Successful transport to the vacuole of heterologously expressed mung bean 8S globulin occurs in seed but not in vegetative tissues

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Abstract

This study investigated the subcellular location of mung bean (Vigna radiata) 8S globulin in transient expression systems as well as in tobacco (Nicotiana tabacum) BY-2 cells and different tissues from a transgenic Arabidopsis (Arabidopsis thaliana) line stably expressing this storage globulin. When transiently expressed in protoplasts from both BY-2 cells and Arabidopsis suspension cultured cells, the 8S globulin located to structures that were neither Golgi nor pre-vacuolar compartments (PVCs). Immunogold electron microscopy of the transgenics reveals the 8S globulin-positive structures to be small, spherical, ribosome-covered endoplasmic reticulum (ER)-derived bodies. In BY-2 cells and all vegetative cells, the 8S globulin was present as a pro-form. However, in Arabidopsis embryos, with the onset of endogenous storage protein synthesis, the 8S globulin exited the ER and passed through the PVC to the protein storage vacuole where it was processed to its smaller mature form. These results clearly demonstrated that, when taken out of context and expressed in vegetative cells, the mung bean 8S storage globulin cannot exit the ER, and indicate that natural targeting of storage proteins to the vacuole should be better studied in the maturing seed.

Key words: 8S globulin, ER body, pre-vacuolar compartment, storage protein.

Introduction

Because of their stored protein reserves, the seeds of legumes are of great nutritional value and therefore of enormous economic importance in world agriculture. For this reason, improving the quality and quantity of seed storage proteins is a major challenge for plant biotechnologists and breeders (e.g. Ufaz and Galili, 2008; Tandang-Silvas et al., 2011). Obviously this goal cannot be achieved without an adequate knowledge of the mechanisms underlying storage protein synthesis and deposition. The major storage proteins in legumes are the salt-soluble globulins, which fall into two major subclasses: 7/8S and 11S globulins (Shewry et al., 1995). Their synthesis occurs asynchronously during cotyledon development, starting about 13 d after flowering (DAF) (Wenzel et al., 1993). The globulins are deposited in a special type of vacuole, the protein storage vacuole (PSV), which arises de novo during cotyledon development and replaces the lytic-type vacuole typical of vegetative tissues (Hoh et al., 1995; Frigerio et al., 2008; Ibl and Stöger, 2012). In the final stages of cotyledon development, the PSV subdivides into hundreds of smaller protein bodies (Herman and Larkin, 1999; Jiang et al., 2002).
Globulins are encoded by multigene families (Shutov et al., 1995), have a signal sequence at their N terminus, and are initially synthesized as a pre-pro-form. The pro-form, which is present in the lumen of the ER, is normally N-glycosylated, although there are exceptions where no glycosylation occurs, such as vicilin in *Pisum sativum* and in *Vicia faba* (Müntz, 1996). The pro-globulins are transported in the lumen of the endomembrane system to the PSV as trimers. In the case of the 11S legumins, the mature form in the PSV is a hexamer, whereas the 7/8S vicilins remain as trimers (Müntz, 1996, 1998). Upon arrival in the Golgi, storage globulins in legumes are segregated from secretory cargo by being sequestered into specialized vesicles (‘dense vesicles’, DVs) at the rim of first cis-cisterna (Hohl et al., 1996; Robinson and Hinz, 1999; Hillmer et al., 2001; Oufattole et al., 2005; Wang et al., 2012). After being released at the trans-Golgi network (TGN), DVs appear to fuse with multivesicular pre-vacuolar compartments, where they become subjected to proteolytic processing (Otegui et al., 2006; Min et al., 2007; Wang et al., 2012). Proteolytic processing involves a family of Asn proteases known as legumains (Müntz and Shutov, 2002) and continues in the PSV leading to the production of numerous smaller mature polypeptides (Müntz, 1998). This can involve a single proteolytic cleavage, e.g. to form α- and β-legumin (which are then linked together by a disulfide bridge), or many cleavages leading to a range of vicilin polypeptides (Müntz, 1996).

