans to complex glycans in the Golgi apparatus. Glycoproteins that have passed through plant Golgi almost invariably contain complex glycans with fucose and xylose residues. The presence of such glycoproteins can be ascertained by the use of βF-antiserum that specifically detects complex N-linked glycans (Laurière et al., 1989). PSV fractions contain two glycosylated polypeptides (M_r 43,000 and 24,000) which are not present in the purified CCV fraction (compare lanes 5 and 6; Fig. 1h). These probably correspond to vicilin polypeptides, as pea legumin is not glycosylated (Chrispeels et al., 1982). Salt treatment of the PSV (3 times washing with 1 M NaCl or 1 M KI) considerably reduced the strength of the signal in the immunoblot of the PSV with the βF-antibody, but no additional polypeptides became visible (data not shown).

In the CCV fraction (lane 5, Fig. 1h) there is one major (M_r 90,000) and three minor (M_r 48,000, 38,000 and 27,000) glycosylated polypeptides. In a less-purified CCV fraction (lane 2, Fig. 1h) the 90 kDa glycoprotein is present at a higher concentration, and the 27 kDa glycoprotein seems to be lower in amount. There is also a 36 kDa rather than a 38 kDa glycoprotein present. This suggests that the 90, 48 and 36 kDa glycoproteins are not carried by CCV, but rather by contaminants still remaining in the purified CCV fraction whereas the 38 kDa and

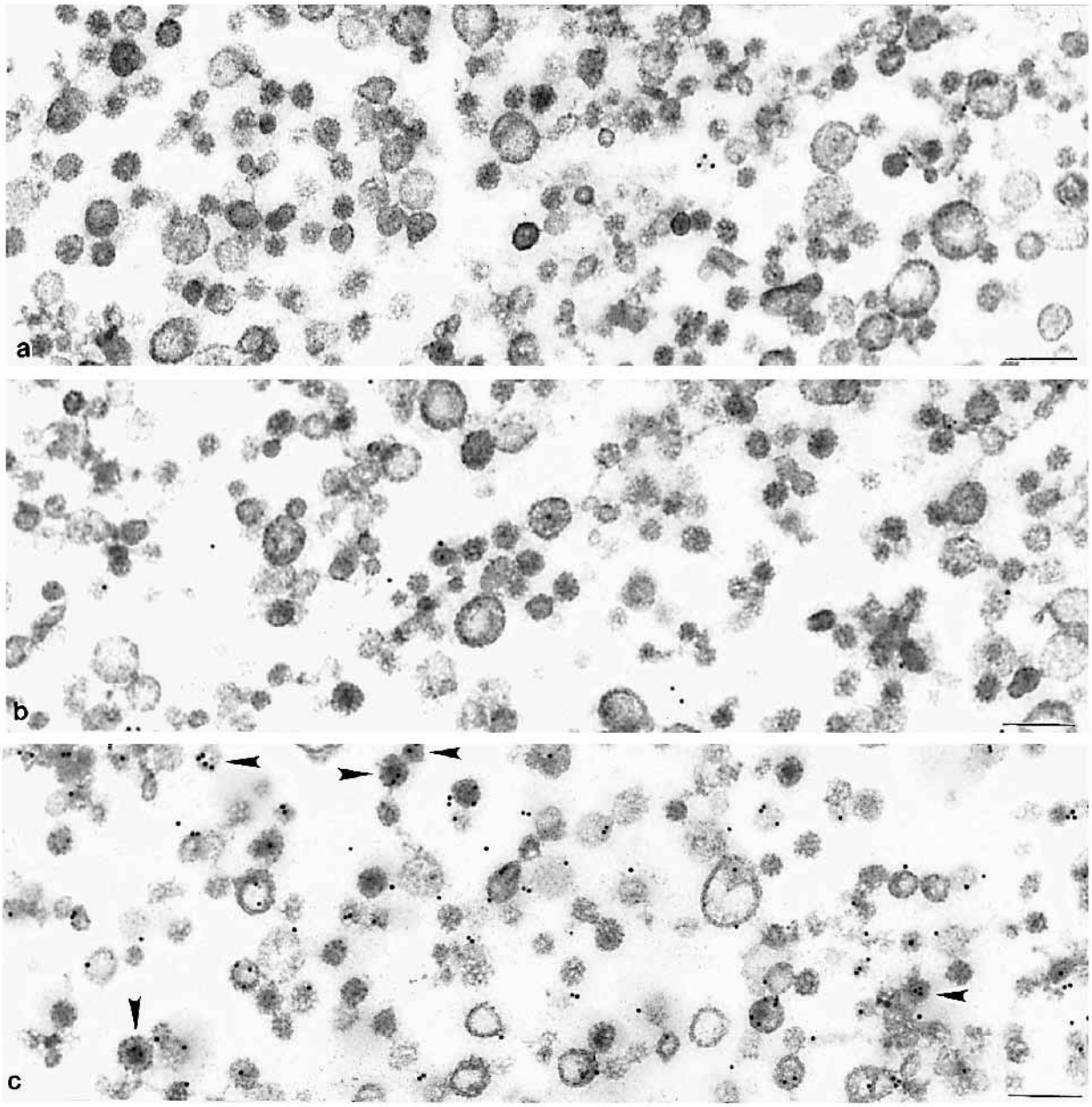

Fig. 2. Postembedding immunogold staining of purified CCV fractions (corresponding to lane 5 of Fig. 1) with antibodies against (a) 50 kDa vicilin, (b) legumin and (c) βF complex glycan. Arrowheads in c point to clustered gold particles lying on CCV. Bars, 200 nm.

