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The Maize γ-Zein Sequesters α-Zein and Stabilizes Its Accumulation in Protein Bodies of Transgenic Tobacco Endosperm

Craig E. Coleman,[a,1] Eliot M. Herman,[b] Koichi Takasaki,[a,2] and Brian A. Larkins[a,3]

[a] Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721
[b] United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20705

Zeins are seed storage proteins that form accretions called protein bodies in the rough endoplasmic reticulum of maize endosperm cells. Four types of zeins, α, β, γ, and δ, aggregate in a distinctive spatial pattern within the protein body. We created transgenic tobacco plants expressing α-zein, γ-zein, or both to examine the interactions between these proteins leading to the formation of protein bodies in the endosperm. Whereas γ-zein accumulated in seeds of these plants, stable accumulation of α-zein required simultaneous synthesis of γ-zein. The zein proteins formed accretions in the endoplasmic reticulum similar to those in maize endosperm. Protein bodies were also found in protein storage vacuoles. The accumulation of both types of zeins peaked early in development and declined during maturation. Even in the presence of γ-zein, there was a turnover of α-zein, suggesting that the interaction between the two proteins might be transitory. We suggest that γ-zein plays an important role in protein body formation and demonstrate the utility of tobacco for studying interactions between different zeins.

INTRODUCTION

Seeds contain one or more proteins whose primary role is the storage of amino acids for protein anabolism during seedling growth. These storage proteins have no known enzymatic function, but their highly conserved structures imply that certain features are essential for synthesis and storage of the amino acids that they contain. For example, storage proteins generally contain large amounts of glutamine and asparagine, facilitating accumulation of nitrogen. Some storage proteins contain a high concentration of cysteine and methionine, which promotes sulfur storage. Storage proteins commonly have neutral pI values, and this may facilitate their concentration in an anhydrous state, which is important for stability during seed desiccation and storage.

The most common types of storage proteins are the saline-soluble 7S and 11S globulins, which are found in embryos, and the alcohol-soluble prolamins, which are unique to cereal endosperms (Shewry et al., 1995). The multisubunit 7S and 11S globulins are synthesized as precursors that are transferred into the lumen of the rough endoplasmic reticulum (RER), where they can undergo glycosylation in the course of being transported into specialized protein storage vacuoles (PSVs). Proteolytic processing within the PSV is often required for the assembly of these proteins, which form dense, insoluble accretions that eventually fill the PSV (Dickinson et al., 1989). Prolamins are unlike most storage proteins, because they generally form accretions (protein bodies) directly within the lumen of the RER. Prolamin protein bodies in maize and rice remain within the lumen of the RER, whereas in wheat, barley, and oats, they can be transported into PSVs by what is thought to be an autophagic process (Levanony et al., 1992).

The mechanism by which prolamins assemble into protein bodies is poorly understood. Prolamins do not contain the canonical KDEL or HDEL signals for retention in the ER; thus, it appears they have novel structural features that promote their retention within the RER. It was initially thought that hydrophobic interactions between the proteins might be sufficient to promote their aggregation into dense protein masses (Argos et al., 1982). It was later suggested that molecular chaperones, such as BiP, may play an important role in prolamin folding (Boston et al., 1991) and perhaps assembly into protein bodies (Li et al., 1993), implying that there is an ordered process involved in their deposition. In addition, temporal changes in the organization and distribution of different types of prolamins within protein bodies suggest that interactions between these proteins may be important for protein body assembly (Lending and Larkins, 1989; Rechinger et al., 1993).

[1] Current address: Department of Botany and Range Science, Brigham Young University, Provo, UT 84602.
[2] Current address: Frontier Technology Research Institute, Tokyo Gas Company, Ltd., Yokohama 230, Japan.
[3] To whom correspondence should be addressed.

Maize protein bodies are composed of four structurally distinct types of prolamins: the α-, β-, γ-, and δ-zeins (Thompson and Larkins, 1989). At early stages of development, the protein bodies are 0.2-μm spherical accretions of β- and γ-zeins. These structures become enlarged through the accumulation of α-zeins. Initially, the α-zeins penetrate the network of β- and γ-zeins, forming small locules that eventually coalesce, filling the central region of the protein body and expanding it to a diameter of 1.0 to 2.0 μm (Lending and Larkins, 1989). Assembly of the protein body appears to be a BiP-mediated process. In the maize *floury2* (*fl2*) mutant, which contains a defective α-zein with a signal peptide that is not cleaved (Coleman et al., 1995), there is a 100-fold increase of BiP, which becomes complexed at the surface and within the protein body (Zhang and Boston, 1992). This mutant also contains enhanced levels of another chaperone, peptide disulfide isomerase (Li and Larkins, 1996).

To investigate the mechanism by which maize prolamin protein bodies form and determine the role of zein interactions in this process, we created transgenic tobacco plants that synthesize α- and γ-zein proteins in the endosperm. Comparison of seeds producing α-zein, γ-zein, or both revealed that γ-zein can stabilize α-zein accumulation during seed development.

RESULTS

Tobacco Transformation

Transformation of tobacco plants was accomplished using the recombinant plasmids pGBαZ and pGBγZ. These vectors contain cDNAs for a 22-kD α-zein gene and a 27-kD γ-zein gene, respectively, fused to the promoter of the rice glutelin gene, *GluB1*. This promoter directed endosperm-specific expression of the zein genes and ensured that they both followed a similar pattern of spatial and temporal expression during seed development. After infection of leaf explants with Agrobacterium containing pGBαZ and pGBγZ, we selected several plants that were resistant to kanamycin. These plants were screened for insertion of the α- and γ-zein genes by the polymerase chain reaction (PCR) assay of genomic DNA (data not shown). Plants that accumulated the largest amount of α- or γ-zein protein in seeds, based on immunoblotting with specific antisera, were selected for further analysis.