Developing mung bean (*Vigna radiata*) cotyledons synthesize a mixture of 8S vicilin-type (89.0%), 7S basic (3.4%), and 11S legumin (7.6%) globulins (Mendoza et al., 2001). The native molecular mass for the mature 8S globulin is around 200 kDa, with the major 8S globulin polypeptide having a molecular mass of 51 kDa. This has a single glycosylation site about 90 aa residues from the C terminus (Bernardo et al., 2004). However, the presence of N-linked glycans is not essential for the assembly or the conformational stability of the 8S globulin (Garcia et al., 2006). We have shown previously that the 8S globulin is transported to the PSV via the Golgi apparatus and multivesicular PVCs, even during early stages in cotyledon development when DVs have not yet been formed (Wang et al., 2012). In another study, we showed that transport of the *Arabidopsis* seed storage protein 2S albumin was also mediated by PVCs in tobacco (*Nicotiana tabacum*) and *Arabidopsis* suspension culture cells (Miao et al., 2008). This prompted us to determine the localization of mung bean 8S globulin when expressed heterologously in stably transformed tobacco BY-2 cells and different tissues of *Arabidopsis thaliana*, the question being whether other types of storage proteins followed the same pathway in suspension-cultured cells.

Initial transient co-expression of 2S albumin and 8S globulin in BY-2 and *Arabidopsis* protoplasts showed that the two storage proteins localized to different compartments. Further co-transient expression of 8S globulin with organelle markers showed that the 8S globulin-positive compartments were distinct from known secretory and endocytic organelles. In order to unravel the identity of the 8S globulin-positive compartments, an 8S globulin–green fluorescent protein (GFP) and 8S globulin tobacco BY-2 transgenic cell lines as well as an *Arabidopsis* transgenic plant were generated. Immunogold electron microscopy (IEM) with anti-8S globulin and anti-GFP antibodies revealed the endoplasmic reticulum (ER) nature of the 8S globulin–GFP compartments. Such ‘ER bodies’ were observed in BY-2 cells and all vegetative tissues of the *Arabidopsis* plant, including leaf, stem, and root. Further IEM showed that, in young *Arabidopsis* embryos prior to the expression of endogenous storage proteins, 8S globulin–GFP still located to ER bodies. However, upon expression in intermediate and mature *Arabidopsis* embryos, 8S globulin went to PSVs via storage PVCs, as in developing mung bean cotyledons. Western blot results were consistent with IEM studies showing that that 8S globulin–GFP remained full length in BY-2 cells and vegetative tissues, whilst GFP was removed from 8S globulin in mature seeds. These results indicated tissue-specific targeting of 8S globulin, and also that correct targeting of storage proteins to the vacuole can only be studied properly in developing seed tissue during a period when the native sorting machinery for such proteins is also expressed.

### Materials and methods

#### General methods for construction and characterization of recombinant plasmids, maintenance of tobacco BY-2 and *A. thaliana* suspension culture cells, and growth of *Arabidopsis* plants

General methods for construction and characterization of recombinant plasmids, maintenance of tobacco BY-2 and *A. thaliana* suspension culture cells, and growth of *Arabidopsis* plants have been described previously (Jiang and Rogers, 1998; Jiang et al., 2000, 2001; Tse et al., 2004; Wang et al., 2009a, 2010; Miao et al., 2011; Gao et al., 2012).

#### Plant materials and transformation

Transient expression in protoplasts prepared from tobacco BY-2 and *Arabidopsis* suspension culture cells was carried out as described previously (Miao and Jiang, 2007). Transgenic BY-2 cells were generated using *Agrobacterium tumefaciens*-mediated transformation with strain LBA4404 as described previously (Tse et al., 2004; Lam et al., 2007a, 2008). Transformation and generation of transgenic *Arabidopsis* (ecotype Columbia-0) plants were performed via the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998).

