

Two Structural Domains Mediate Two Sequential Events in γ -Zein Targeting: Protein Endoplasmic Reticulum Retention and Protein Body Formation

M. Isabel Geli, Margarita Torrent, and Dolores Ludevid¹

Departamento de Genética Molecular, Centro de Investigación y Desarrollo de Barcelona, Consejo Superior de Investigaciones Científicas, C/ Jorge Girona Salgado 18-26, 08034 Barcelona, Spain

γ -Zein is a maize storage protein synthesized by endosperm cells and stored together with α - and β -zeins in specialized organelles called protein bodies. Previous studies have shown that in maize there is only one type of protein body and it is derived directly from the endoplasmic reticulum (ER). In this article, we describe the domains of γ -zein involved in ER retention and the domains involved in protein body formation. To identify the signal responsible for γ -zein retention in ER-derived protein bodies, DNAs encoding various deletion mutants of γ -zein were constructed and introduced into *Arabidopsis* as a heterologous system. By using pulse-chase experiments and immunoelectron microscopy, we demonstrated that the deletion of a proline-rich domain at the N terminus of γ -zein puts an end to its retention in the ER; this resulted in the secretion of the mutated protein. The amino acid sequence of γ -zein necessary for ER retention is the repeat domain composed of eight units of the hexapeptide PPPVHL. In addition, we observed that only those γ -zein mutants that contained both the proline-rich repeat domain and the C-terminal cysteine-rich domain were able to form ER-derived protein bodies. We suggest that the retention of γ -zein in the ER could be a result of a protein-protein association or a transient interaction of the repeat domain with ER membranes.

INTRODUCTION

During seed development, plant seed storage proteins are synthesized on the rough endoplasmic reticulum (ER) but are then sequestered by different cellular pathways in the protein bodies (PBs; Galili et al., 1993). PB biogenesis occurs within specific organelles to which storage proteins are targeted; they accumulate and form large multimers or aggregates surrounded by a single membrane. Various origins of PBs have been identified in developing seed of cereals. In rice seed, for example, storage proteins are retained in different protein bodies: vacuolar PBs (containing globulins) and ER PBs (containing prolamins, the proline-rich storage proteins) (Krishnan et al., 1986; Yamagata and Tanaka, 1986). In wheat seed, storage proteins are deposited in two different types of PBs inside vacuoles (Rubin et al., 1992). Two separate routes to the vacuole have been postulated with regard to the transport of gliadins and glutelins: one is via the Golgi complex and the other is by way of an autophagy-like pathway (Simon et al., 1990; Levanony et al., 1992). Noncovalent interaction and disulfide bond cross-links between glutelins and/or gliadins seem to direct PB formation in the wheat endosperm (Shani et al., 1992).

The key to understanding the biogenesis of PBs in plant cells could lie in an analysis of the influence of both specific multimers and aggregation of storage proteins in this context. The

parallelism existing between the biogenesis of secretory granules in animal cells and protein bodies in plant cells should be taken into account. Recently, Voorberg et al. (1993) demonstrated that selective domain self-association of the von Willebrand factor protein resulted in its multimer assembly, which is required for secretory granule formation. It is not known whether proteins in storage granules contain (1) independent signals for sorting and for protein condensation, (2) cooperative signals, or (3) only one signal for both events.

In developing maize endosperm cells, the accumulation of zeins (α -, β -, and γ -zeins) involves the importation of newly synthesized proteins into the ER (Larkins et al., 1979; Torrent et al., 1986) by means of an N-terminal signal peptide and a signal recognition particle component (Campos et al., 1988). Once there, the zeins are retained within the rough ER lumen and assemble, giving rise to PBs delimited by ER membranes (Larkins and Hurkman, 1978). Contrary to the well-documented sorting events in vacuolar storage proteins, little information is available concerning the signals in zein polypeptides responsible for protein retention in the ER and protein assembly in PBs. Zeins may possess an ER retention signal even though they lack the KDEL motif, which has been described as the retention signal for ER resident proteins (Munro and Pelham, 1987). It has been proposed that the retention of highly hydrophobic α -zeins in the ER occurs as a result of their subsequent

¹ To whom correspondence should be addressed.

aggregation inside the ER, which is produced by interaction among the α -zeins themselves (Argos et al., 1982).

γ -Zein is a sulfur-rich prolamin that is soluble in aqueous solutions in the presence of a reducing agent. It is distinct from the main group of maize prolamins, α -zeins, but structurally related to other cereal storage proteins (reviewed in Shewry and Tatham, 1990). The γ -zein gene is represented in one or two copies by the haploid maize genome (Boronat et al., 1986), and analysis of cDNA (Prat et al., 1985) and genomic (Boronat et al., 1986) sequences reveals that the protein contains three characteristic structural domains: (1) an N-terminal proline-rich tandem repeat after the signal peptide, (2) a proline-Xaa (P-X) linker domain, and (3) a hydrophobic C-terminal cysteine-rich domain. Immunocytochemical studies of isolated maize PBs reveal the presence of γ -zein surrounding the dense central aggregate of α -zeins (Ludevid et al., 1984). During maize endosperm development, there is a sequential pattern of accumulation of α -zeins and γ -zeins within PBs; γ -zeins are deposited before α -zeins (Lending and Larkins, 1989). However, until now, little information has been provided about the role of zein structural domains in ER retention and PB formation. In a previous paper, we defined a putative domain involved in γ -zein retention inside *Xenopus* oocytes (Torrent et al., 1994).

In this study, we analyze the function of three domains in γ -zein. First, we constructed mutant γ -zein cDNAs by the deletion of specific structural domains, and then full-length γ -zein and mutant cDNAs were expressed in a heterologous plant system (*Arabidopsis*). The subcellular localization of the various proteins was analyzed in the respective transgenic plants. From these studies, we provide evidence that the proline-rich tandem repeat domain is necessary for γ -zein retention in the ER of *Arabidopsis* cells. However, this repeat domain is not sufficient for the formation of γ -zein-containing PBs. The hydrophobic C-terminal domain is required for PB formation. In addition, our data revealed that the accumulation of γ -zein is not associated with the cell type in which it is expressed. γ -Zein accumulates in PB-like structures inside the *Arabidopsis* leaf cells despite the fact that it is a maize seed storage protein.

RESULTS

Expression of γ -Zein and γ -Zein-Mutated Proteins in *Arabidopsis*

The structure of γ -zein is similar to that of other sulfur-rich cereal prolamins. Structural studies of cereal seed proteins have shown that despite considerable diversity in amino acid compositions, such proteins have common structural elements that have probably evolved from a common ancestral protein (reviewed in Shewry and Tatham, 1990). The biological significance of the conservation of these structural elements is unknown. To ascertain whether such domains are essential for the sorting pathway and accumulation of storage proteins in the developing grain, we generated γ -zein mutants by deletion of three domains in γ -zein previously defined by Prat et

al. (1985): a cysteine-rich C-terminal domain, a proline-rich tandem repeat domain, and a linker region, P-X, located between those two domains. These mutants and the wild-type γ -zein were expressed in *Arabidopsis*, and their subcellular distribution inside the cells was studied.