Expression of Recombinant Gene Constructs in Transgenic Tobacco Seed

We assessed the level of zein synthesis in mature seeds of the self-pollinated transgenic tobacco plants by extracting reduced, alcohol-soluble proteins, separating them by SDS-PAGE, and identifying the proteins by immunoblotting. Figure 1 shows an analysis of the γ-zein protein from seeds of five of the plants transformed with pGBγZ. Seeds from two of these plants contained a detectable level of γ-zein (Figure 1, lanes 1 and 5), whereas other plants produced seeds with lesser or undetectable amounts (Figure 1, lanes 2 to 4). This variation in the accumulation of the γ-zein protein may have been caused by differences in the number of functional gene copies or the location of these genes in the genome (position effect). In contrast to γ-zein protein accumulation, the α-zein protein could not be detected in mature seeds of any of the transgenic plants containing this gene (data not shown).

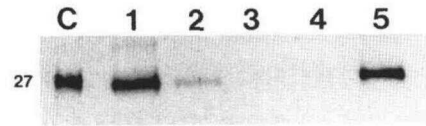

Figure 1. Detection of the γ-Zein Protein in Mature Transgenic Tobacco Seeds.

Each lane was loaded with extract from 200 μg of mature seed harvested from self-pollinated, kanamycin-resistant T_0 plants. Alcohol-soluble protein from 5 μg of maize endosperm flour was loaded in lane C. The blot was immunoreacted with the γ-zein polyclonal antiserum. The molecular mass in kilodaltons is indicated at left.

To examine the expression of the recombinant α-zein gene in the transgenic plants, seeds at various stages of development were analyzed for the presence of α-zein transcripts. Figure 2A shows a gel blot of total RNA from seeds of a transgenic plant expressing the α-zein gene. The results show that α-zein mRNA accumulated in seeds during early stages of endosperm development. The developmental accumulation of the α-zein mRNAs in tobacco seeds was very similar to that of γ-zein transcripts (Figure 2B), as would be expected, because both genes are regulated by the same promoter. Transcripts of both types of zein genes began accumulating by 7 days after pollination (DAP), peaked at 9 DAP, and subsequently declined during later developmental stages. The largest reduction in the level of zein transcripts occurred 13 DAP.

The fact that α-zein mRNAs are present in seeds during early stages of development suggested that α-zein protein can be synthesized in immature seeds but that it is absent in mature seeds because of instability. To examine α-zein accumulation at an early developmental stage, we extracted alcohol-soluble proteins from seeds harvested 11 DAP from self-pollinated plants transformed with pGBαZ. The proteins were separated by SDS-PAGE, and α-zein was visualized by immunoblotting (Figure 3). This analysis showed that the α-zein protein accumulated at 11 DAP in seeds of some plants (Figure 3, lanes 2 and 3) but not in others (Figure 3, lanes 1 and 4). Variation in the level of α-zein accumulation may reflect gene copy number or location.

Zein Protein Accumulation in Seeds of Selfed and Reciprocally Crossed Transgenic Plants

In maize endosperm, protein bodies begin as aggregates of β- and γ-zein proteins and subsequently expand through accumulation of α-zeins in their interior (Lending and Larkins,

1989). This suggests that γ-zein may play a role in localizing and organizing α-zeins in protein bodies. To investigate the interaction of these proteins, we analyzed the alcohol-soluble proteins from seeds of reciprocal crosses between plants synthesizing α- or γ-zein to determine whether simultaneous expression of the two genes had an effect on accumulation of α-zein. For these analyses, we used seed from T_1 plants derived from the T_0 plants that accumulated the highest amount of α- or γ-zein in their seeds. Immunoblots of α- and γ-zein from seeds of the self-pollinated parents that were used to make the reciprocal crosses are shown in Figures 4A and 4B, respectively. There was a small quantity of α-zein in seeds of the selfed plants (αα) at early to middle stages of development (Figure 4A, lanes 9, 13, and 17), but none was detected late in development or at maturity (Figure 4A, lanes 21 and 25). In contrast, in seeds of the selfed γ-zein plant (γγ), γ-zein protein was found at each developmental stage examined (Figure 4B). When we analyzed the zein protein from developing seeds of the reciprocal crosses of these plants by immunoblotting, we found that there was more α-zein than in the seeds of the self-pollinated parent (Figures 4C and 4E). This increase was detected at each developmental stage whether the α-zein plant was the female or male parent. Immunoblots of γ-zein from developing seeds of the reciprocal crosses showed large amounts of protein at all stages (Figures 4D and 4F). Apparently, the γ-zein protected or stablilized the accumulation of α-zein.

Because immunobloting does not quantitatively measure protein, we used ELISA to estimate the relative amount of α- and γ-zein in developing seeds of the self-pollinated parents and their reciprocal crosses (Figure 5). Because the endosperm is triploid, the homozygotes and heterozygotes created a gene dosage series (0 to 3). The highest level of γ-zein protein was measured in seeds of the self-pollinated plant (Figure 5A). There was a proportional decrease in γ-zein in seeds of the reciprocal crosses with the α-zein plant, depending on the direction of the cross. Thus, there was more γ-zein in seeds of the cross using the γ-zein plant as the female parent than in seeds of the cross using the same γ-zein plant as the male parent. For α-zein, the relationship between the amount of protein and gene dosage was observed only when α-zein and γ-zein were simultaneously synthesized (Figure 5B). Accordingly, the highest level of α-zein in seeds was observed when the α-zein plant was the female parent in a cross with the γ-zein plant. The peak level of α- and γ-zein accumulation occurred in seeds harvested 13 DAP. There was a subsequent decrease in the amount of both proteins beginning at 17 DAP, suggesting that they became unstable in the maturing endosperm. The more dramatic decline in α-zein accumulation suggests that this protein was broken down faster than γ-zein.

