Coexpression of the Maize δ-Zein and β-Zein Genes Results in Stable Accumulation of δ-Zein in Endoplasmic Reticulum-Derived Protein Bodies Formed by β-Zein

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Zeins, the major seed storage proteins of maize, are of four distinct types: α, β, δ, and γ. They are synthesized on the rough endoplasmic reticulum (ER) in a sequential manner and deposited in ER-derived protein bodies. We investigated the potential for producing sulfur-rich β-zein and δ-zein proteins in leaf and seed tissues by expressing the corresponding genes in a constitutive manner in transgenic tobacco. The δ-zein and β-zein, when synthesized individually, were stable in the vegetative tissues and were deposited in unique, zein-specific ER-derived protein bodies. Coexpression of δ-zein and β-zein genes, however, showed that δ-zein was colocalized in β-zein-containing protein bodies and that the level of δ-zein was fivefold higher in δ-/β-zein plants than in δ-zein plants. We conclude that δ-zein interacts with β-zein and that the interaction has a stabilizing effect on δ-zein.

INTRODUCTION

Seed storage proteins are synthesized on the rough endoplasmic reticulum (ER), translocated into the lumen of the ER, and then sequestered by different cellular pathways into protein bodies (Galili et al., 1993). Although the mechanism by which these proteins are translocated into the lumen of the ER is well understood, the subsequent steps in storage protein processing and aggregation are not as clearly defined (Shewry et al., 1995). Similar to many unfolded proteins that enter the lumen of the ER, storage proteins must be protected from aggregation and be maintained in a folding-competent manner; consequently, the ER contains a high concentration of molecular chaperones that appear to promote protein translocation, folding, and oligomerization (Gething and Sambrook, 1992).

Zeins, the storage proteins of maize seeds, belong to the prolamin class of storage proteins and are deposited in ER-derived protein bodies (Larkins and Hurkman, 1978). Zeins consist of several types of polypeptides, the α-, β-, γ-, and δ-zeins, which are structurally distinct (Thompson and Larkins, 1989). The β-zein and γ-zein proteins share regions of homology with other prolamins. δ-Zein has some sequence similarity with dicot storage proteins, such as the 2S albumin of Brazil nut (Kirihara et al., 1988). α-Zeins, which are the most abundant, have a unique structure (Argos et al., 1982).

In developing maize endosperm cells, the accumulation of zeins involves transport of the newly synthesized proteins into the ER by means of an N-terminal signal peptide and a signal recognition particle component (Campos et al., 1988). The proteins are retained in the rough ER lumen, where they are assembled into protein bodies (Larkins and Hurkman, 1978). Little information is available concerning the signals in zein polypeptides responsible for protein retention in the ER or assembly into protein bodies. Zeins do not possess the well-characterized KDEL ER retention motif (Pelham, 1990; Denecke et al., 1992); therefore, they must contain an as-yet-uncharacterized signal that is responsible for their retention in the ER. It has been suggested that prolamins are retained in the ER because of their interaction with an ER-resident chaperone, such as BiP (Li et al., 1993a). BiP is a cognate of the 70-kD heat shock protein located in the lumen of the ER; it has a C-terminal ER retention signal, and it functions as a chaperone (Haas and Wabl, 1983).

Studies on zein protein body biogenesis have shown that the β-zein and γ-zein proteins are the first to be deposited, followed by the accumulation of α-zeins within the matrix of the β- and γ-zeins. As the interior of the protein body fills with α-zein, the β- and γ-zeins form a more or less continuous layer around the periphery (Lending and Larkins, 1989). Along with α-zein, δ-zein is exclusively located in the core of the protein body (Esen and Stetler, 1992). It has been proposed that γ-zein or β-zein or both are necessary for the assembly of the protein bodies (Lending and Larkins,
Some studies suggest that an ER transmembrane domain exists in γ-zein that acts as a nucleating factor in protein body formation (Torrent et al., 1994), but a recent report refuted the presence of a transmembrane region in the γ-zein protein (Lee et al., 1995). Little is known about the interactions among the different zeins and how the interactions contribute to the formation of protein bodies.

The γ-zein and β-zein coding sequences have been expressed individually in transgenic Arabidopsis (γ-zein) and tobacco (β-zein) plants by using the cauliflower mosaic virus (CaMV) 35S promoter, and in both cases, the proteins were retained in ER-derived protein bodies (Geli et al., 1994; Bagga et al., 1995). In the case of γ-zein, Geli et al. (1994) determined that a proline-rich region composed of repeats of a hexapeptide (PPPVPVL) in the N-terminal domain is responsible for ER retention, whereas both the proline-rich repeat and the C-terminal cysteine-rich domains are needed for protein body biogenesis. β-Zein, which can form protein bodies alone, does not have these proline-rich repeats, but it does contain a short cysteine-rich domain (Shewry et al., 1995). Transgenic tobacco plants expressing an α-zein gene driven by the CaMV 35S promoter showed accumulation of α-zein in all tissues, except for those of embryos of developing seeds (Schernthaner et al., 1988). These plants were not analyzed by using electron microscopy, and as such it is not known whether α-zein is capable of forming protein bodies on its own. More recently, Coleman et al. (1996) demonstrated that when α-zein was made in an endosperm-specific manner in transgenic tobacco, it was unstable and protein bodies could not be detected. However, when coexpressed with γ-zein, the stability of α-zein increased, and it was colocalized with γ-zein in ER-derived protein bodies.