27 kDa may be present in the CCV. This is supported by immunocytochemistry with βF antibodies in situ (Fig. 5a-f), as well as on isolated CCV fractions (Fig. 2c), whereby gold particles are occasionally found lying directly over sectioned CCV. We have performed a statistical analysis of the βF immunogold labelling in the CCV fraction and have found that roughly 20% of the labelled vesicles in this fraction are definitely clathrin coated (Table 2). However, the proportion of label in CCV in this fraction could rise to 50% when the labelled smooth-surfaced vesicles whose diameter is less than 100 nm are regarded as uncoated CCV. The nature and origin of the larger contaminants in the CCV fraction is unclear. However, βF-immunogold labelled smooth-surfaced vesicles in this size category are seen both at the TGN (Fig. 5a) as well as directly attached to the cisternae of the Golgi apparatus (Fig. 5g,h).

In contrast to the storage protein antisera the cisternae of the Golgi apparatus are quite heavily labelled with βF immunogold (Figs 5f and 6b). Label is also present in the cell wall (Fig. 6b) and in the LV (Fig. 6a). The storage protein deposits in the PSV are only weakly labelled (Fig. 6b) and, in agreement with this, label is sometimes absent from dense vesicles (compare Fig. 5f with Fig. 6a,b). Thus there are three compartments into which Golgi-derived glycoproteins can be transported and there are three vesicle types in which glycoproteins can be detected.

DISCUSSION

Targeting domains, receptors and vesicles in plants

The pathway and mechanism of vacuolar protein transport in

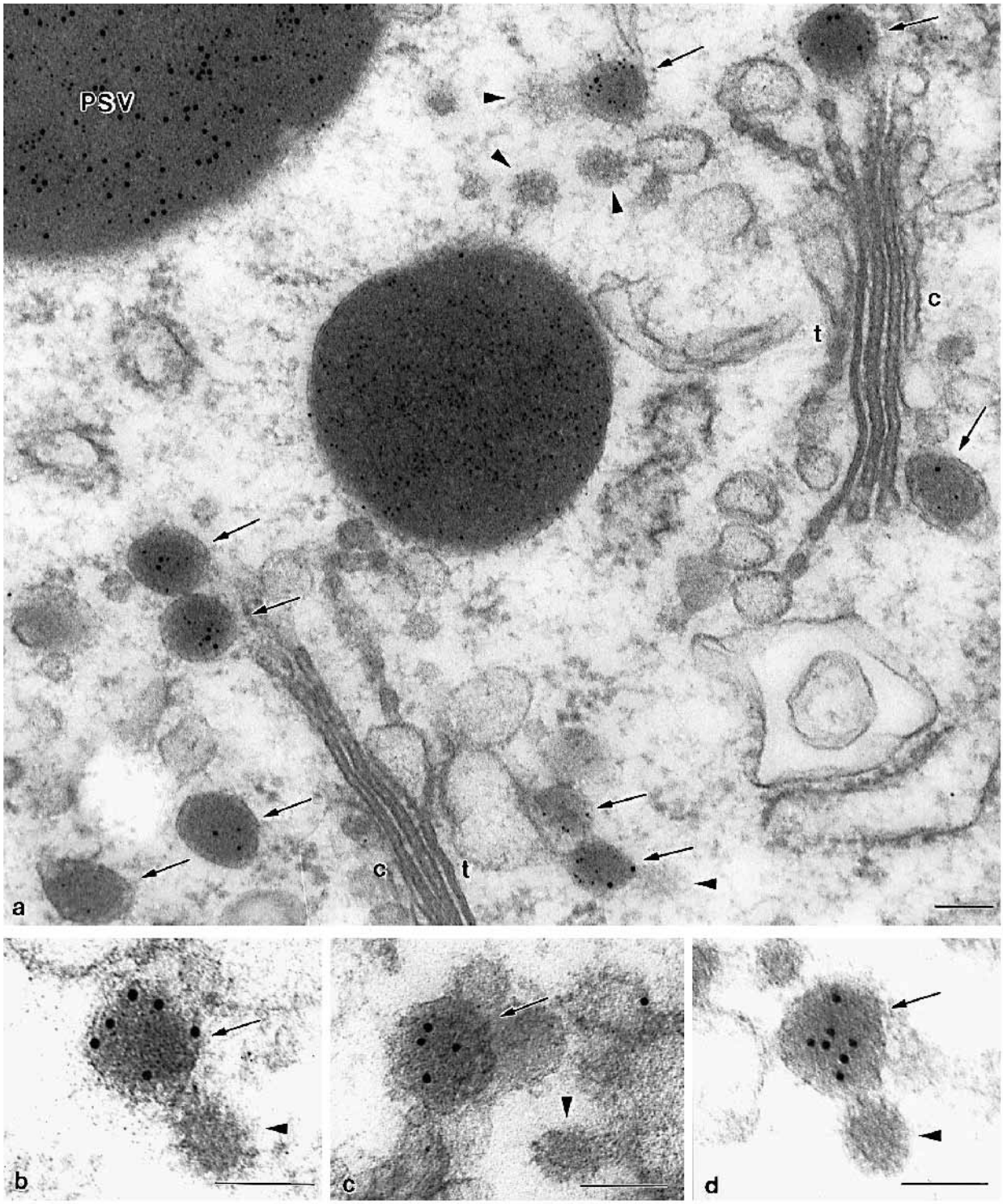

Fig. 3. Postembedding immunogold staining of developing pea cotyledon cells with antibodies against (a) legumin (10 nm gold) and 50 kDa vicilin (5 nm gold), (b,c,d) 50 kDa vicilin (12 nm gold). Arrowheads point to CCV. Arrows indicate dense vesicles. c, *cis* and t, *trans* poles of the Golgi apparatus. PSV, protein storage vacuole. Bars, 100 nm.

plant cells have been studied in a number of laboratories, and there is considerable information about the nature of the targeting domains, but much less about the proteins that interact with these domains and the identity of the vesicles that carry proteins from the TGN to the vacuole. Vacuolar targeting information is contained in propeptides at the N or C terminus of the protein, or in internal segments of the mature protein as is the case with legumin and phytohemagglutinin (Chrispeels and Raikhel, 1992; Neuhaus, 1996), and not in phosphorylated mannose residues of N-linked glycans as in the lysosomal