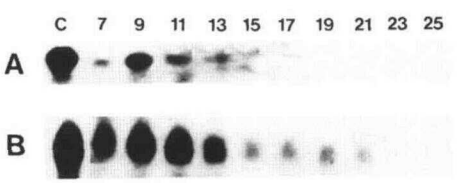

Figure 2. Detection of Zein Transcripts in RNA from Developing Transgenic Tobacco Seed.

(A) RNA gel blot of seeds harvested from a self-pollinated, kanamycin-resistant plant transformed with pGBαZ. Each lane was loaded with 20 μg of total RNA; the numbers above the lanes indicate the developmental stage (DAP) at which the seed was harvested. Lane C contains 10 μg of total RNA from a normal maize endosperm. The blot was hybridized with a 22-kD α-zein cDNA probe.

(B) Same gel blot as shown in **(A)**, except that the RNA was extracted from seeds of a plant transformed with pGBγZ, and the blot was hybridized with a 27-kD γ-zein cDNA probe.

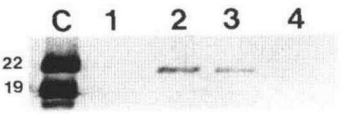

Figure 3. Detection of the α-Zein Protein in 11-DAP Seeds of Transgenic Tobacco Plants.

Each lane was loaded with extract from 3 mg of seed harvested 11 DAP from self-pollinated, kanamycin-resistant T_0 plants. Alcohol-soluble protein from 5 μg of maize endosperm flour was loaded in lane C. The blot was immunoreacted with the anti–α-zein polyclonal antiserum. Molecular masses in kilodaltons are indicated at left.

Subcellular Localization of the Zein Protein

To investigate the location of α- and γ-zein in the cell, immunogold labeling of zein proteins was performed, as shown in Figures 6 and 7. For this assay, seeds were harvested at 19 DAP from self-pollinated F_2 plants expressing both the α- and γ-zein genes. Gold particle labeling was observed on electron-dense bodies in the cytoplasm when treated with either anti–γ-zein (Figures 6A and 6C) or anti–α-zein antisera (Figure 6B). These bodies were 0.1 to 0.5 μm in diameter and had an appearance very similar to maize endosperm protein bodies at early stages of development. The protein bodies consisted of a number of less-electron-dense locules embedded in a matrix of more-electron-dense material (arrowheads in Figures 6B and 6C). Gold particles were closely associated with the more-electron-dense matrix when treated with anti–γ-zein antiserum (Figures 6A and 6C). The anti–α-zein antiserum labeled discrete locations within the protein bodies that appeared to be correlated with the less-electron-dense locules embedded within the protein body matrix (Figure 6B). The size of the locules was sufficiently small that they lay entirely within an ultrathin section, and therefore, some of the best-visualized locules are entirely within the plastic section and can be inaccessible to antibody label. Locules that are exposed

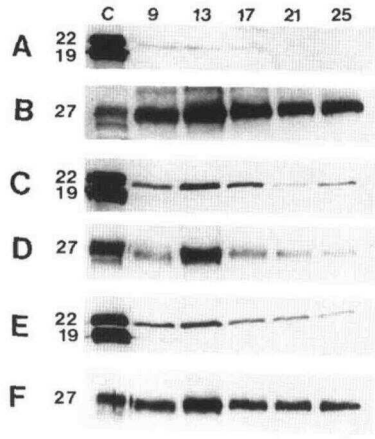

Figure 4. Immunodetection of α-Zein and γ-Zein in Developing Seeds of Selfed and Reciprocally Crossed Transgenic Tobacco Plants.

For each blot, lanes were loaded with alcohol-soluble proteins extracted from seven seeds harvested at 9, 13, 17, 21, and 25 DAP. Molecular masses in kilodaltons are indicated at left.
(A) Immunodetection of the α-zein protein from seeds harvested from a self-pollinated T_1 plant expressing a 22-kD α-zein gene.
(B) Immunodetection of the the γ-zein protein from seeds of a self-pollinated T_1 plant expressing a 27-kD γ-zein gene.
(C) Immunodetection of the α-zein protein from seeds of a cross between the two T_1 plants, with the α-zein plant as female.
(D) Immunodetection of the γ-zein protein from seeds of the cross described in **(C)**.
(E) Immunodetection of α-zein protein from seeds of a cross between the two T_1 plants, with the γ-zein plant as female.
(F) Immunodetection of the γ-zein protein from seeds of the cross described in **(E)**.

to the section surface are labeled with gold particles (Figure 7B), which appears to indicate that the discrete distribution of α-zein is the result of its sequestration into domains within the protein bodies. Both antisera gave highly specific labeling reactions on the plastic sections. Only background gold particle labeling was present on subcellular structures, such as the PSVs and oil bodies (Figure 6C) as well as the nucleus and cell wall (data not shown). Most protein bodies were observed within the cytoplasm, appressed between the oil bodies that were prevalent in the endosperm cells of mid-development-stage tobacco seeds (Figures 6A to 6C). Although a distinct membrane appeared to surround the cytoplasmic protein bodies, it is unclear whether this membrane is continuous with the ER network.