Although α-zein and γ-zein appear to interact physically during protein body formation (Coleman et al., 1996), we still do not know either the role of β-zein or the way δ-zein is assembled into the protein body. δ-Zein is rich in methionine and cysteine residues and shares few structural features with the other zeins. This protein appears to be deposited late during protein body biogenesis, and it is found throughout the core of the protein body, probably interspersed with α-zeins (Esen and Stetler, 1992). To determine how δ-zein behaves by itself and whether it interacts with other zeins, transgenic tobacco plants expressing the δ-zein gene alone and in combination with β-zein were created. Analysis of these transgenic plants showed that although δ-zein is fairly stable in the vegetative tissues of transgenic plants, it becomes even more stable when the δ-zein gene is coexpressed with the β-zein gene. Our data also show that δ-zein by itself forms protein bodies distinct from those formed by β-zein. However, in the presence of β-zein, δ-zein is colocalized in the β-zein-containing protein bodies. Because BIP has been associated with protein body biogenesis, some preliminary work was done to determine whether zein synthesis in transgenic plants is associated with the induction of BIP. Our data indicate that there is increased accumulation of BIP in the transformants expressing the zein genes.

### RESULTS

**δ-Zein Accumulates to High Levels in the Vegetative Tissues of Tobacco Transformants Containing the δ-Zein Gene Driven by the CaMV 35S Promoter**

Tobacco plants were transformed with the gene construct pM10Z, consisting of the δ-zein gene driven by the 35S promoter (Figure 1A). Protein from leaves of seven randomly selected independent transformants was subjected to immunoblot analysis to measure the accumulation of δ-zein (Figure 1B). All of the transformants showed two immunoreactive proteins: one comigrated with the 10-kD δ-zein from maize seeds (Figure 1B) and the other as a 29-kD band. The
latter did not comigrate with the higher molecular mass immunoreactive band seen in maize seeds (Figure 1C). The 29-kD immunoreactive band obtained from leaves of transgenic plants probably represents an aggregate of $\delta$-zein and another endogenous leaf protein. Similarly, the 20-kD immunoreactive protein band in maize seeds may represent an aggregate of $\delta$-zein with an endogenous maize protein. The accumulation of the 10- and 29-kD proteins differed by $\sim$10-fold among the different transformants. Transformant 4 showed almost negligible levels of both proteins. Differences in the accumulation of $\delta$-zein in the various transformants can be attributed to position effect or the number of copies of the integrated gene.

To determine the accumulation of the $\delta$-zein protein among different plant parts, equal amounts of protein extract (equivalent of 50 $\mu$g of PBS-soluble protein) from the leaf, stem, root, and seeds of transformant 6 were subjected to immunoblot analysis along with maize seed extract (the equivalent of 2 $\mu$g of PBS-soluble fraction) (Figure 1C). The leaves showed the highest level of accumulation of $\delta$-zein, with stems having the next highest level of accumulation. The seeds had an $\sim$10-fold lower level of $\delta$-zein compared with leaves, as was the case with $\beta$-zein in transgenic tobacco (Bagga et al., 1995). Taken together, our results suggest that $\delta$-zein accumulates to significant levels in all of the organs of tobacco plant, as we had reported earlier for $\beta$-zein (Bagga et al., 1995).

$\delta$-Zein Is Stable in Germinating Tobacco Seeds

It was shown previously that $\beta$-zein in transgenic tobacco seeds is not proteolytically digested during germination (Hoffman et al., 1987; Bagga et al., 1997). To determine whether $\delta$-zein behaves in a similar manner, seeds from a $\delta$-zein plant were allowed to germinate for different time periods (0 to 10 days), and the seeds/seedlings were harvested and their ethanol-soluble proteins extracted and analyzed by immunoblotting using the $\delta$-zein antibodies (Figure 2). The level of $\delta$-zein remained essentially unchanged for the first 4 days after germination, after which the level showed a dramatic increase in concentration. A slight drop in the level of $\delta$-zein was observed between 0 and 1 day after germination (DAG), and that level was maintained until 4 DAG. The drop observed with the 3-DAG sample was not consistent within different experiments and is attributed to lower load of the protein extract in that lane. The 4-DAG time point coincided with the appearance of the first set of green leaves and may be related to the activation of the CaMV 35S promoter in the developing seedling. The immunoreactive $\delta$-zein band also appeared to be fairly diffuse in SDS gels of proteins from the seedling stage, as has been observed with the leaf sample, suggesting that the leaves have some material in the ethanol-soluble protein fraction that interferes with the mobility of $\delta$-zein. These results suggest that $\delta$-zein is not degraded during germination of tobacco seeds.

