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Protein Body Biogenesis in Cereal Endosperms

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Abstract Developing cereal endosperms accumulate storage proteins as a reserve to support the early postgerminating phase of plant growth and development. These storage proteins are concentrated and packaged into a specialized organelle, the protein body, which are derived from the endoplasmic reticulum lumen or vacuole. In this chapter, we describe the biochemical and cellular events that underlies the basis for the use of these two intracellular sites for storage protein deposition and accumulation.

1 Introduction

Storage proteins, which serve as a reservoir of carbon, nitrogen, and sulfur for the postgerminating seedling, constitute up to 90% of the total protein fraction in developing seeds. Because of their large quantities, plants have utilized the endomembrane system for sequestering the storage proteins into a highly compact form, the protein body (PB). Most plants, including several of the major cereals, use the protein storage vacuole (PSV) as the site of deposition. Other cereals have exploited the lumen of the endoplasmic reticulum (ER) as an alternative storage site. In this chapter we describe the processes involved in PB formation in the major cereals wheat, barley, maize, and rice, with particular emphasis on how each of these cereals has contributed to our knowledge in this topic area. The study of each of these cereals has shed light on specific aspects of PB formation which, when viewed in total, have resulted in a better understanding of how the storage proteins are packaged in the cell. The reader should refer to two monographs on the subject (Larkins and Vasil 1997; Shewry and Casey 1999) for other topics not covered here.

2
PB Formation in Wheat and Barley

The storage proteins of the *Triticeae* are homologous in structure (Shewry and Casey 1999). The wheat prolamines consist mainly of two types: monomeric gliadins solubilized by alcohol solution and polymeric glutenins solubilized by alcohol solution in the presence of a reducing agent. The barley prolamines are classified by their electrophoretic mobility into three groups: B hordein, C hordein, and D hordein. The B hordein and D hordein subunits are rich in cysteine residues and are present as monomeric and polymeric species, while sulfur-poor C hordein subunits are present as monomers.

The prolamines of wheat (Campbell et al. 1981) and barley (Cameron-Mills and von Wettstein 1980) are deposited within the PSV (Fig. 1a,d). The earliest event of PB formation in these cereal grains is the synthesis of the storage protein on the ER and their cotranslational import into the ER lumen. Despite their close phylogenetic relationship, different mechanisms have been suggested for how prolamines are exported from the ER lumen to the PSV and the involvement of the Golgi apparatus in PB formation in these plant species.

2.1
Golgi-Dependent Pathway in Wheat PB Formation

Consistent with the secretory pathway unraveled in animal cells, the Golgi apparatus was suggested to play a prominent role in mediating the transport of wheat storage proteins from the ER to the PSV (Buttrose 1963). Evidence for a direct role of the Golgi apparatus in wheat PB formation was obtained by immunocytochemical studies of the gliadins during endosperm development (Kim et al. 1988). When viewed by electron microscopy the earliest indication of PB formation was the packaging of gliadins within small (0.2–0.3 μm in diameter) electron-dense vesicles connected to the Golgi cisternae (Fig. 1c). The absence of antigen signals within the ER lumen and their presence in (protein accumulating) vesicles suggested that the gliadins were rapidly exported from the ER lumen and concentrated within dense vesicles. The Golgi observed during the mid-stage of grain filling were atypical from the four to seven closely stacked cisternae structure commonly observed in other plant tissues. Instead the Golgi apparati were highly modified in containing two or three highly curved cisternae with associated electron-dense and electron-lucent vesicles. Dense vesicles in the cytoplasm were commonly observed associated with larger electron-lucent vesicles or vacuoles. Also distributed in the cytoplasm were small to medium size vacuoles containing one or more gliadin inclusion bodies, suggesting the coalescence of smaller inclusion granules to form larger ones which, in turn, merged with the larger PSV (Bechtel et al. 1982; Kim et al. 1988).