#### Plasmid construction

The full-length mung bean **8S**GLOBULIN (α-isoform, GenBank accession no. DQ538333) cDNA was amplified by RT-PCR using mRNA extracted from developing mung bean cotyledons with forward primer 5′-CTGACTAGTAGTTGAGACGACAGGATCTCAGT-3′ and reverse primer 5′-AGTTCGGAGTCAGTACACAAATGGACCTT-3′. The amplified DNA fragments were cloned into the pBI221-GFP or pBI121-GFP vector using *Xhol/Xhol* restriction cutting sites to generate **8S** globulin–GFP fusion constructs for transient expression or stable transformation, respectively. To generate the untagged **8S** globulin construct, full-length **8S** globulin cDNA was amplified using the forward primer 5′-CTGACTAGTAGTTGAGACGACAGGATCTCAGT-3′ and reverse primer 5′-AGTTCGGAGTCAGTACACAAATGGACCTT-3′, and cloned into pBI121 vector with *Xhol/Xhol* restriction cutting sites for stable transformation. The expression of all **8S** globulin–GFP fusions and untagged **8S** globulin was under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator.

#### Antibodies

The generation and characterization of **8S** globulin antibody has been described previously (Wang et al., 2012). Polyclonal antibodies
against VSRa1 and Man1 have been characterized previously (Li et al., 2002; Tse et al., 2004). The sources for calnexin and complex glycan antibodies have been described elsewhere (Jiang and Rogers, 1998). The Arabidopsis Syp61 antibody was generously provided by Professor Natasha Raikhel (University of California, Riverside, CA, USA). The Arabidopsis Sar1p antibody (Yang et al., 2005) was kindly provided by Professor David Robinson (University of Heidelberg, Germany). For protein immunoblotting and confocal immunofluorescent analysis, antibodies were used at 4 μg ml⁻¹ (Wang et al., 2007, 2009a). Polyclonal Alexa Fluor 568-conjugated goat anti-rabbit IgG antibodies were purchased from Molecular Probes for immunofluorescent detection as the secondary antibody.

**Analysis of protein expression**

Protein extraction and immunoblotting analysis with antibodies were performed as described previously (Sohn et al., 2003; Tse et al., 2004; Wang et al., 2007, 2009b). Arabidopsis seedlings (2 weeks old) were used for protein extraction of leaves, stems, and roots. For protein analysis in developing seeds, total proteins were isolated from different developmental stages of Arabidopsis seeds: young (~5–6 DAF), intermediate (~11–12 DAF), and mature seeds.

**Confocal immunofluorescent studies**

Fixation and immunolabelling of transgenic BY-2 cells was carried out as described previously (Tse et al., 2004; Miaow et al., 2006; Lam et al., 2007a,b; Wang et al., 2010; Cai et al., 2011, 2012). The immunolabelled cells were subjected to confocal fluorescent imaging using an Olympus FV1000 laser-scanning confocal system.

**Electron microscopy studies**

The general procedures for the preparation of high-pressure frozen/freeze-substituted samples and ultrathin sectioning of resin-embedded sample blocks have been described previously (Tse et al., 2004; Lam et al., 2007a,b; Wang et al., 2007, 2009a, 2010, 2012). For high-pressure freezing, the leaves and stems of Arabidopsis seedlings were degassed in hexadecane and immediately subjected to a high-pressure freezing apparatus (EMP2; Leica, Bensheim, Germany). For Arabidopsis embryos, seed coats were peeled off and embryos were applied to a high-pressure freezer. Subsequently, substitution, HM20 resin infiltration, embedding, and UV polymerization were performed stepwise in a Leica AFS freeze substitution unit. Immunogold labelling on HM20 ultrathin sections was carried out as described previously (Tse et al., 2004; Wang et al., 2012) with anti-8S globulin (10 μg ml⁻¹), anti-GFP (40 μg ml⁻¹), anti-VSRa1 (40 μg ml⁻¹), anti-calnexin (40 μg ml⁻¹) or anti-complex glycan (40 μg ml⁻¹) antibodies. Sections were post-stained with aqueous uranyl acetate/lead citrate and examined using transmission electron microscope (Hitachi H-7650) with a CCD camera operating at 80 kV.