$\delta$-Zein Accumulates in Novel Protein Bodies

Electron microscopy and immunocytochemical studies of the transgenic tobacco plants expressing the $\beta$-zein gene showed that this protein accumulated in special ER-derived protein bodies in the leaves as well as in the seeds (Bagga et al., 1995). Electron microscopy was also performed using leaf tissue from transgenic tobacco plants expressing $\delta$-zein to determine whether any protein bodies were present. As shown in Figures 3A and 3B, protein bodies very different from those formed by $\beta$-zein alone (Figure 3C) were detected in the leaves of the $\delta$-zein plants. The protein bodies in the $\delta$-zein plants were very osmophilic, the osmophilia being concentrated along the circumference of the bodies. In some cross-sections, the osmophilia appeared to radiate in discrete spokes from a central hub (Figure 3B). The protein bodies in the $\beta$-zein plants did not exhibit this extreme osmophilia (Figure 3C). In some of the leaf sections, the $\delta$-zein protein bodies were found to be associated with the ER; however, in most cases, because of the large size of the bodies, the ER membranes appeared to be disjoined. Based on immunolocalization, $\delta$-zein was found to be evenly distributed in these unique protein bodies, suggesting that they result from the assembly of $\delta$-zein (Figure 3D).

$\delta$-Zein Accumulates to Higher Levels in Seeds and Leaves of Plants Coexpressing $\delta$-Zein and the $\beta$-Zein Genes

Based on the temporal pattern of deposition of the different zeins during protein body biogenesis in maize endosperm, it has been proposed that $\gamma$-zein and $\beta$-zein may play a role in
Figure 3. Ultrastructure and Immunogold Localization of δ-Zein in Transgenic Tobacco Leaves.

(A) Regions of two mesophyll cells showing several δ-zein protein bodies (indicated by arrowheads) in a δ-zein transformant.

(B) Higher magnification of δ-zein protein bodies (indicated by arrowheads).

(C) Structure of a β-zein protein body (indicated by arrowheads) in the mesophyll cell of a β-zein transformant.

(D) Immunolocalization of δ-zein in the leaf cells of a δ-zein transformant with 5-nm gold particles (indicated by arrowheads).

localizing and organizing α-zein and δ-zein in protein bodies (Lending and Larkins, 1989; Esen and Stetler, 1992). To investigate how β-zein might interact with δ-zein, a δ-zein transformant was crossed with a β-zein transformant, and the seeds obtained from this cross were germinated on high levels of kanamycin (200 μg/mL). The seedlings expressing both of the zein genes (δ-/β-zein plants) were selected. The δ-/β-zein cross should have the same genomic context as the parents with regard to the β-zein and δ-zein genes. Thus, any differences in expression pattern between the parents and their progeny should not be due to position effects.

Protein extracts from the leaves of two independent δ-/β-zein plants and their respective δ- and β-zein parents were analyzed by immunoblotting using both δ- and β-zein antibodies. The accumulation of β-zein appeared similar in the parents and the δ-/β-zein crossed plants, whereas the accumulation of δ-zein was many fold higher in the δ-/β-zein plants compared with the corresponding δ-zein parent (data not shown).

To determine the exact level of increase of δ-zein due to coexpression with β-zein, equal amounts of protein extracts from the leaves and seeds of one of the δ-zein plants, a β-zein plant, and the corresponding δ-/β-zein cross were analyzed by immunoblotting followed by quantitation of the immu-
noreactive bands by using a Biolmage Intelligent quantifier (Biolmage, Ann Arbor, MI) (Figure 4). This quantitative analysis showed that the \( \beta \)-zein levels in both the seeds and the leaves of the \( \delta-/\beta \)-zein plants were essentially similar to those in the \( \beta \)-zein parent plant (Figures 4C and 4D). However, the amount of \( \delta \)-zein was four- to fivefold higher in the leaves and seeds of the \( \delta-/\beta \)-zein cross compared with that of the parent plant (Figures 4A and 4B). The levels of \( \beta \)-zein and \( \delta \)-zein in the cross or the parental lines cannot be compared directly with each other because of differences in the antigenicity of the two antibodies and the concentration of the antibodies used for developing the blots. These results also confirm previous studies indicating that the leaves accumulate more of the zein proteins than do seeds. In the case of the \( \delta \)-zein protein (Figures 4A and 4B), the amount of protein loaded on the gel is 10 \( \mu \)g for the leaf samples and 50 \( \mu \)g for the seed samples.

**Increased Accumulation of \( \delta \)-Zein in Leaves of the \( \delta-/\beta \)-Zein Plants Is Not Due to Increased Availability of the \( \delta \)-Zein Transcripts**

Our data suggest that \( \delta \)-zein accumulates to higher levels in the \( \delta-/\beta \)-zein cross than in the \( \delta \)-zein parent. To determine whether this increase is due to increased stability of the \( \delta \)-zein protein in the cross or an increase in transcript availability, we analyzed total RNA isolated from leaves of the parents and the cross (at two developmental stages) with RNA gel blotting using gene-specific probes for \( \delta \)-zein and \( \beta \)-zein genes followed by quantitation of the radioactive bands using a Biolmage Intelligent quantifier (Figures 5A and 5B). The blots were also analyzed with an rRNA probe to standardize RNA loads. The levels of \( \delta \)-zein and \( \beta \)-zein RNA were identical between the parents and the cross as well as between leaves from young and mature plants (Figure 5). Taken together, the data suggest that the increased accumulation of \( \delta \)-zein in the leaves of the \( \delta-/\beta \)-zein plant is not due to an increase in the corresponding RNA. Moreover, these data also suggest that there is no difference in the zein transcript level due to the maturity of the leaves.