Protein bodies were also observed to be sequestered within the PSVs (Figures 6C and 7A to 7D). Some of the intravacuolar protein bodies appear to be morphologically identical to the cytoplasmic protein bodies (Figures 6C, 7A, and 7B), with a distinct membrane boundary at the protein body surface (Figures 6C, 7A, and 7B) and locules containing α-zein (Figures 6C and 7B). Other intravacuolar protein bodies appear to be less distinct in morphology, lacking the sharp membrane boundary at the surface and distinct α-zein locules (Figures 7C and 7D). The intravacuolar proteins with intact boundary and α-zein locules were labeled with both anti-γ-zein (Figures 6C and 7A) and anti-α-zein (Figure 7B) antisera with approximately the same density of gold particles as observed on the cytoplasmic protein bodies (Figures 6A to 6C). The intravacuolar proteins with an indistinct structure were labeled by the anti-γ-zein antibodies and gold secondary antibodies at a much lower particle density (Figures 7C and 7D) than was observed on either the cytoplasmic (Figures 6A to 6C) or intact intravacuolar protein bodies (Figures 6C, 7A, and 7B). The indistinct structure of these vacuolar sequestered protein bodies as well as the lower abundance of antigen suggest that these structures may have been partially degraded by vacuolar hydrolases. The presence of protein bodies within the vacuole as well as their apparent partial degradation indicate that they have been sequestered within the vacuole by autophagy after budding off of the ER network.

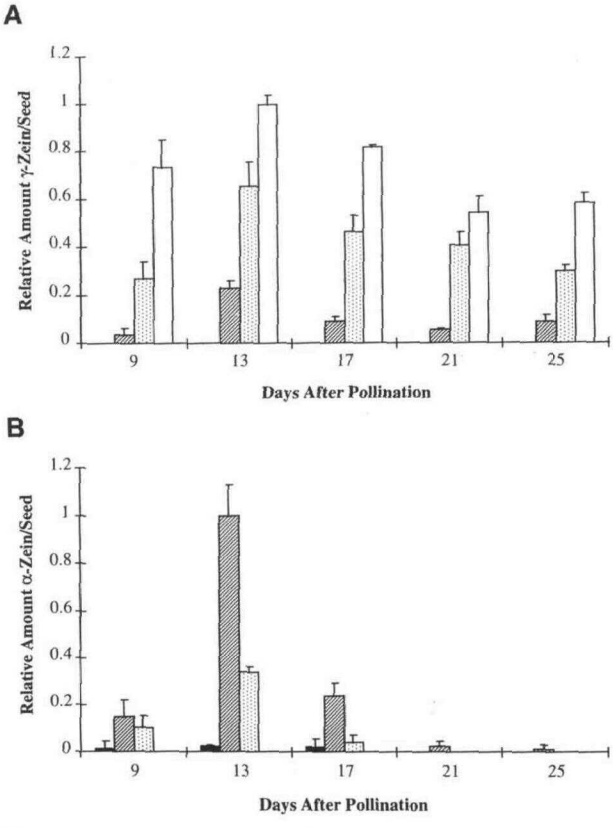

Figure 5. ELISA Measurement of Zein Proteins in Developing Transgenic Tobacco Seed.

(A) Relative amounts of α-zein in seeds harvested at 9, 13, 17, 21, and 25 DAP from a self-pollinated T_1 plant expressing the α-zein gene (filled bars) or γ-zein gene (open bars) and their reciprocal crosses (α × γ, hatched bars; γ × α, stippled bars). Data are presented as means +SE of three replicates of seeds from the same pod.
(B) Relative amounts of γ-zein in plants as described in **(A)**.

