Protoplasma (2012) 249:379–392
DOI 10.1007/s00709-011-0288-z

ORIGINAL ARTICLE

The formation, function and fate of protein storage compartments in seeds

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Received: 5 April 2011 / Accepted: 12 May 2011 / Published online: 26 May 2011
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Abstract Seed storage proteins (SSPs) have been studied for more than 250 years because of their nutritional value and their impact on the use of grain in food processing. More recently, the use of seeds for the production of recombinant proteins has rekindled interest in the behavior of SSPs and the question how they are able to accumulate as stable storage reserves. Seed cells produce vast amounts of SSPs with different subcellular destinations creating an enormous logistic challenge for the endomembrane system. Seed cells contain several different storage organelles including the complex and dynamic protein storage vacuoles (PSVs) and other protein bodies (PBs) derived from the endoplasmic reticulum (ER). Storage proteins destined for the PSV may pass through or bypass the Golgi, using different vesicles that follow different routes through the cell. In addition, trafficking may depend on the plant species, tissue and developmental stage, showing that the endomembrane system is capable of massive reorganization. Some SSPs contain sorting signals or interact with membranes or with other proteins en route in order to reach their destination. The ability of SSPs to form aggregates is particularly important in the formation or ER-derived PBs, a mechanism that occurs naturally in response to overloading with proteins that cannot be transported and that can be used to induce artificial storage bodies in vegetative tissues. In this review, we summarize recent findings that provide insight into the formation, function, and fate of storage organelles and describe tools that can be used to study them.

Keywords Seed storage protein · Protein trafficking · Vesicle · Endomembrane system · Secretory pathway · Endoplasmic reticulum · Protein accumulation

Abbreviations

At-ELP	*Arabidopsis thaliana* EGF receptor-like protein
CCV	Clathrin-coated vesicle
DIP	Dark intrinsic protein
DV	Dense vesicle
ER	Endoplasmic reticulum
ERvt	ER to vacuole trafficking
GFP	Green fluorescent protein
GPA1	G-protein alpha subunit 1
HMW	High molecular weight
i-ER	Induced ER (body)
KV	KDEL-tailed cysteine proteinase-accumulating vesicle
LV	Lytic vacuole
MBP	Maltose binding protein
MVB	Multivesicular body
PAC	Precursor-accumulating (vesicle)
PB	ER-derived protein bodies
PCD	Programmed cell death
PPI	Peptidyl-prolyl *cis–trans* isomerase
PSV	Protein storage vacuole
PVC	Prevacuolar compartment
rER	Rough endoplasmic reticulum
RMR	Receptor homology-transmembrane-RING H2

Handling Editor: David Robinson

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RNAi	RNA interference
SBP	Sucrose-binding protein
SH-EP	Sulfhydryl endopeptidase
SNARE	Soluble *N*-ethylmaleimide-sensitive-factor attachment receptor
SSP	Seed storage protein
TGN	*Trans*-Golgi network
TIP	Tonoplast intrinsic protein
VAMP	Vesicle associated membrane protein
VSR	Vacuole sorting receptor

Introduction

Plants store amino acids in the form of seed storage proteins (SSPs), which are deposited into specialized membrane-bound storage organelles that prevent their degradation (Muntz 1998). Storage proteins accumulate primarily in protein storage vacuoles (PSVs) and in protein bodies (PBs) derived from the endoplasmic reticulum (ER). Several factors help to determine the cellular compartment in which SSPs accumulate, including targeting sequences, membrane association, and aggregation (Muntz 1998). All SSPs include an N-terminal signal peptide that mediates translocation across the ER membrane into the lumen during protein synthesis on membrane-bound polysomes. The signal peptide is cleaved and the SSP is glycosylated and its folding subjected to a quality control by chaperones, and then transported to the appropriate storage compartment.

In this sense, the dynamic and pleomorphic ER (Sparkes et al. 2009) can be considered as the "gateway to the secretory pathway". The ER has a crucial role in the folding and assembly of its resident proteins, and also proteins destined for other locations (Vitale and Denecke 1999). SSPs can either bud from the ER to form ER-derived PBs, or follow one of several routes to the PSV. Some proteins travel through the Golgi en route to the PSV, while others are packed into ER-derived vesicles and bypass the Golgi complex (Hara-Nishimura et al. 1998) (Fig. 1). In this review, we consider recent studies focusing on SSPs and summarize recent developments in the understanding of seed storage organelles and SSP trafficking in the context of seed maturation.

Seed storage proteins in different plant species

SSPs have two special characteristics: first, they accumulate in massive amounts and second, their special aggregation characteristics allow efficient desiccation during seed maturation and rehydration during seed germination (Galili 2004).

Seeds generally synthesize different classes of storage proteins: prolamins, albumins, and globulins (Shewry et al. 1995; Galili 2004). The 2S albumins are widely distributed in dicotyledonous seeds. They are compact globular proteins synthesized as single precursors that are proteolytically cleaved. Prolamins are restricted to grasses and are the major protein components of most cereal grains. The prolamin superfamily of the Triticeae is classified into three groups: the S-rich, S-poor, and the high molecular weight (HMW) prolamins. Similarly, three groups of prolamins are found in rice, and four groups known as zeins (α, β, γ, and δ) are found in maize. Globulins are present not only in dicots but also in monocots and fern spores. They fall into two groups: the 7S vicilin-type and the 11S legumin-type. The 11S globulins are the major storage proteins in many dicots (especially in legumes) but are also found in some cereals. They are synthesized as precursor proteins that are proteolytically cleaved after the formation of disulfide bonds, and they are usually not glycosylated. The 7S globulins are typically trimeric proteins that undergo significant posttranslational processing (proteolysis and glycosylation). In rice, 60–80% of the total seed protein is composed of globulins known as glutelins, and 20–30% is prolamins (Kawakatsu et al. 2010). *Brachypodium distachyon* grain was recently reported to contain prolamins and globulins like other cereals, with 11S globulins as the most abundant storage proteins (Larre et al. 2010).