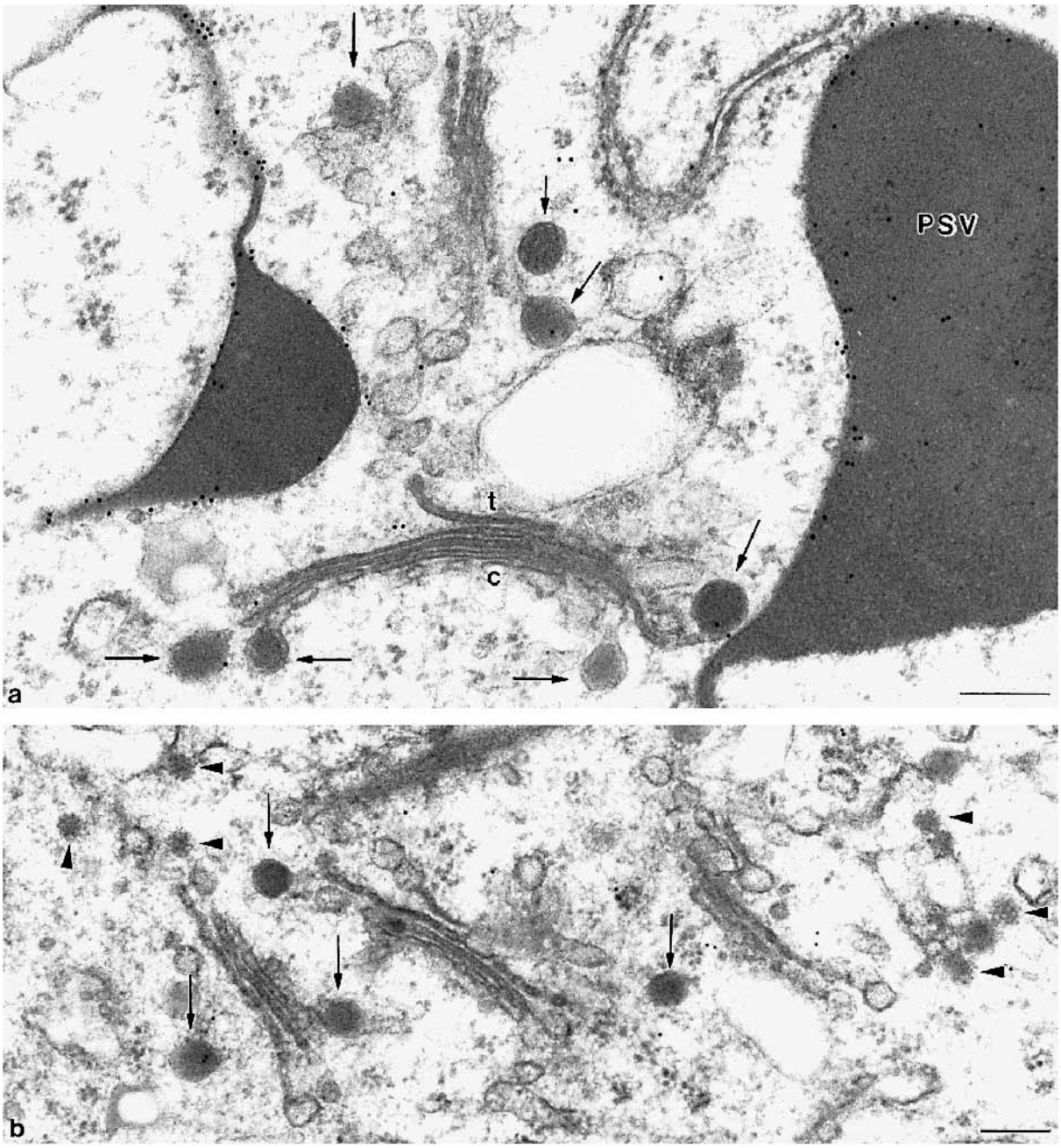

Fig. 4. (a,b) Postembedding immunogold staining of the Golgi apparatus developing pea cotyledon cells with antibodies against α-TIP. Arrowheads point to CCV. Arrows indicate dense vesicles. c, *cis* and t, *trans* poles of the Golgi apparatus. PSV, protein storage vacuole. Bars, 200 nm.

enzymes of mammalian cells. Candidate proteins for the transport machinery have also been isolated. BP-80, a protein obtained from CCV-rich fractions of pea cotyledons binds to certain C and N-terminal targeting domains of vacuolar proteins, but not to mutated domains that are unable to target proteins in situ (Kirsch et al., 1994, 1996). BP-80 has a putative transmembrane domain and could be a transport receptor. The cDNA of another candidate protein of the vacuolar transport machinery has recently been obtained from *Arabidopsis*. This protein, Pep12, is an *Arabidopsis* syntaxin homologue that is functionally active in yeast (Bassham et al., 1995). Syntaxins are transmembrane proteins in the destination membrane where they mediate the docking and fusion of the arriving vesicle but it is not yet known in which membrane compartment this particular *Arabidopsis* syntaxin is located.

As early as 1972, Dieckert and Dieckert suggested that dense vesicles budding from the Golgi apparatus were involved in protein transport. Indeed, when investigated by immunocytochemistry, these vesicles have invariably been found to contain vacuolar proteins (mostly storage proteins and lectins). Although, in frozen sections from cotyledons that had been fixed only with aldehydes, Golgi derived vesicles are not

electron dense (Greenwood and Chrispeels, 1985), the electron density of the material in the dense vesicles in OsO_4-fixed tissue resembles that found in the PSV and is probably a consequence of the aggregation or condensation of the storage proteins. Such aggregation is not usually seen earlier in the transport pathway (e.g. in the cisternae of the ER or the Golgi; see, however, Robinson et al., 1995). Protein-protein interactions may lead to the physical aggregation of storage polypeptides which, as a result, become segregated in the secretory pathway. Such aggregation occurs in wheat endosperm with the prolamine-type storage polypeptides. They are retained in the ER by a proline-rich region at the N terminus of the protein (Geli et al., 1994). This domain allows for an interaction between adjacent polypeptides which subsequently aggregate through disulfide bridges located at the C termini. Storage proteins have an acidic pI and acidification of the transport vesicles may aid in the aggregation of the proteins. In this respect it will be interesting to learn whether dense vesicles possess V-ATPase or pyrophosphatase acitvities.