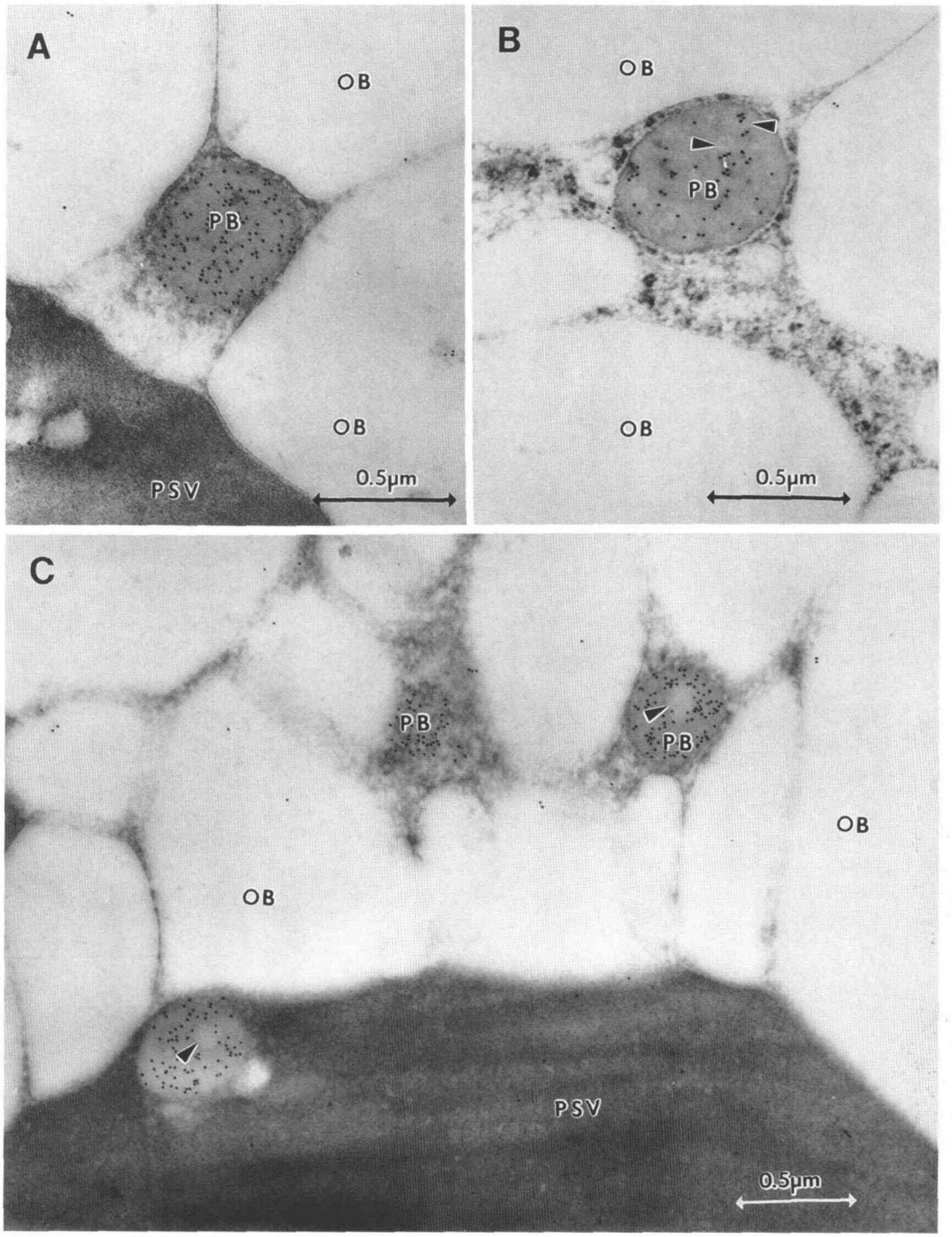

Figure 6. Immunogold Localization of α- and γ-Zeins to Cytoplasmic and Intravacuolar Protein Bodies in the Tobacco Endosperm.

(A) Gold particle labeling of γ-zein in a cytoplasmic protein body.
(B) Gold particle labeling of α-zein in a cytoplasmic protein body.
(C) Gold particle labeling of γ-zein in cytoplasmic and vacuolar protein bodies.
Arrowheads identify locules of less-electron-dense material embedded in the matrix of more-electron-dense material. OB, oil body; PB, protein body; PSV, protein storage vacuole.

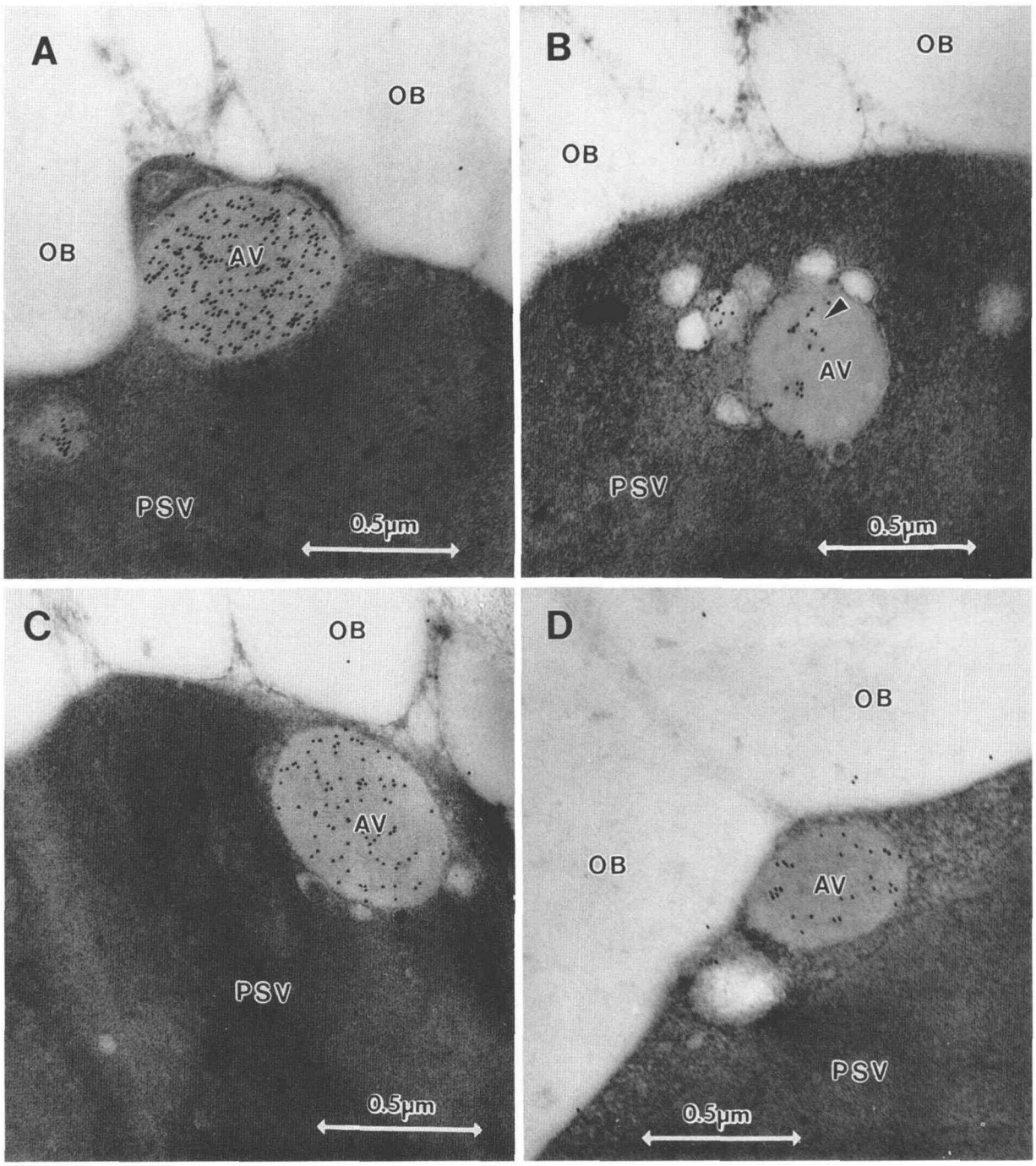

Figure 7. Immunogold Localization of α- and γ-Zeins to Intravacuolar Protein Bodies in the Tobacco Endosperm.