**Results**

**Transiently expressed 8S globulin and 2S albumin do not co-localize**

We performed a series of co-expression experiments on tobacco BY-2 and Arabidopsis protoplasts with fluorescently tagged mung bean 8S globulin and Arabidopsis 2S albumin and various organelle marker proteins. As seen in Fig. 1A and G, the fluorescent punctae representing 8S globulin–GFP did not overlap with signals for the pre-vacuolar compartment (PVC) [monomeric red fluorescent protein conjugated to vacuolar-sorting receptor 2 (mRFP–VSR2)]. This contrasted with the situation for 2S albumin–GFP where a considerable signal overlap with the PVC was observed (Fig. 1B, H). The 8S globulin–GFP punctae also did not co-localize with signals for the cis-Golgi (Man1–mRFP) and TGN (mRFP–Syp61) (Fig. 1D, E, J, K). On the other hand, the 8S globulin–GFP punctae appeared to lie over the signal for mRFP–HDEL, an ER marker (Fig. 1F, L). The lack of co-localization between the 8S globulin–GFP signal and these organelle markers was confirmed by immunofluorescence (Fig. S1 at JXB online). The difference between the locations of 8S globulin and 2S albumin was also shown in transient expression experiments performed in the presence of Sec12, which showed that the punctate fluorescence of 8S globulin–GFP was unaffected whereas the punctate signals for 2S albumin–GFP and Man1–mRFP became reticulate, indicating their retention in the ER (Fig. S2 at JXB online). Overexpression of this protein titrates out the guanine nucleotide exchange factor Sar1 causing elimination of COPII-mediated ER export (Phillipson et al., 2001). All proteins normally localizing to a post-ER compartment are therefore, after Sec12 overexpression, restricted to the ER.

**The location of transiently expressed 8S globulin is not affected by brefeldin A or wortmannin**

To confirm that the subcellular location of transiently expressed 8S globulin was neither the Golgi apparatus nor the PVC, we applied well-known inhibitors of the secretory pathway at the end of the expression period. Firstly, brefeldin A was used, an inhibitor of adenosine ribosylation factor 1 (ARF1) activity, which, depending on the location of the corresponding ARF guanine nucleotide exchange factor, may interfere with vesicle-mediated trafficking at the cis-Golgi or the TGN (Nebenführ et al., 2002; Lam et al., 2009; Langhans et al., 2011). Secondly, wortmannin was used, a PI3-kinase inhibitor, which results in swelling of the PVC (Tse et al., 2004; Robinson et al., 2012; Ding et al., 2012). In both BY-2 and Arabidopsis protoplasts, brefeldin A treatment caused the formation of large aggregates of the Golgi markers Man1–RFP and RFP–Syp61, but the punctate 8S globulin signals remained unaffected by this treatment (Fig. 2A, B, D, E). In contrast, treatment with wortmannin lead to the production of ring-like signals for mRFP–VSR2, which are typical of dilated PVCs (Fig. 2C, F). Again, the 8S globulin–GFP punctae were unaffected. Thus, from our transient expression experiments, we concluded that 8S globulin, unlike 2S albumin, localizes neither to the Golgi apparatus nor to the PVC.

