

Review

Protein Sorting to the Storage Vacuoles of Plants: A Critical Appraisal

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The vacuole of plant cells is no longer considered to be a single compartment with multifunctional properties. A lot of evidence now points to the presence of multiple functionally distinct vacuolar compartments, some existing side by side in the same cell. As a consequence, the plant Golgi apparatus is faced with the problem of recognizing proteins destined for lytic and storage vacuoles and segregating them individually from the flow of secretory proteins to the cell surface. In contrast to acid hydrolases, which are sorted by BP-80-like receptors at the *trans*-Golgi of plant cells, the identification of receptors for storage proteins has in many ways resembled 'the search for the Holy Grail'. There are several candidates for storage protein receptors, but in no single case is the evidence entirely convincing. Much of the problem lies in the lack of consensus, sorting sequences in the proteins investigated. Other difficulties stem from 'out-of-context' heterologous expression studies. Evidence is now accumulating for the participation of hydrophobic sequences in inducing the formation of protein aggregates in the early Golgi apparatus, for which classical sorting receptors do not appear to be necessary. This review critically examines the current situation and contrasts the differences between data obtained *in situ* and data obtained transgenically. It highlights the so-called 'dense-vesicle' pathway and culminates with a discussion on the hitherto neglected problem of the intracellular transport of storage protein processing enzymes.

Key words: BP-80, *cis*-Golgi sorting, clathrin-coated vesicles, dense vesicles, hydrophobic sequences, lytic vacuoles, protein-storage vacuoles, RMR, storage globulins, vacuolar sorting sequences/receptors

List of abbreviations and glossary of special botanical terms:
aleurone – a secretory tissue surrounding the endosperm and embryo in germinating cereal grains; apoplast – the space outside of the plasma membrane which includes the cell wall; BP-80 – binding protein of 80 kDa; cotyledons – embryonic leaves in the seed representing the principal storage tissue in legumes; crystalloid – a crystalline protein inclusion in some protein bodies; ELP – epidermal growth factor-like protein; endosperm – a triploid storage tissue in seeds which is used up during germination; leaf mesophyll – the major photosynthetic tissue in leaves; RMR – receptor-like protein of the ReMemBR-H₂ protein family.

Received 17 February 2005, revised and accepted for
publication 2 May 2005, published on-line 2 June 2005

Through the course of evolution, higher plants have developed the ability to store copious amounts of proteins in a stable form in their seeds. These act as a source of amino acids for various synthetic activities during germination, but also represent immensely important nutritional sources for humans and ruminants. Storage proteins accumulate in specialized vacuoles, protein storage vacuoles (PSV), which are intrinsically different from lytic-type vacuoles (LV). During seed development, especially in many legumes, both PSV and LSV can exist side-by-side, increasing the complexity of protein sorting within the secretory pathway of these cells (1). Although considerable progress has been made in understanding the sorting machinery required for successful targeting to LV, the corresponding mechanisms responsible for transport to the PSV remain unclear.

Protein Transport to Lytic Compartments: A Short Discourse

In mammalian cells, sorting of acid hydrolases destined for the lysosome is facilitated by the mannose-6-phosphate receptor (MPR) (2). Mannose-6-phosphate receptor–ligand complexes are recruited into CCV at the *trans*-Golgi network (TGN), a process which is mediated by ADP-ribosylation factor (ARF), the monomeric GGAs (Golgi-localized, γ -ear containing ARF-binding proteins), and the tetrameric AP1 adaptor complex (3). This involves interactions with tyrosine (YXX \emptyset) and dileucine (LL) motifs in the cytosolic tail of the MPR (2). There are also cell-type-specific transport pathways to the lysosome or lysosome-related organelles which are independent of the MPR (4). Saposins, which are involved in the degradation of glucosylceramide lipids, are sorted instead by sortilin (5,6). This is a type I membrane protein with high homology to the yeast vacuolar sorting receptor (VSR) Vsp10p (7), but with no clear homologs in the plant databases.

In yeast, sorting and delivery to the lytic vacuole is very similar to the MPR pathway of mammalian cells, in that it is clathrin-dependent and involves an interaction between the cytoplasmic tail of Vps10p, the carboxypeptidase sorting receptor and an AP1 adaptor complex (8), as well as GGAs (9).

Proteins targeted to the LV of plant cells require sequence-specific vacuolar sorting determinants (ssVSD), most commonly of the NPIR type which are usually (but not always) located toward the N-terminus (10). These are recognized by members of the BP-80 family of VSRs (11). The luminal domain of pea BP-80 has two binding sites which recognize two different ligands (12). The NPIR-binding site is situated within the N-terminal (two-third portion) and binds with high affinity to the NPIR ligand (11). The other (non-NPIR-specific) binding site is comprised of the central domain and the three epidermal growth factor domains preceding the transmembrane helix. The binding affinity is rather low in the μM range (11). The NPIR-binding site has been recognized as a protease-associated (PA) domain, which is also present in the transferrin receptor and a number of proteases (13). The PA domain appears to constitute a protein-protein interaction domain in addition to mediating substrate recognition for peptidases.

BP-80 not only recognizes ssVSD in the N-terminus, but also in the C-terminus of some proteins (e.g. in 2S albumin) (12,14), as well as internally located VSD (e.g. in ricin) (15,16). PV72, a closely related VSR in developing pumpkin seeds, also recognizes sorting signals situated within the N- and C-termini (17). Interestingly, however, the BP-80 family of VSRs, do not recognize the sorting motifs located within the C-terminus of barley lectin and tobacco chitinase (18,19,20). Conclusive evidence for the role of BP-80 and its homologs in the sorting of acid hydrolases comes from transgenic expression studies in yeast (21), and, more recently in *Arabidopsis thaliana* (22) where the expression of a VSR-HDEL construct leads to cargo retention in the endoplasmic reticulum (ER).