(A) An apparently intact intravacuolar protein body labeled with the anti–γ-zein antiserum.
(B) A similar intravacuolar protein body labeled with the anti–α-zein antiserum. The arrowhead identifies a less-electron-dense locule.
(C) and **(D)** *Partially degraded intravacuolar protein bodies labeled with anti–γ-zein antiserum.*
AV, autophagic vesicle; OB, oil body; PSV, protein storage vacuole.

DISCUSSION

The temporal and spatial changes in zein distribution in protein bodies of developing maize endosperm cells suggest that these proteins have intrinsic properties that cause them to be retained in the ER and that direct their organization within the protein body. The β- and γ-zeins appear to play an important role in these processes. Protein bodies initially consist exclusively of β- and γ-zeins, with the α- and δ-zeins subsequently penetrating this complex and expanding the developing protein body (Lending and Larkins, 1989; Esen and Stetler, 1992). The β- and γ-zeins are rich in cysteine and are cross-linked by intermolecular and/or intramolecular disulfide bonds (Larkins et al., 1989). These proteins cannot be extracted without a reducing agent, which makes it difficult to dissect their interactions.

Although little is known about the structure and function of the β-zein, Bagga et al. (1995) showed that synthesis of this protein in leaves and seeds of transgenic tobacco plants resulted in accretions that are stably retained within the lumen of the ER. These bodies consist of a series of interconnected lobes, rather than distinct spheres, like the protein bodies in maize endosperm. Using site-directed mutagenesis, Geli et al. (1994) have begun to dissect functional domains of the γ-zein protein. This protein is proline rich (25%), due in part to a series of hexapeptide repeats (PPPVHL) at the N terminus (Prat et al., 1985). These repeated peptides appear to be important for the retention of γ-zein in the ER lumen, because in transgenic Arabidopsis their removal caused the truncated protein to move through the secretory pathway of leaf cells (Geli et al., 1994). Neither γ-zein nor any other zeins have the canonical ER retention peptides HDEL or KDEL, so it is possible that these proline-rich repeats of the γ-zein are responsible for its retention in the ER. Because the γ-zeins are located at the surface of the protein body, the hexapeptide repeats may also provide the mechanism for retention of the entire protein body complex.

The results of this study imply that there is an interaction between γ-zein and α-zein proteins that promotes α-zein retention within the protein body. Although it is possible that γ-zein mRNA somehow stabilizes α-zein mRNA, it is difficult to imagine the mechanism whereby this would occur. Based on studies of zein mutants, there is no evidence that such an interaction occurs in maize endosperm (Kodrzycki et al., 1989; Dannenhoffer et al., 1995). In transgenic tobacco endosperm, this interaction appears to enhance retention of α-zein in the ER, as shown by the presence of α-zein in cytoplasmic protein bodies, and this stabilizes its accumulation in the seed. Sequestration of α-zein by γ-zein within the protein body may also protect it from degradation by proteases within the ER. In the absence of γ-zein, only trace amounts of α-zein were found in developing endosperm, and the protein could not be detected in the mature seed. We were unsuccessful at localizing α-zein on these sections despite multiple attempts to do so. We surmise that this is because the accretions of α-zein were too small or too few to be detected by immunocytochemistry, or perhaps because the protein was rapidly transported to the vacuole, where it was degraded.

Based on the analysis of zein accumulation in seeds of plants expressing α- and γ-zein genes and the reciprocal crosses between them, there was a direct relationship between gene dosage (gene expression) and the steady state level of zein synthesis in the transgenic tobacco seeds. This relationship was quite clear for the γ-zein plants, but it was also true for the α-zein plants, providing γ-zein was present. γ-Zein did not appear to have a catalytic effect on α-zein accumulation; rather, there appeared to be some proportionality between the two proteins. It has not yet been possible to create plants with a constant amount of α-zein and variable amounts of γ-zein, or vice versa, to investigate the stoichiometry of this relationship. Nevertheless, it is clear that the synthesis of even a small amount of γ-zein significantly increased the stability of α-zein.

The interaction between α-zein and γ-zein in tobacco endosperm appears to be transient. Maximum levels of both proteins were observed 13 DAP, after which their steady state levels decreased to a relatively constant level. The level of γ-zein 17 DAP was ~80% of that 13 DAP, whereas the level of α-zein decreased by 75% over the same developmental period; thus, γ-zein is more stable than α-zein in developing tobacco seeds. It has been shown that the inner cell layers of tobacco endosperm are catabolized during embryo development (Erdelská, 1985), and this could account for the temporal reduction in α-zein and γ-zein levels 13 DAP. The degradation of zeins can also be explained by autophagic accumulation of protein bodies in the protein storage vacuoles, with subsequent proteolysis. The fact that α-zein turned over more rapidly than γ-zein suggests that it is more sensitive to vacuolar proteases and that it is not stably sequestered by γ-zein within the protein body. Perhaps the stability of α-zein in the protein body is enhanced through interactions with other proteins, such as β-zein. Because β-zein is hydrophobic and appears to be disulfide linked with γ-zein, it is conceivable that it may form a stable interaction with α-zein in the protein body.