Seeds generally provide two classical transport routes for storage proteins. Albumins and globulins are often transported as soluble proteins from the ER via the Golgi to the vacuoles where they finally accumulate, whereas the prolamins aggregate in the ER and are generally deposited into ER-derived protein bodies (Shewry et al. 1995; Galili 2004). However, the situation has turned out to be more complex because some storage proteins are present in both ER-derived and vacuolar storage organelles (Levanony et al. 1992; Rubin et al. 1992; Reyes et al. 2011). Moreover, some ER-derived SSP structures seem to bypass the Golgi apparatus and finally deliver their cargo to the PSV (Levanony et al. 1992; Hara-Nishimura et al. 1998). The flexibility of this system is also revealed by the variable composition of the protein bodies: prolamin and globulin storage proteins are present in separate storage organelles in rice, but they form separate phases within the same PSV in other cereals (Levanony et al. 1992; Rechinger et al. 1993; Shewry et al. 1995).

The protein storage vacuole

Plant vacuoles are classed either as lytic vacuoles (LVs) or storage vacuoles (Bassham and Raikhel 2000). Consistent with this, distinct transport vesicles have been described for

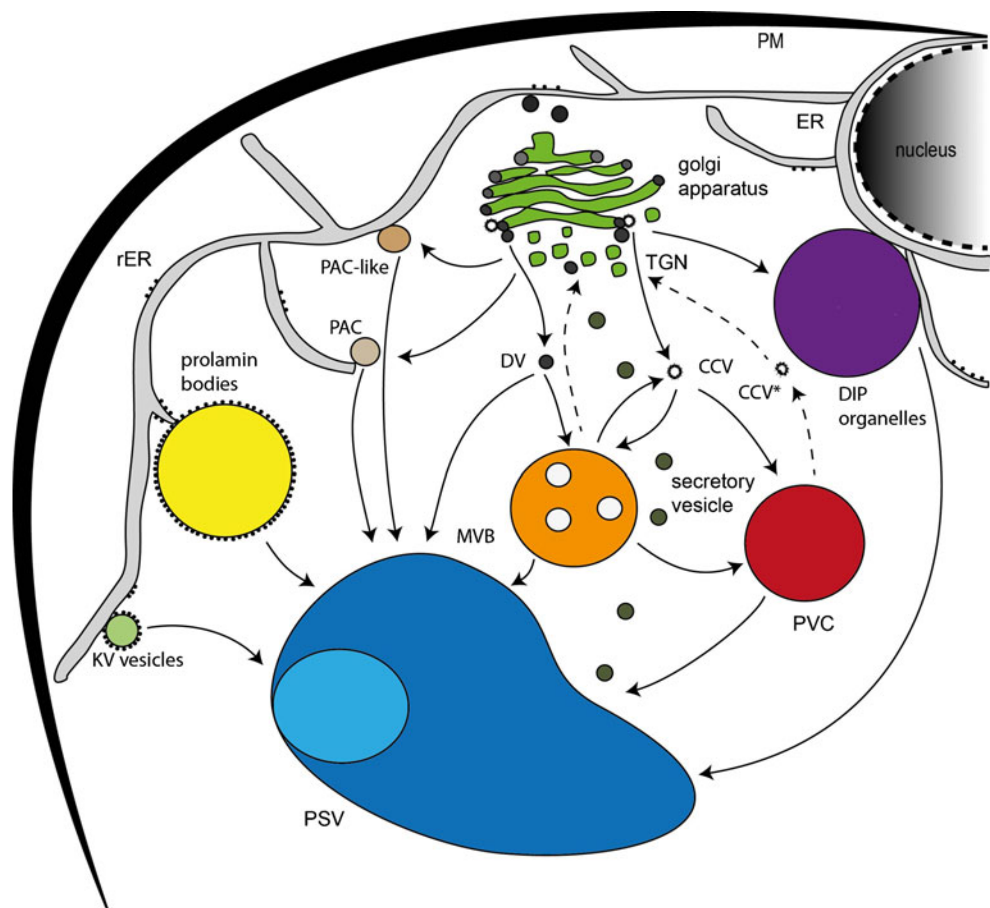

Fig. 1 Schematic summary representing the diverse storage organelles found in embryonic or endospermous seed tissues of different plant species and developmental stages, and possible pathways followed by storage proteins. *ER* endoplasmic reticulum, *rER* rough ER, *TGN trans*-Golgi network, *PAC vesicles* precursor-accumulating vesicles, *MVB* multivesicular bodies, *CCV* clathrin coated vesicles, *CCV** CCV-like vesicles, *PVC* prevacuolar compartment, *DV* dense vesicles, *DIP organelles* dark intrinsic protein organelles

both organelles, and their membranes are marked by the presence of different tonoplast intrinsic proteins (TIPs) that function as transmembrane channels for water and various low-molecular-weight substances (Gattolin et al. 2010; Wudick et al. 2009; Jiang et al. 2001). However, three different TIP isoforms fused to yellow fluorescent protein all localized to the tonoplast of protein storage vacuoles in embryos from developing, mature, and germinating *Arabidopsis* seeds (Hunter et al. 2007). In addition, the three TIP isoforms were differentially expressed during development, which calls into question their use as markers to identify coexisting vacuoles in single cell types (Hunter et al. 2007).