Although plant VSRs have no homology to the MPRs or Vps10p, they are concentrated in clathrin-coated vesicles (CCV) (23,24). However, whereas the cytosolic domain of VSR-PS1 from peas does not possess a dileucine motif, it does have a tyrosine-based sorting motif, YMPL, and this has recently been shown to interact with μA -adaptin, one of the five μ -adaptins from *Arabidopsis* (25). As this adaptin is part of an AP1-like complex and localizes to the TGN (25), it indicates that the basic mechanism for the sorting of acid hydrolases in the secretory pathway is conserved between plants, fungi and mammals.

Receptor-mediated traffic to the lytic compartment in yeast and mammalian cells entails the dissociation of the ligand from the receptor in a low pH-intermediate organelle and subsequent recycling of the receptor back to the Golgi apparatus (26,27,28). This multivesicular prevacuolar compartment (PVC) is therefore characterized by the presence of the transport receptor, a feature that has recently also been demonstrated in tobacco BY-2 cells for VSR-At1 (29). Having said this, it must be pointed out that in yeast and mammalian cells, there is also a direct route to the vacuole/lysosome, which bypasses the PVC. This pathway

involves a different adaptor complex (AP3), which does not associate with clathrin. In mammals, both dileucine as well as tyrosine motifs are recognized by AP3, whereas in yeast only dileucine signals are involved (30). It is not yet known whether a non-clathrin AP3-based trafficking pathway to the vacuole exists in plant cells.

Protein Storage Vacuoles: A Different Kind of Vacuole

The classic cell biology textbook puts a single 'vacuole' in all eukaryotic cell types, but this is an oversimplification not held up by recent research. First of all, many different cell types in the immune system possess a lysosome which also contains secretory proteins that can be released to the cell exterior by regulated exocytosis (31). In some cells, e.g. platelets, neutrophils and melanosomes, there is good evidence that such hybrid organelles co-exist in the same cell with conventional lysosomes (32,33). Conversely, under some conditions, mammalian cells can store large amounts of the proform of a lysosomal enzyme (the cysteine protease cathepsin L) in a multivesicular endosome, which is distinct from the lysosome containing the active form of this enzyme (34). It is now recognized that plant cells may also contain several, functionally distinct vacuolar compartments (35). These vacuoles differ in terms of their luminal contents and processing enzymes (36), as well as on the basis of the type of integral proteins in their membranes (tonoplast intrinsic proteins, TIPs) (37).

The plant PSV contains lectins, albumins, globulins and defense proteins such as the toxin ricin in castor beans. Protein-storage vacuoles are normally found in the cotyledons, aleurone or endosperm of seeds, where they form *de novo* during seed development (38,39), but the presence of small PSVs has also now been established for typically vegetative tissues, e.g. root tips (40) and leaf mesophyll (41,42). In contrast to the very acidic LV, it has been claimed that the pH of the PSV would appear to be closer to neutral (41,43). However, in developing pumpkin seed cotyledons, the optimum pH for the enzymes responsible for the processing of 11S proglubulins is pH 5 (44). Therefore, it has been claimed that the pH in developing cotyledons is slightly acidic.

Sorting Determinants in Proteins of the PSV

In contrast to acid hydrolases of the LV, there appears to be no consensus-sorting motif for proteins of the PSV, and no general location for the sorting sequences which have been elucidated (10) (Table 1). Although some vicilins possess NPIR-like sequences within the N-terminus, e.g. -NPFYFNS- in phaseolin and -NPFYFRS- in conglycinin, such sequences are absent from the 11S pea legumin (45). NPIR-related sequences have also been shown to participate in the sorting of some storage proteins, e.g.

Table 1: Sorting determinants in proteins of the plant seed storage vacuole

Protein	Sequence	Location and type		System and tissue	Reference
Ricin <i>Ricinus communis</i>	SLLIRPVVPNFN	Internal, ssVSD	Necessary and sufficient	Heterologous leaf	15
2S albumin <i>R. communis</i>	STGEEVLRMPGDEN	Internal, ssVSS	Necessary and sufficient	Heterologous leaf	14
Phytohemagglutinin <i>Phaseolus vulgaris</i>	Long (32 AA) loop	Internal, psVSD	Necessary and sufficient	Heterologous leaf	53
B-type legumin <i>Vicia faba</i>	Multiple segments of the α -chain	Internal, psVSD	Necessary and sufficient	Heterologous seed	45
Phaseolin <i>P. vulgaris</i>	AFVY	C-terminal, ct-VSD	Necessary and sufficient	Heterologous leaf	51
α' subunit of β -conglycinin <i>Glycine max</i>	PLSSILRAFY	C-terminal, ct-VSD	Necessary and sufficient	Homologous seed	52
β -subunit of β -conglycinin <i>G. max</i>	PFPSILGALY	C-terminal	Necessary and sufficient	Heterologous seed	99
2S albumin <i>Bertholletica excelsa</i>	16 AA long C-terminal stretch including IAGF	C-terminal, ctVSD	Necessary and sufficient	Heterologous leaf	49
2S albumin <i>C. maxima</i>	KARNLPSMCGIRPQRCDF	C-terminal, ssVSD	Sufficient	Heterologous BY2 cells	98
Lectin <i>Hordeum vulgare</i>	VFAEIAIANSTLVAE	C-terminal, ctVSD	Necessary and sufficient	Heterologous leaf	97

N-X-I and LRMP behind the N-termini of sweet potato sporamin and castor bean 2S albumin, respectively (14,46,47), as well as the internally located IRPV of castor bean ricin (48). Interestingly, the N-terminal located VSD of sporamin is also effective when transposed to the C-terminus of this protein (47). Similarly, the IRPV of ricin is capable of directing a secretory reporter protein to the vacuole, when attached either to the C-terminus or inserted behind the signal peptide at the N-terminus (16). However, a mutation of this sequence leading to the secretion of a reporter was ineffective when the mutated signal was transferred to the C-terminus. This indicates that the position of NPIR-like VSD is important but not crucial for successful targeting to the PSV.