A PvuI-XbaI DNA fragment of a γ -zein genomic clone (Boronat et al., 1986) containing the γ -zein coding sequence (669 bp), which is flanked by a short 5' sequence upstream from the start codon (11 bp) and the polyadenylation signals (175 bp), was appropriately engineered to delete sequences corresponding to the P-X domain (63 bp), the tandem repeat (174 bp), and the cysteine-rich domain (282 bp). All these constructs were subcloned into the plant expression vector pBin19 (Bevan, 1984) under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 1) to transform *Arabidopsis* root explants. Figure 2A is a schematic representation of the proteins encoded by p19 γ Z and the truncated gene constructs p19HbP, p19DC, and p19RcP. p19 γ Z encodes the wild-type γ -zein (223 amino acids); p19HbP encodes a γ -zein derivative, the HbP protein. This protein was deleted in the P-X domain, resulting in an HbP protein that was 21 amino acids shorter than γ -zein (deletion of amino acids 91 to 102). The other two deletion genes (p19DC and p19RcP) encode two proteins that are almost complementary in relation to the wild-type γ -zein. Thus, the DC protein contained the tandem repeat domain and the P-X domain, whereas RcP contained only the cysteine-rich domain. It should be noted that all truncated proteins had the signal peptide, a short amino acid sequence (10 amino acids) contiguous to the signal peptide, and a C-terminal tail of 17 amino acids in common.

To determine whether the γ -zein and truncated proteins were expressed in *Arabidopsis*, at least 10 transgenic plants for each construct were screened by using protein gel blot analysis with an antiserum (Ludevid et al., 1985) that recognized the γ -zein and truncated proteins (Torrent et al., 1994). Expression of γ -zein and the mutants was investigated in three different tissues: leaves, roots, and mature seed (Figure 2B). Protein extracts of identical tissues but from nontransgenic plants (Figure 2B, lanes labeled C) were always used as controls. Figure 2B shows that in the four transformation experiments, the γ -zein and mutated proteins (HbP, DC, and RcP) accumulated in leaves (see lanes 1) and roots (see lanes 2). The electrophoretic bands corresponding to the γ -zein, HbP, and DC proteins migrated according to the apparent molecular masses (24, 23, and 17 kD, respectively) previously observed in *in vitro* translation/translocation experiments (Torrent et al., 1994), as did the RcP protein (data not shown). The high molecular mass bands observed in Figure 2B correspond to positions expected for oligomers. Surprisingly, no traces of zein proteins were detected in seed. Moreover, RNA gel blot analysis (data not shown) revealed that the corresponding transcripts were present in root and leaf tissues but not in seed. Thus, the absence of expression in seed could be a result of the instability of these mRNAs in dicotyledonous seed.

As can be seen in immunoblots using a γ -zein antibody (Figure 2B, arrowheads), both γ -zein and the HbP mutant migrated

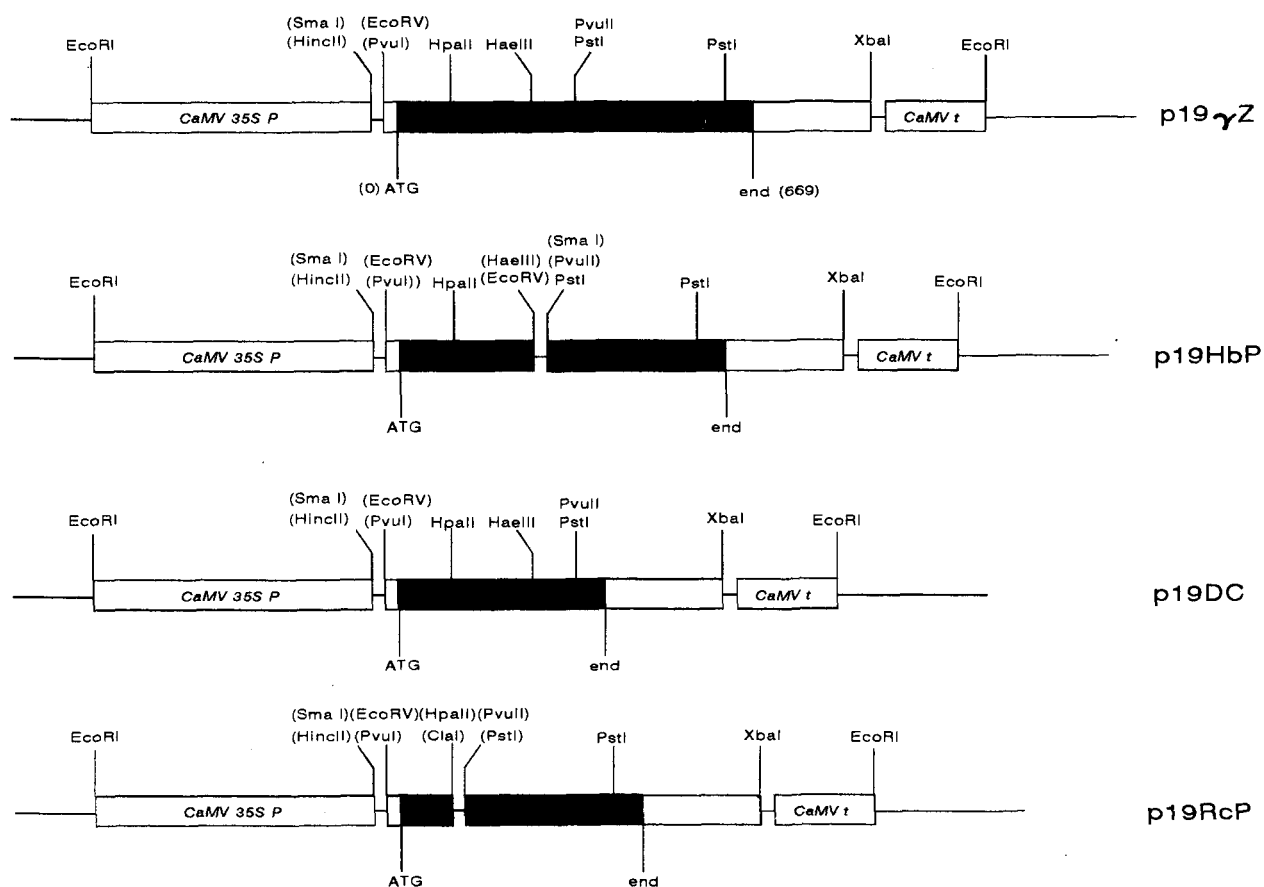


Figure 1. Schematic Diagram of Chimeric Gene Constructs Containing the Coding Sequences of γ -zein and the Truncated Mutants HbP, DC, and RcP. All constructs were cloned in pBin19 under the control of CaMV 35S promoter (*CaMV 35S P*) and the polyadenylation signals (*t*) of the CaMV. The black boxes correspond to the coding regions of γ -zein (p19 γ Z), HbP (p19HbP), DC (p19DC), and RcP (p19RcP) proteins. Restriction sites used in the various cloning steps are indicated.