**8S globulin localizes to ER-derived bodies in BY-2 cells and cells of vegetative organs in Arabidopsis**

In order to confirm our suspicions that 8S globulin was present in the ER, we first generated a stable transgenic BY-2 line expressing 8S globulin–GFP under the control of the CaMV 35S promoter. By CLSM, fluorescent punctae representing 8S globulin were distributed uniformly throughout the cytoplasm, but no signal was seen in the vacuole (Fig. 3A). A similar punctate distribution was seen by immunofluorescence with anti-8S globulin antibodies in transgenic BY-2 cell lines.
Fig. 1. 8S globulin–GFP localizes as discrete punctae in the cytosol. The punctae were separate from 2S albumin–mRFP and markers for the cis-Golgi (mRFP) and TGN. The 8S globulin–GFP signal largely overlapped with the ER network. Tobacco BY-2 and Arabidopsis protoplasts were co-electroporated with 8S globulin–GFP and the DNA of organelle markers or 2S albumin–mRFP as indicated. After 12–16 h incubation, the protoplasts were imaged using confocal laser-scanning microscopy (CLSM). DIC, differential interference contrast. Bar, 50 μm.
Fig. 2. The formation of 8S globulin–GFP punctae is not affected by brefeldin A or wortmannin treatments. Tobacco BY-2 and Arabidopsis protoplasts were co-electroporated with 8S globulin–GFP and organelle marker DNA as indicated, followed by incubation for 12–16 h and treated with either brefeldin A or wortmannin for 1 h before being imaged by CLSM. Bar, 50 μm.
Fig. 3. 8S globulin–GFP fusion localizes to ER-derived compartments in transgenic tobacco BY-2 cells. (A) The fluorescent signal patterns of 8S globulin–GFP in tobacco BY-2 transgenic cells were similar to that observed by transient expression in protoplasts. Bar, 20 μm. (B–D) Immunogold labelling of ultrathin sections cut from high-pressure frozen/freeze-substituted 8S globulin–GFP-expressing tobacco BY-2 cells. Both anti-8S globulin and anti-GFP antibodies labelled the ER-derived compartments (indicated by asterisks), instead of multivesicular bodies (m) (B). Immunogold labelling with anti-calnexin antibody confirmed that the 8S globulin–GFP-localized compartments were derived from the ER (C, D). (This figure is available in colour at JXB online.)
expressing untagged 8S globulin (Fig. 4A, B). In addition, in these transgenic cell lines, the fluorescent punctae did not co-localize with the PVC marker anti-VSR, nor was a vacuolar signal detected (Fig. 4C, panels 3–5). IEM with the 8S globulin–GFP transgenics revealed that the gold label was restricted to a population of single-membrane-bound organelles that lacked internal membranes (Fig. 3B). To confirm that the globulin detection by CLSM and IEM was the same, we checked by immunofluorescence using anti-8S globulin and anti-GFP antibodies that the GFP fluorescence from the 8S globulin was indeed 8S globulin (Fig. S3 at JXB online).

The 8S globulin-positive organelles had a circular profile in section with a relatively constant diameter of 200–600 nm. Based on the presence of ribosome-like particles at their surface and a positive labelling of the boundary membrane with calnexin antibodies (Fig. 3C, D), the 8S globulin-containing organelles were therefore derived from the ER. The same conclusion was reached with IEM studies on an untagged 8S globulin transgenic BY-2 cell line, with gold label being detected only in the swollen ER and not in Golgi stacks or in multivesicular PVCs (Fig. 5).

We also generated a stable Arabidopsis line expressing 8S globulin–GFP and examined cells in the cotyledonal leaves, stems, and root tips. Using CLSM, a punctate fluorescent pattern was again observed (Fig. 6A, panels 1–3), and after IEM these proved to have the same morphology and size as those described for BY-2 cells (Fig. 6B, panels 4–6). Additional evidence that the 8S globulin-containing bodies were not a post-Golgi compartment was provided by IEM with complex glycan antibodies. Whilst these antibodies gave rise to a clear positive labelling of the Golgi apparatus and multivesicular bodies (i.e. PVCs), there was no gold label over the ER bodies (Fig. 6C, D). Although the majority of ER bodies were unconnected to the ER network, we were able to find examples of a positive labelling of the ER with anti-8S globulin antibodies (Fig. 6E), supporting the notion that the ER bodies must somehow bud off from the ER.

**8S globulin is present in a processed form only in seeds**

To ascertain the molecular condition of the 8S globulin in the various tissues from the transgenic Arabidopsis line, we carried out immunoblotting with globulin and GFP antibodies on total cell extracts. As seen in Fig. 7A, 8S globulin

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**Fig. 4.** 8S globulin expression in stably transformed tobacco BY-2 cells. (A) Detection of 8S globulin by Western blotting in a number of different lines (1–8). (B) Punctate distribution of 8S globulin in cells of line 4 as revealed by immunofluorescence. (C) No co-localization of fluorescent signals for 8S globulin and the PVC marker VSR. Bars, 50 μm.
was present in its pro-form (molecular mass 75 kDa) in all vegetative organs (leaf, stem, and roots), but in mature and dry seeds, it existed in its processed form of 50 kDa (Fig. 7B). During seed development, there was a clear transition from 8S globulin in its pro-form to the mature form as maturation proceeded (Fig. 7B). The lack of a GFP signal in the immunoblots of mature seeds was indicative of vacuolar processing, with the GFP tag being removed and degraded.