Some sorting determinants are located at the C-terminus. In brazil nut 2S albumin for example, the last 16 AA including the C-terminal IAGF sequence is responsible for targeting to the vacuole (49). Similarly, the C-terminus of barley lectin is also important for its targeting to the vacuole with the two stretches of amino acids FAEAL and LVAE being chiefly responsible for this (50). Two further examples for C-terminal located VSD are AFVY in the common bean 7S globulin phaseolin (51) and SILRAVY in the soybean globulin β -conglycinin alpha subunit (52). The C-terminal tetrapeptide AFVY of phaseolin causes the secretory form of GFP to be redirected to the vacuole in tobacco and *Arabidopsis* leaf protoplasts (15). It would then seem that AFVY is indeed a true VSD for storage proteins. However, a search in the *Arabidopsis* database reveals that 93 protein accessions carry a AFVY motif, but

none of these proteins are designated as storage proteins, and none of them have this motif at their C- or N-terminus!

Besides these defined VSDs, there are also internal or protein structure-related signals, as is the case for the common bean lectin phytohemagglutinin where a longer, internal domain of 30 AA forming an exposed loop at the surface is necessary for proper sorting (53). A more extreme example is that of the 11S globulin legumin from pea which even needs the presence of the complete α -chain for proper sorting to the vacuole (45).

It is difficult to judge the true value of the above observations, because most of them have been obtained in 'out-of-context' situations. By this we mean that, with notable exceptions, nearly all of the pertinent data were obtained by transient expression in tobacco mesophyll or BY2 cells which do not have a prominent PSV. Nevertheless, it must be said that the Golgi apparatus in these cells is able to discriminate between two vacuolar compartments: GFP constructs containing the aleurain NPIR signal were targeted to the lytic vacuole and GFP constructs containing the C-terminal motif of tobacco chitinase were targeted into small neutral (putative storage) vacuoles (41).

Sorting of Storage Proteins: Tissue/Cell Specificity and Heterologous Expression

One aspect of storage protein sorting that is often neglected in discussions on sorting determinants and

possible receptors is tissue specificity. It is possible that sequences in a storage protein are recognized in the transgenic situation, which plays no role in the native, *in vivo* situation and *vice versa*. Similar problems have been encountered when plant proteins are expressed in yeast (53,54,55). There are also examples where plant storage proteins are correctly targeted to the vacuole in one part of the plant, but secreted into the cell wall in others. Thus, when expressed in roots, the lectin phytohemagglutinin E is localized to the vacuole in regions immediately distal and proximal to the root meristem of bean seedlings, but is deposited into the cell wall in the elongation zone and beyond (56). Another example which points to caution is the case of the diagnostic vacuolar proteins, the TIPs. Whereas these intrinsic membrane proteins are sorted along the same route as storage proteins in seeds (16,57), they seem to take different routes when expressed in non-storage tissues (58,59).

A most interesting case is that of vacuolar phytase from *Aspergillus niger*. When expressed with a signal peptide in tobacco and rice, this enzyme is secreted into the apoplast in leaves, but is either retained in ER-derived protein bodies or deposited in the PSV in the rice endosperm (60; Stoeger et al., personal communication). Interestingly, the glycosylation pattern of phytase corresponds to its final location: the secreted form bearing secretion-type glycans and the vacuolar form having typical vacuolar glycans. This indicates that when exported out of the ER, phytase in the transgenic situation always moves through the Golgi apparatus, but the capacity to actively sort into the PSV is restricted to the Golgi apparatus in seed tissue. One might therefore summarize that only this particular type of Golgi possesses the necessary sorting machinery. However, it may also be a question of the other cargo molecules present: if aggregation-based sorting is already in operation as discussed below, e.g. in the cereal endosperm Golgi apparatus, the phytase reporter protein might well coaggregate. Thus, the successful sorting of a storage protein into an undefined vacuolar compartment in cells that do not normally store proteins may be irrelevant to the true situation in storage tissue, and may lead to the identification of spurious targeting signals. One might also question the validity of comparing data obtained with vegetative and seed-storage proteins in the transgenic context, as e.g. sporamin is a protein deposited in a LV in vegetative storage tissue. One could therefore argue that in terms of targeting, sporamin is an LV- rather than PSV-type protein.

The usefulness of heterologous expression studies in the determination of sorting domains or identification of putative receptors has also been questioned in the animal literature because of the dangers of failing to recognize targeting signals/receptors in an inappropriate tissue. A good example for cell-specific sorting events in mammals is the transport of chromogranin A, a regulated secretory

protein. Chromogranin A contains two sorting motifs which are utilized independently when expressed in different cell types (61). A second example is cathepsin L, a lysosomal protease with an intrinsic M6P-sorting signal (62). Depending on the cell type, cathepsin L may become either sorted into the lysosome (63), secreted (62,64) or targeted via 'dense core vesicles' into multivesicular endosomes where it is stored (65,66). Secretion of cathepsin L can be induced by upregulating its expression after transformation of normal cells into tumor cells. Targeting and endosomal storage seems to be due to the activity of an alternative, dense vesicle (DV)-mediated, MPR-independent pathway. It is clear that this novel sorting mechanism would not have been recognized in heterologous expression studies involving cells of another type or physiological status.

Dense Vesicles: A Transport Vesicle for Storage Proteins that is Unique to Plants?

In contrast to the prolamins of many cereal grains which accumulate in ER-derived protein bodies, legume storage proteins (globulins) pass through the Golgi apparatus before being deposited in the PSV (67,68). Previous work in our group has established that proteins of the PSV in developing pea cotyledons exit the Golgi at the TGN via DV (Figure 1A) (57,67,69,70). Dense vesicles form initially at the periphery of the first *cis*-cisterna and appear to be carried to the *trans*-face of the Golgi stack via cisternal progression (Figure 1B) (69). Dense vesicles are not COP-coated vesicles (69), but are frequently seen to bud CCV at their surface (1,67).