in the form of two electrophoretic bands (24 and 26 kD for γ -zein and 23 and 25 kD for HbP). In an attempt to establish whether these two bands correspond to different gene products, poly(A)⁺ RNA was isolated from γ -zein and HbP transgenic plants and in vitro translated in separate experiments. After immunoprecipitation of the translation products by using the γ -zein antibody, a unique, specific band was detected in both cases (Figure 3A). These bands migrated in the same position as the lower bands observed in the immunoblots (see Figure 2B). Interestingly, the γ -zein antibody recognized the proteins located in both the lower and upper bands (Figure 3B, lanes 1), whereas the antibody raised against the synthetic peptide composed of eight units of the hexapeptide PPPVHL (α R; see Methods) recognized only those proteins located in the lower bands (Figure 3B, lanes 2). This result suggests that the upper bands could be the product of post-translational modifications within the tandem repeat sequence present in the γ -zein and HbP polypeptides. Hydroxylation of specific

prolyl residues could explain the differences in apparent molecular weights of the two immunoreactive bands of the γ -zein and HbP proteins. This post-translational modification has been previously described in many proline-rich proteins (Sticher et al., 1993).

Subcellular Localization of γ -Zein and the Deletion Mutants

Expression of γ -zein and the two γ -zein deletion mutants (HbP and DC) in *Xenopus* oocytes demonstrated that the deletions of the cysteine-rich and P-X domains did not affect protein stability and routing (Torrent et al., 1994). We analyzed expression of γ -zein and the mutated proteins (HbP, DC, and RcP) in plant cells and found initial evidence that, despite structural alterations caused by deletions, substantial amounts of the different polypeptides accumulated in transgenic plants (see Figure 2B).

To determine the time course of this accumulation and the route followed by the different proteins, protoplasts from transgenic plants were pulse labeled for 45 min and then chased for different periods of time in the presence of an excess of unlabeled methionine and cysteine (Figure 4). Analysis of immunoprecipitated proteins from protoplast extracts and incubation media revealed that the newly synthesized γ -zein, HbP, and DC proteins remained inside the cells and did not seem to lose stability during the chase. In contrast, the RcP protein, the only truncated protein lacking the tandem repeat domain (composed

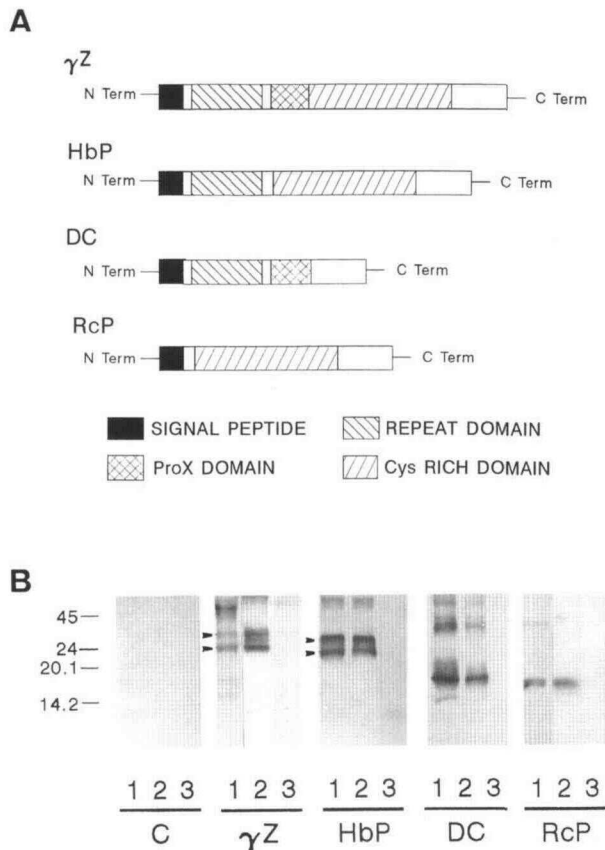


Figure 2. Protein Expression in Transgenic Arabidopsis Plants.

(A) Schematic representation of the different domains of γ -zein (γ Z) and the truncated proteins (HbP, DC, and RcP). Term, terminal; ProX DOMAIN, proline-Xaa linker domain.

(B) Expression of γ -zein (γ Z) and the truncated proteins (HbP, DC, and RcP) in transgenic Arabidopsis plants that were analyzed by SDS-PAGE and immunoblotting using α G2 antiserum. Proteins were extracted from leaves (lanes 1), roots (lanes 2), and mature seeds (lanes 3) of plants transformed with p19 γ Z, p19HbP, p19DC, and p19RcP. Wild-type plants were used as controls (lanes labeled C). Total protein extracted from leaves and roots of wild-type, γ -Z, and RcP plants (20 μ g) or HbP and DC plants (2 and 1 μ g, respectively) was loaded in lanes 1 and 2. Total protein (100 μ g) extracted from mature seed was loaded in lanes 3. The two electrophoretic bands detected for γ -zein and the HbP mutant are indicated (arrowheads). Molecular mass markers (in kilodaltons) are indicated at left.

of eight units of the hexapeptide PPPVHL), gradually disappeared from protoplasts and finally appeared in the medium. The half-time of secretion of the RcP protein was \sim 4 hr. Furthermore, the RcP polypeptide that had accumulated in the medium after 12 hr of chase was 2 kD smaller (14 kD) than that detected inside protoplasts over shorter chase periods (16 kD). This suggests that the newly synthesized RcP was proteolytically processed during the time course of the secretion.

Immunoelectron microscopy was used to examine the subcellular localization of the γ -zein and truncated proteins in Arabidopsis (Figure 5). Ultrathin sections of transgenic Arabidopsis leaves expressing the γ -zein, HbP, DC, and RcP proteins were incubated with a γ -zein antibody and protein A-gold. PB-like organelles strongly labeled with the γ -zein antibody were observed in cells expressing the γ -zein and HbP proteins (Figures 5C and 5D, respectively). These dense vesicles had different sizes, probably reflecting the different protein expression levels, and were clearly surrounded by a membrane. In contrast, the DC protein (Figure 5E) accumulated inside organelles that were clearly distinct from PB-like vesicles. These organelles consisted of big tubular/reticular structures enveloped by a membrane that penetrated the cytoplasm. They assumed an amorphous appearance depending on the plane of the section. Consistent with our pulse-chase experiments, after incubation of ultrathin sections of Arabidopsis cells expressing the RcP protein with the γ -zein antibody, labeling was found in the intercellular spaces outside the cells. Instead of being dispersed over the intercellular spaces, the RcP protein was densely accumulated in specific areas (Figure 5F).