The discovery that numerous CCV are present at the TGN and that CCV-enriched fractions contain precursors of vacuolar storage proteins led to the idea that they, and not the

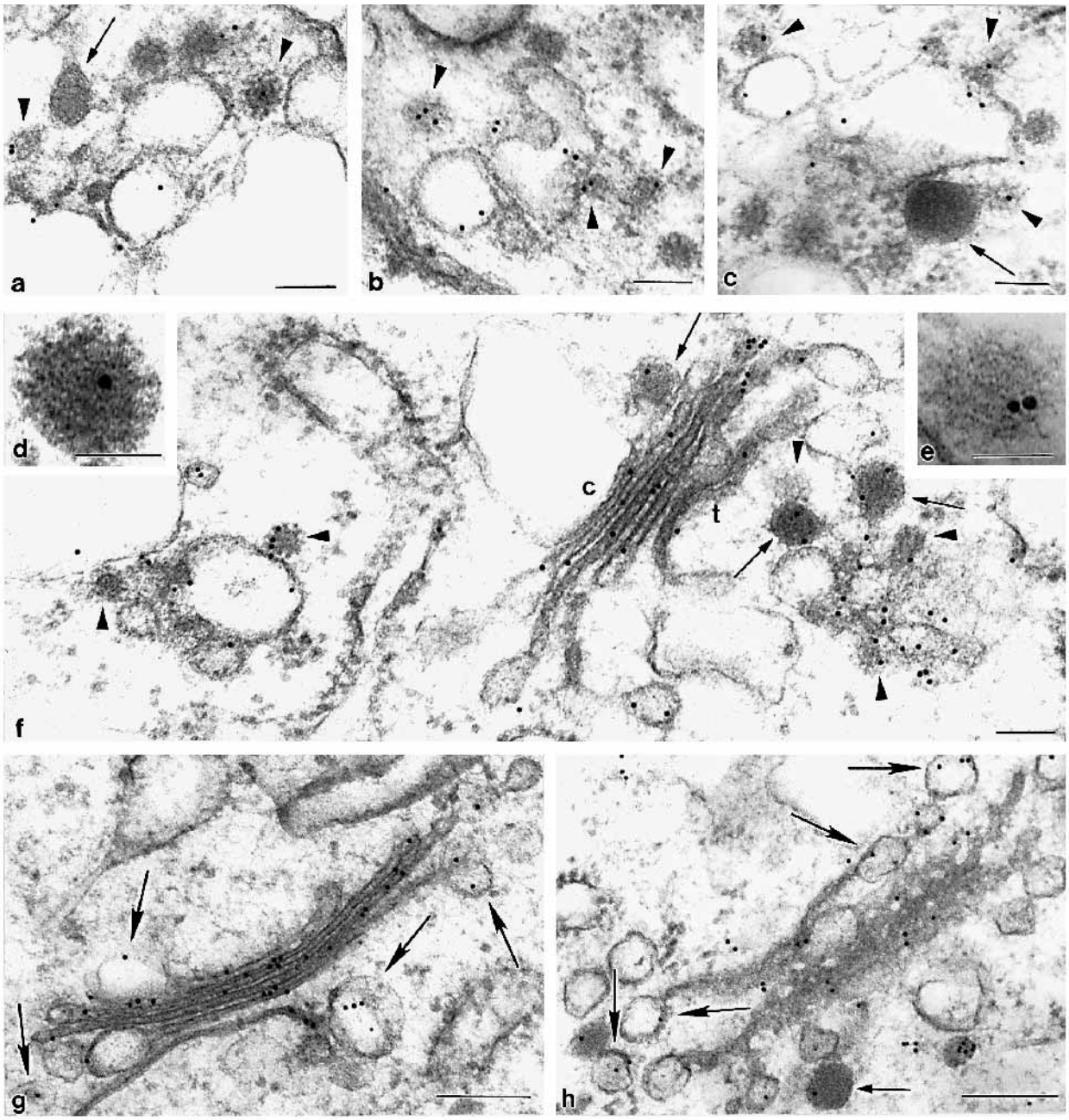

Fig. 5. βF-immunogold staining of the Golgi apparatus and surrounding elements. (a-c) Labelling of CCV and tubular/vesicular elements of the TGN; (d,e) label in lumen of CCV; (f) general distribution of label. The cisternal stack and TGN are heavily labelled, whereas dense vesicles (small arrows) are barely labelled. (g,h) Labelling of the cisternal stack and associated smooth-surfaced vesicles (large arrows). Arrowheads point to CCV. c, *cis* and t, *trans* poles of the Golgi apparatus. Bars, 100 nm.

Table 2. Quantitation of F-immunogold labelling on this sections of purified pea cotyledon CCV fractions*

	Gold labelled vesicles†		
	Total no. of vesicles labelled	Total no. of gold particles	No. of gold particles per vesicle
CCV‡	62	85	1.4
Small (<100 nm diameter) smooth vesicles	117	237	2.0
Large (>200 nm diameter) smooth vesicles	151	348	2.3

*Counting was performed on 12 micrographs of randomly chosen areas of sectioned CCV pellets as presented in Fig. 2c.

†For this analysis vesicles which were counted as being labelled were vesicles which were sufficiently cross sectioned to allow for detection of a lumenal antigen. Because of their size (around 80 nm) and the thickness of their coat (20 nm) only a small proportion of the total CCV population fall into this category.