In many seeds, PSVs contain three morphologically distinct regions: the matrix, crystalloid, and globoid (Jiang et al. 2001). This coincides with the presence of integral membrane proteins in the PSV crystalloid, strongly arguing for the presence of a distinct storage compartment within PSVs (Jiang et al. 2000, 2001).

It is interesting to consider the occasional co-existence of different types of vacuoles in the same cell and the question of how one type of vacuolar compartment is replaced with another during development. Although the ontogeny of vacuoles in general is still a matter of debate, it has been shown in pea seed cotyledons that LVs are succeeded by PSVs in a clear developmental sequence. For a short time, both types of vacuoles co-exist in the same cell. The PSV appears to be formed de novo as a tubular structure that engulfs preexisting LVs in an autophagy-like process (Hoh et al. 1995). Similar observations were made in *Arabidopsis* and *Medicago truncatula* embryos (Frigerio et al. 2008). Recent observations indicate that VAMP727, a seed-specific component of the SNARE complex, plays a crucial role in PSV biogenesis and the transport of storage proteins by regulating membrane fusion in the vacuolar transport pathway during seed development (Ebine et al. 2008).

Interestingly, the inverse transition, i.e., from PSV to LV does not seem to involve the simultaneous presence of the two vacuole types but involves a transformation event including a transition state containing both PSV and LV marker proteins (Olbrich et al. 2007). The transformation from PSV to LV was described in detail in recent studies of vacuole biogenesis in tobacco seedling root tip cells. The results suggested that root tip cell LVs are derived from PSVs through cell type-specific sets of transformation events (Zheng and Staehelin 2011), involving PSV fusion, storage protein degradation, and the gradual replacement of the PSV marker protein α-TIP with the LV marker protein γ-TIP. These and other studies demonstrate that vacuolar structures may change rapidly during the cell cycle (Segui-Simarro and Staehelin 2006) and development (Oda et al. 2009),

revealing that vacuoles are dynamic organelles regulated by cell type-specific pathways. The LV and PSV sorting machinery may therefore share certain similarities and may recognize the targeting signals on particular seed proteins (Watanabe et al. 2004).

Although such indications imply that the distinction between pathways, signals, and ultimately between different vacuoles may not be as clear cut as initially assumed, some distinct sorting routes and mechanisms have been identified for vacuolar proteins (Vitale and Raikhel 1999). Two different types of vacuolar sorting receptors are known to mediate the selective recognition and sorting of cargo proteins in plants: the BP-80 family and the receptor homology-transmembrane-RING H2 domain (RMR) (Kirsch et al. 1994; Neuhaus and Paris 2006; Paris and Neuhaus 2002; Jiang et al. 2000; Park et al. 2005, 2007). Recent studies have shown that RMR proteins are located in the *cis* and medial cisternae of the Golgi apparatus and in the dense vesicles (DVs), supporting a role for them in SSP trafficking (Hinz et al. 2007).

In contrast, BP-80 was found to be enriched in clathrin-coated vesicles (CCVs) and was postulated to be necessary for protein transport to the prevacuolar compartment (PVC). Notably, however, very recent results suggest that the transport of cargo molecules towards the lytic vacuole downstream of the TGN may be receptor-independent (Niemes et al. 2010a, b). In addition, VSR proteins have also been found at the plasma membrane, where they may bind missorted ligands and retrieve them to the early endosome by endocytosis (Saint-Jean et al. 2010). Finally, there seems to be a kind of synergic interplay between the different sorting mechanisms. In this context, CCVs also develop and bud off from the surface of DVs, suggesting that VSR-At1 (a member of the BP-80/AtELP family) may selectively remove proteins from DVs, and that this process might be required for the DV–PSV pathway to function correctly (Hinz et al. 2007).

Routes to the PSV

Wherever a SSP is destined, the ER serves as the starting point of the journey through the cell. There are also indications that the ER may comprise subdomains that specialize in the synthesis of proteins directed to different compartments of the plant endomembrane system (Takahashi et al. 2005).

Receptor-mediated sorting and targeting to the vacuoles requires specific N-terminal or C-terminal propeptide signals or a signal within the mature portion of the cargo protein (Bednarek and Raikhel 1992). Recently, it was shown that vacuolar sorting receptors (VSRs) and their ligands are able to interact in the lumen of the ER prior to selective export. When transport is complete, VSRs recycle back to the ER in a retromer-dependent manner (Niemes et al. 2010a, b).

Given the strategic importance of the ER, an ER quality control system exists in plants to retain incompletely or incorrectly folded proteins and degrade those that are irreversibly misfolded (Vitale and Denecke 1999). Having passed through the ER, SSPs move further along the secretory pathway and may reach the PSV via additional membrane vesicles such as DVs, multivesicular bodies (MVBs), and precursor-accumulating (PAC) vesicles.

PAC vesicles

PAC vesicles were first characterized by immunoelectromicroscopy in maturing pumpkin seeds (Hara-Nishimura et al. 1998). PAC vesicles are 200–400 nm in diameter and transport large quantities of proprotein precursors such as 11S globulin, 2S albumin, and 51-kDa protein from the rough endoplasmic reticulum (rER) to PSVs. They are frequently observed close to the rER and are sometimes surrounded by ribosomes, suggesting that they originate from proprotein aggregates in the ER. Golgi-derived glycoproteins may also be incorporated into the mature PAC vesicles after release from the ER. PV72/82, a homolog of VSR BP-80, binds to pro2S albumin and co-exists with pro2S albumin in PAC vesicles, so PV72/82 is probably a receptor for vacuolar protein sorting in plants (Shimada et al. 1997; Hara-Nishimura et al. 1998). The type I integral membrane protein PV72 was found on PAC vesicle membranes and in the Golgi complex (Shimada et al. 2002). Because PV72 on PAC vesicles contains a complex type oligosaccharide, it seems clear that PV72 is delivered from the Golgi complex to the PAC vesicles, suggesting that PV72 is responsible for recruiting a small proportion of pro2S albumin molecules that have escaped aggregation in the ER and have therefore entered the bulk flow from the Golgi complex to the PAC vesicles (Vitale and Hinz 2005).