γ -Zein and Tandem Repeat-Containing Mutants Accumulate in the ER

The above mentioned experiments revealed the localization of γ -zein and the DC and HbP mutant proteins within specific organelles inside the cell. Hence, it was important to determine whether these organelles were ER-derived vesicles. In dicotyledonous seed, storage proteins accumulate inside vacuoles (for review, see Chrispeels, 1991; Bednarek and Raikhel, 1992). Therefore, the possibility that γ -zein and derivatives had been targeted to this organelle in Arabidopsis plants cannot be excluded. For this reason, subcellular fractionation experiments using sucrose gradients were performed on transgenic plant homogenates. Antibodies against an immunoglobulin binding protein (BiP) and against a tonoplast intrinsic protein (TIP) were used as ER and tonoplast markers, respectively. The occurrence of the γ -zein mutants and protein markers in the gradient fractions was determined by quantification of immunoblots.

The distribution patterns of BiP and TIP through the sucrose gradients (20 to 70% sucrose) as compared with those of the γ -zein, HbP, DC, and RcP proteins are shown in Figure 6. In the four sucrose gradients, TIP sedimented between fractions 5 and 14, reaching a peak at 30% sucrose (fractions 8 and 9). Because TIP did not cosediment with the γ -zein, HbP, DC,

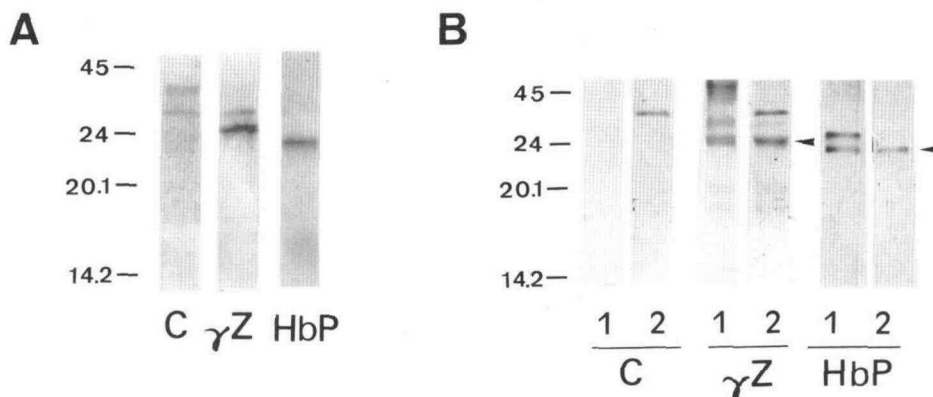


Figure 3. Analysis of the Two Electrophoretic Bands Corresponding to the γ -Zein and HbP Proteins Detected in Arabidopsis Plants Transformed with p19 γ Z and p19HbP.

(A) In vitro translation of poly(A)⁺ RNA from Arabidopsis plants transformed with p19 γ Z (γ Z) and p19HbP (HbP). Wild-type plants (lane C) were used as controls. Translation products labeled with ³⁵S-methionine were immunoprecipitated with the α G2 antiserum, electrophoresed, and fluorographed. It should be noted that the antiserum recognized two high molecular mass proteins in the control experiment. Molecular mass markers (in kilodaltons) are indicated at left.

(B) Immunoblot of protein extracts from wild-type plants (lanes labeled C; 20 μ g) and from plants transformed with p19 γ Z (lanes labeled γ Z; 20 μ g) and p19HbP (lanes labeled HbP; 2 μ g) using α G2 (lanes 1) or α R (lanes 2) antibodies. The α R antiserum recognizes only one electrophoretic band corresponding to the γ -zein and HbP proteins (lanes 2, arrowheads). Molecular mass markers (in kilodaltons) are indicated at left.

or RcP proteins, this result could indicate that the γ -zein and mutated proteins are not located inside vacuoles. In fact, we observed that the γ -zein and truncated proteins were absent in vacuoles isolated from protoplasts of the respective transgenic plants (data not shown). Significant portions of the γ -zein, HbP, DC, and RcP proteins sedimented at densities higher than 50% sucrose (fractions 17 to 21), but in these fractions only the DC protein cosedimented with BiP. It seems likely that γ -zein and the nonsecreted mutants sedimented in more than one structure; the γ -zein and HbP proteins were recovered from low-density structures (fractions 10 to 16) associated with BiP and from high-density structures (fractions 17 to 21) not associated with BiP (Figures 6A and 6B). These high-density structures may be the ones identified by immunoelectron microscopy as PB-like structures (Figures 5C and 5D). The concomitant occurrence of the DC protein and BiP detected even in high-density fractions (Figure 6C) indicates that DC accumulates in the ER, forming dense structures such as those detected by immunoelectron microscopy (Figure 5E). These results seem to indicate that γ -zein and the nonsecreted mutated proteins are not related to tonoplast or soluble fractions.

To demonstrate that the proteins are located in the ER, we performed double-labeling immunoelectron microscopy on leaf sections of transgenic plants using γ -zein and BiP antibodies (Figure 7). Consistent with our previous morphological studies (Figure 5), the γ -zein and HbP proteins accumulated in PB-like vesicles. We were able to detect γ -zein and HbP labeling close to BiP in specific structures spread over all the cytoplasm (Figure 7A). These structures may represent an initial phase of ER PB formation (pre-PBs), after which they will give rise to PB-like independent organelles. In these mature PBs, few

gold particles labeling the BiP antibody were observed (Figure 7B). The low levels of BiP in mature PBs could be a result of the KDEL receptor-mediated retrieval of this protein toward the ER (Lewis and Pelham, 1992). Pre-PBs containing BiP

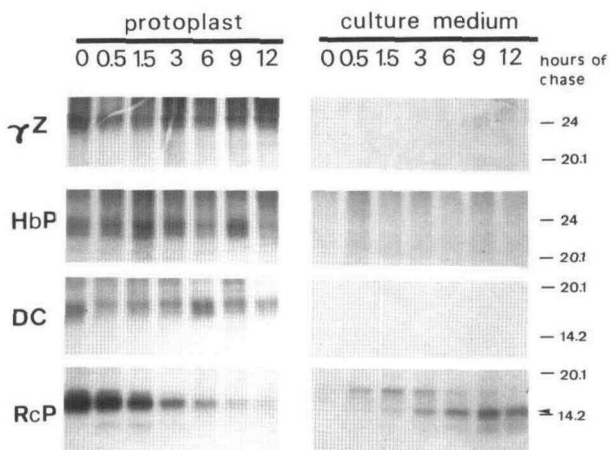


Figure 4. Pulse-Chase Labeling Experiment with Protoplasts Expressing γ -Zein and the γ -Zein Truncated Proteins.

Protoplasts were pulse labeled for 45 min and chased for the indicated times. Radiolabeled samples from both cell extracts and media were immunoprecipitated using α G2 antiserum and electrophoresed on 12.5 (γ -zein [γ Z] and HbP samples) or 15% (DC and RcP samples) SDS-polyacrylamide gels. The position of the secreted RcP polypeptide in the media is indicated (arrowhead). Molecular mass markers (in kilodaltons) are indicated at right.