‡With an estimated lumen diameter of 40 nm we estimate that maximally only two 10 nm antibody bound gold particles can collect over a cross-sectioned CCV.

dense vesicles, are responsible for transport between the TGN and the vacuole (reviewed by Beevers, 1996). However, the data presented here show that the CCV-enriched fractions used in earlier studies were contaminated at least with dense vesicles. On the basis of the immunoblots and immunocytochemical evidence presented here, we now maintain that CCV do not play a role in transport of storage proteins to the PSV, at least not in developing legume cotyledons.

Multiple routes to multiple vacuoles?

Our finding that CCV do not carry storage proteins to the PSV is not necessarily in contradiction to the findings of others that CCV contain BP-80, a possible vacuolar transport receptor (Kirsch et al., 1994, 1996). Plant cells contain multiple types of vacuoles and protein storage cells have been shown to contain at least two types that differ not only in their soluble proteins but also in their tonoplast proteins. Immunoblotting of subcellular fractions and immunocytochemistry with antibodies to aquaporins have shown that legume cotyledons contain PSV that have storage proteins and the aquaporin α-TIP in their tonoplast as well as LV which do not have storage

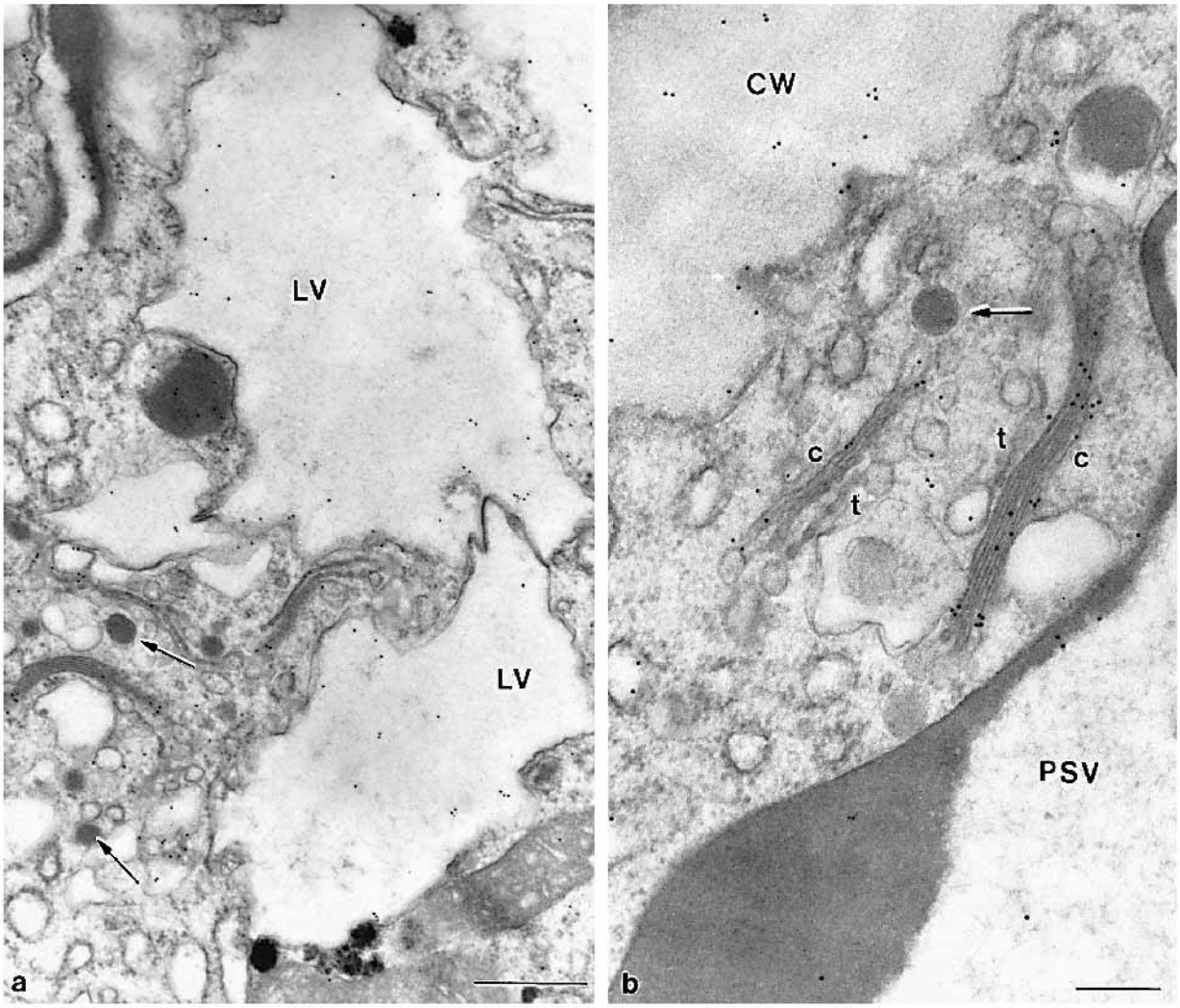

Fig. 6. Postembedding immunogold staining of developing pea cotyledon cells with antibodies against βF complex glycan. (a) Labelling LV; (b) labelling of cell wall (CW), PSV and Golgi apparatus. Dense vesicles are indicated by arrows. c, *cis* and t, *trans* poles of the Golgi apparatus. Bars, 200 nm.