More recently, PAC-like vesicles have also been identified in rice endosperm cells (Takahashi et al. 2005). These vesicles are directly derived from the rER and accumulate both glutelin and α-globulin. As in pumpkin, PAC-like vesicles in rice endosperm may mediate an alternative pathway directly to the PSV, bypassing the Golgi. PAC vesicles may deliver their cargo to PSVs by one of two mechanisms: autophagy by the PSVs or membrane fusion between the PAC vesicle and the PSV (Hara-Nishimura et al. 1998).

DIP vesicles

In transgenic tobacco roots and seeds, small organelles derived from the ER are marked by a unique tonoplast intrinsic protein, known as dark intrinsic protein (DIP)

(Jiang et al. 2000; Jurgens 2004). These DIP vesicles contain an additional protein that acquires Golgi-specific oligosaccharide modifications, so they must also be the destination of vesicular trafficking from the Golgi apparatus. DIP vesicles may be surrounded by a membrane that fuses with the PSV tonoplast to deliver the internal, DIP-containing membranes to the vacuole interior, and they may therefore be equivalent to PAC vesicles.

Dense vesicles

The microscopic analysis of pea cotyledon storage parenchyma cells revealed the presence of dense vesicles (DVs) at the *trans*-Golgi network (TGN), which contained storage proteins en route to the PSV (Hohl et al. 1996). The DVs originate in the *cis*-cisternae and undergo maturation as they pass through the Golgi stack (Robinson et al. 1997; Hillmer et al. 2001). DVs have an average diameter of 130 nm and contain an electron-dense core but no coat (Hinz et al. 1999; Hillmer et al. 2001). Studies in developing pea seeds indicate that the sucrose-binding protein (SBP), which makes up only 2–4% of the total seed protein, is transported to the PSV in DVs together with the major storage proteins legumin and vicilin (Wenzel et al. 2005). Pro-legumin and α-TIP, but not the vacuolar sorting receptor BP-80, are located in the DVs of developing pea cotyledons, so multiple vesicular vacuolar sorting mechanisms may exist in the same cell. An aggregation process may be required to direct proteins into DVs, so SSP aggregation and vesicle formation must be coincidental events. Notably, CCVs have been shown to bud from DVs at the pea cotyledon TGN, indicating a complex interplay between the targeting of storage protein precursors and a sorting mechanism in the secretory system, involving either the removal of mistargeted proteins or the recycling of sorting receptors (Hinz et al. 1999; Hillmer et al. 2001; Hohl et al. 1996; Robinson and Hinz 1997; Robinson et al. 1997; Wenzel et al. 2005).

Multivesicular bodies

Multivesicular bodies (MVBs) in plant cells are prevacuolar compartments that are labeled by vacuolar sorting receptors and are involved in degradation, intracellular recycling and secretion (Tanchak and Fowke 1987; Samuels et al. 1995; Otegui et al. 2001; Segui-Simarro et al. 2004; Segui-Simarro and Staehelin 2006). It is now clear that MVBs are also involved in the trafficking of SSPs to the PSV. In legume seeds, MVBs filled with SSPs were identified as large (1 μm) intermediate compartments en route between DVs and the PSV (Robinson et al. 1998), ultimately fusing with the PSV (Robinson and Hinz 1999). Because MVBs arise in close proximity to the *trans* side of the Golgi stacks and TGN cisternae, it has been suggested that they are produced from freshly budded DVs and other small vesicles (Otegui et al. 2006). Recent studies suggest that MVBs in germinating seeds function dually as a storage compartment for proteases that are physically separated from the PSVs in the mature seed and as an intermediate compartment for VSR-mediated delivery of proteases from the Golgi apparatus to the PSV for protein degradation during seed germination (Wang et al. 2007). The SSPs and their processing proteases are segregated within the Golgi cisternae and packaged into separate secretory vesicles, which appear to fuse into MVBs (Otegui et al. 2006). In agreement with the above, the proteolytic processing of 2S albumin storage proteins begins inside MVBs.

The ER body in plant cells

In animal cells, the formation of ER-derived structures is usually associated with pathological states (Carlson et al. 1989; Mattioli et al. 2006). In contrast, the ER in plants gives rise to multiple ER-derived compartments that are either involved in defense mechanisms and stress responses or in the sequestration of reserve compounds, such as SSPs and oils (Galili 2004; Herman 2008). Recent studies have shown that the actin cytoskeleton helps to establish the tubular ER morphology and mediates the localization and transport of mRNAs targeted to distinct ER domains (Crofts et al. 2010; Herman 2008). The coupled translation of targeted mRNAs and the conglomeration of triglycerides, proteins, or rubber then results in the formation of an ER body, which accumulates its insoluble cargo until it reaches a critical size. At this point, the mature body detaches from the ER and often enters the direct ER to vacuole trafficking pathway (ERvt), although it can also remain as a cytoplasmic organelle (Herman and Schmidt 2004).

Although ER bodies occur most frequently in storage tissues, there are some exceptions. A spindle-shaped ER body that functions as a defense system in cotyledons and hypocotyls after germination has been identified in *Arabidopsis* (Matsushima et al. 2003). Defense-related ER bodies can also be induced (i-ER bodies) by wounding and stress in *Arabidopsis* rosette leaves, which have no ER bodies under normal conditions (Matsushima et al. 2003). Furthermore, it seems that i-ER bodies also play a role in resistance against fungal infections (Sherameti et al. 2008; (Yamada et al. 2009). Typically however, ER-derived organelles are found in seeds where they serve as storage compartments. Especially in cereal species, ER-derived protein bodies are often found in addition to PSVs and their main role appears to be the storage of prolamins.