Dense vesicles received their name on the basis of their electron-opaque, osmiophilic contents which represent highly condensed storage globulins (57). In Golgi stacks of developing pea cotyledons, storage globulins are present in a non-aggregated form only in the *cis*-most cisternae (69). In the other cisternae, the globulins are aggregated and restricted to DV at the rims of the cisternae. This indicates that these proteins are separated from other proteins in the *cis*-cisternae and rapidly transported to the periphery of the cisternae where their accumulation and condensation leads to the formation of DV. Thus, the sorting of globulins seems to precede their aggregation, but in rapid procession and within the same cisterna. It also means that aggregation and DV formation are tightly coupled. On the other hand, as mature DVs also contain complex glycoproteins whose oligosaccharide processing occurs later in the stack, protein sorting into DV in general is not restricted to the *cis*-cisternae, but is obviously an ongoing process throughout the stack as the DV mature and proceed toward the TGN (67,69). Thus, although DVs superficially resemble the dense-core granules recorded many times in the animal literature (34,71), the sorting/aggregation process is initiated much earlier in the Golgi apparatus. If receptors are required for this event, the

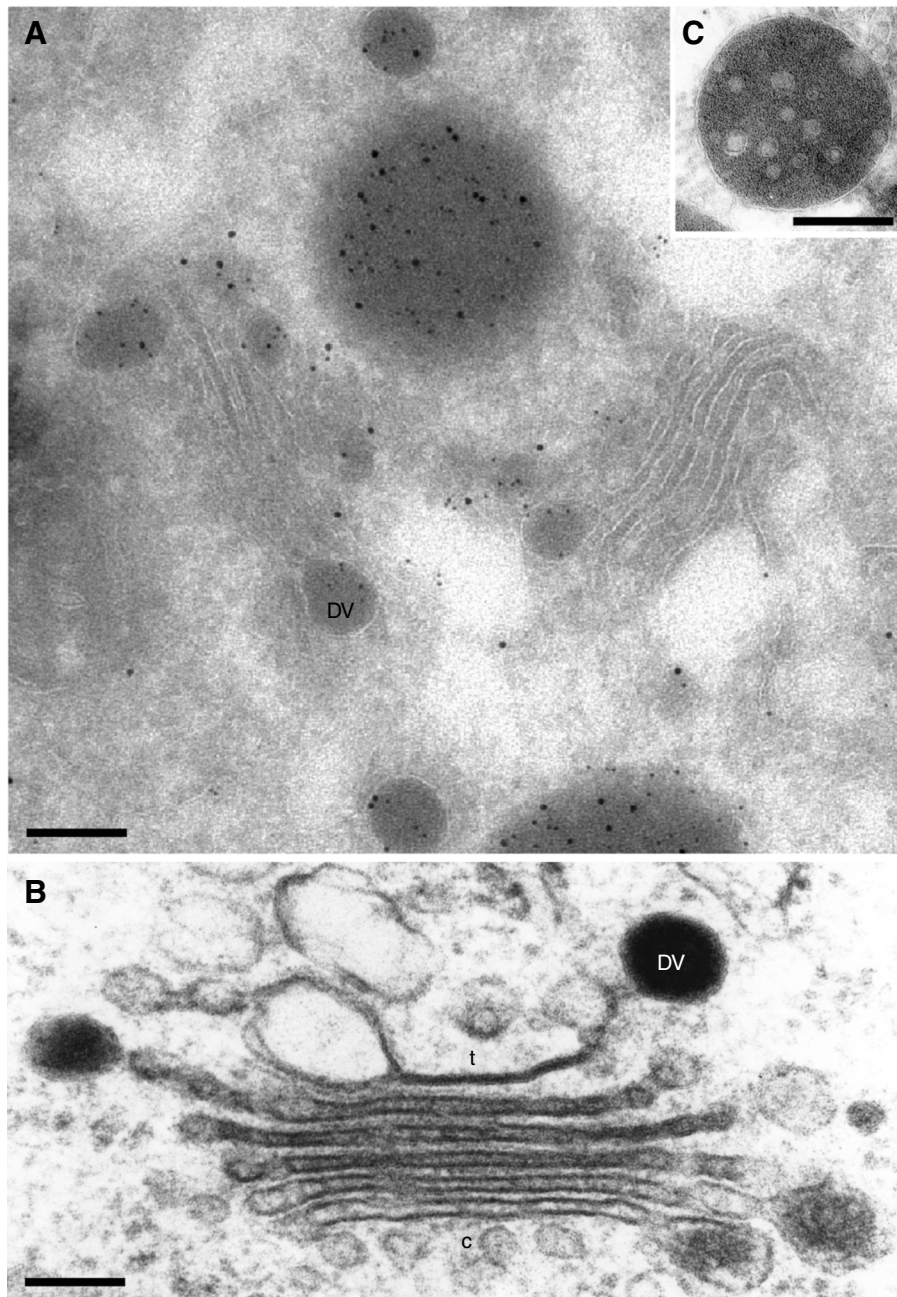


Figure 1: The Golgi apparatus and prevacuolar compartment in developing pea (*Pisum sativum* L.) cotyledons. A) Cryosection revealing Golgi stacks and dense vesicles (DV). Double immunolabeling with legumin (5 nm gold) and sucrose-binding protein (10 nm gold) antisera. Note the peripheral distribution of the sucrose-binding protein in the DV. Bar = 200 nm. B) Median section through a pea cotyledon Golgi stack revealing a progressive *cis* – *trans* osmophilicity in immature DV at the rims of the cisternae. Conventional fixation. Bar = 150 nm. C) Cryosection revealing a multivesicular body (MVB). Bar = 400 nm.

question then arises as to how they are retained in and respectively recycled back to the *cis*-cisterna as the individual DV-bearing cisternae are shunted upwards through the stack?