**Rate of \( \delta \)-Zein Synthesis Is Higher in the \( \delta-/\beta \)-Zein Cross than in the Corresponding \( \delta \)-Zein Parent**

To determine whether the increased steady state level of \( \delta \)-zein in the \( \delta-/\beta \)-zein cross is due to enhanced protein accumulation, we performed a pulse-labeling experiment. Leaf discs from a \( \delta \)-zein plant, a \( \beta \)-zein plant, and the corresponding \( \delta-/\beta \)-zein cross were incubated in \( ^{35} \)S-methionine for 2 hr, and the EtOH-soluble protein fraction from these discs was analyzed by SDS-PAGE and autoradiography. The protein bands corresponding to \( \delta \)-zein and \( \beta \)-zein along with that of endogenous tobacco proteins were subjected to quantitative image analysis using the Biolmage Intelligent quantifier. By using the endogenous tobacco protein bands as internal standard (Figure 6A), the values for \( \delta \)-zein and \( \beta \)-zein were calculated in each of the samples and plotted on a graph (Figure 6B).

The labeling of the zein proteins far exceeded that of any other proteins in the EtOH-soluble fraction, and the amount of \( \delta \)-zein labeling exceeded that of \( \beta \)-zein in the \( \delta-/\beta \)-zein cross. This was as expected, based on the methionine content of \( \delta \)-zein and \( \beta \)-zein (21 and 11%, respectively). A comparison of the radioactivity incorporated into the two zein proteins of the parent plant and the cross, relative to incorporation into endogenous tobacco proteins, indicated that there was an almost twofold increase in the amount of labeled \( \delta \)-zein in the \( \delta-/\beta \)-zein cross compared with that of the \( \delta \)-zein plant; the difference in the labeling of \( \beta \)-zein between the \( \delta-/\beta \)-zein cross and the \( \beta \)-zein plant, however, was not significant. These data suggest that either there is increased synthesis of \( \delta \)-zein in the \( \delta-/\beta \)-zein cross or there is a faster turnover of \( \delta \)-zein in the absence of the \( \beta \)-zein protein.
Figure 5. Analysis of δ-Zein and β-Zein Transcripts in the Leaves of the δ-Zein, β-Zein, and δ-/β-Zein Tobacco Transformants.

(A) Fifteen micrograms of total RNA isolated from the leaves of young (Y) and mature (M) plants (δ-zein, β-zein, and δ-/β-zein plants and a non-transformed [NT] plant) was subjected to gel blot analysis using a 470-bp EcoRI-XbaI fragment containing the δ-zein coding region, a 690-bp BglII-BamHI fragment containing the β-zein coding region, or a 28S rRNA gene fragment from soybean.

(B) Quantification of intensity of the bands hybridizing to the δ-zein and β-zein probes, using the Biolmage Intelligent quantifier.

δ-Zein Is Found in the Same ER-Derived Protein Bodies as the β-Zein in Plants Coexpressing the δ-Zein and β-Zein Genes

The increased accumulation of δ-zein in the δ-/β-zein cross compared with that of the δ-zein plant alone is suggestive of an interaction between δ-zein and β-zein. Electron microscopy and immunocytochemistry of leaf tissue from a δ-/β-zein plant revealed only the ER-derived protein bodies typical for β-zein (Figure 7A). We did not observe any protein bodies similar to those detected in the δ-zein plants. However, immunolocalization of δ-zein showed that the protein was exclusively confined in the β-zein protein bodies (Figure 7B). To determine whether both δ-zein and β-zein were located in the β-zein protein bodies, we performed double-labeling immunocytochemistry with leaf and seed sections of the δ-/β-zein plant by using monoclonal antibodies raised against δ-zein and polyclonal antibodies raised against β-zein. Both δ-zein (represented by the larger 10-nm gold particles) and β-zein (represented by the smaller 5-nm gold particles) were immunolocalized in the same β-zein protein bodies (Figures 7C and 7D).

δ-Zein and β-Zein Can Be Localized in Both Cytoplasmic and Intravacuolar Protein Bodies in the Seeds of δ-/β-Zein Crosses

Seed storage proteins in tobacco, as in most dicotyledonous plants, are stored in protein bodies derived from vacuoles (Shewry et al., 1995), whereas the zeins are deposited in ER-derived protein bodies (Larkins and Hurkman, 1978).
In an earlier study, we showed that β-zein accumulates in ER-derived protein bodies in both tobacco leaves and seeds (Bagga et al., 1995); however, Hoffman et al. (1987) reported that β-zein, when expressed in a seed-specific manner in transgenic tobacco, accumulated in vacuolar protein bodies. To investigate the δ-zein accumulation pattern in seeds of transgenic tobacco, seeds of both the δ-zein and the δ/β-zein plants were analyzed by using electron microscopy and immunocytochemistry. The tissue sections from the δ-zein seeds showed the same typical protein bodies observed in the leaves of the δ-zein plants. These protein bodies were interspersed with the much larger vacuole-derived protein bodies (data not shown). Electron microscopic analysis of seed sections from the δ/β-zein cross, however, showed the rosette-shaped structure typical of the β-zein protein bodies, and these were dispersed in the cytoplasm along