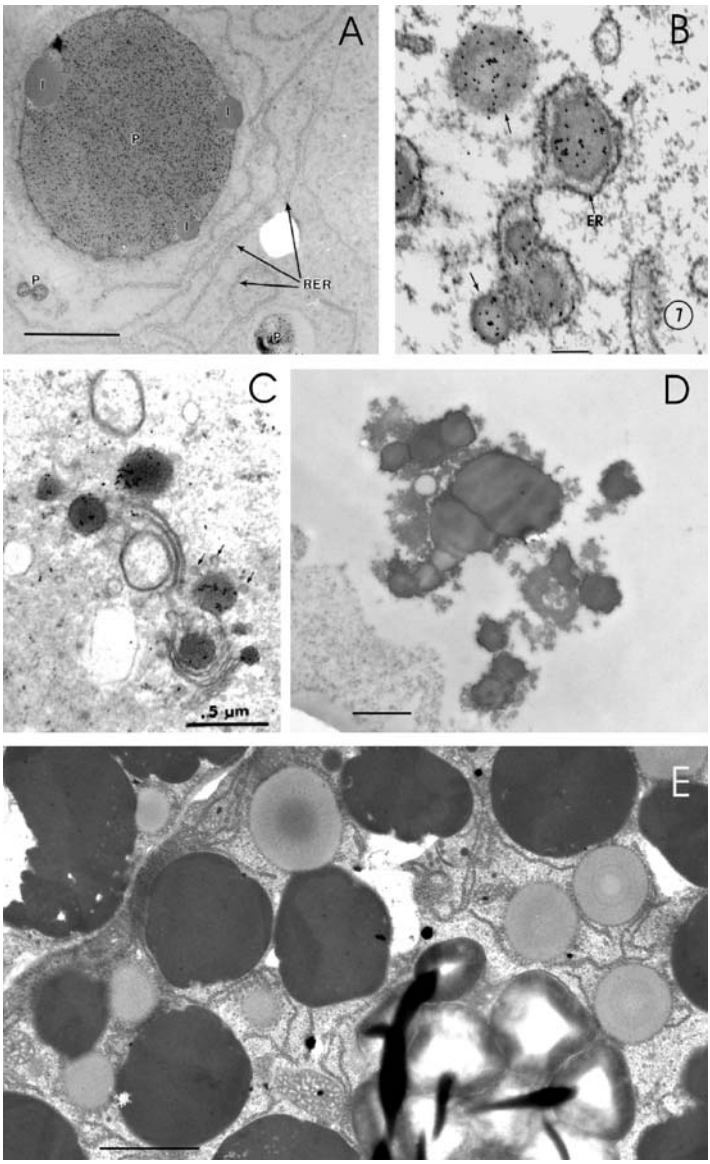

Fig. 1 Morphology of cereal PBs. **a** Wheat PB immunolabeled with gliadin antibodies and protein A gold particles (*scale bar*: 2 μm). **b** Small intracisternal inclusions in developing wheat endosperm (*scale bar*: 0.5 μm; taken from Krishnan et al. (1991) with permission from the author and NRC Research Press). **c** Unique Golgi structures containing highly curved cisternae with associated dense vesicles and electron-lucent vesicles in developing wheat endosperm. **d** Barley PB containing electron-light and -dense staining globules as well as a fibrillar matrix (*scale bar*: 1 μm). **e** Rice electron-lucent spherical prolamine-containing PBs and irregularly shaped, electron-dense glutelin-containing PSVs (*scale bar*: 2 μm)

2.2 Golgi-Independent Pathway in Wheat PB Formation

The role of the Golgi apparatus in wheat PB formation has been a subject of controversy ever since the first electron micrographic studies were undertaken in the early 1960s (for a review, see Parker 1982). The principal reason for dismissing the involvement of this organelle in wheat PB formation was that it was rarely detected throughout seed development or during the biosynthetic period when protein accumulation rates were at their highest (Bechtel et al. 1982; Campbell et al. 1981; Parker 1982). Moreover, in several studies (Bechtel et al. 1982; Campbell et al. 1981; Krishnan et al. 1991; Levanony et al. 1992; Parker 1982) but not all, large intracisternal inclusion granules bounded by rough ER were observed (Fig. 1b). These inclusion granules were thought to be too large to be exported from the ER to the Golgi via transport vesicles and were assumed to be transported to the PSV (Rubin et al. 1992). Lastly, the lumenal chaperone binding protein BiP was observed trapped within the PB inclusions (Levanony et al. 1992). As this protein normally resides in the ER lumen, its presence in the PB inferred the initial assembly of the inclusion granules in the ER lumen. Collectively, these three reasons suggested that the Golgi played, at best, a minor role in wheat PB formation. In turn, various novel transport processes from the ER to the PSV were proposed.

The latest and generally accepted rendition of a Golgi-independent pathway for wheat PB formation was proposed by Galili's laboratory in a series of studies (Galili 1997; Galili et al. 1995; Levanony et al. 1992). Although a Golgi-dependent pathway was acknowledged to exist, only small amounts of prolamines were transported from the ER to the storage vacuole by this pathway. The bulk of the proteins were assembled to form inclusion granules within the ER lumen which was then released into the cytoplasm, a proposal first suggested by Miflin et al. (1981). Once in the cytoplasm the inclusion granules were seen to be engulfed by small vesicles through a process akin to autophagy to form small vacuoles containing PB, which then merged with each other and eventually the large PSV. In addition to electron micrographs suggesting the presence of ER inclusion granules and remnant rough ER membranes within the PSV, the PSV contained the lumenal chaperone BiP, a marker of the ER lumen.