Prolamin bodies—endogenous or recombinant

Prolamins are water-insoluble storage proteins, which are mainly found in cereal endosperm. They are synthesized by membrane-bound polyribosomes, and an N-terminal signal peptide ensures they are transported co-translationally into the ER lumen, where they form aggregates that bud from the main ER network. Their composition, size, number, and distribution within the cell are species-dependent (Shewry et al. 1995; Galili 2004). In maize, for example, prolamin bodies are highly organized structures that contain α-, ß-, γ-, and δ-zeins distributed in specific domains of the ER body. In the final stages of PB formation, α-zeins fill most of the core and are surrounded by a thin layer of ß- and γ-zeins (Lending and Larkins 1989). Prolamins do not contain a KDEL signal for ER retention, so other properties are responsible for their accumulation and aggregation in the ER (see below), and some members of the prolamin family are able to initiate PB formation (Coleman et al. 1996). Whatever the mechanism of PB formation, it can also be induced in non-seed tissues as shown by the expression of recombinant zeins in tobacco leaves and seeds (Coleman et al. 1996; Bagga et al. 1995). Similarly, PBs have been induced in tobacco cells by the overexpression of a HMW-glutelin subunit (Saumonneau et al. 2011) and in rice leaves by overexpressing rice 13-kDa prolamin fused to a fluorescent reporter protein (Saito et al. 2009).

After formation and budding, PBs may remain in the cytosol but they can also be sequestered into PSVs, probably by an autophagy-like process, as shown in wheat, barley, and oats (Levanony et al. 1992; Herman and Larkins 1999; Galili 2004; Herman and Schmidt 2004). Vacuolar sequestration of prolamins has not been reported in rice and maize endosperm, with the exception of maize aleurone cells (Reyes et al. 2011). It is therefore surprising that zein aggregates induced by heterologous expression in tobacco seeds are sometimes sequestered into PSVs (Bagga et al. 1995; Coleman et al. 2004).

Gamma-zein is able to induce the formation of ER-derived PBs in dicot tissues in the absence of other zein subunits (Coleman et al. 1996). Its retention in the ER and its capacity for self-assembly are attributed to the presence of a highly repetitive proline-rich sequence and a Pro-X motif (Geli et al. 1994), and to N-terminal cysteine residues (Llop-Tous et al. 2010). Consequently, the N-terminal portion of 27 kDa gamma-zein including these sequences is sufficient to induce the formation of protein bodies when fused to another protein. This has been implemented as Zera technology to stabilize recombinant proteins expressed in plants and increase their overall yields (Mainieri et al. 2004; Ludevid Mugica et al. 2007; Torrent et al. 2009; Abhary et al. 2011).

Additional amphipathic or hydrophobic sequences that can self-assemble into supramolecular structures have been identified and used as fusion partners for protein stabilization and the induction of PBs in vegetative tissues (Conley et al. 2011). Elastin-like peptides and hydrophobins, the former from mammals and the latter from fungi, can both induce ER-derived PBs in plant cells (Floss et al. 2009; Joensuu et al. 2010).

Russell-like bodies

Occasionally, recombinant proteins are deposited in ER-derived vesicular structures, although they are not hydrophobic in nature and do not contain an assembler peptide. One example is a single-chain variable fragment (scFv-Fc) antibody recognizing the maltose-binding protein (MBP), which was expressed in *Arabidopsis* seeds. This protein carried a KDEL sequence for ER retrieval, and although it assembled into IgG-like dimers through the formation of disulfide bonds between cysteine residues present in the Fc hinge region, a portion of the recombinant protein was found in ER-derived compartments delimited by ribosome-associated membranes (Van Droogenbroeck et al. 2007).

Similarly, another KDEL-tagged scFv-Fc molecule derived from the hepatitis antibody HA78 was deposited in newly formed ER-derived vesicles. Remarkably, a scFv-Fc antibody derived from the IgG 2G12, which lacked a KDEL sequence, was also found in newly formed ER-derived vesicles (Loos et al. 2011).

The appearance of these vesicles was very similar to Russell bodies, which occur in connection with human ER storage diseases and have been described in multiple myelomas as ER-derived structures containing accumulations of condensed mutant immunoglobulins (Russell 1890; Hsu et al. 1982). In this context, it is interesting to note that the scFv-Fc antibodies used in the studies discussed above consisted of the variable antibody regions fused to a human IgG1 Fc domain including the hinge, C_H2 and C_H3 domains, but lacking the C_H1 domain that binds to BiP during normal antibody assembly (Munro and Pelham 1986). Russell bodies on the other hand can be induced in lymphoid and non-lymphoid cells by overexpressing a mutant IgM or other immunoglobulin isotypes lacking the C_H1 domain (Valetti et al. 1991; Kaloff and Haas 1995).

These parallels are very interesting and suggest that plant cells may also be able to form Russell bodies as a self-protection mechanism, mirroring the situation in mammalian plasma cells when the production of a transport-incompetent molecule exceeds the cell's degradative capacity (Mattioli et al. 2006). It has been proposed that Russell bodies originate as SOS compartments, where abnormal proteins that cannot be secreted, but have

escaped intracellular degradation, can accumulate without blocking the normal secretory pathway (Valetti et al. 1991). Indeed, plasma cells containing Russell bodies, which are sometimes known as Mott cells, remain viable (Weiss et al. 1984). It is possible that Russell body formation occurs in a similar manner in plant cells confronted with large quantities of abnormal or otherwise detrimental proteins. Indeed, we have observed similar ER-derived vesicles in *Arabidopsis* seeds producing potentially harmful recombinant proteins (E. Arcalis, unpublished results).