proteins but contain γ-TIP in their tonoplast (Hoh et al., 1995). Similarly, two populations of vacuoles have recently been identified in root tip cells of barley and pea. Whereas one vacuole type seemed to have a storage function, being characterized by barley lectin and α-TIP, the other, containing the thiol protease aleurain and having γ-TIP in its tonoplast appeared to represent a lytic compartment (Paris et al., 1996). Proteins must be sorted to be targeted to these two types of vacuoles and this sorting probably occurs at the *trans*-face of the Golgi. Using wortmannin, an inhibitor of phospholipid biosynthesis, Matsuoka et al. (1995) have demonstrated the presence of two different vacuolar sorting mechanisms in tobacco cells. At a low concentration (0.33 μM) wortmannin inhibited delivery of proteins to the vacuole mediated by a C-terminal signal, but not by an N-terminal signal. In addition, soluble and membrane proteins of the vacuole may not travel together by the same route as is the case for lysosomal proteins in animal cells (Braulke, 1996). This is indeed suggested by the experiments of Gomez and Chrispeels (1993) who examined the kinetics of transport and its sensitivity to inhibitors of vesicle transport for two proteins, phytohemagglutinin and α-TIP in mesophyll protoplasts of transgenic tobacco. Thus we postulate that the storage proteins use an aggregation mechanism for sorting and travel in dense vesicles to the next compartment, whereas other proteins, e.g. acid hydrolases are bound by specific receptors. These and their attached ligands may be transported by CCV. We cannot discount the possibility that α-TIP is also borne by dense vesicles, but certainly neither α-TIP nor γ-TIP are carried by CCV. Unfortunately antisera directed against pea cotyledon hydrolases are not available so that their positive identification as cargo for CCV is not yet possible.

Our data relating to the detection of N-linked complex glycans in CCV may also be taken as supporting the notion that there are two modes of exit from the Golgi apparatus for vacuolar proteins in pea cotyledons. The modification of high-mannose glycans to complex glycans is mediated by Golgi enzymes (Sturm et al., 1987). It is therefore not surprising that the Golgi cisternae stain abundantly with anti-βF, a serum that is specific for these xylose and fucose-containing glycans (Laurière et al., 1989). Besides resident glycoproteins some of the labelling could represent hydrolases destined for the LV whilst others may be secretory proteins. Thus the presence of complex glycoproteins in CCV and smooth vesicles that are in close proximity to the Golgi stack in pea cotyledons could be taken, respectively, as cargo hydrolases en route to the LV, and secretory proteins to the cell wall. We cannot, however, eliminate the possibility that part of the complex glycoprotein labelling in isolated CCV might represent a subpopulation of plasma membrane-derived CCV which contain endocytosed glycoproteins from the apoplast.

Intra-Golgi transport of storage proteins

Immunogold labelling of the pea cotyledon Golgi apparatus with vicilin and legumin antibodies is restricted to the dense vesicles, an observation also made for some storage polypeptides in cereal grains (Krishnan et al., 1986; Kim et al., 1988; Levanony et al., 1992). Since, by contrast, βF antibodies heavily label the cisternae and only seldom the dense vesicles, we assume that storage proteins are efficiently and rapidly segregated to the periphery of the cisternae after entering the Golgi apparatus. According to current concepts of Golgi function in animal and yeast cells (reviewed by Rothman, 1994; Schekman and Orci, 1996) intra-Golgi transport is facilitated by COP vesicles. This idea is, however, not without criticism (see Becker and Melkonian, 1995, for a review) and there are a number of lower organisms, e.g. scale algae, in which a cisternal progression through the stack is clearly the preferred way (Becker et al., 1995). In pea cotyledon Golgi, dense vesicles were observed attached to cisternae at all levels in the stack as well as at the TGN. They are larger and do not have the typical nap-like coat of COP-vesicles. On the other hand we have no evidence for a gradual enlargement of the dense vesicle in a *cis*→*trans* direction, nor for a maturation of their contents. Since legumin is unglycosylated and only a portion of the vicilin polypeptides become glycosylated (Kummer and Rüdiger, 1988) it is possible that most of the unglycosylated forms of these storage proteins exit the Golgi apparatus in *cis* and medial cisternae-derived dense vesicles. An early exit from the plant Golgi apparatus is also implied in the immunocytochemical studies of Staehelin and coworkers who detected the pectin rhamnogalacturonan I exclusively in *cis* and median cisternae of sycamore cells (Moore et al., 1991).

The interesting observation that CCV seem to bud directly from TGN located dense vesicles (see also Fig. 2 of Hinz et al., 1993) could reflect a situation analogous to that recorded for neuropeptide-secreting endocrine cells. In these cells immature secretory granules, also with dense contents, are partially coated with clathrin triskelions (Tooze and Tooze, 1986) and with the AP1-adaptor complex (Dittié et al., 1996). It has been suggested that CCV derived from the immature secretory granule may salvage proteins which were missorted at the TGN (Kuliawat and Awan, 1994). In the light of the present results the removal of missorted hydrolases from TGN-derived dense vesicles by CCV formation and fission at their surface is an attractive postulate.

We thank Dorothee Dassbach for help with the photographic work and Heike Freundt for typing the manuscript. Supported by funds from the Deutsche Forschungsgemeinschaft (Hi 488/1-2) to G. Hinz and the European Economic Community (ERB 405PL PL 32532) to D. G. Robinson and the National Science Foundation (Cell Biology Program) to M. J. Chrispeels.

REFERENCES

Bassham, D. C., Gal, S., Conceicao, A. D. and Raikhel, N. V. (1995). An *Arabidopsis* syntaxin homolog isolated by functional complementation of a yeast pep 12 mutant. *Proc. Nat. Acad. Sci. USA* **92**, 7262-7266.

Bassham, D. C. and Raikhel, N. V. (1996). Transport proteins in the plama membrane and the secretory system. *Trends Plant Sci.* **1**, 15-20.

Bechtel, D. B. and Gaines, R. L. (1982). The presence of protease digestible material in Golgi vesicles during endosperm development of selected cereals. *Am. J. Bot.* **69**, 880-884.

Becker, B., Böllinger, B. and Melkonian, M. (1995). Anterograde transport of algal scales through the Golgi complex is not mediated by vesicles. *Trends Cell Biol.* **5**, 305-306.

Becker, B. and Melkonian, M. (1995). Intra-Golgi transport mediated by vesicles? *Botanica Acta* **108**, 172-173.

Beevers, L. (1996). Clathrin-coated vesicles in plants. *Int. Rev. Cytol.* **167**, 1-35.