The existence of two pathways leading to PB formation in wheat was also inferred by biochemical studies. Wheat PBs consist of a low-density type (light PB) and a high-density type (dense PB) (Rubin et al. 1992). The gliadins were present in both light PBs and dense PBs, while the high molecular weight (HMW) glutenins were localized only in the dense PBs, suggesting that both prolamines are transported and deposited within PSV by different processes, one Golgi-dependent and the other Golgi-independent. Pulse–chase experiments showed that the dense PBs were formed from the light PBs, but the

dense PBs were formed not by a gradual increase in density but by a quick process of storage protein aggregation.

2.3 PB Formation in Barley

The barley PSVs are highly complex as they contain electron-light and electron-dense staining globules, as well as a fibrillar matrix and vesicles (Fig. 1d) (Cameron-Mills and von Wettstein 1980). The electron-dense globules and the fibrillar matrix predominated in Risø 1508, a mutant line deficient in B and C hordeins, indicating that the electron-light staining globules were made up of B and C hordeins (Cameron-Mills and von Wettstein 1980), a conclusion later confirmed by immunogold labeling studies (Rechinger et al. 1993). Unlike wheat, where several studies demonstrated the existence of ER inclusion bodies, no such entities have been described in barley except in the Nevsky barley line, which is discussed below. Instead, dense vesicles containing hordeins associated with the Golgi were prominently observed (Galili and Herman 1997; Møgelsvang and Simpson 1998). In addition to those contained within the PSV, globules were also detected free in the cytoplasm. These cytoplasmic globules were not surrounded by a rough ER-like membrane. The cytoplasmic globules varied in size (0.34 μm on average) and were smaller than those present in the PSV (average size of 1.15 μm). Overall, these microscopic observations discount the existence of a direct ER to PSV pathway in developing barley endosperm. Instead, PB formation in barley is mediated by the Golgi, where the hordeins are initially concentrated into dense vesicles, and later in cytoplasmic globules which combine to form larger species and eventually with the PSV.

In the barley line Nevsky, which lacks γ_3-hordein (a minor B hordein), nearly all of the B and C hordeins are observed as globules contained within the ER lumen in the central starchy endosperm (Rechinger et al. 1993). These observations suggest a possible role for γ_3-hordein in conferring transport competence of B and C hordeins from the ER to the Golgi. Interestingly, subaleurone cells exhibited a normal PB phenotype of globules contained within the PSV and were nearly devoid of intracisternal inclusion granules in the ER. This apparent dichotomy depending on the cell type suggests that the extent of hordein assembly within the ER depends on relative rates of synthesis, of protein folding, and of their export from the ER (Tooze et al. 1989). In young subaleurone cells, the rate of hordein synthesis is lower than its export rate from the ER, thereby favoring hordein accumulation in the PSV. In the bulky endosperm the high rates of hordein synthesis exceed its capacity for export, conditions leading to elevated levels in the ER lumen and their assembly to form an inclusion granule.

3
PB Formation in Maize

The major storage proteins in maize kernels are the alcohol-soluble zeins (Coleman and Larkins 1999; Shotwell and Larkins 1989). Maize also contains smaller quantities of legumin-1, an 11S globulin (Yamagata et al. 2003), which is deposited in small PSVs. The zeins are composed of four major classes, α, β, γ, and δ, which are readily distinguished by their different molecular sizes and their dependence on reducing agents for their solubility in alcoholic solutions. The α-zeins are sulfur-deficient polypeptides of relative molecular mass (M_r) 19 000 and 22 000, and constitute the most abundant class (70%) of the prolamine fraction. The other zein classes are sulfur-rich and consist of the cysteine-rich γ-zeins, the second most abundant class, with major species at 16 and 27 kD as well as a third minor species of 50 kD identified by expressed sequence tag (EST) analysis of a maize developing endosperm library (Woo et al. 2001). The two remaining classes consist of β-zeins of M_r 15 000 and δ-zeins of M_r 10 000 (some inbred lines also have a δ-zein of M_r 18 000). These zein classes are sulfur-rich in containing a high mole percentage of cysteine and methionine residues. Sequence alignments indicate that the β-zeins belong to the γ-zein family in sharing the conserved N-terminal and C-terminal peptides (Woo et al. 2001). Both the γ-zeins and β-zeins require a reducing agent for their solubilization in alcoholic solution and exist as disulfide-linked protein complexes. The δ-zeins are the most hydrophobic and exist as monomeric species, readily extractable in the absence of reducing agents. Further details on the physical properties and protein gene structures can be found in Coleman and Larkins (1999) and Woo et al. (2001).