It is even more tempting to speculate that the mechanism responsible for the formation of Russell bodies in mammalian cells may be similar to the one for prolamin body formation in plant cells, particularly since prolamin bodies can be induced in animal cells (Ludevid Mugica et al. 2007). However, the ER-resident and/or membrane proteins that orchestrate this process remain to be identified in both animals and plants.

Investigation of storage organelle formation

Roles of SSPs in protein body formation

Several sequence-dependent and intrinsic features of SSPs have been identified that are responsible for sorting and/or PB formation, particularly sorting signals and receptors required for trafficking to the PSV (Vitale and Hinz 2005). The sorting of proteins into the vacuole can be saturated (Frigerio et al. 1998), confirming that sorting depends on interactions with specific receptors. Notably, the sorting machinery benefits from cumulative information, because sorting efficiency appears to depend on the number of sorting signals. The ratio between the number of vacuolar sorting determinants and the size of cargo proteins may also play a role in the efficiency of sorting (Holkeri and Vitale 2001).

The formation of aggregates in the ER and Golgi apparatus and their interaction with membranes may also influence the trafficking and deposition of storage proteins (Banc et al. 2009; Galili 2004). In the case of cereal prolamins, the aggregation of LMW glutenin within the rough ER of wheat endosperm cells seems to influence the route it takes, and also seems to be induced by the higher protein synthesis rate that results in a higher concentration of protein within the ER lumen (Tosi et al. 2009). A high local concentration of prolamins within the ER may also be promoted by the targeting of prolamin mRNA to specific, PB-forming areas of the ER, as has been shown mainly in rice (Crofts et al. 2004, 2010).

In maize, the selective oligomerization of γ-zeins directed by protein–protein interactions involving the repeat domain has long been considered a potential retention mechanism (Geli et al. 1994). Although membrane interactions (involving the repeat domain) (Kogan et al. 2004) and/or association with other proteins may also facilitate the packaging of γ-zein into PBs, it has recently been shown that the formation of disulfide bridges between cysteine residues is pivotal for the retention of γ-zein in the ER (Pompa and Vitale 2006).

Interactions between individual maize prolamins have been investigated using the yeast two-hybrid system, revealing strong affinities among the different γ-zeins and the 15-kDa γ-zein, and also suggesting an important role for the 16-kDa γ-zein and the 15-kDa γ-zein in the binding and assembly of γ-zeins within the PB (Kim et al. 2002). In agreement with this, interactions between storage proteins in the secretory pathway have been shown to influence the sorting process in soybean and barley (Kinney et al. 2001; Rechinger et al. 1993). Furthermore, interactions between storage proteins appear to contribute to the shape and organization of protein storage organelles, as revealed by the aberrant morphology that occurs when particular SSPs are missing (see below). The interaction with storage proteins may also influence the sorting and deposition of recombinant proteins, as shown for the Cp7 peptide, which is retained in ER-derived PBs because it forms disulfide bridges with rice prolamins (Wakasa et al. 2009).

Mutants and transformants with altered protein storage organelles

A number of maize mutants with quantitave differences in zein protein profiles also show specific changes in protein body morphology, which in turn influences the texture and vitreous phenotype of the mature endosperm. For example, the *opaque2* (*o2*) mutant accumulates low levels of α-zeins and has much smaller protein bodies than wild-type plants (Geetha et al. 1991). The *Defective endosperm B30* (*De-B30*) and *floury2* (*fl2*) mutants have single amino acid substitutions preventing cleavage of the signal peptide in two different α-zeins, and contain small, misshapen protein bodies (Lending and Larkins 1992; Kim et al. 2004). All three of the mutants are associated with an unfolded protein response (UPR) as indicated by elevated BiP accumulation in the ER and PBs (Hunter et al. 2002). The *floury1* (*fl1*) mutant is also associated with a subtle alteration in zein body architecture. The mutation affects a PB transmembrane protein required for the correct targeting of the 22-kDa α-zein (Holding et al. 2007). However, most of these storage protein mutants have pleiotropic effects, which make it difficult to determine the specific role of the storage proteins in PB formation.

Diverse null and knock-down lines for SSPs have therefore been generated to investigate storage organelle formation (Wu and Messing 2010; Kawakatsu et al. 2010). In maize, RNA interference (RNAi) was used to generate plants deficient for specific storage proteins (α-, ß-, γ-, and δ-zeins) (Segal et al. 2003; Wu and Messing 2010). Subcellular analysis of these lines revealed changes to PB morphology and size, e.g., a substantial reduction in the level of 22-kDa α-zeins generated PB protuberances, whereas the knock-down of ß- and γ- zeins confirmed their redundant and specific role stabilizing the highly ordered PB architecture (Wu and Messing 2010). Interestingly, and in contrast to results obtained in heterologous systems (Coleman et al. 2004; Bagga et al. 1997), the lack of both ß- and γ-zeins did not reduce the accumulation of α- and δ-zeins in the maize endosperm, but it did result in dramatic changes to PB morphology (Wu and Messing 2010).

Similarly, RNAi has been used to knock down prolamin accumulation in rice, changing PB morphology and revealing individual roles for the different storage proteins. The knock-down of 13-kDa prolamins reduced the size of endoplasmic reticulum-derived PBs and also altered the rugged peripheral structure, whereas suppression of the 10- or 16-kDa prolamins reduced the size of the PBs slightly. Extreme increases or reductions in the low-sulfur prolamins resulted in the production of small PBs, suggesting that the ratio of individual prolamins is crucial for proper aggregation and folding (Kawakatsu et al. 2010).