Braulke, T. (1996). Origin of lysosomal proteins. In *Biology of the Lysosome. Subcellular Biochemistry*, vol. 27 (ed. J. B. Lloyd and R. W. Mason). Plenum Press, New York (in press).

Casey, R. D. (1979). Immunoaffinity chromatography as a means of purifying legumin from *Pisum* (pea) seeds. *Biochem. J.* **177**, 509-520.

Chrispeels, M. J., Higgins, T. J. V. and Spencer, D. (1982). Assembly of storage protein oligomers in the endoplasmic reticulum and processing of the polypeptides in the protein bodies of developing pea cotyledons. *J. Cell Biol.* **93**, 306-313.

Chrispeels, M. J. (1983). The Golgi apparatus mediates the transport of phytohemagglutinin to the protein bodies in bean cotyledons. *Planta* **158**, 140-151.

Chrispeels, M. J. (1991). Sorting of proteins in the secretory system. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 21-53.

Chrispeels, M. J. and Raikhel, N. V. (1992). Short peptide domains target proteins to plant vacuoles. *Cell* **68**, 613-616.

Craig, S. and Goodchild, D. J. (1984). Periodate-acid treatment of sections permits on-grid immunogold localization of pea seed vicilin in ER and Golgi. *Protoplasma* **122**, 35-44.

Craig, S. (1988). Structural aspects of protein accumulation in developing legume seeds. *Biochem. Physiol. Pflanzen* **183**, 159-171.

Demmer, A., Holstein, S. E. H., Hinz, G., Schauermann, G. and Robinson, D. G. (1993). Improved coated vesicle isolation allows better characterization of clathrin polypeptides. *J. Exp. Bot.* **44**, 23-33.

Denecke, J., Goldman, M. H. S., Demolder, J., Seurinck, J. and Botterman, J. (1991). The tobacco luminal binding protein is encoded by a multigene family. *The Plant Cell* **3**, 1025-1035.

Depta, H. and Robinson, D. G. (1986). The isolation and enrichment of coated vesicles from suspension-cultured carrot cells. *Protoplasma* **130**, 162-170.

Dieckert, J. W. and Dieckert, M. C. (1972). The deposition of vacuolar proteins in oilseeds. In: *Symposium Seed Proteins* (ed. G. E. Inglett), pp. 52-85. Avi. Publ. Comp. Inc., Westport.

Dittié, A. S., Hajibagheri, N. and Tooze, S. A. (1996). The AP-1 adaptor complex binds to immature secretory granules from PC12 cells and is regulated by ADP-ribosylation factor. *J. Cell Biol.* **132**, 523-536.

Drucker, M., Herkt, B. and Robinson, D. G. (1995). Demonstration of a β-type adaptin at the plant plasma membrane. *Cell Biol. Intern.* **3**, 191-201.

Geli, M. I., Torrent, M. and Ludevid, D. (1994). Two structural domains mediate two sequential events in γ-zein targeting: protein endoplasmic reticulum retention and protein body formation. *Plant Cell* **6**, 1911-1922.

Gomez, L. and Chrispeels, M. J. (1993). Tonoplast and soluble vacuolar proteins are targeted by different mechanisms. *The Plant Cell* **5**, 1113-1124.

Greenwood, J. S. and Chrispeels, M. J. (1985). Immunocytochemical localization of phaesolin and phytohemagglutinin in the endoplasmic reticulum and Golgi complex of developing bean cotyledons. *Planta* **164**, 295-302.

Griffing, L. R. (1991). Comparisons of Golgi structure and dynamics in plant and animal cells. *J. Electron Microsc. Tech.* **17**, 179-199.

Harley, S. M. and Beevers, L. (1989). Coated vesicles are involved in the transport of storage proteins during seed development in *Pisum sativum* L. *Plant Physiol.* **91**, 674-678.

Harris, N., Grindley, H., Mulchrone, J. and Croy, R. D. (1989). Correlated *in situ* hybridisation and immunochemical studies of legumin storage protein deposition in pea (*Pisum sativum* L.). *Cell Biol. Int. Rep.* **13**, 23-35.

Herman, E. M. (1994). Multiple origins of intravacuolar protein accumulation of plant cells. *Advan. Struct. Biol.* **3**, 243-283.

Hinz, G., Hoh, B. and Robinson, D. G. (1993). Strategies in the isolation of storage protein receptors. *J. Exp. Bot.* **44** (Suppl.), 351-357.

Höfte, H., Hubbard, L., Reizer, J., Ludevid, D., Herman, E. M., Chrispeels, M. J. (1992). Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of *Arabidopsis thaliana*. *Plant Physiol.* **99**, 561-570.

Hoh, B., Schauermann, G. and Robinson, D. G. (1991). Storage protein polypeptides in clathrin coated vesicle fractions from developing pea cotyledons are not due to endomembrane contamination. *J. Plant Physiol.* **138**, 309-316.

Hoh, B., Hinz, G., Jeong, B.-K. and Robinson, D. G. (1995). Protein storage vacuoles form de novo during pea cotyledon development. *J. Cell Sci.* **108**, 299-310.

Holstein, S. E. H., Drucker, M. and Robinson, D. G. (1994). Identification of a β-type adaptin in plant clathrin-coated vesicles. *J. Cell Sci.* **107**, 945-953.

Johnson, K. D., Herman, E. M. and Chrispeels, M. J. (1989). An abundant, highly conserved tonoplast protein in seeds. *Plant Physiol.* **91**, 1006-1013.

Kim, W. T., Franceschi, V. R., Krishnan, H. B. and Okita, T. W. (1988). Formation of wheat protein bodies: involvement of the Golgi apparatus in gliadin transport. *Planta* **176**, 173-182.

Kirsch, T., Paris, N., Bulter, J. M., Beevers, L. and Rogers, J. C. (1994). Purification and initial characterization of a potential plant vacuolar targeting receptor. *Proc. Nat. Acad. Sci. USA* **91**, 3403-3407.