α-Zein synthesis and assembly into protein bodies appear to be BiP-mediated processes that occur at the β-zein–γ-zein surface (Fontes et al., 1991; Marocco et al., 1991). The molecular mechanisms that coordinate this process are poorly understood; furthermore, the mechanisms that limit the size and number of protein bodies in the endosperm also remain unknown. Based on this and other studies, it appears that γ-zein synthesis plays an important role in initiating protein body formation and perhaps in determining the size and number of protein bodies as well. The γ-zein accretions that form in transgenic tobacco endosperm are spherical structures of 0.2 to 0.5 μm in diameter, which is approximately the size of incipient protein bodies in maize endosperm (Lending and Larkins, 1992). In the absence of other zeins, it appears that the interactions between these proteins are such that only a certain number of γ-zein molecules are able to assemble into a complex, and this takes on a spherical shape. This property undoubtedly relates to the unusual structural features of γ-zein

proteins (Rabanal et al., 1993). Normally, the α-zeins penetrate this network, and perhaps because of their hydrophobic nature, they partition to the interior of the protein body. This suggests that the hydrophilic properties of the γ-zein are important for maintaining the spherical orientation of the protein body. If this organization is disrupted, it causes asymmetric growth of the protein body, resulting in an irregular shape and, perhaps, a soft endosperm texture.

Evidence for this notion comes from the maize *fl2* mutant, which results from a mutation in the signal peptide of a 22-kD α-zein protein (Coleman et al., 1995). This defect causes the protein to become anchored to the ER membrane, thereby disrupting the outer β-zein–γ-zein shell and creating a hydrophobic surface of α-zein proteins (Lending and Larkins, 1992; J.W. Gillikin, F. Zhang, C.E. Coleman, B.A. Larkins, and R.S. Boston, unpublished data). As a consequence, protein bodies in the *fl2* mutant are irregularly lobed, with pockets of β- and γ-zein partitioned into the interior (Zhang and Boston, 1992). What limits the growth of protein bodies, which are uniformly 1 to 2 μm in diameter in maize endosperm? Perhaps the size of the protein bodies is determined by the extent to which γ-zein can form a proteinaceous network, which in turn determines the amount of α-zein that can be complexed.

Storage protein transport to the PSVs of dicotyledonous seeds, such as tobacco, occurs by trafficking through the Golgi apparatus and deposition by secretory pathway processes. In contrast, certain monocotyledonous seeds deposit storage proteins within the vacuole by secretory system trafficking through the Golgi apparatus as well as by autophagy of preassembled protein bodies (Galili and Herman, 1996). The results presented here demonstrate that a dicotyledonous seed can be induced to produce protein bodies virtually identical to those of cereal plants. This result suggests that the selective sequestration of protein bodies in the vacuole may occur as a consequence of producing this "new" organelle. PSVs have previously been shown to selectively sequester cytoplasmic organelles during germination to alter the composition of the cell (Herman et al., 1981) and the tonoplast (Melroy and Herman, 1991). This raises a number of interesting questions regarding how vacuoles selectively recognize cell constituents for autophagy. Experiments such as those conducted here may provide important model systems to explore the regulation of autophagy in plant cells.

A number of different seed storage proteins have been expressed in seeds of transgenic plants. Whereas many of these proteins were correctly processed, targeted, and accumulated in the protein storage vacuoles (Herman et al., 1989), other proteins, such as α-zein (Schernthaner et al., 1988; Williamson et al., 1988; Ohtani et al., 1991), or engineered proteins, such as a high-methionine (HiMet) phaseolin (Hoffman et al., 1988), were post-translationally unstable. The HiMet phaseolin is apparently degraded in the PSV after trafficking through the secretory system, indicating that the vacuole contains or is induced to contain proteases that selectively degrade the labile protein (Pueyo et al., 1995). The results presented here indicate that labile zeins are likely degraded by vacuolar proteases after selective autophagy of protein bodies. This result indicates that post-translationally unstable storage proteins expressed in seeds are selectively degraded in the vacuole independent of whether the proteins are deposited within the vacuole after endomembrane trafficking or by autophagy of assembled structures.

The demonstration that protein bodies similar to those in maize endosperm can form in transgenic tobacco seed shows that this system can be used to investigate zein interactions that lead to protein body formation. It should now be possible to dissect the structures of zein proteins and determine the role of specific protein domains in protein body assembly. Because zeins are devoid of lysine, an essential amino acid for monogastric animals (Nelson, 1969), there has been interest in genetically engineering the genes encoding these proteins to increase their lysine content. Although lysine-containing zeins aggregated into dense, protein body–like structures in Xenopus oocytes (Wallace et al., 1988), the proteins did not stably accumulate in transgenic tobacco seed (Ohtani et al., 1991). Our results suggest that the instability of the engineered α-zein can be overcome by coexpression of γ-zein. It should therefore be possible to reexamine the potential for genetically engineering α-zein proteins to improve their nutritional value.

METHODS

Plasmid Constructs

The plasmid pGBαZ was constructed by fusing a 1325-bp DNA fragment containing the promoter of the rice glutelin gene *GluB1* (Takaiwa et al., 1991) with a 995-bp DNA fragment containing the coding sequence of a 22-kD α-zein gene from the genomic clone gz22.8 (Thompson et al., 1992) in the binary vector pBI101 (Jefferson, 1987). A 1.6-kb Sau3A fragment of the *GluB1* promoter was cloned into the BamHI site of pUC18. This plasmid was digested with AflII and SmaI to remove ~250 bp of the 3′ end of the fragment, and the termini were repaired by treatment with the Klenow fragment of DNA polymerase I. The remaining *GluB1* promoter region extends from positions −1307 to +18 relative to the transcription start site. The 995-bp α-zein gene sequence was generated by polymerase chain reaction (PCR) amplification and extends from positions −51 to +944 relative to the translation start site. It includes 46 nucleotides of the 3′ noncoding sequence. The PCR product was inserted behind the *GluB1* promoter in pUC18. The entire fusion cassette was removed by digestion with HindIII and SstI restriction enzymes and subsequently inserted into the site created by removal of the β-glucuronidase cassette from pBI101.