Zeins are packaged as accretions, spherical inclusion granules 1–2 μm in diameter, within the ER lumen (Lending et al. 1988). The zeins lack the canonical HDEL/KDEL retention/retrieval signal, and hence their retention and localization within the ER has been a subject of many studies over the past decade (Coleman and Larkins 1999). Although suggestions have been made for a peptide signal functional equivalent to HDEL/KDEL, the retention of zeins at the ER is likely due to their ability to assemble into a stable intracisternal inclusion granule (Geli et al. 1994; Kim et al. 2002) at a rate significantly higher than its capacity for ER export.

Based on its spherical shape and the lack of an unfolded protein response with accompanying high amounts of the lumenal chaperone BiP, zeins must be assembled and packaged in a highly ordered structure. Indeed, Lending and Larkins (1989) have shown in a series of elegant electron micrographs that the zein protein bodies undergo a series of morphological changes during their enlargement to a mature organelle. Young PBs, found in subaleurone cells, are small and electron dense and contain only γ- and β-zeins. During the maturation of the endosperm cell, the enlarging PB contains small,

electron-lucent locules containing α- and δ-zeins (Esen and Stetler 1992; Lending and Larkins 1989). As additional α- and δ-zeins accumulate in older endosperm cells, the electron-lucent locules fuse to form a large central core of α- and δ-zeins surrounded by a thinner shell of β- and γ-zeins. Thus, the development of the zein PB occurs by a strict temporal expression pattern where the γ- and β-zeins are accumulated first, followed by the deposition and assembly of α- and δ-zeins to form the core region and, in turn, the displacement of γ- and β-zeins to the periphery. The spatial location of the various zeins within the inclusion granule is likely directed by their intrinsic physical properties.

3.1 The Role of Protein to Protein Interactions in Maize PB Formation

The biochemical basis for zein PB formation has been explored by expressing various zeins in a wide variety of heterologous hosts and plant tissues (Coleman and Larkins 1999). Despite the limitations of these systems, valuable information has been obtained from these studies, which have provided novel insights into how these proteins are retained and assembled to form a spherical intracisternal inclusion granule 1–2 μm in diameter.

The 27-kD γ-zeins form ER inclusion granules when expressed in *Arabidopsis* leaves (Geli et al. 1994). The γ-zein containing ER inclusions, however, were much smaller (< 0.5 μm) and somewhat amorphous in shape than those seen in maize. The 27-kD γ-zein has a modular structure containing an N-terminal domain with PPPVHL tandem repeats, and a conserved cysteine-rich C-terminal domain. Removal of the N-terminal proline-rich tandem repeat domain resulted in the export of the truncated γ-zein from the ER to the extracellular space, whereas removal of the conserved C-terminal domain transformed the small ER inclusions to large reticulated ER-bound structures. These observations indicate that the two domains have different roles in PB formation. The proline-rich tandem repeat domain is required for retention of the γ-zeins within the ER lumen by promoting protein to protein interactions and their assembly into a higher order protein conformation. The conserved C-terminal peptide is essential for the formation of a highly ordered spherical granular structure mediated by intermolecular disulfide bonds.

The β-zeins also have the capacity to form PBs in tobacco seeds and leaf tissues (Bagga et al. 1995), although the PB morphology was quite distinct from that seen for γ-zeins. In leaves, the PBs appeared as highly lobed, rosette-like structures. In seeds, the PBs resembled those found in maize endosperm, although multilobed PBs and multiple inclusion granules bounded by a single ER membrane were also apparent. In contrast to an earlier report (Hoffman et al. 1987), no significant levels of β-zein were observed in the crystalloid areas of the PSV although, in rare instances, a spherical β-zein PB

was detected in the matrix region, an event suggested to occur by autophagy. A more recent study (Coleman et al. 2004), however, showed that the formation of ER-bound PBs is a relatively rare event and that the bulk of the β-zein is exported to the PSV (discussed below).

The β- and γ-zeins are each capable of forming PB-like structures, whose morphology reflects the intrinsic physical properties of each protein. In neither case were these PBs, formed by homotypic interactions of a single zein class, identical in morphology to the spherical compact accretions observed in maize. In light of their coordinate expression during the early endosperm development and colocalization in an early immature PB, it is likely that the interactions and assembly of γ- and β-zeins is a prerequisite for normal PB formation in maize.