How can aggregation mediate sorting into a DV? On the basis of morphological observations, it has been suggested that DV formation in plants resembles the formation of dense-core/immature secretory granules (ISG) at the *trans*-Golgi of cells in mammalian glands (1,57,69). The most obvious similarities are the highly aggregated state of the cargo and the formation of CCV at the surface

before release from the Golgi stack (1,67,72,73). Currently, there are two models for the formation of ISG (74,75). In the 'sorting for entry model', secretory proteins are actively sorted into the ISG through an interaction with certain receptor proteins in the TGN. By contrast, in the so-called 'sorting by retention' model, a significant portion of the volume of the TGN is transformed into the ISG, which still contains lysosome-destined proteins. The latter are later removed by MPR in the membrane of the ISG and collected into CCV, whereas the secretory proteins are held back by virtue of their highly aggregated state (76). According to this model, the secretory granule is

nothing, but a transformed TGN out of which all non-regulated secretory proteins are removed by successive receptor-mediated sorting steps.

On the basis of observation that a transgenically expressed constitutive secretory protein was excluded from secretory granules (77), it is possible that regulated secretory proteins may contain some specific sorting information for entry into the ISG. On the other hand, a secretory GFP construct bearing only a signal peptide was found to be entrapped into secretory granules. However, the latter result may have arisen out of GFP's ability itself to form cross-linked polymers (78). Together, these results indicate that if regulated secretory proteins carry positive sorting signals they do not constitute an absolute requirement, but only improve the efficiency of the sorting process (75).

Aggregation plays a crucial role in the sorting of a number of regulated secretory proteins, e.g. chromogranins, porphyrin, zymogens, neural peptides and insulin (74,79). It is also heavily supported by studies on the sorting of procathepsin L. Cathepsin L is a lysosomal hydrolase which is normally sorted as a proform via the MPR-dependent pathway into the lysosome. However, when the synthesis of procathepsin L is upregulated, it is targeted to small dense-core vesicles and is eventually secreted (34,65,66). In these vesicles, procathepsin is present in a highly aggregated oligomeric form. Because the procathepsin-secreting cells still express MPR, it has been suggested that the procathepsin aggregates have a limited number of

MPR-binding sites, thus reducing their chances of interacting with the receptor (34).

Aggregation-based sorting is known to be facilitated by factors which enhance the condensation process *per se*, e.g. ions (zinc and calcium), low pH and proteins which support aggregation, so-called assembly factors (34,76,79,80), as well as by proteins which might act like chaperones (81). However, the attachment of the aggregates to the membrane of the granule is also important, and may start before the condensation process is initiated at the TGN (34). In this regard, the aggregated secretory proteins in the ISG of the rat pancreas are surrounded by a proteoglycan matrix which is linked to certain lectins. These lectins appear to recruit the condensing secretory proteins (82). Interestingly, there is also a stratification of proteins within the pea cotyledon DV (70). Furthermore, the binding of storage proteins to the membranes in an enriched Golgi fraction appears to be mediated by certain peripheral membrane proteins (von Lüpke and Hinz, unpublished results). Thus, a scenario similar to that occurring in the ISG can well be envisaged for the developing DV in pea cotyledons (Figure 2).

Candidate Sorting Receptors for Storage Proteins

VSR-At1

It is known that there are seven isoforms of BP-80 in the *Arabidopsis* genome, and closely related proteins in other

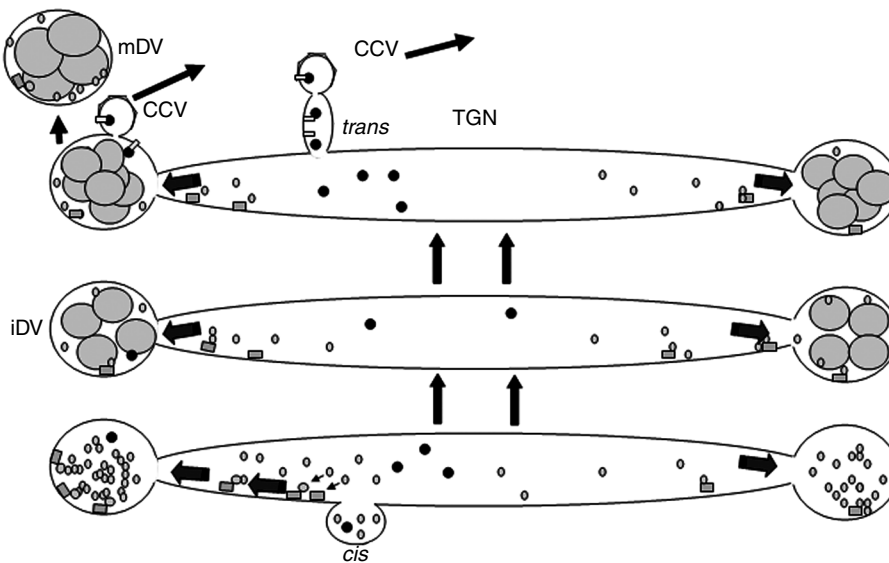


Figure 2: Proposal for a *cis*-Golgi-based sorting mechanism for globulin-type storage proteins. Upon delivering to the Golgi apparatus via putative COP II-vesicles, the globulins are recognized by an 'unconventional type of receptor protein linker' situated at the luminal surface of the first or second *cis*-cisternae. The receptor-cargo complexes are then transported laterally into the forming dense vesicles (DV) at the periphery of the cisternae, where neighboring cargo molecules interact with one another via externally exposed hydrophobic sequences. These act as nucleation centers for the subsequent aggregation of incoming globulins. Condensation of the globulin aggregates in the DV continues as the cisternae move in a *trans*-direction. Missorted acid hydrolases are postulated to be recovered from mature DV at the *trans*-Golgi network through the formation of clathrin-coated vesicles.