**Figure 7.** Subcellular Localization of δ-Zein and β-Zein in Leaves of δ/β-Zein Plants.

(A) Conventionally fixed and stained sections of leaves from a δ/β-zein plant. Arrowheads indicate the protein bodies formed in the cytoplasm. (B) Immunolocalization of the δ-zein protein by using mouse anti-δ-zein antibody diluted 1:50 followed by labeling with 10 nm in diameter gold-conjugated goat anti-mouse IgG. The arrowheads indicate the protein bodies that are labeled with the antibodies. (C) Coimmunolocalization of δ-zein and β-zein by using the mouse anti-δ-zein antibody and the rabbit anti-β-zein antibodies followed by labeling with 10 nm in diameter gold-conjugated goat anti-mouse IgG and 5-nm gold-conjugated goat anti-rabbit IgG. (D) A higher magnification of a region showing double-labeling from (C). The arrowheads indicate the 10-nm gold particles; the arrows point to the 5-nm gold particles.
with the vacuolar-derived protein bodies (Figures 8A and 8B). Immunolocalization studies with the δ-zein and β-zein antibodies showed double labeling in these rosette-shaped protein bodies, indicating the colocalization of the two proteins (Figure 8D). On rare occasions, the ER-derived protein bodies containing zein proteins were found sequestered in the matrix of the vacuolar protein bodies (Figure 8C), as was reported earlier for the β-zein protein bodies (Bagga et al., 1995). These intravacuolar protein bodies appear to be morphologically similar to the cytoplasmic protein bodies, but they lacked a sharp membrane boundary on the surface.

**BiP Is Induced in Transgenic Plants Expressing Zein Genes**

Because BiP has been implicated in prolamin protein body biogenesis (Zhang and Boston, 1992; Li et al., 1993a), it fol-

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**Figure 8.** Analysis of the Endosperm Ultrastructure of a δ-/β-Zein Plant.

(A) Conventionally fixed and stained endosperm cells showing the novel ER-derived protein bodies (arrowheads) and the typical vacuolar-derived protein bodies.

(B) Higher magnification of the ER-derived protein body in (A).

(C) Higher magnification of a vacuole-derived protein body containing the ER-derived protein bodies in (A) (shown by an arrowhead).

(D) Coimmunolocalization of the δ-zein and β-zein by using the mouse anti-δ-zein antibody and the rabbit anti-β-zein antibodies followed by labeling with 10 nm in diameter gold-conjugated goat anti-mouse IgG and 5-nm gold-conjugated goat anti-rabbit IgG. The arrowheads indicate the 10-nm gold particles; the arrows point to the 5-nm gold particles.
ows that BiP may play a role in the formation of zein protein bodies in transgenic tobacco plants. If so, it is possible that BiP is increased in plants making zein protein bodies. To test this hypothesis, protein samples from leaves of δ-zein, β-zein, and δ/β-zein plants were subjected to quantitative immunoblot analysis using a maize BiP antibody (Zhang and Boston, 1992). The PBS-soluble sample (100 μg) from a control plant and three transgenic plants (δ-zein, β-zein, and δ/β-zein plants) at the same developmental stage, all grown under the same conditions, and 1 μg of purified BiP from maize were subjected to SDS-PAGE followed by immunoblot analysis (Figure 9A). The immunoreactive bands were then analyzed using the Biolmage Intelligent quantifier. The graph in Figure 9B shows the relative intensity of the immunoreactive bands. All three transgenic plants showed a significantly higher level of BiP accumulation when compared with the control; the levels of BiP were more or less similar in the three transformants. These results suggest that synthesis of the zein proteins in transgenic plants induces the synthesis or stable accumulation of BiP.

DISCUSSION

We have demonstrated that δ-zein, like β-zein (Bagga et al., 1995) and γ-zein (Geli et al., 1994), is capable of forming protein bodies by itself. The δ-zein protein bodies, similar to those of β-zein and γ-zein, appear to be retained within the ER. The δ-zein protein bodies, however, are morphologically distinct from the protein bodies formed by β-zein (Bagga et al., 1995) or γ-zein (Geli et al., 1994) or those formed in maize endosperm (Lending et al., 1988), suggesting that each type of zein has intrinsic structural properties that influence the morphology of the protein body. Moreover, the results presented here demonstrate that vegetative tissues of a dicot plant can be induced to produce protein bodies that are typical of seeds of cereal plants. It is intriguing that when δ-zein was coexpressed with β-zein, the protein bodies resembled those formed by β-zein alone; this suggests that intermolecular interactions between the two zeins are dominated by β-zein. In addition, in two δ/β-zein crosses, we did not observe any δ-zein protein bodies or protein bodies uniquely labeled with either the δ- or β-zein antibodies alone. Work is in progress to create plants with a constant amount of BiP accumulation when compared with the control; the levels of BiP are more or less similar in the three transformants. These results suggest that synthesis of the zein proteins in transgenic plants induces the synthesis or stable accumulation of BiP.

Figure 9. Analysis of the Accumulation Pattern of BiP in the δ-Zein, β-Zein, and δ-/β-Zein Tobacco Transformants.

(A) The PBS-soluble extracts (100 μg of protein) from leaves of control (NT), δ-zein, β-zein, and δ-/β-zein plants were subjected to SDS-PAGE followed by immunoblotting using an antibody raised against BiP from maize. A positive control lane containing purified BiP (1 μg) was included in the gel.

(B) Shown are the results of band quantification using the Biolmage Intelligent quantifier.
"protein body ER" and that these proteins need not have any structural determinants for retention in the ER. In rice endosperm, two distinct kinds of ER can be differentiated: the cisternal ER and protein body ER (Li et al., 1993b). Whereas the former is involved in the secretory pathway, the latter delimits the ER-derived protein bodies, and the destination of the protein into these two kinds of ER is determined by the signal peptide.