BiP is thought to play a major role in prolamin retention and deposition (Li et al. 1993). This is supported by the observation that BiP is located in the periphery of both endogenous and induced PBs (Li et al. 1993; Saito et al. 2009), and by the changes in SSP accumulation and deposition that occur in response to changing levels of BiP expression. The overexpression of BiP1 in the endosperm of transgenic rice lines not only altered the morphology of ER-derived prolamin bodies, but also generated an additional ER-derived population of unusual PB-like structures containing glutelin together with BiP and prolamins in areas of different electron density (Yasuda et al. 2009). Interestingly, lowering BiP1 expression induced other chaperones, reduced the accumulation of SSPs, and produced distorted protein storage organelles, whereas moderate upregulation of BiP1 increased the accumulation of SSPs while maintaining normal organelle structure (Wakasa et al. 2011).

Other mutants have revealed that intramolecular and intermolecular disulfide bonds play a role in the control of SSP deposition. The rice *esp2* mutant, which lacks oxidoreductase PDIL1;one from the protein disulfide isomerase (PDI) family, deposits glutelin precursors together with prolamines in the ER (Takemoto et al. 2002; Onda et al. 2009), whereas the knock-down of oxidoreductase PDIL2;3 inhibited the accumulation of cysteine-rich 10-kDa prolamin in the core of PBs. The two oxidoreductases are not functionally redundant and therefore play distinct roles in the formation of PBs (Onda et al. 2011).

Mutations can also affect the morphology of PSVs. Rice glutelin knock-down lines with very low glutelin levels contained fewer and smaller PSVs, indicating that PSV formation and development may correlate with the quantity of PSV-resident proteins (Kawakatsu et al. 2010). The 57H rice mutant *gp1* accumulates 57-kDa pro-glutelins in the endosperm, and mistargeted glutelins are also found in *gpa1* endosperm cells indicating a defect in the organization of the endomembrane system in *gpa1* seeds (Wang et al. 2010). *GPA1* corresponds to *OsRab5a*, a small GTPase that is necessary for the vacuolar trafficking of SSPs via the Golgi. Notably, considerable amounts of glutelins were correctly transported to the PSV in *gpa1* mutants via PAC vesicles derived from the ER.

Powerful tools for the investigation of vacuolar biogenesis and sorting have been developed in *Arabidopsis*, including transgenic lines expressing tonoplast-localized proteins and vacuolar signal peptides fused to GFP. These can be mutagenized and screened for vacuolar biogenesis mutants by confocal microscopy (Avila et al. 2003; Fuji et al. 2007). Another approach was developed to identify chemicals that interfere with vacuolar sorting (Zouhar et al. 2004). In this manner, Sortin1 was identified as a specific drug that may interfere with vesicle budding or fusion or another essential process in vacuole biogenesis in *Arabidopsis* seedlings (Zouhar et al. 2004). Although the *Arabidopsis* markers and assays provide some insight into vacuolar trafficking in cereals, it remains a challenge to optimize the direct analysis of SSP sorting in cereal seeds. Stable transgenic cereal lines can be generated by *Agrobacterium*-mediated transformation (Frame et al. 2002; Mohanty et al. 2009a, b) or particle bombardment (Altpeter et al. 2005), but the relatively long generation time and small number of resulting plants is a disadvantage. Therefore, transient transformation approaches including particle bombardment and protoplast transfection (Gallie and Young 1994; Peters J. unpublished data) have been developed for the analysis of SSP sorting. In maize, the agroinfection of in vitro grown endosperm tissue has been developed and optimized for the aleurone layer (Reyes et al. 2010). The analysis of maize starchy endosperm cells could perhaps be optimized by combining particle bombardment, protoplast transfection, and in vivo fluorescence dye studies of maize kernels.

Factors that affect the intracellular trafficking of SSPs

Species and tissue dependency

Protein trafficking often appears to depend on the plant species and tissue, as observed particularly when com-

paring the expression of recombinant proteins in different systems. Microscopy and biochemical analysis in wheat plants expressing a recombinant glycoprotein containing a signal peptide showed that the protein was sequestered into PBs and PSVs in endosperm cells (Arcalis et al. 2004). However, the same protein was efficiently secreted from tobacco leaves (Verwoerd et al. 1995) and from *M. truncatula* seed cotyledons (Abranches et al. 2008).

Also, on a number of other occasions, recombinant proteins that are otherwise secreted have been shown to accumulate in storage organelles when expressed in cereal seeds, e.g., a secretory antibody and lysozyme produced in rice (Nicholson et al. 2005; (Yang et al. 2003). Microscopy and glycoanalysis have shown that the recombinant reporter glycoprotein phytase accumulates in PBs and PSVs in rice endosperm but is efficiently secreted from rice leaves (Drakakaki et al. 2006).

There can even be differences between adjacent tissues in the same organ. For example, electron microscopy and molecular analysis have shown that zeins are expressed not only in starchy endosperm, but also in aleurone cells. Surprisingly, the zeins are not stored in ER bodies in the aleurone layer but are transported to the aleurone PSVs (Reyes et al. 2011). The difference between aleurone and starchy endosperm cells may reflect fundamental differences in protein trafficking between the cell types, lower expression levels or altered zein ratios in the aleurone, or the greater abundance of PSVs in the aleurone cells. Indeed, Reyes et al. (2011) did not show any storage protein-containing vacuolar compartments in starchy endosperm. Nevertheless, PSV-like compartments containing SSPs have been identified in the starchy endosperm as well although their contents have not been determined in detail (Woo et al. 2001; Arcalis et al. 2010). In summary, these data suggest that the specialized architecture of endosperm cells, which have evolved for storage, affects the intracellular trafficking and deposition of endogenous and recombinant proteins.