plants (83). BP-80 possesses a low affinity for storage proteins (12), and virtually, all of the previously published data on BP-80 itself, or on AtELP, has been strongly indicative of the participation of these two receptors in targeting to LV, rather than PSV (84). However, the recent demonstration of Shimada et al. (85) that a knock-out mutant of VSR-At1 (=AtELP) leads to partial missorting of a 12S storage globulin (cruciferin) in developing *Arabidopsis* cotyledons necessitates renewed thought about the exact role of this family of receptors in vacuolar protein sorting. In this investigation, cruciferin was only partially secreted, with the rest correctly targeted into the PSV. This is not the expected phenotype if AtELPs were essential for the sorting of cruciferin to the PSV; it is more like a situation where the vacuolar sorting machinery had gradually become saturated. Nevertheless, further, albeit indirect support for the notion that a BP-80/AtELP-like VSR may be involved in sorting to the PSV has come from recent experiments on the intracellular transport of ricin and 2S albumin in castor bean endosperm (16). Both of these proteins traverse the Golgi apparatus, and both bind to AtELP *in vitro*. Thus, it has in our opinion not yet been unequivocally demonstrated that AtELP is a receptor for storage protein sorting, and it is certainly premature to contemplate a dual role for AtELP in targeting to LV and PSV.

In developing pea cotyledons, BP-80 itself is present in the Golgi stack but is not present in mature DV (57). However, we cannot rule out the presence of related BP-80 family members in immature DV. In analogy to mammalian cells which produce secretory granules, such receptors in DV may be necessary for the retrieval of missorted acid hydrolases (73,86). In this respect, the formation of CCV at the surface of TGN-attached DV (67) closely resembles the situation seen in the immature secretory granule (86). Osmiophilic vesicles attached to the rims of Golgi cisternae have been reported in developing *Arabidopsis* cotyledons (87), but evidence that these DV actually carry the 11S globulin cruciferin is at present lacking. However, for the reasons just given, the direct *in situ* demonstration of VSR-At1 in Golgi-associated, cruciferin-containing DVs in *Arabidopsis* cotyledons would not necessarily be in support of the idea that the VSR-At1 receptor participates in the sorting of globulins.

RMR

In their study on the biogenesis of the crystalloid in the PSV of tobacco seeds, Jiang and coworkers (88) discovered a novel transmembrane protein of the PSV. This protein, termed RMR, has a 50 amino acid cytosolic tail, which bears a RING-H₂ domain. In addition, it has a luminal domain homologous to the domain of BP-80, which is responsible for ligand binding. Proteins containing the RING-H₂ domain are involved in membrane traffic to the vacuole in yeast (89). RMR is known to bind to the C-terminus of barley lectin, but not the N-terminal NPIR signal of aleurain (90). Furthermore, reporter constructs

containing the transmembrane domain of RMR and its cytoplasmic domain traffic through the Golgi to the vacuole in transgenic tobacco cells (88). Therefore, it is not unreasonable to think that RMR might play a role in protein transport to the PSV.

Recently, Hwang and colleagues (personal communication) have expressed HA-tagged RMR in *Arabidopsis* protoplasts transiently expressing phaseolin. They have found that the majority of the RMR is localized to a putative PVC, with a smaller amount present in the Golgi apparatus. Expression of various mutant RMR forms leads to an accumulation of phaseolin in the Golgi apparatus, but has no effect on the transport of sporamin:GFP or AALP:GFP to the LV. Another piece of evidence in favor of RMR as a storage protein receptor is the demonstration that RMR and phaseolin coimmunoprecipitate, but not when the C-terminal AFVY motif is deleted from phaseolin. As RMR is endogenously expressed in various tissues in tobacco (88) and *Arabidopsis*, the question is now what its native ligand(s) might be, and whether they might differ between storage and vegetative tissues? Nevertheless, as Jiang et al. (88) themselves have pointed out – the fact that RMR is concentrated in the vacuole membrane and in the PSV crystalloid strongly suggests that this molecule does not recycle to the Golgi apparatus from the PVC like the MPR, Vps10p- or BP-80-type VSRs.

Sorting into the Dense Vesicle Pathway: A Role for Hydrophobic Sequences?

Several independent observations strengthen the hypothesis that aggregation is an integral part of the sorting process for seed globulins. Prolegumin in peas is tightly bound to the membrane of the DV, forming detergent-resistant aggregates (70,90), and shows a higher degree of aggregation as compared to prolegumin from isolated ER/Golgi vesicles (57). Correspondingly, DV attached to the Golgi stack reveal an increase in electron opacity from the *cis* to the *trans* side of the stack, indicating a gradual filling of nascent DV with condensing cargo (91). When expressed in transgenic tobacco, newly synthesized bean phaseolin is also strongly bound to membranes in ER/Golgi fractions and even forms SDS-resistant aggregates (92). Interestingly, a mutant form of phaseolin lacking the C-terminal AFVY motif, does not produce such aggregates and is transported to the cell wall rather than to the vacuole (92).

It is important to note that globulin aggregation and as a consequence the physical sorting of these proteins from the rest of the flow of secretory proteins is an early event in the endomembrane system. Hillmer et al. (69) demonstrated by immunogold electron microscopy that this process is initiated in the periphery of the very first *cis*-cisterna of the Golgi apparatus in developing pea