Kirsch, T., Saalbach, G., Raikhel, N. V. and Beevers, L. (1996). Interaction of a potential vacuolar targeting receptor with amino- and carboxy-terminal targeting determinants. *Plant Physiol.* **111**, 469-474.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483-525.

Krishnan, H. B., Franceschi, V. R. and Okita, T. W. (1986). Immunochemical studies on the role of the Golgi complex in protein-body formation in rice seeds. *Planta* **169**, 471-480.

Kuliawat, R. and Arvan, P. (1994). Distinct molecular mechanisms for protein sorting within immature secretory granules of pancreatic β-cells. *J. Cell Biol.* **126**, 77-86.

Kummer, H. and Rüdiger, H. (1988). Characterization of a lectin-binding storage protein from pea (*Pisum sativum*). *Biol. Chem. Hoppe-Seyler* **369**, 639-646.

Laurière, M., Laurière, C., Chrispeels, M. J., Johnson, K. D. and Sturm, A. (1989). Characterization of a xylose-specific antiserum that reacts with the complex asparagine-linked glycans of extracellular and vacuolar glycoproteins. *Plant Physiol.* **90**, 1182-1188.

Levanony, H., Rubin, R., Altschuler, Y. and Galili, G. (1992). Evidence for a novel route of wheat storage proteins to vacuoles. *J. Cell Biol.* **119**, 1117-1128.

Lycett, G. W., Delauney, A. J., Gatehouse, J. A., Gilroy, J., Croy, R. R. D. and Boulter, D. (1983). The vicilin gene family of pea (*Pisum sativum* L.): a complete cDNA coding sequence for provicilin. *Nucl. Acids Res.* **11**, 2367-2380.

Lycett, G. W., Croy, R. R. D., Shirsat, A. H. and Boulter, D. (1984). The complete nucleotide sequence of a legumin gene from pea (*Pisum sativum* L.). *Nucl. Acids Res.* **12**, 4493-4506.

Mäder, M. and Chrispeels, M. J. (1984). Synthesis of an integral protein of the protein body membrane in *Phaseolus vulgaris* cotyledons. *Planta* **160**, 330-340.

Marty-Mazars, D., Clémencet, M.-C., Dozolme, P. and Marty, F. (1995). Antibodies to the tonoplast from the storage parenchyma cells of beetroot recognize a major intrinsic protein related to TIPs. *Eur. J. Cell Biol.* **66**, 106-118.

Matsuoka, K., Bassham, D. C., Raikhel, N. V. and Kakamura, K. (1995). Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. *J. Cell Biol.* **130**, 1307-1318.

Moore, P. J., Swords, K. M. M., Lynch, M. A. and Staehelin, L. A. (1991). Spatial orgniaztion of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J. Cell Biol.* **112**, 589-602.

Müntz, K. (1996). Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. *J. Exp. Bot.* **47**, 605-622.

Neuhaus, J.-M. (1996). Protein targeting to the plant vacuole. *Plant Physiol. Biochem.* **34**, 217-221.

Okita, T. W. and Rogers, J. C. (1996). Compartmentation of proteins in the endomembrane system of plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 327-350.

Paris, N. and Rogers, J. (1996). The role of receptors in targeting soluble proteins from the secretory pathway to the vacuole. *Plant Physiol. Biochem.* **34**, 223-228.

Paris, N., Stanley, C. M., Jones, R. L. and Rogers, J. C. (1996). Plant cells contain two functionally distinct vacuolar compartments. *Cell* **85**, 563-572.

Qi, X., Tai, C.-Y. and Wasserman, B. P. (1995). Plasma membrane intrinsic proteins of *Beta vulgaris* L. *Plant Physiol.* **108**, 387-392.

Record, R. D. and Griffing, L. R. (1988). Convergence of the endocytic and lysosomal pathways in soybean protoplasts. *Planta* **176**, 425-432.

Robinson, D. G. and Kristen, U. (1982). Membrane flow via the Golgi apparatus of higher plant cells. *Int. Rev. Cytol.* **77**, 89-127.

Robinson, D. G., Balusek, K. and Freundt, H. (1989). Legumin antibodies recognize polypeptides in coated vesicles from developing pea cotyledons. *Protoplasma* **150**, 79-82.

Robinson, D. G. and Hillmer, S. (1990). Coated pits. In *The Plant Plasma Membrane* (ed. C. Larsson and I. M. Møller), pp. 233-255. Springer Verlag.

Robinson, D. G., Hoh, B., Hinz, G. and Jeong, B.-K. (1995). One vacuole or two vacuoles: do protein storage vacuoles arise de novo during pea cotyledon development? *J. Plant Physiol.* **145**, 654-664.

Robinson, M. S. (1994). The role of clathrin, adaptors and dynamin in endocytosis. *Curr. Opin. Cell Biol.* **6**, 538-544.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Schekman, R. and Orci, L. (1996). Coat proteins and vesicle budding. *Science* **271**, 1526-1533.

Schmid, S. L. and Damke, H. (1995). Coated vesicles: a diversity of form and function. *FASEB J.* **9**, 1445-1453.

Sturm, A., Johnson, K. D., Szumilo, T., Elbein, A. D. and Chrispeels, M. J. (1987). Subcellular localization of glycosidases and glycosyl-transferases involved in the processing of N-linked oligosaccharides. *Plant Physiol.* **85**, 741-745.

Tooze, J. and Tooze, S. A. C. (1986). Clathrin-caoted vesicular transport of secretory proteins during the formation of ACTH-containing secretory granules in AtT_{20} cells. *J. Cell Biol.* **103**, 839-850.

zur Nieden, U., Manteuffel, R., Weber, E. and Neumann, D. (1984). Dictyosomes participate in the intracellular pathway of storage proteins in developing *Vicia faba* cotyledons. *Eur. J. Cell Biol.* **34**, 9-17.

(Received 8 May 1996 – Accepted 19 July 1996)