BiP has been shown to facilitate the folding and retention of the rice prolamin in the ER (Li et al., 1993a), and BiP has also been proposed to play a role in protein body biogenesis in the maize endosperm (Marocco et al., 1991; Zhang and Boston, 1992). Our data show that there is a higher accumulation of BiP in transgenic tobacco plants expressing the zein genes, suggesting that the presence of the zein protein in the ER triggers the induction of BiP. Several studies have shown that the induction of chaperones, such as BiP, is triggered by the presence of nonnative structures and/or misassembled proteins in the ER (Gething and Sambrook, 1992).

Induction of BiP in zein-expressing plants may be a result of the accumulation of a foreign protein in the ER or may imply that it plays a role in the folding and assembly of zein protein bodies in transgenic tobacco. Our data on BiP induction are consistent with a recent report showing that BiP is induced in transgenic tobacco plants expressing antibody genes and that BiP was also found to copurify with the antibodies (J. Ma, personal communication). This suggests that BiP is involved in the assembly of antibodies in transgenic plants.

Our results demonstrate that δ-zein, like β-zein, when expressed by itself in a constitutive manner, is stable and accumulates to high levels in both vegetative and seed tissues. γ-Zein has also been shown to accumulate to high levels in vegetative tissues in transgenic Arabidopsis plants (Gelli et al., 1994). The stability of the zeins in the vegetative tissues of transgenic plants implies that they are not very accessible to proteases. This could be attributed to the fact that the zeins are retained in the ER and not shuttled into the vacuoles. Plant vacuoles are known to have powerful proteases (Pueyo et al., 1995), and proteins that are stored in vacuoles are exposed to a highly proteolytic environment. We cannot, however, rule out the possibility that indigestibility of the zeins is due to the intrinsic hydrophobic nature of the proteins and their insolubility in vivo. It is interesting that zeins, including δ-zein, remain more or less undigested during germination of transgenic seeds, whereas the tobacco seed proteins are readily utilized (Bagga et al., 1997). It is possible that specific proteases are required to digest the zeins and that such proteases are not present in the germinating tobacco seeds, or that the protein bodies containing zeins are not accessible to the proteases in germinating seeds. Of course, we have not ruled out the possibility that the 3SS promoter is active in germinating seeds and that new synthesis of zein proteins keeps up with turnover of zein proteins.

One of the intriguing results of this study is that δ-zein accumulation is increased manyfold when it is cosynthesized with β-zein, whereas β-zein accumulation is not significantly changed in the presence of δ-zein. This suggests that even though δ-zein is stable by itself, its accumulation is increased when it interacts with β-zein. A similar observation was made by Coleman et al. (1996) when they coexpressed γ-zein with α-zein in a seed-specific manner. When synthesized by itself, α-zein showed no significant accumulation, but when coexpressed with γ-zein, α-zein accumulated in the mid-maturation seeds.

We have ruled out the possibility that δ-zein mRNA is stabilized by β-zein mRNA in the δ-/β-zein cross; however, our data cannot prove conclusively that there is no increase in the rate of synthesis of the δ-zein protein in the δ-/β-zein cross. Labeling studies showed that over a period of 2 hr, the amount of radioactivity incorporated into δ-zein in the δ-/β-zein plant was twofold higher than in the δ-zein plant, whereas there was no significant difference in the incorporation of label into β-zein in the δ-/β-zein plant and the β-zein parent plant. It is difficult to envision preferential transcript recruitment into the polysomes as the basis for increased accumulation of δ-zein in the δ-/β-zein plants, and there is no precedent for this in the literature. One possible explanation is that the rate of turnover of the newly formed δ-zein is lower in the δ-/β-zein cross. If this is true, it would mean that the interaction between δ-zein and β-zein has a stabilizing effect on δ-zein.

The data presented in this study suggest that δ-zein by itself is not as stable as it is when it is cosynthesized with β-zein; however, because δ-zein accumulates in the δ-zein plants, it follows that in the absence of β-zein, δ-zein is probably stabilized by an interaction with some chaperone protein, such as BiP in the ER. Our data indicate that there is induction of BiP in the δ-zein-producing plants; however, it is possible that the BiP level in the δ-zein plants is not adequate to allow the folding and stabilization of all of the δ-zein protein that is made, with δ-zein in excess of BiP being rapidly degraded. In this regard, it would be interesting to study the effects of overexpressing the BiP gene in plants producing the δ-zein protein. Nevertheless, we cannot rule out the possibility that δ-zein by itself is more accessible to the proteases present in the ER.

As is true of the β-zein plants, seeds of the δ-zein and δ-/β-zein plants contained both the ER-derived protein bodies and the vacuole-derived protein bodies. δ-Zein and β-zein were found exclusively in the ER-derived protein bodies in the δ-/β-zein plants. On rare occasions, some of the protein bodies containing zein proteins were found inside the vacuolar protein bodies. Similar observations were made when γ-zein was expressed in an endosperm-specific manner in transgenic tobacco (Coleman et al., 1996). It is not known how zein protein bodies become engulfed in the vacuolar protein bodies, but it could occur in a manner similar to autophagy, as has been described for prolamine proteins in wheat seeds (Levanony et al., 1992).