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CAA21203gammaVPE  MATMTTRVSVGVVFLVLLVSLVAVSAARSGPDDVILKLP[SQASRFFRPAENDDDDNSNGTRW 60
AAC31241          ----MTTVVSVFLALFLFLV-----AAVSG--DVIKLP[SLASKFFRPTENDDDD---TKW 45
S49175sv         --MGSSQLSTLLFFTIIVVFTLVVSSGRDLPGDYLRL[SETSRFFREPKNDDDFEG-TRW 57
AAF19550         ----MAKSCYFRPALLLLVLVHVAESRGRFEPK[ILMPTEEAN---PADQDEGGV-TRW 52
AAM60827         ----MSSPLGHFQILVFLHALLIFSAESRK--TQLLNDNDVE----SSDKSAK-TRW 47
BAB01880         ----MSSPLGHFQILVFLHALLIFSAESRK--TQLLNDNDVE----SSDKSAK-TRW 47
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CAA21203gammaVPE  AVLVAGSSGYWNYRHQADI CHAYQLLRKGGGLKEENIVVFMYYDDIANNYENPRPGTI INSP 120
AAC31241         AVLVAGSSGYWNYRHQADV CHAYQLLRKGGGKKEENIVVFMYYDDIAKNEENPRPGVI INSP 105
S49175sv         AILLAGSNYWNRYRHQSDV CHAYQLLRKGGGKKEENIVVFMYYDDIASNEENPRPGVI INKP 117
AAF19550         AVLVAGSSGYGNRYRHQADV CHAYQILRKGGLKEENIVVLMYYDDIANHPLNPRPGTLINHP 112
AAM60827         AVLVAGSNEYNYRHQADI CHAYQILRKGGLKDENIVVFMYYDDIAFSSENPRPGVI INKP 107
BAB01880         AVLVAGSNEYNYRHQADI CHAYQILRKGGLKDENIVVFMYYDDIAFSSENPRPGVI INKP 107
          * : * : * * * * * : * : * : * : * : * * * * * * * * * * * * * * * * * * * * * * *

CAA21203gammaVPE  HGKDVYQGVV----- 130
AAC31241         NGEDVYNGV----- 115
S49175sv         DGDDVYAGV----- 127
AAF19550         DGDDVYAGVPKALHNNYSDSDCRDI CYGKPNFMCQPF IGIAPRFLIATICSVIYVLKYL 172
AAM60827         DGEDVYKGV----- 117
BAB01880         DGEDVYKGV----- 117
          . * . * * * * *

CAA21203gammaVPE  KDYTGDDVNDVNLFAVILGDKTAVKGGSGKVVDSDGPNDFI FIFYSDHGGPGVLGMPTSPY 190
AAC31241         KDYTGDEVNDVNDLAVILGNKTKALKGGSGKVVDSDGPNDFI FIIYSDHGGPGVLGMPTSPN 175
S49175sv         KDYTGAEVHADNFYALLGKNSALTGGSGKVVDSDGPNDFI FVYITDHHGGPGVLGMVPGPY 187
AAF19550         QDYTGSSVTAANFYAVLLGDQKAVKGGSGKVIASKPNDFI FVYIADHGGPGVLGMPTPH 232
AAM60827         KDYTKAVNVNQFNYNVLLGNESGVTGGNGKVVKSGPNDFI FIIYADHGAPGLIAMPTGDE 177
BAB01880         KDYTKAVNVNQFNYNVLLGNESGVTGGNGKVVKSGPNDFI FIIYADHGAPGLIAMPTGDE 177
          : * * * * * * * * * * * : : * * * * * * * * * * * * * * * * * * * * * * *

CAA21203gammaVPE  LYANDLNDVLLKHKHALGTYKSLVVFYLEACESGSI FEGLLPEGLNIYATTASNAEESSWGT 250
AAC31241         LYANDLNDVLLKKYASGTYKSLVVFYLEACESGSI FEGLLPEGLNIYATTASNAEESSWGT 235
S49175sv         LYANDLNEVLLKHKHASGTYKSLVVFYLEACESGSI FEGLLPDDLNIYATTASNAEESSWGY 247
AAF19550         IYAADF IETLKKHKHASGTYKEMVIYVEACESGSI FEGIMPKDLNIYVTTASNAQESSYGT 292
AAM60827         VMAKDFNEVLEKMHKRIYNKMVIYVEACESGSMFEGILKKNLNIYAVTAANSKESSWGV 237
BAB01880         VMAKDFNEVLEKMHKRIYNKMVIYVEACESGSMFEGILKKNLNIYAVTAANSKESSWGV 237
          : * * * * * : : * * * * * * * * * * * * * * * * * * * * * * *

CAA21203gammaVPE  YCPGEEPPSPPEYETCLGDLYSVAWMEDSGMHNLTQETLHQQYELVKKRTAPV-GYSYGS 309
AAC31241         YCPGEDPSPPEYETCLGDLYSVAWIEDSEKHNLTQETLHQQYELVKKRTAGS-GKSYGS 294
S49175sv         YCPGDKPSPPEYETCLGDLYSVAWEDSEVHNLTQETLHQQYKLVKRNITIS--EPYGS 304
AAF19550         YCPGMNPPSPPEYETCLGDLYSVAWMEDSETHNLTQETLHQQYVTKMRTSNYNTYSGGS 352
AAM60827         YCPESYPPSPPEYETCLGDLYSVAWEDSETHNLTQETLHQQYVHVVKRRVGS--VPETS 295
BAB01880         YCPESYPPSPPEYETCLGDLYSVAWEDSETHNLTQETLHQQYVHVVKRRVGS--VPETS 295
          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

CAA21203gammaVPE  HVMQYGDVIGISKDNLDMGTNPANDNFTFADANS---LKP[SRVTNQRDADLVHFWEK 365
AAC31241         HVMEFGDIGLSKEKLVLFMGTNPADENFTFVNENS---IRPP[SRVTNQRDADLVHFVHK 350
S49175sv         HVMEYGDIGLSKNDLYQYLGTPANDNNSFVDETENSLKLRTPSAAVNQRDADLVHFWEK 364
AAF19550         HVMEYGNNSIKSEKLYLQGFDPATVNLPLN-ELP---VKSKI[GVVNQRDADLVLFWHM 407
AAM60827         HVCRF[TEKMLKDYLSYI[GRNPENDNFTFTESFSS--PISNSGLVNP[DIPLLYLQ[RK 352
BAB01880         HVCRF[TEKMLKDYLSYI[GRNPENDNFTFTESFSS--PISNSGLVNP[DIPLLYLQ[RK 352
          * * . * * : : * * * * * * * * * * * * * * * * * * * * * * *