Unlike the γ- and β-zeins, the α-zeins failed to accumulate to any appreciable extent when expressed in most heterologous hosts (Coleman and Larkins 1999). Coexpression with the 27-kD γ-zein, however, elevated the expression of a 22-kD α-zein by stabilizing protein levels (Coleman et al. 1996). Immunocytochemical analysis showed that both zein classes were located in spherical PBs similar in appearance to those observed during the early stages of maize development. The PBs were 0.1–0.5 μm in diameter and consisted of α-zein-associated electron-lucent locules contained within an electron-dense γ-zein matrix. The results support the view that γ-zeins help retain α-zeins within the ER by sequestering them and preventing their export from this compartment.

The accumulation of α-zein was also stabilized by coexpression with β-zein in tobacco seeds (Coleman et al. 2004). Closer examination showed that accumulation of α-zein but not β-zein was temporally regulated, suggesting that α-zein was more prone to proteolysis. Electron microscopic studies showed the presence of both multilobed and single spherical PBs. Interestingly, the multilobed PBs contained only β-zein while the single spherical PBs harbored both α- and β-zeins, although their spatial distribution was just the opposite from that seen in maize. The electron-lucent locules labeled with α-zein antibody were located on the periphery and surrounded the electron-dense β-zein core. Hence, β-zeins are capable of interacting with α-zeins and retaining them within the ER, but are not responsible for their spatial distribution within the protein body. This role is probably assumed by γ-zeins, which are capable of sequestering α-zeins to the core region.

Indirect immunofluorescence studies showed that although a few small PBs were observed, most of the α- and β-zeins were localized to the PSV (Coleman et al. 2004). The PSVs showed uniform fluorescence for both zein species, indicating that the zeins were randomly dispersed within this compartment. Therefore, the increase in α-zein accumulation in these transgenic plants was not due to their retention in the ER but, instead, to their protection by β-zeins from proteolysis within the PSV. In addition, these observations indicate that the α- and β-zeins are capable of ER export and at rates much

higher than their capacity to form inclusion granules. The γ-zeins may also be exported from the ER efficiently when expressed in *Arabidopsis* and tobacco and only a small proportion may form ER-derived PBs.

It is unclear why the α- and β-zeins exit the ER so efficiently in tobacco. It is possible that the interaction of γ- and β-zeins and their assembly retards their export from the ER and that these two zein species work in unison to sequester α-zein within this compartment, as suggested by yeast two-hybrid analysis (discussed below). The existence of a receptor-like protein that retards the export of β-zein, however, cannot be excluded.

The 10-kD but not the 18-kD δ-zeins are capable of forming PBs in tobacco leaves and seeds (Bagga et al. 1997; Hinchliffe and Kemp 2002). These PBs, which differ considerably in structure from the γ-zein and β-zein types, contained amorphous inclusions surrounded by a thick layer of osmophilic material. Coexpression with β-zein resulted in a four- to five-fold increase in δ-zein accumulation. These results support the interaction between β- and δ-zeins where formation of the complex stabilizes δ-zein from proteolytic turnover. Interestingly, the mixed zein PBs were identical to that observed in the β-zein expressing plants, indicating that the structural properties of β-zein dictate the morphology of this inclusion granule.

The putative interactions between the various zein classes are supported by results obtained by yeast two-hybrid analysis. In particular, the protein binding specificity of the 16-kD γ-zein and 15-kD β-zein suggest that they play important roles in PB formation. These two zeins showed strong homotypic interaction with themselves, and strong heterotypic interactions with each other as well as with other zeins. The 16-kD γ-zein interacts with the 50- and 27-kD γ-zeins as well as with the 19- and 22-kD α-zeins and 10-kD δ-zein. Likewise, the 15-kD β-zein interacts with the 22-kD α-zein and 10-kD δ-zein. The interacting properties of the 16-kD γ-zein and 15-kD β-zein overlapped in specificity, suggesting that they play a dominant role in the assembly and formation of the PB.

3.2
RNA Localization in Maize

The formation of ER-bound PBs in rice has been suggested to be facilitated by the specific targeting of prolamine RNAs to the PB-ER (see Sect. 4.1). Using an in situ hybridization technique to visualize the density of RNAs on thin secretions of developing maize endosperm, Kim et al. (2002) showed that the 22-kD α-zein and 27-kD γ-zein RNAs were not concentrated but, instead, distributed uniformly on the cisternal ER and PB-ER membranes. This random distribution of RNAs was consistent with earlier biochemical fractionation studies (Larkins and Hurkman 1978), which showed that microsomal membranes enriched for cisternal ER and PB-ER membranes had about the same translational capacity for zein synthesis. These results led to the conclusion

that zein protein interactions determine PB assembly and that RNA localization of zein RNAs played, at best, a minor role in this process.