Our data indicate that leaves accumulate more δ-zein than do mature seeds (Figure 1C). The difference between the levels of δ-zein from seeds and vegetative tissues was quite
pronounced when δ-zein levels were compared between germinating seeds and emerging seedlings (Figure 2). Similar observations have been made for other zeins (Scherthnaner et al., 1988; Gehl et al., 1994; Bagga et al., 1995). In our studies, the discrepancy in protein levels between leaves and seeds cannot be attributed to differential transcript levels, because previous work in which the β-zein gene behind the CaMV 35S promoter was expressed in transgenic tobacco showed that the seeds and leaves contained the same level of the β-zein transcript (Bagga et al., 1995).

Ultrastructural analysis of the β-zein and δ-zein transformants showed that the ER-derived protein bodies are not as abundant in seeds as they are in leaves and that the number of protein bodies decreased as the seeds matured and filled with the vacuolar-derived protein bodies containing the tobacco storage proteins. Thus, lower levels of zeins in seeds may be due to competition between zein mRNA and tobacco storage protein mRNAs for the recruitment of the ER for translation. Lower levels of zeins in seeds could also be due to the fact that some of the protein bodies in seeds become engulfed by vacuolar protein bodies, where they are digested by proteases present in the vacuoles. Coleman et al. (1996) observed that some of the intravacuolar protein bodies showed lower labeling with zein antibodies when compared with the ones in the cytosol, suggesting degradation of the zein proteins by vacuolar hydrolases. Hoffman et al. (1987), however, reported a significant accumulation of β-zein in seeds of plants expressing the β-zein gene when a β-phaseolin promoter was used, even though the zein in that case was being directed into the vacuolar protein bodies.

It is interesting that despite the heavy load on the protein trafficking system caused by the accumulation of the zein protein in the ER, the tobacco plants seemed to perform normally. Plants homozygous for both the β-zein and δ-zein genes were identified in the F2 generation. These plants accumulated high levels of the two zeins, yet they showed no phenotypic difference when compared with control plants. These results will have a significant impact on our long-term goal of increasing the methionine content in forage tissues of plants.

METHODS

Recombinant DNA Techniques

Standard procedures were used for recombinant DNA manipulations (Maniatis et al., 1982). Plasmid pMZEllOk containing the β-zein cDNA isolated from a maize endosperm cDNA library (Kihara et al., 1988) was a gift from J. Messing (Rutgers University, Piscataway, NJ). A 470-bp EcoRI-Xbal fragment containing the entire coding region was removed from pUC119 and cloned into the EcoRI and Xbal sites of pSP73. The stop codon for the δ-zein gene is contained within the Xbal site. The δ-zein gene was then recovered as a Bglli-Xhol fragment and inserted into the Bglli and Xhol sites in the polylinker of pMON316 (Rogers et al., 1987). The translation terminator following the stop codon of the δ-zein gene is the nopaline synthase terminator. The resulting plasmid was called pM10Z (Figure 1A). Plasmid pMEZ is as described by Bagga et al. (1995).

Plant Transformation and Regeneration

The plasmid pM10Z was mobilized from Escherichia coli DH5α into the Agrobacterium tumefaciens receptor strain pTiT37FAE by triparental mating (Rogers et al., 1987). Nicotiana tabacum cv Xanthi was transformed by the leaf disc procedure (Horsch et al., 1985). Transformants were selected and regenerated on Murashige and Skoog media (Murashige and Skoog, 1962) containing 100 μg/mL kanamycin. Shoots appeared within 4 to 6 weeks after inoculation. The shoots were rooted on the same media without hormones and transferred to the soil.

To obtain the plants containing both of the zein genes driven by the cauliflower mosaic virus (CaMV) 35S promoter, tobacco transformants containing either pM10Z or pMEZ were crossed, and the seeds obtained were germinated on media containing 200 μg/mL kanamycin. Immunoblot analysis was performed with protein extracts from seedlings, using both β-zein and δ-zein antibodies. Plants expressing both of the zein genes (δ-/β-zein plant) and the parent plants (δ-zein and β-zein plants) were used in all comparative analyses.

Zein Extraction and Immunoblot Analysis

Plant tissues were ground and extracted in PBS and centrifuged. The supernatant was used for protein determination using the Bradford assay (Bio-Rad). The pellet was incubated in 70% ethanol containing 1% w/v acetic acid at 65°C for 30 min to extract the zein proteins. For immunoblot analysis, the EtOH-extractable fraction equivalent to a known amount of the PBS-soluble protein extract was subjected to SDS-PAGE (Laemmli, 1970), followed by electroblotting onto a nitrocellulose membrane. The membrane was blocked for 1 to 2 hr with 1% BSA in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 [Sigma]), followed by overnight incubation in the same solution containing the appropriate antibodies. The δ-zein monoclonal antibodies were kindly provided to us by DEKALB Genetics Corporation (Mystic, CT), the δ-zein polyclonal antibodies by J. Messing, and the β-zein polyclonal antibodies by B. Larkins (University of Arizona, Tucson, AZ). The protein bands reacting with the antibodies were visualized by using an alkaline phosphatase–linked secondary antibody (goat antibody to rabbit IgG in the case of the polyclonal antibody or mouse IgG in the case of the monoclonal antibody) and the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, according to the manufacturer’s instructions (Promega). Both the polyclonal and monoclonal antibodies raised against δ-zein gave similar results; however, the polyclonal antibodies showed some degree of cross-reactivity with β-zein, and as such, in all of our comparative analyses involving the parents and the crossed progeny, only the monoclonal antibodies were used.