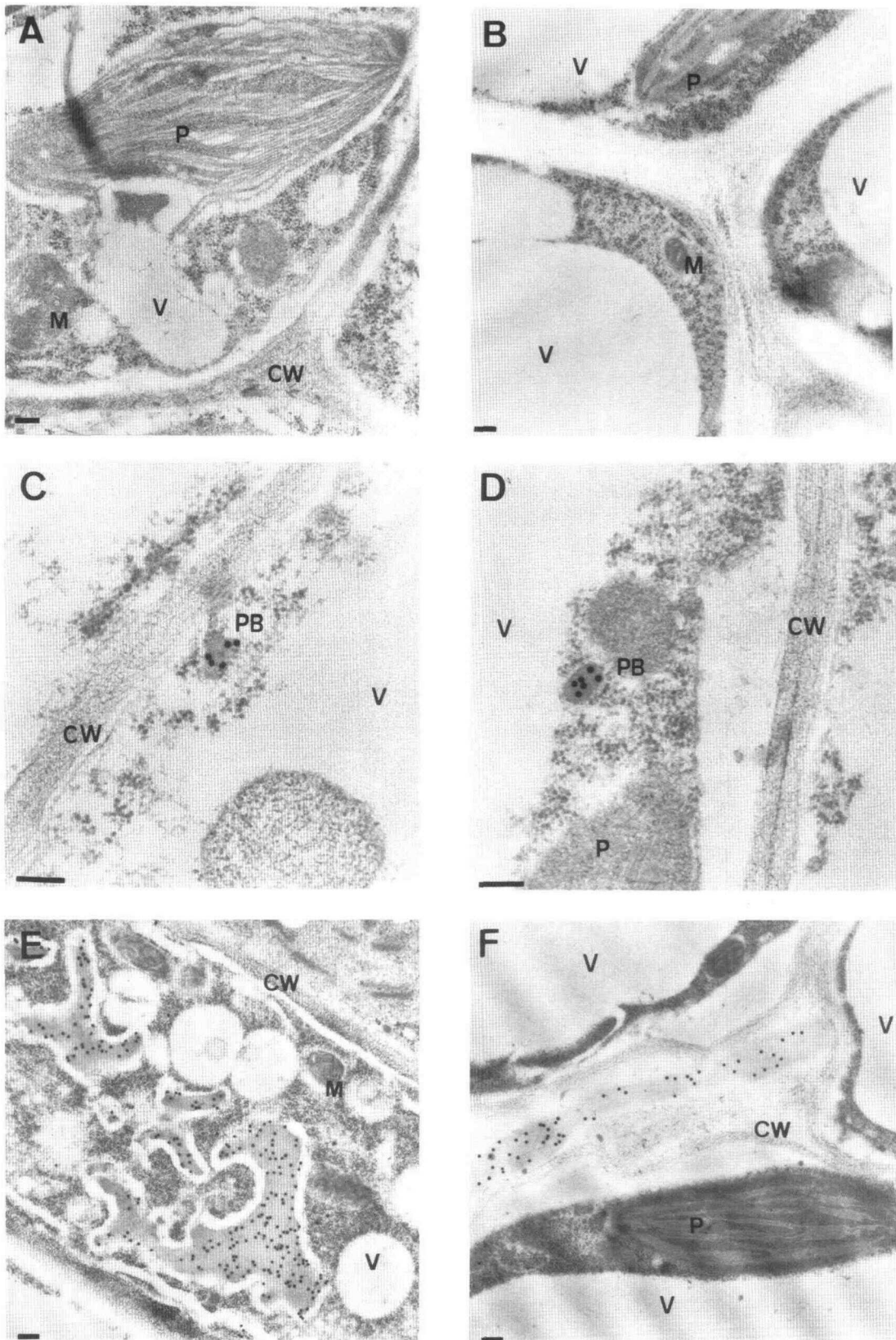


Figure 5. Subcellular Localization of the γ -Zein, HbP, DC, and RcP Proteins in Ultrathin Sections of Leaves from Transgenic Arabidopsis Plants.

would be less dense than mature PBs, which could explain the distribution pattern of the γ -zein and HbP proteins in sucrose gradients (Figures 6A and 6B). In transgenic DC plant sections, DC labeling was always associated with BiP inside large structures (Figure 7C). We concluded that γ -zein and the mutated proteins containing the repeat domain accumulate in ER-derived compartments. The DC protein remains in specific regions of the ER associated with BiP, whereas the HbP and γ -zein proteins stay in ER-derived PBs.

DISCUSSION

In this study, we have attempted to map the γ -zein sequences that determine its retention in the ER. Our results show that after *Arabidopsis* has been transformed with a full-length γ -zein coding sequence, the γ -zein expressed is found in ER-derived PBs. This result is consistent with the general concept that the molecular machinery of traffic and secretion is very similar between species (Bennett and Scheller, 1993). We have previously shown that in maize endosperm cells, the γ -zein is located in ER-derived PBs (Ludevid et al., 1984). A fundamental observation from our results is that the molecular events necessary for the PB formation, which occurs in the highly specialized cells of maize endosperm, are also present in *Arabidopsis* leaves. Thus, accumulation of γ -zein in ER-derived PBs is probably the result of the intrinsic features of the protein.

The ER Retention Signal of γ -Zein

Proteins resulting from the deletion of different structural domains of γ -zein were well expressed in *Arabidopsis* plants using the constitutive CaMV 35S promoter (0.01 to 0.1% of total protein). Immunoelectron microscopy and subcellular fractionation studies with transgenic plants revealed that γ -zein and the HbP proteins were located in ER-derived PBs and that DC protein was retained in the ER. Despite the obvious structural changes caused by the deletions, the truncated HbP and DC proteins entered the secretory pathway and remained stable in the cells. Pulse-chase experiments and immunocytochemical analysis indicated that deletion of the repeat domain and P-X domain of γ -zein resulted in a complete loss of its potential

to target inside the cell. Thus, according to the "default pathway" theory (Wieland et al., 1987), the autonomous C-terminal region of γ -zein (the RcP protein) did not contain any sorting signal and was secreted outside the cell. The sorting signal may be located in the N-terminal proline-rich domains (i.e., in both the tandem repeat and the P-X domain). Attempts to identify the retention sequence in greater detail showed that the tandem repeat (composed of eight units of the hexapeptide PPPVHL) is necessary for ER retention. Deletion of the P-X sequence flanking the tandem repeat did not interfere with retention in the ER because no trace of the HbP protein was detected either inside the vacuoles or outside the cells. The HbP protein, even though it lacks the P-X domain, was correctly sorted to the ER and packaged within PBs.

The question remains why the PPPVHL repeated sequence causes retention of γ -zein in the ER. One explanation for this sorting could be the aggregation of γ -zeins resulting from the existence of multiple unfolded states created by proline-rich sequences. In the case of some proteins, it has been reported that *cis-trans* isomerization of prolyl peptide bonds is a rate-limiting step in protein folding (reviewed in Jaenicke, 1991). If this is true, unfolding as a retention event can only relate to the N-terminal half of γ -zein, in particular, the proline-rich repeat domain. Unfolding of the proline-rich repeat would facilitate the formation of random, insoluble aggregates, but aggregation seems unlikely because the DC protein was soluble in aqueous media without detergent or reducing agents (data not shown).