An identical procedure was used to construct pGBγZ, except that a 787-bp DNA fragment containing a gene encoding a 27-kD γ-zein was fused to the *GluB1* promoter. This fragment was generated by PCR amplification of a genomic clone, gz27.3 (Lopes et al., 1995). It extends from positions −25 to +762 relative to the translation start site and includes 93 nucleotides of the 3′ noncoding sequence.

Tobacco Transformation

Plasmids pGBαZ and pGBγZ were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Bevan, 1984), and the bacteria were used to infect leaf explants from *Nicotiana tabacum* cv Xanthi (Horsch et al., 1985). Transformed plants were selected for resistance to kanamycin, and insertion of the *GluB1* promoter and zein genes was confirmed by PCR amplification of genomic DNA extracted from leaf tissue. The primers used for PCR amplification were the same as those used to generate the Ti plasmid constructs as described above.

Protein Isolation, Immunoblotting, and ELISA Measurement

The T_0 plants were self-pollinated, and seeds were harvested 11 or 25 days after pollination (DAP; maturity). For the protein assays of developing seeds, we used T_1 plants grown from the seed of the self-pollinated T_0 plants with the highest level of α- or γ-zein accumulation. The T_1 plants were selfed or reciprocally crossed, and seed was collected at 9, 13, 17, 21, and 25 DAP. The seeds were frozen in liquid nitrogen and stored at −80°C before protein extraction. Zein was extracted using a procedure developed for maize endosperm flour (Wallace et al., 1990). Seeds were weighed, and 1 mL of borate buffer was added per 50 mg of seed. The tissue was ground with a Kontes pestle in a microcentrifuge tube and incubated for 16 hr at 37°C with agitation. Insoluble material was pelleted by centrifugation, and the supernatant was diluted with ethanol to 30%. The mixture was incubated for 1 hr at 37°C, and alcohol-insoluble material was pelleted by centrifugation.

For the analysis of zein proteins from seeds of self-pollinated T_0 plants, an aliquot of the alcohol extract equivalent to 1 mg of seeds was vacuum dried, and the residue was resuspended in sample buffer (Laemmli, 1970). For the analysis of zein proteins from developing seeds of T_1 plants, the concentration of protein in the alcohol extracts was converted to a per seed basis after weighing and counting a sample of seeds from each developmental stage. An aliquot of the alcohol extract equivalent to seven seeds was vacuum dried, and the residue was resuspended in Laemmli buffer (Laemmli, 1970). Proteins were separated on 12.5% SDS–polyacrylamide gels and transferred to nitrocellulose by using a semidry apparatus. Zein proteins were detected after reaction with either anti–α- or anti–γ-zein rabbit polyclonal antisera (Lending et al., 1988). Relative amounts of α- and γ-zein were measured by ELISA using the procedure developed by Moro et al. (1995), except alcohol extracts were first adjusted to a concentration of 350 seeds per mL and then diluted 1 to 25 (v/v) for measurement of α-zein and 1 to 800 (v/v) for measurement of γ-zein.

RNA Gel Blot Analysis

Seeds were harvested from self-pollinated plants at 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 DAP, quick frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated from seeds as previously described (Takaiwa et al., 1991). For each developmental stage, 10 μg of glyoxal-denatured total RNA was separated in a 1.2% agarose gel and transferred to a nylon membrane by using 10 mM NaOH as a transfer buffer. Gel blots were hybridized to digoxigenin-labeled DNA probes generated by PCR amplification of α-zein or γ-zein gene template DNA (McCreery and Helentjaris, 1993a). Detection of the probe that hybridized with zein mRNA was accomplished by using a chemiluminescent assay (McCreery and Helentjaris, 1993b).

Tissue Fixation and Embedding

Transgenic seed tissue was harvested at 13 or 19 DAP and immediately placed in 4% formaldehyde, 0.6% gluteraldehyde, and 0.1 M sucrose in 33 mM phosphate buffer, pH 7.3 (Bagga et al., 1995). Individual seeds were cut in half with a razor blade and incubated in fixative for 2 hr on ice. The tissue was washed three times with 7% sucrose in 33 mM phosphate buffer, pH 7.3, stored overnight at 4°C, and dehdryated using a series of alcohol washes. Seed halves were embedded in LR White resin (Electron Microscopy Sciences, Fort Washington, PA), according to the manufacturer's instructions.

Immunocytochemistry

Antisera specific for α-zein and γ-zein have been described previously (Lending et al., 1988). For α-zein labeling, thin sections mounted on nickel grids were directly labeled with a 1-to-25 dilution of the antiserum in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween 20) for 1 hr without prior blocking steps. For γ-zein labeling, the antiserum was diluted in TBST 1 to 25 to which 20% (v/v) normal tobacco seed extract was added to eliminate pseudospecific cell wall labeling. The tobacco seed extract was produced by grinding nontransformed tobacco seeds in liquid nitrogen and then adding 10 volumes per unit weight of distilled water. The extract was boiled and then added directly to the assay mixture. The assay mixture of FBS/TBST (10% [v/v] fetal bovine serum with TBST), seed extract, and antisera was preincubated for 30 min at room temperature and then clarified by centrifugation in a microcentrifuge for 5 min. The clarified assay mixture was used to label thin sections mounted on nickel grids for 1 hr at room temperature. After labeling with primary antiserum, the grids were washed with TBST and indirectly labeled with 10 nm of anti–rabbit IgG colloidal gold (Ted Pella Inc., Tustin, CA) diluted 1 to 1 with FBS/TBST for 10 min at room temperature. The grids were washed with TBST and stained with 5% (w/v) uranyl acetate for 20 min before examination and photography with Hitachi H500 (Tokyo, Japan) and Phillips 400T (Eindhoven, The Netherlands) electron microscopes.

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