Analysis of BiP

The PBS-soluble extracts from the leaves of the different plants were subjected to SDS-PAGE followed by immunoblot analysis using the polyclonal antibodies for maize BiP (kindly provided to us by R. Boston,
North Carolina State University, Raleigh, NC), using the procedure described in the previous section.

In Vivo Labeling of Leaf Discs

Four leaf discs (7 mm in diameter) from young expanding leaves were incubated in 120 μL of labeling mix (1 mM potassium phosphate, pH 6.1, 1% sucrose, and 50 μg/mL of chloramphenicol) containing 120 μCi of 35S-methionine (specific activity, 1047 Ci/mmol) for 2 hr in the light. The discs were then washed well with incubation buffer, and the samples were ground in cold PBS. The samples were extracted in PBS, and protein estimates were made in the PBS-soluble fraction. The zeins were recovered from the pellet, as described by Bagga et al. (1995). The proteins were analyzed by SDS-PAGE followed by electrophotography onto a polyvinylidifluoride membrane (Millipore Corp., Bedford, MA). The membrane was sprayed with Enhance (Du Pont-New England Nuclear, Beverly, MA), air dried, and exposed to x-ray film.

RNA Isolation and Analysis

Total RNA was isolated using a lithium chloride precipitation method (de Vries et al., 1982). The RNA was fractionated on a 1% agarose-formaldehyde gel, transferred to a nitrocellulose membrane, and probed with radiolabeled fragments, as described in the legend to Figure 5A. Hybridization was in 50% formamide at 42°C by using standard conditions (Maniatis et al., 1982). The filters were washed three times with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 42°C for 15 min each, followed by two washes with 0.1 × SSC and 0.1% SDS at 42°C for 20 min, and exposed to x-ray film.

Electron Microscopy

Small pieces of leaf and seed tissue were fixed in 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer for 2 hr and then postfixed in 1% aqueous osmium tetroxide for 1 hr. The samples were dehydrated in ETOH and embedded in Spurr's resin (Electron Microscopy Sciences, Fort Washington, PA) at 70°C. Silver sections on copper grids were then stained in uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). The grids were examined in a Hitachi transmission electron microscope (model H7000; Nissel Sanyo America Ltd., Mountain View, CA).

Immunoelectron Microscopy

Small pieces of leaf and seed tissue were fixed for 2 hr on ice in 4% paraformaldehyde and 0.6% glutaraldehyde in 0.33 M sodium/potassium phosphate buffer, pH 7.3, containing 0.1 M sucrose. The tissue was washed in three changes of buffer containing 7% sucrose and kept in the third wash overnight at 4°C. Two different protocols were used at this stage. The fixed tissue was dehydrated in ETOH and infiltrated with Lowicryl (Electron Microscopy Sciences) at −10°C, and the resin was polymerized under UV light at −10°C for 24 hr and then at room temperature for 24 hr. In the second protocol, the tissue was dehydrated in ETOH and embedded in either Spurr's resin or London Resin White (Electron Microscopy Sciences) and polymerized at 50°C. The remaining steps were all done at room temperature. Silver sections on nickel grids were first incubated in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 [Sigma]) containing 1% BSA (Sigma). This buffer mixture was used for all of the remaining steps. For immunolabeling seed sections, 5 to 20% normal serum from the animal source of the antibody was added to the blocking solution to reduce nonspecific staining.

Immunolabeling with the α-Zein Antibodies

The grids of sections from the α-zein and α-β-zein crosses were drained and incubated with the monoclonal antibody raised against α-zein diluted 1:50 in buffer for 45 min to 4 hr. Controls were incubated in nonimmune mouse IgG. They were then washed in the buffer and placed in gold-labeled goat anti-mouse IgG with a diameter of 10 nm (Sigma) diluted 1:50 in buffer for 45 min. In the case of the sections from the α-zein plant, the grids were incubated with the polyclonal rabbit anti-α-zein antibody diluted 1:1000 in buffer for 60 min. They were then washed and incubated in a solution of gold-conjugated anti-rabbit IgG with a diameter of 5 nm and diluted 1:50 for 60 min. Because the polyclonal anti-α-zein antibody cross-reacted with β-zein, in the case of the α-β-zein cross, the grids were incubated with the α-zein monoclonal antibody followed by gold-conjugated anti-mouse IgG with a diameter of 10 nm. The grids were washed with TBST and then with double-distilled water. The grids were then left unstained or lightly poststained in uranyl acetate and lead citrate and examined.

Double-Labeling with the α- and β-Zein Antibodies

The grids were incubated in rabbit anti-β-zein antibody diluted to 1:100 for 45 to 60 min followed by incubation in gold-conjugated goat anti-rabbit IgG with a diameter of 5 nm, diluted 1:50 for 45 to 60 min. After thorough washing in the buffer, the grids were incubated with mouse anti-α-zein diluted 1:50 in buffer for 45 to 60 min. They were then washed in TBST followed by distilled water and incubated in gold-conjugated goat anti-mouse IgG with a diameter of 10 nm, diluted 1:50 in buffer for 45 min. After washing the grids in TBST followed by double-distilled water, they were examined unstained or lightly poststained in uranyl acetate and lead citrate.

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