In our opinion, selective oligomerization of γ -zeins, directed by protein-protein interactions through the repeat domain, may constitute a retention mechanism. The selective oligomers would be unable to exit from the ER. In fact, it has been proposed that proteins containing proline-rich regions (frequently found as multiple tandem repeats) have an important role in protein-protein binding. This binding cannot be highly specific, but it can be both rapid and remarkably strong (reviewed in Williamson, 1994). γ -Zein and BiP could interact, forming transient oligomers. Immunocytochemical analysis using both γ -zein and BiP antibodies demonstrated that BiP protein colocalized with γ -zein and HbP and DC proteins. We have observed that only at very early stages of γ -zein and HbP accumulation within the ER (pre-PBs) did these proteins colocalize with BiP. In contrast, the anti-BiP gold label was always present in the structures where DC protein accumulated. Thus, repeat domain-BiP binding could be the first sorting

Figure 5. (continued).

Immunocytochemistry was performed using the α G2 antiserum and protein A-colloidal gold (20 nm).

(A) and (B) Wild-type leaf sections essentially showing intracellular (A) and intercellular (B) spaces.

(C) Immunolocalization of γ -zein in p19 γ Z-transformed plants.

(D) Immunolocalization of HbP protein in p19HbP-transformed plants.

(E) Immunolocalization of DC protein in p19DC-transformed plants.

(F) Immunolocalization of RcP protein in p19RcP-transformed plants.

CW, cell wall; M, mitochondria; P, chloroplast; PB, protein body; V, vacuole. Bars = 0.2 μ m.

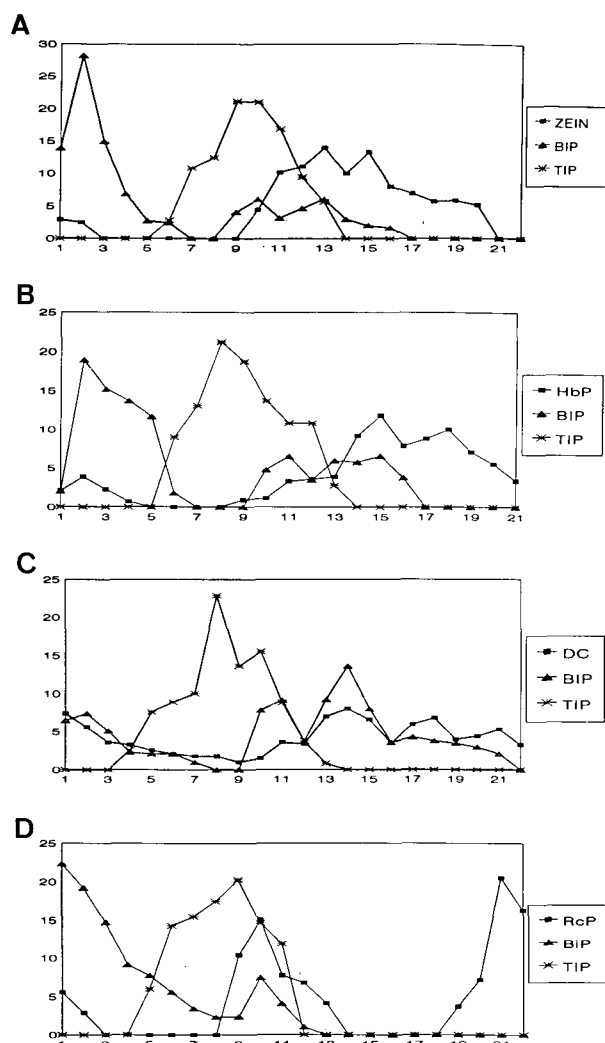


Figure 6. Subcellular Fractionation of Leaf Homogenates from p19 γ Z-, p19HbP-, p19DC-, and p19RcP-Transformed Plants.

Fractions isolated from 20 to 70% sucrose gradients were analyzed by immunoblotting as described in Methods using α G2, α BiP, and α TIP antisera. The different immunodetected proteins were quantified by densitometric analysis. The protein content per fraction is given as the percentage of the total amount of the corresponding protein in the gradient.

- (A) Profiles of γ -zein, BiP, and TIP protein content per fraction.
 (B) Profiles of HbP, BiP, and TIP protein content per fraction.
 (C) Profiles of DC, BiP, and TIP protein content per fraction.
 (D) Profiles of RcP, BiP, and TIP protein content per fraction.

event in γ -zein retention. Our observations are consistent with the results recently described by Li et al. (1993). In their study, the authors demonstrate that rice prolamin retention in the ER lumen is mediated by interaction with BiP.

Independent evidence for the relevance of N-terminal proline-rich tandem repeats with regard to ER retention was recently

obtained from *Xenopus* oocytes by expressing γ -gliadin, a wheat storage protein, and deletion mutants (Altschuler et al., 1993). These authors demonstrated that (1) γ -gliadin was secreted in oocytes and (2) the γ -gliadin mutant containing only the highly hydrophobic N-terminal proline-rich repeat was retained in the ER. In this context, if ER retention involves a tandem repeat-BiP association, such an association may be nonspecific because the γ -zein repeat sequence differs from the general hydrophobic repeats of the gliadins and other cereal prolamins.

Alternatively, retention in the ER could be a result of interaction between the proline-rich repeat and ER membrane. Using circular dichroism spectroscopy, it has been observed that the synthetic peptide composed of eight units of PPPVHL adopted a preferred conformation known as the polyproline II helix (Rabanal et al., 1993). These authors suggest that such a conformation results in an amphipathic helix, where histidine residues would be on the hydrophilic face of the helix and valine and leucine residues on the hydrophobic face. Thus, we can speculate that there is interaction between γ -zein and the ER membrane in two possible ways: (1) insertion through the lipid bilayer in such a way that the histidine residues face the lumen of the ER and the prolines bind to a membrane protein(s) and (2) interaction in such a way that the histidines face the negatively charged phospholipids of the membrane and the prolines bind to other γ -zein polypeptides. Such interaction would explain the particular localization of γ -zeins within maize PBs (Ludevid et al., 1984). We are working on these hypotheses. Preliminary *in vitro* experiments (unpublished data) point toward close but noncovalent interaction of γ -zeins with microsomal membranes. Finally, we cannot exclude the possibility that both protein-BiP association and membrane interaction are involved in the γ -zein retention process. These two events could operate in a cooperative manner.

Protein Body Formation

In transgenic plants, morphological differences between the γ -zein-, HbP-, and DC-containing organelles revealed that only γ -zein and the HbP truncated protein accumulate in PB-like structures. In contrast, the DC protein was located in reticular/amorphous structures. This observation probably reflects a process of hypertrophy/swelling of the ER during DC protein accumulation. It was surprising to find that the DC mutant lacking the cysteine-rich domain contained the necessary information to be retained within the ER but was unable to direct PB formation. ER retention does not seem sufficient to enable the sorting process to reach completion.