Curiously, when expressed heterologously in developing rice endosperm, the 10-kD δ-zein RNA displayed asymmetric localization to the PB-ER (Washida and Okita, unpublished data). Further studies showed that this RNA contained zip code sequences that targeted green fluorescent protein (GFP) RNA to the PB-ER (Washida and Okita, unpublished data). To resolve these apparent differences in RNA localization in maize and rice, the localization of RNA coding for the various zein classes together with a legumin-like protein were reexamined in maize endosperm (Washida et al. 2004). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that the various zein RNAs had restricted localization to the PB-ER, whereas legumin RNAs were targeted to the cisternal ER. These results support a role for RNA localization in PB formation in maize.

The basis for the apparent differences in results between these two studies (Kim et al. 2002; Washida et al. 2004) remain to be resolved. Further studies must be conducted in maize to show whether these zein RNAs actually contain zip codes that direct RNA localization to the PB-ER, and whether PB formation is dependent on the targeting of zein transcripts to these membranes.

Overall, maize PB formation is dependent on the temporal expression patterns of the various zein classes, and their intrinsic properties to interact and assemble with each other and with other zein classes to form a higher ordered, stable inclusion granule. The initial deposition of γ- and β-zeins in early development and the propensity of these zeins to form intracisternal inclusion granules by homotypic protein to protein interactions support the dominant role of these proteins in initiating PB formation. Both zein types interact with α- and δ-zeins, which enables these zeins to be retained in the ER lumen so that they eventually interact with themselves to form locules and then later the central PB core.

4 PB Formation in Rice

Unlike other plants which store predominantly a single class of storage proteins, rice accumulates three major types (Muench et al. 1999). The most abundant on a weight basis are the glutelins, storage proteins homologous to 11S globulins, which constitute up to 60% of the total protein. Two other classes are the alcohol-soluble prolamines ($\sim$ 20%) and α-globulins ($\sim$ 10%). These storage proteins are transported to and accumulated in different sites of the endomembrane system (Krishnan et al. 1986; Tanaka et al. 1980). When viewed by electron microscopy, two types of PBs are evident in the bulky endosperm: irregularly shaped, electron-dense PSVs containing glutelins and

globulins, and spherical ER-bound inclusion granules containing prolamines (Fig. 1e).

The rice prolamines, which lack repetitive sequences, are composed of sulfur-rich (10-, 13-, and 16-kD molecules) and sulfur-poor (13-kD molecule) species (Ogawa et al. 1987). The sulfur-rich species comprise about 60% of the total prolamine fraction and possess the ABC modular structure of the prolamine superfamily where each domain contains a single or a pair of conserved cysteine residues (Shewry et al. 1995). When viewed by electron microscopy, the spherical ER-derived PBs possess a high electron density core with a surrounding lamellar structure (Bechtel and Juliano 1980; Ogawa et al. 1987; Yamagata and Tanaka 1986). Immunocytochemical analysis indicates that the sulfur-rich prolamines reside in the electron-dense core region surrounded by sulfur-poor prolamines (Ogawa et al., unpublished data). Temporal expression studies (Ogawa, unpublished data) show that the sulfur-rich 10- and 13-kD prolamines are initially deposited within the ER lumen to form the electron-dense core followed by the accumulation of the other prolamine species which form the peripheral lamellar structure. This spatial relationship between sulfur-rich and sulfur-poor prolamines within the intracisternal inclusion granule is just the opposite to that seen in maize, where the sulfur-poor zeins constitute the core which, in turn, is surrounded by the sulfur-rich species (Lending et al. 1988). The PBs found in the endosperm of sorghum (Shull et al. 1992) and *Setaria* (Rost 1971) resemble the rice prolamine PBs, suggesting that they are formed by a similar process.

The mechanism by which prolamine polypeptides are assembled into a structurally ordered stratified inclusion granule remains largely unexplored. As suggested for maize zein PB formation, specific protein to protein interactions among the various rice prolamine classes are likely responsible for the ordered spatial organization of the PBs. In addition to homotypic and heterotypic interactions, there is compelling evidence that the lumenal chaperones are actively involved in this cellular process. Rice endosperm contains unusually high amounts of the lumenal chaperone BiP, which can be easily detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of PB extracts (Li et al. 1993a). This lumenal chaperone was found to be associated with nascent prolamine chains bound to polysomes, free prolamine polypeptides, and on the surface of the prolamine inclusion granule. The association with the latter was ATP dependent. Moreover, immunocytochemical studies showed that BiP was not uniformly distributed to the ER but, instead, concentrated on the periphery of the prolamine PBs (Muench et al. 1997). Overall, these observations indicate a role for BiP in prolamine polypeptide folding and assembly to form an intracisternal inclusion granule.