Dependency on seed developmental stage

The investigation of zein distribution during PB formation revealed variations in PB size and composition dependent on the position of the PB in the endosperm and the stages of cell maturity (Lending and Larkins 1989). The smallest zein bodies were observed in the subaleurone cells, consisting primarily of ß- and γ-zeins, whereas larger protein bodies containing α- and δ-zeins in addition to ß- and γ- zeins were found in the subaleurone and starchy endosperm cells. The localization of DIP vesicles within the PSV is also developmentally regulated, the crystalloid forming when 1–2-μm DIP vesicles are taken up from the cytoplasm (Jiang et al. 2000). Several studies also show that the route taken by individual SSPs on their way to the PSV can change during seed development. This has been proposed for 2S and 11S storage protein trafficking in pumpkin and castor bean (Vitale and Hinz 2005), as well as for wheat prolamins (Tosi et al. 2009; Shy et al. 2001). For example, LMW glutenin may aggregate within the ER or translocate to the PSV via the Golgi depending on its concentration and/or the developmental stage of the tissue (Tosi et al. 2009).

The structure of N-glycans is a useful indicator for intracellular trafficking, allowing the route taken by a glycoprotein to be determined from its glycan profile (Vitale and Hinz 2005). A combination of microscopy and N-glycan analysis in maize confirmed changes in the route taken by a recombinant glycoprotein and also a reduction in the number of vacuolar storage organelles during seed maturation. Two endogenous non-glycosylated storage proteins were shown to share the same intracellular fate, indicating that the diversion of storage proteins may be a common strategy in seed development (Arcalis et al. 2010).

Changes during preparation for germination

The synthesis and mobilization of storage proteins, starch, and oil is organized not only during seed maturation, but also as the seed prepares for germination (Angelovici et al. 2010). The mobilization of storage reserves during germination involves the induction of programed cell death (PCD) and senescence (Gietl and Schmid 2001).

In castor bean endosperm tissue, ER-derived precursor protease vesicles (ricinosomes), emerge as cell organelles in plant tissues undergoing developmentally regulated PCD (Schmid et al. 1998; Gietl and Schmid 2001; Schmid et al. 2001; Greenwood et al. 2005). In the cotyledons of germinated *Vinga mungo* seeds, cysteine proteinase (sulfhydryl endopeptidase, SH-EP) has a major role in the breakdown of seed globulins (Toyooka et al. 2000). The SH-EP precursor is synthesized in the ER lumen and packaged into 200–500 nm KDEL-tailed cysteine proteinase-accumulating vesicles (KVs) at the fringes of the ER. When filled with proSH-EP, the KVs bud from the ER bypass the Golgi and fuse with the PSV to release proSH-EP inside. The KDEL sequence is essential for the correct intracellular sorting of SH-EP. Heterologous expression of normal SH-EP and a variant lacking the KDEL tail in *Arabidopsis* showed that wild-type SH-EP is packed into KV-like vesicles, whereas such vesicles are not formed in the cells expressing the KDEL deletion mutant, perhaps indicating that a local threshold concentration is required for vesicle genesis (Okamoto et al. 2003). Notably, the asparaginyl endopeptidase VmPE-1,

which processes proSH-EP, is transported separately to the PSV via the Golgi complex, indicating that *V. mungo* seed cotyledon cells use two sorting pathways to transport proteolytic enzymes from the ER to the PSV.

Finally, during the germination of mung bean seeds, the localization of VSR proteins shifts from the peripheral membrane to the lumen of MVBs, indicating that VSR proteins have distinct functions during seed germination. Whereas during seed development, VSR proteins seem to transport proteases to MVBs where they are physically separated from PSV-localized storage proteins, upon seed germination, VSR proteins mediate their transport to the PSVs via MVBs for protein degradation (Wang et al. 2007).

Conclusions

The regulation of protein storage and mobilization in seeds is a complex and dynamic process, based on the ability of the endomembrane system to undergo massive reorganization during seed development. The system must cope with a vast quantity of storage proteins and must distinguish different types of cargo vesicles in order to avoid mistargeting. Seed cells contain PSVs rather than LVs, and there are several specialized transport pathways leading to this organelle, and the direct ER-PSV route is more pronounced in seeds than other tissues (Fig. 1). In many cell types, the ER can store proteins and generate ER-derived PBs that serve as additional storage organelles in cereals. Striking differences in protein trafficking and deposition are observed between different species and tissues, and protein trafficking pathways also appear to be developmentally regulated in some cases. The intrinsic properties of storage proteins, their interactions with each other, and their interactions with additional proteins en route also influence sorting and thus determine their fate, as well as controlling the morphology of protein bodies. The potential for aggregation influences the formation of ER-derived protein bodies, a mechanism that appears universal, and can be used to induce protein storage bodies, protecting the protein from degradation on one hand and protecting the secretory pathway from negative effects or blockage on the other.

Seed tissues are not easy to study as they can be difficult to handle and adapt for some microscopy procedures. Genome-scale analysis, mutants, marker lines, and screening techniques have been developed primarily in *Arabidopsis*, a species with miniscule seeds containing only a single layer of endosperm cells, which limits its usefulness as a model for endospermous seeds. Legumes and many cereal species remain recalcitrant to genetic transformation, so marker lines are rare, although there are some notable exceptions (Onda et al. 2009; Mohanty et al. 2009b). The availability of mutants is limited, but increasingly, this bottleneck is being circumvented by the development of strategies based on transient expression and/or RNAi.

Conflict of interest The authors declare that they have no conflict of interest.

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