It could be that the cysteine-rich domain plays an important role in the condensation of γ -zeins and HbP inside the ER. It is known that the ER lumen provides an appropriate oxidizing environment for disulfide formation and for the protein disulfide isomerase to promote disulfide links (Freedman, 1989). Apparently, interchain cross-links by means of disulfide bonds could be necessary for the nucleation of the PB

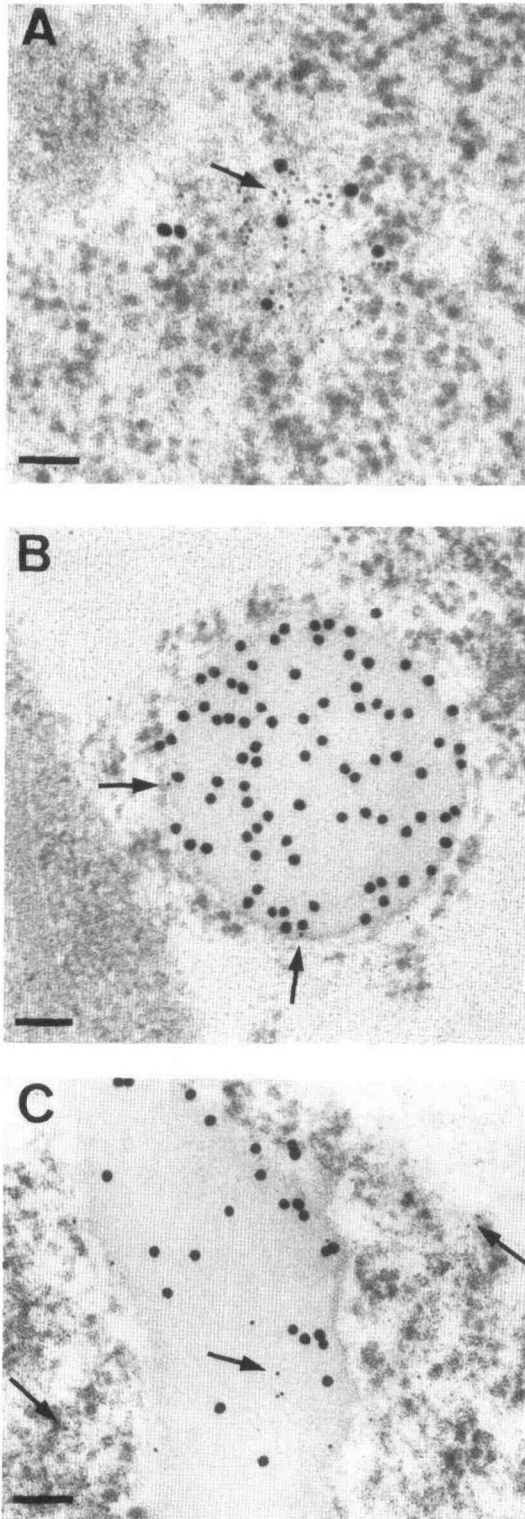


Figure 7. Colocalization of BiP with the γ -Zein, HbP, and DC Proteins on Ultrathin Sections of Leaves from Transgenic Arabidopsis Plants.

containing γ -zeins. Thus, the progressive formation of disulfide bonds with γ -zein retained in the ER could create a microenvironment within the ER lumen, giving rise to PB formation. As has already been reported in the case of secretory granule biogenesis, disulfide-mediated multimerization of von Willebrand factor and chromogranin B proteins appears to be required for the formation of secretory granules in the trans-Golgi network (Bauerfeind and Huttner, 1993; Voorberg et al., 1993). Thus, it may be suggested that the cysteine-rich domain of γ -zein is necessary to direct PB formation.

In conclusion, the sorting of maize γ -zein in PBs could depend on a two-step process: (1) ER retention via the tandem repeat domain as the result of membrane interaction and/or protein-protein association and (2) formation of large multimers by means of disulfide cross-links in the C-terminal region. Two questions remain to be answered. Is the repeat domain sufficient for ER retention? Do residues flanking this domain cooperate in the retention mechanism?

Methods

Plasmid Constructs

DNA sequences encoding γ -zein and the γ -zein derivatives HbP and DC were first assembled in pBluescript KS- (Stratagene) as described by Torrent et al. (1994) before being inserted into a plant expression cassette. Plasmid KSG2 (Torrent et al., 1994), containing the coding sequence of γ -zein, was used for the construction of derivative KSRcP11. Plasmid KSRcP11 was generated by two cloning steps: (1) the 120-bp Sall-HpaI restriction fragment from pKSG2 was cloned into the Sall and ClaI restriction sites of pBSKS (pKSC10), and (2) the 600-bp PvuII-XbaI fragment from pKSG2 was inserted into the restriction sites PstI (blunt-ended) and XbaI of pKSC10. The new construct, pKSRcP11, contained the coding sequence of γ -zein where the proline-rich repeat and the P-X domains were deleted in frame (RcP; Figure 2). All constructs were sequenced to ensure that reading frames were maintained.

For stable plant transformation, γ -zein and mutant derivative coding sequences were inserted as HincII-XbaI fragments into pDH51 (Pietrzak et al., 1986) containing the cauliflower mosaic virus (CaMV) 35S promoter and the signals for 3' end formation and polyadenylation.

Immunocytochemistry was performed on ultrathin sections using antibodies α BiP and α G2 and protein A coupled to 5 or 15 nm colloidal gold.

(A) Pre-PB inside a leaf cell of a p19 γ Z-transformed plant immunolocalized by double labeling using α BiP and α G2 antibodies.

(B) Mature PB immunolocalized in a p19HbP-transformed plant by double labeling. The organelle shows a high HbP protein accumulation but very few α BiP gold label.

(C) Colocalization of DC protein and BiP protein in p19DC-transformed plants by double labeling using α BiP and α G2 antibodies. Both proteins accumulate in large reticular structures.

Arrows indicate BiP localization corresponding to 5-nm gold particles. The γ -zein, HbP, and DC proteins are labeled with 15-nm gold particles. Bars = 0.1 μ m.

tion of the CaMV 35S. The chimeric genes were inserted into the polylinker (as EcoRI fragments) of the binary vector pBin19 (Bevan, 1984). The new plasmids were called p19 γ Z, p19HbP, p19DC, and p19RcP (Figure 1).

***Arabidopsis thaliana* Transformation and Protein Extraction**

Binary vectors containing the coding sequences for γ -zein and the γ -zein mutated proteins (p19 γ Z, p19HbP, p19DC, and p19RcP) were transferred to the LBA4404 strain of *Agrobacterium tumefaciens*. *Arabidopsis* ecotype RLD plants were transformed following the method described by Valvekens et al. (1988). Transformants were selected in a medium containing kanamycin (50 μ g/mL). For each construct, 10 transgenic plants were screened by immunoblot analysis using an antiserum raised against γ -zeins (α G2; Ludevid et al., 1985). Plants with the highest transgene product levels in the F₁ generation were chosen to obtain the F₂ generation. These plants were also screened for protein expression, and for each construct one plant was selected to perform further studies taking into account both protein expression level and viability.