The location of 8S globulin changes during seed development in transgenic Arabidopsis

We performed IEM on developing embryos from the transgenic Arabidopsis line expressing 8S globulin under the control of the CaMV 35S promoter. At early developmental stages when the 8S globulin was present in the pro-form, the positively-labelled organelle was the ER body, as seen in vegetative tissues (Fig. 8A–C). In intermediate stages of embryo development, ER bodies were no longer present. Instead, the enlarging PSV was labelled, together with smaller storage protein-filled organelles (Fig. 8D, E, F, H). Based on their positive labelling with anti-VSR antibodies (Fig. 8G, H; see Wang et al., 2012), we assumed that these were PVCs. In mature seeds, the protein bodies were positively labelled with anti-8S globulin antibodies (Fig. 8I, J). Thus, the site of subcellular accumulation of the 8S globulin changed from the ER to the PSV with the onset of the synthesis of endogenous seed proteins. During these stages of seed development, the 8S globulin was then capable of exiting the ER and entering the secretory pathway.

Discussion

Protein accumulation in the ER

Depending on the nature of the stored protein, two types of ER-based protein accumulation are known in plants. In both cases, the compartments are formed as a result of protein aggregation. In the first type, encountered mainly in epidermal cells of leaves and roots, a β-glucosidase (PYK10), which has an ER-retention motif (KDEL) at its C terminus, is present. This is synthesized and accumulates in response to stress or pathogen attack (Matsushima et al., 2003; Nagano et al., 2005). The second type is restricted to...
Fig. 6. The fluorescent patterns and organelle identity of 8S globulin–GFP-containing structures in various tissues from a transgenic Arabidopsis plant. (A) 8S globulin–GFP localized as discrete punctae in the cytosol of leaf, stem, and root tip cells. Bar, 50 μm. (B) 8S globulin–GFP localized to ER-derived bodies of leaf, stem, and root tip cells. (C, D) Complex glycan antibodies were positively labelled on Golgi stacks (G) and multivesicular bodies (m) but not ER bodies (asterisks). (E) Positive labelling of the ER indicateds that ER bodies must arise from the ER. V, vacuole; CW, cell wall.
The most well-known example is found in the endosperm of cereal grains where the accumulation of prolams eventually leads to the production of ER-derived protein bodies (Herman and Larkins, 1999). In addition to achieving high local concentrations through targeting of prolamin mRNA to specific ER domains (Crofts et al., 2004, 2010), protein–protein interactions via disulfide bridge formation seem to be the underlying mechanism for prolamin retention in the ER, at least for the zeins (Geli et al., 1994; Pompa and Vitale, 2006). In addition, both the relative amounts of individual prolams and the chaperone BiP also play important roles in the folding and aggregation process (Li et al., 1993; Kawakatsu et al., 2010). Interestingly, the self-aggregation properties of zein and a chimaeric construct of zein and the globulin phaseolin (zeolin) have been used successfully to induce the formation of ER-derived protein bodies in vegetative tissues (Mainieri et al., 2004; Takahashi et al., 2005; de Virgilio et al., 2008; Francin-Allami et al., 2011).

Globulins, however, do not normally accumulate in the ER, although there are some exceptions to this rule. Small protein bodies with a ribosomal covering have been recorded on several occasions at later stages in legume cotyledon development (Adler and Müntz, 1983; Craig, 1988; Robinson et al., 1995). Whether these eventually fuse with the PSV is unclear. Similar protein bodies, which survive as such until seed desiccation, are also formed in transgenic soybean (Glycine max) cotyledons when the ratio of glycmin to β-conglycinin is disturbed through gene silencing of the latter (Kinney et al., 2001). Another exception, although only of temporary nature, are the PAC (precursor-accumulating) vesicles seen in maturing pumpkin (Cucurbita maxima) and castor bean (Ricinus communis) seeds. These are derived from the ER and contain aggregates of the pro-forms of 11S globulins and 2S albumin (Hara-Nishimura and Shimada, 2006). On their way to the PSV, PAC vesicles receive a contribution from the Golgi apparatus, as they also contain complex glycans (Hara-Nishimura et al., 1998; Jiang and Sun, 2002).