CAA21203gammaVPE  YRKAPEGSARKTEAQKQVLEAMSHRHLIDNSVILVKGILFGISRGPEVLNKRVSAGQPLV 425
AAC31241         YQKAPEGSARKVEAQKQVLEAMSHRHLVDNSILLIGILFGL-EGHAVLNKVRPSGPELV 409
S49175sv         FRKAPEGSSQKNEAEKQVLEAMSHRKHIDNSVKLIGQLLFGIEKGT[ELD[VVRPAGSPLV 424
AAF19550         YRTSEDDGSRKDDTLKELTETTRHRKHLDAVELIATILFPG--TMNVLN[VREPLPLV 465
AAM60827         IQKAPMGLESKEAQKLLDEKNHRKQIDQ[ITDILRLSVKQTNVNLNLTSTRTTGQPLV 412
BAB01880         IQKAPMGLESKEAQKLLDEKNHRKQIDQ[ITDILRLSVKQTNVNLNLTSTRTTGQPLV 412
          : : * * . : : * : : * * * * * : : . * * * * * * * * * * * * *

CAA21203gammaVPE  DDWNLK[NOVRAFERHCGSL[SYGIKHMR[SFANICNAGIQMEQMEEAASQACTTLP[TPGW 485
AAC31241         DDWDLK[SLVRAFERHCGSL[SYGIKHMR[SIANMCNAGIQMRQMEEAAMQACTTIP[TPSPW 469
S49175sv         DNWDCLK[TMVKTFFETHCGSL[SYGKMHMR[SFANICNAGIPNEPMAEASAQACASIPANPW 484
AAF19550         DDWECLK[SMVRVFEHCGSL[TOYGMKHMRAFANVCNNGVSKELMEEA[TAACGGYSEARY 525
AAM60827         DDWCFK[TLVNSFKNHCGATVHYGLKYTGALANICNMGVDVQ[TVSAIEQACSM----- 466
BAB01880         DDWCFK[TLVNSFKNHCGATVHYGLKYTGALANICNMGVDVQ[TVSAIEQACSM----- 466
          * : * : * * * * * : * * * * * : : * * * * * * * * * * * * * * * *

CAA21203gammaVPE  SSLN--RGFSA 494
AAC31241         SSLD--RGFSA 478
S49175sv         SSLQ--GGFSA 493
AAF19550         TVHPSILGSA 536
AAM60827         -----
BAB01880         -----

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Figure 3: Sequence alignment of vacuolar legumain-like proteases. Highlighted in black are N-terminal NP[IR-like sorting determinants, and in grey a C-terminal hydrophobic type motif. Identical amino acids are indicated by an asterisk, and similar residues are marked by a colon or a dot.

cotyledons. This has recently been corroborated biochemically for bean phaseolin by Castelli and Vitale (92) who have shown that aggregation begins before glycan processing, i.e. is an event occurring upstream of the medial Golgi cisternae. Together, these data point to a spatial

segregation of sorting events for proteins destined to LV and PSV within the plant Golgi apparatus.

The ability of the C-terminal AFVY sequence of phaseolin to redirect some proteins into the vacuolar-sorting

pathway may well lie in its hydrophobicity. Interestingly, the C-terminus of soybean β -conglycinin alpha subunit also possesses a hydrophobic sequence: SILRAVY (52) and a number of the previously mentioned storage VSD also contain hydrophobic amino acids: IAGF in brazil nut 2S albumin, FAEAI and LVAE in barley lectin, and IRPV in castor bean ricin. Short hydrophobic sequences of these types are not present in pea legumin, but structural studies performed on the related trimer of bean proglycinin (an 11S globulin with 60% sequence similarity to legumin) show that this complex has a triangular shape with one side having exposed acidic, hydrophilic residues. In contrast, the other face is highly hydrophobic (93). Conform to this is the fact that pea prolegumin is much more hydrophobic as compared to the processed mature protein and that processing of the mature protein involves an combination of two trimers into a hexamer (90). Legumin may therefore constitute a case of sorting via a hydrophobic macrostructure.

Hydrophobicity by itself may not constitute a sorting signal, but by causing neighboring proteins to interact with one another, it can be an important factor in promoting aggregation. In this respect, it is interesting to note that the transport form of phaseolin is trimeric, so that there are three accessible hydrophobic motifs, one on each subunit. Holkeri and Vitale (94) have shown that a single AFVY is insufficient for the proper sorting of phaseolin, and that sorting efficiency increases with the number of hydrophobic motifs present in the trimer. This suggests that complex protein-protein or protein-membrane interactions are necessary for the sorting of these proteins.

What is the transport pathway for storage protein processing enzymes?

The prototype of the VSR BP-80 was identified in CCV which were isolated from developing pea cotyledons (18), and CCV are to be seen at the TGN in the cells of this tissue (67). As during cotyledon development the lytic compartment decreases in size whereas the PSV increases in volume (38), the question arises as to the role of CCV. Instead of transporting acid hydrolases to the lytic vacuole, it is possible that CCV carry storage protein processing enzymes? According to this scenario, the hydrolases will be recognized by the BP-80 receptor in the TGN and become packaged into CCV. Dense vesicles and CCV would then be transported separately to the PVC, whose acidic pH might then trigger the onset of storage protein processing. The only alternative is that the processing enzymes must coaggregate with their substrates in the DVs and somehow become activated in the PVC or PSV. The question is therefore: do DVs contain processing enzymes? This should be easy to answer if specific antibodies are available.

Legumin (asparagine-specific endopeptidase) proteases are an evolutionary conserved group of proteases which

cleave legumin-type storage globulins (95). A database search reveals that there are two such proteases in *Arabidopsis* with high sequence homology (69 to 70%) to the best characterized legumin of *Vicia narbonensis* (CAA21203 – At4g32940; AAC31241 – At2g25940). The first in fact corresponds to the γ VPE of *Arabidopsis thaliana* (96). Most intriguingly, a sequence alignment (Figure 3) reveals these proteins to have a NPIR/LRMP/IRPV-like sorting sequence at the N-terminus, but also a hydrophobic type motif at the C-terminus. Thus, investigating the transport of these proteins might well be a most rewarding project in itself.

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