To examine the expression of γ -zein and the γ -zein mutated proteins, seeds from transgenic plants were germinated in a solid culture medium (Murashige and Skoog salts [Duchefa, Biochemie BV, Haarlem, The Netherlands], 10 g/L sucrose, 50 μ g/mL kanamycin, 0.5 g/L 2-(*N*-morpholino)ethane-sulfonic acid, pH 5.8, 7 g/L agar) at 22°C for 3 weeks. Transgenic plants from each construct were collected. Whole plants, leaves, roots, and mature seeds were homogenized in liquid nitrogen, lyophilized, and delipidized using acetone-hexane (49:51). Total proteins were extracted in a buffer containing 50 mM Tris-HCl, pH 8, 50 mM NaCl, 1% SDS, 5% β -mercaptoethanol, and protease inhibitors (10 μ g/mL aprotinin, 1 μ g/mL pepstatin, 0.5 μ g/mL leupeptin, 1 μ g/mL E64, 0.1 mM phenylmethylsulfonyl fluoride). Protein extracts were quantified by using the Bradford assay (Bio-Rad) and analyzed by SDS-PAGE and immunoblotting.

Protein Gel Blot Analysis and Immunoprecipitation

For protein gel blot analysis, we used the antisera α G2, α R, α BiP, and α TIP. α G2 is a polyclonal rabbit antiserum raised against purified γ -zeins (Ludevid et al., 1985). α R is a polyclonal rabbit antiserum raised against a synthetic peptide (composed of eight units of the hexapeptide PPPVHL) corresponding to the tandem repeat sequence of γ -zein. This peptide was synthesized by using solid-phase peptide synthesis (Celma et al., 1992). Antibodies were produced in rabbits by injection at 15-day intervals of 300 μ g of peptide (without carrier). α BiP is a polyclonal antibody against the 77-kD BiP from yeast generously provided by H. Höfte (CNRS-INRA, Versailles, France), and α TIP is a polyclonal antibody against a tonoplast intrinsic protein from *Arabidopsis* (Höfte et al., 1992). Immunoblotting and SDS-PAGE were performed essentially as described by Ludevid et al. (1985). Nitrocellulose sheets were incubated with α G2 (1:2000 dilution), α R (1:200 dilution), α BiP (1:500 dilution), or α TIP (1:1500 dilution), and alkaline phosphatase conjugated to secondary antibodies (Dakopatts, Dako A/S, Glostrup, Denmark) was used for protein detection. Immunoprecipitation was performed using the α G2 antiserum as described previously (Torrent et al., 1994). Immunocomplexes were recovered by using protein A-Sepharose CL4B (Pharmacia), eluted with SDS sample buffer, and analyzed by SDS-PAGE and fluorography.

Poly(A)⁺ RNA Isolation and in Vitro Translation

Poly(A)⁺ RNA was isolated from γ -zein, HbP, and wild-type plants by using oligo(dT) cellulose type 7 (Amersham, Aylesbury, U.K.)

following standard methods. One microgram of each poly(A)⁺ RNA was in vitro translated using a wheat germ extract (Amersham) and ³⁵S-methionine as a labeled precursor. Translation products were immunoprecipitated with α G2 antiserum, electrophoresed in a 15% SDS-polyacrylamide gel, and fluorographed (Torrent et al., 1994).

Pulse and Chase

Protoplasts were prepared from leaves of wild-type and transformed *Arabidopsis* plants as described by Damm and Willmitzer (1988) with the exception that after digestion they were recovered by flotation in a medium containing 0.4 M sucrose and 10 mM CaCl₂. Protoplasts were resuspended in a B5a medium (Damm and Willmitzer, 1988) to a concentration of 4 × 10⁶ protoplasts per mL. After a 12-hr incubation at 22°C in darkness, 2 × 10⁶ protoplasts per point were pulse labeled at room temperature for 45 min using ³⁵S-Met and ³⁵S-Cys (in vitro cell labeling mix; Amersham) to a final concentration of 280 μ Ci/mL in the presence of protease inhibitors. Unlabeled methionine and cysteine were added to a concentration of 6 and 3 mM, respectively, and after 30 min of recruitment, protoplasts were recovered at different chase times. Every sample was centrifuged at 600g for 10 min to separate cells and culture media. Protoplasts were homogenized in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitors. Protoplasts and media were immunoprecipitated using α G2 antiserum (Torrent et al., 1994). Immunocomplexes were eluted with SDS sample buffer, electrophoresed in SDS-PAGE (12.5% acrylamide for γ -zein and HbP samples and 15% acrylamide for DC and RcP samples), and fluorographed.

Electron Microscopy

Leaves from wild-type and transgenic plants were fixed by vacuum infiltration with 2% glutaraldehyde and 2.5% paraformaldehyde in 20 mM phosphate buffer, pH 7.2, for 1 hr at room temperature. After washing with 20 mM phosphate buffer, samples were dehydrated through an ethanol series and embedded in Lowicryl K4M. Immunocytochemistry was performed as described by Moore et al. (1991). Ultrathin sections were incubated with α G2 (dilution 1:1000) for 2 hr at room temperature. Protein A-colloidal gold (20-nm diameter) was used for antibody detection. Sections were stained with uranyl acetate and lead citrate and examined under an electron microscope (Phillips EM301, Eindhoven, The Netherlands). For double labeling, the same procedure was followed although with some modifications: ultrathin sections were first incubated with α BiP (dilution 1:200), and protein A-colloidal gold (5-nm diameter) was used for antibody detection. After washing, grids were incubated with 0.15 mg/mL protein A for 20 min to saturate immunoglobulins that might have remained uncomplexed. Finally, the sections were incubated with α G2 (dilution 1:1000), and protein A-colloidal gold (15-nm diameter) was used for antibody detection.

Subcellular Fractionation

One gram of leaves from wild-type and transgenic plants was homogenized at 4°C in 2 mL of buffer F (50 mM triethanolamine, pH 7.5, 250 mM sucrose, 50 mM potassium acetate, 6 mM magnesium acetate, and 1 mM EDTA). After removing cellular debris by centrifugation at 2500g for 10 min at 4°C, 300 μ L of supernatant was loaded on 20 to 70% linear sucrose gradients in buffer F. Samples were centrifuged at 4°C for 12 hr at 33,000 rpm in an SW 40Ti rotor (Beckman),

and fractions of 500 μ L were recovered from the top. Each sample was diluted three times with water, precipitated in 15% trichloroacetic acid, electrophoresed in SDS-polyacrylamide gels (12.5% acrylamide for the wild-type, γ -zein, HbP, and DC samples and 15% acrylamide for RCP samples), and immunoblotted. Immunodetected proteins were measured by densitometry using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

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