Glutelin synthesis is detected in 5-day-old developing seeds, whereas the onset of prolamine accumulation occurs 5 days later (Yamagata and Tanaka 1986). Glutelins are initially synthesized on ER-bound polysomes (Yamagata and Tanaka 1986) as a 57-kD precursor, which is then cleaved to acidic and

basic subunits within the PSV (Yamagata et al. 1982). Transport from the ER to the PSV is through the Golgi apparatus, an organelle containing two highly curved cisternae with attached glutelin-containing dense vesicles (Krishnan et al. 1990). Larger dense vesicles were evident in the cytoplasm, suggesting that they were formed by fusion of smaller vesicles (Oparka and Harris 1982). The mature PSV is composed of glutelin crystalline inclusions separated by an electron-dense matrix (Bechtel and Juliano 1980; Krishnan et al. 1986).

Few studies have been directed at understanding the expression of the third most abundant rice storage protein, the α-globulins. These proteins are synthesized on the ER and exported to the Golgi apparatus where they form dense vesicles, which are then transported to the PSV (Krishnan et al. 1992). The globulins accumulate in the matrix located at the periphery and the border regions between the glutelin crystalline inclusions. This partitioning of α-globulins from glutelins within the PSV suggests that these proteins are not cotransported together to the PSV but, instead, use different pathways.

Despite its solubility in saline solutions, the 26-kD α-globulin is a member of the prolamine superfamily (Krishnan and Pueppke 1993). Kawagoe et al. (2005) showed in an elegant study that the conserved cysteine residues in the motifs LxxC (A domain), CCxQL (B domain), and PxxC (C domain) formed two intra-disulfide bonds, which were essential for proper polypeptide folding and intracellular localization. GFP fusions containing the AB domain were found to be mislocalized to the prolamine PB, while the same protein containing a C79S (CC_{79}xQL of the B domain) replacement was properly sorted to the PSV. As cysteine-79 forms a disulfide bond with the conserved cysteine (PxxC) of the C domain, the unreacted residue interacts with a sulfur-rich prolamine resulting in its retention within the ER lumen. Replacement of cysteine-79 enables the GFP fusion to fold properly and gain competence for protein export.

4.1 Localization of Rice Storage Protein RNAs

The presence of a signal peptide and translation by membrane-bound polysomes indicates the synthesis of these storage proteins on the ER (Muench et al. 1999; Takaiwa et al. 1999). Early studies have suggested that the storage proteins were not randomly synthesized on this membrane complex but distributed to specific subdomains. In vitro translation of $polyA^+$-RNA isolated from a highly enriched prolamine PB fraction produced only prolamine, indicating that prolamine mRNA was highly enriched on the PB-ER (Yamagata et al. 1986). In contrast, glutelins were present at more than twofold greater levels than prolamine transcripts RNA in a microsomal fraction enriched for cisternal ER vesicles (Kim et al. 1993). The nonrandom distribution of these storage protein RNAs on the ER subdomains was unequivocally demonstrated by results obtained by biochemical and high-

resolution in situ hybridization studies (Li et al. 1993b). Prolamine RNAs were highly enriched on the PB-ER while glutelin RNA predominated on the cisternal ER. By assessing the spatial location of RNA transcripts from synthetic prolamine gene constructs in transgenic developing endosperm tissue, targeting to PB-ER was dependent on signals contained within the prolamine RNA and not its primary sequence (Choi et al. 2000). Interestingly, protein synthesis was required for PB-ER localization, although this requirement could be provided by translatable reporter gene sequences such as β-glucuronidase (GUS) or GFP independent of prolamine sequences. Deletion analysis confirmed the presence of two partially redundant *cis* elements or zip codes, located in the prolamine RNA coding sequence and 3′ untranslated region, which were required for PB-ER targeting (Hamada et al. 2003a). RNA transport was shown to occur by the formation and movement of a particle, the latter dependent on intact actin filaments but not on intact microtubules (Hamada et al. 2003b).

4.2 Storage Protein Mutants

Three mutants *esp1*, *esp3*, and *Esp4*, generated by *N*-methyl-*N*-nitrosourea mutagenesis, contained significant alterations in prolamine accumulation. The *esp1* mutant was deficient in sulfur-poor 13-kD polypeptides, while *esp3* and *Esp4* contained depressed and elevated levels of the sulfur-rich prolamines, respectively (Kumamaru et al. 1987, 1988). The spherical PBs in *esp3* and *Esp4* mutations lacked the typical lamellar structure, although *Esp4* PBs showed an enlarged electron-dense core surrounded by a region of uniform electron density while PBs of *esp1* had the same structure as wild type (Ogawa et al. 1989), suggesting that sulfur-rich prolamines are essential for the core formation of ER-derived PBs.