Why does mung bean 8S globulin not pass through the Golgi when expressed in vegetative tissues?

The ER bodies produced in vegetative tissues of Arabidopsis in response to heterologous expression of mung bean 8S globulin are different to the stress-induced β-glycosidase bodies. Morphologically, they are roughly spherical rather than spindle-shaped and are relatively immobile. Unlike β-glycosidase, 8S globulin also lacks a KDEL motif so that even temporary retention in the ER should not take place. Nevertheless, the 8S globulin of mung bean, like other globulins, can form aggregates as judged by the electron-dense contents of DVs, storage PVCs and PSVs in developing mung bean cotyledons.
Fig. 8. Subcellular localization of 8S globulin–GFP changes during seed development. Immunogold labelling of ultrathin sections cut from high-pressure frozen/freeze-substituted 8S globulin–GFP Arabidopsis transgenic seeds. (A–C) Immunogold labelling with anti-8S globulin and anti-GFP antibodies showed that 8S globulin localized to ER-derived compartments (indicated by asterisks) in young seeds. V, vacuole; CW, cell wall; P, plastid. (D–H) Immunogold labelling with anti-8S globulin or anti-GFP antibodies showed that 8S globulin–GFP localized to PSVs (D) and storage PVCs (E, F, H), which is labelled with anti-VSR antibody (G, H) in the intermediate stage of developing seeds. Immunogold double labelling confirmed that 8S globulin–GFP and VSR proteins localized to the same storage PVC (H). (I, J) Immunogold labelling with anti-8S globulin antibody showed that 8S globulin–GFP localized in PSVs of mature seeds.
(Wang et al., 2012), so its accumulation in the ER is probably the consequence of high local concentrations.

The accumulation of 8S globulin–GFP in the ER could be a consequence of inability of the fusion protein to fold and assemble correctly and would therefore be retained by the ER quality-control machinery (Pedrazzini et al., 1997). However, as untagged 8S globulin also accumulates in the ER of transgenic BY-2 cells, this explanation does not seem probable. We feel it is more likely to be a consequence of the inability of COPII-vesicles to export storage protein aggregates out of the ER. That the mung bean 8S globulin does enter the Golgi in Arabidopsis embryos and is correctly directed to the PSV suggests, however, that, although globulins may be sorted into DVs via an aggregation-based mechanism at the cis-Golgi, there are factors required for this process that are absent in the Golgi apparatus of cells in vegetative tissues. These factors may include other storage proteins that are necessary for nucleation of the aggregation process, or even specific receptors that are required for capturing the globulins upon entry into the cis-Golgi.

The identity of putative globulin receptors remains controversial. Although data has been presented in support of a role for VSR1 (Shimada et al., 2003; Zouhar et al., 2010), the primary localization of VSRs to the TGN in developing Arabidopsis embryos (Hinz et al., 2007) is not compatible with a cis-Golgi sorting event. The other possible receptors are the receptor homology region transmembrane domain ring H2 (RMR) proteins (Wang et al., 2011), which seem to be a more likely candidate in that they do interact with vicilin-like proteins (Shen et al., 2011) and localize preferentially to early Golgi cisternae and DVs in Arabidopsis embryos (Hinz et al., 2007). However, RMR proteins are not restricted to seed tissue. Whereas all six Arabidopsis RMRs are expressed in seeds, all vegetative tissues have at least one expressed RMR (Neuhaus and Paris, 2005). Therefore, in theory, both types of receptor are present in vegetative tissues and should be able to cope with the sorting of a heterologously expressed storage protein. This is indeed the case with 2S albumin but clearly is not so with mung bean 8S globulin.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. As shown by immunofluorescence and FM4-64 labelling, 8S globulin–GFP punctae are distinct from known secretory and endocytic compartments in transgenic tobacco BY-2 cells.

Fig. S2. The formation of 8S globulin–GFP punctae is not affected by overexpression of Sec12. This contrasts with the behaviour of 2S albumin–mRFP and the Golgi marker Man1–mRFP.

Fig. S3. Both 8S globulin and GFP antibodies specifically recognize the 8S globulin–GFP punctae.

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