Several *glup* mutants have been identified that contain abnormal elevated amounts of the 57-kD glutelin precursor, which is normally processed to acidic and basic subunits in the PSV compartment. One mutant, *esp2*, lacks a functional 60-kD protein disulfide isomerase (PDI) (Takemoto et al. 2002). Electron microscopic analysis showed that PSVs were normal in appearance but that the formation of prolamine-accumulating PBs was significantly altered where numerous small, low electron density ER-derived PBs of uniform size (0.5 μm in diameter) were readily observed in place of the normally larger spherical PBs possessing stratified regions of varying electron density. Immunocytochemical and biochemical studies showed that these abnormally small PBs contained glutelin as well as prolamine bound together by intermolecular disulfide bonds (Takemoto et al. 2002). The retention of glutelins within the ER lumen via their interaction with prolamine polypeptides indicates that this lumenal chaperone is required for intramolecular disulfide bond formation for these storage proteins. Glutelin is not absolutely depen-

dent on PDI for intramolecular S – S bond formation as normal PSVs are readily evident, which may occur especially in 5- to 10-day-old developing seeds where glutelin synthesis occurs in the absence of prolamine. PDI may be essential at 10 days and later where synthesis and accumulation of these storage proteins are occurring at their maximum rates.

Immunocytochemistry of the *glup4* mutant showed a significant disruption in transport of glutelins to the PSV. Instead of normal PSVs 2–3 μm in size, numerous smaller cytoplasmic inclusions were present. Moreover, regions of the ER were highly distended and contained glutelins. Map-based cloning studies showed that the *glup4* gene encodes a Rab5a-like protein, which possesses GTPase activity and functions in vesicle traffic (Satoh, unpublished data). The accumulation of glutelin precursor within the ER in *glup4* endosperm may be due to a defect in the export of glutelins from the ER.

Analysis of the *glup3* mutant showed that the PSVs contained large quantities of unprocessed glutelin precursors, suggesting a defect in proteolytic processing (Kumamaru, unpublished data). Subsequent analysis revealed that the vacuolar processing enzyme (VPE) activity in the developing seeds from three independent *glup3* lines in rice was markedly reduced compared to wild type. DNA sequencing of the VPE gene in these *glup3* lines revealed single or multiple amino acid replacements or the formation of premature stop codons in the coding region. The *glup3* gene encodes a major VPE, which is responsible for the proteolytic processing of glutelin precursor within the PSV.

5 Conclusions

The major cereal grains deposit their reserve protein in the PSV or ER lumen. Irrespective of the endomembrane site used for storage, the processes responsible for the formation of the inclusion granule within the ER lumen or within Golgi-associated dense vesicles are likely to be functionally similar. In the ER lumen, the storage proteins must be correctly folded into a competent state, a process facilitated by chaperones, for subsequent protein to protein interactions or for their export from the ER. Another shared requirement for PB formation is that the storage proteins must be concentrated, a condition conducive for protein to protein interactions, at its initial site of inclusion granule formation, whether it is the ER lumen or Golgi-associated dense vesicles. Localization of RNAs to a defined ER subdomain, such as the PB-ER in rice endosperm, would effectively concentrate the levels of the newly synthesized prolamine polypeptide at this site. The assembly of storage proteins within Golgi-associated dense vesicles is not known, although one obvious mechanism would be the retrograde transport of vesicles from the *cis*-Golgi to the ER resulting in the concentration of the storage proteins within this compart-

ment and their assembly into an inclusion within the dense vesicle. Lastly, the site of final deposition utilized by the different cereals may depend on the relative rates of storage protein synthesis, rates of polypeptide folding, and rates of export from the ER as inferred by the example seen for the barley Nevsky line.

The proposed Golgi-independent, direct ER to PSV pathway requires further study for its role in PB formation in wheat. Questions that remain to be addressed include the biochemical processes involved in the release of the intracisternal inclusion granule from the ER and the autophagic uptake of the intracisternal inclusion granule into a vacuole, the biochemical basis for the trapping of BiP within the inclusion granules, and the determination of the relative contribution of this pathway compared to the Golgi-dependent pathway in PB formation. These studies should provide critical insights into how closely related wheat and barley can exploit distinct cellular pathways for the packaging of nearly identical proteins to the PSV.

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