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Accumulation of 15-Kilodalton Zein in Novel Protein Bodies in Transgenic Tobacco[1]

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Zeins, the seed storage proteins of maize, are a group of alcohol-soluble polypeptides of different molecular masses that share a similar amino acid composition but vary in their sulfur amino acid composition. They are synthesized on the rough endoplasmic reticulum (ER) in the endosperm and are stored in ER-derived protein bodies. Our goal is to balance the amino acid composition of the methionine-deficient forage legumes by expressing the sulfur amino acid-rich 15-kD zeins in their leaves. However, it is crucial to know whether this protein would be stable in nonseed tissues of transgenic plants. The major focus of this paper is to compare the accumulation pattern of the 15-kD zein protein with a vacuolar targeted seed protein, β-phaseolin, in nonseed tissues and to determine the basis for its stability/instability. We have introduced the 15-kD zein and bean β-phaseolin-coding sequences behind the 35S cauliflower mosaic virus promoter into tobacco (*Nicotiana tabacum*) and analyzed the protein's accumulation pattern in different tissues. Our results demonstrate that the 15-kD seed protein is stable not only in seeds but in all nonseed tissues tested, whereas the β-phaseolin protein accumulated only in mid- and postmaturation seeds. Interestingly, zein accumulates in novel protein bodies both in the seeds and in nonseed tissues. We attribute the instability of the β-phaseolin protein in nonseed tissues to the fact that it is targeted to protease-rich vacuoles. The stability of the 15-kD zein could be attributed to its retention in the ER or to the protease-resistant nature of the protein.

Seed storage proteins constitute a potentially useful class of proteins for the improvement of forage crops if they can be made to accumulate in leaves. It is a fairly simple genetic engineering feat to introduce a seed protein-coding sequence behind a strong constitutive promoter into transgenic plants and ensure high rates of synthesis of the corresponding protein. However, stability of the protein in an alien environment is still not clearly defined and has to be treated on a case by case basis.

Seed proteins are synthesized during seed development and accumulate in protein bodies. These proteins are then utilized by the emerging seedling during germination. The major seed protein in dicotyledonous plants are the salt-soluble globulins, which are stored in vacuole-derived protein bodies. Most monocot seed storage proteins are alcohol-soluble prolamines that are stored either in ER-derived protein bodies as in the case of maize and rice or in vacuole-derived protein bodies as in the case of oat and wheat. Irrespective of their final destination, seed storage proteins are synthesized on polysomes associated with the ER and they then enter the lumen by direction of the signal peptide (Chrispeels, 1991). Whereas the ER-targeted storage proteins are retained in the ER, the vacuolar proteins are targeted to the vacuoles via the Golgi complex and involve an additional sorting signal (Chrispeels, 1991). In wheat, however, the deposition of seed storage protein into protein bodies inside vacuoles does not involve transit through the Golgi complex. Instead, the storage proteins aggregate into protein bodies within the ER and are then transported as intact protein bodies to the vacuole by a novel route (Levanony et al., 1992). Retention of many of the ER proteins in the lumen of the ER has been attributed to the presence of an amino acid sequence (KDEL) at the carboxy terminus (Pelham, 1990). However, many of the seed proteins that accumulate in ER-derived protein bodies do not have the KDEL sequence. In the case of rice prolamines and corn zeins, it has been proposed that an ER-resident chaperone, BiP, retains prolamines in the ER lumen by facilitating their folding and assembly into protein bodies (Boston et al., 1991; Li et al., 1993b).

Because of the vacuolar targeting signal, vacuolar seed proteins, if synthesized in nonseed tissue, would enter the protease-rich vacuoles (Van der Valk and Van Loon, 1988) and risk being degraded. ER-retained proteins would thus be more stable in nonseed tissues simply because they are not exposed to vacuolar proteases. In support of this hypothesis is the fact that retention of the vacuolar pea vicilin protein in the ER by engineering a ER retention signal onto the protein stabilized the modified protein 100-fold over unmodified vicilin protein when synthesized in leaves of transgenic plants (Wandelt et al., 1992). However, many vacuolar proteins such as the pea lectin (Edwards et al., 1991), barley lectin (Wilkins et al., 1990), and cowpea trypsin inhibitor (Hilder et al., 1987) have been shown to be stable in leaves of transgenic plants. Thus, it follows that the stability of a protein is not determined solely by its subcellular location but may be controlled by the intrinsic properties of the protein itself.

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Abbreviations: BiP, binding protein; DAP, days after pollination; EtOH, ethanol; NOS, nopaline synthase.

Forage legumes are deficient in sulfur amino acids (Croissant et al., 1976) and have to be supplemented with synthetic amino acids when used to feed livestock. Among the various seed proteins, the 15-kD zein protein of corn meets all of the criterion for being the ideal protein for improving the nutritional quality of forage crop. The 15-kD zein is a member of the sulfur amino acid-rich corn seed storage proteins with a high proportion of Met residues (Giánnazza et al., 1977). The zeins are synthesized by membrane-bound polysomes and transported into the lumen of the ER, where they assemble into protein bodies (Khoo and Wolf, 1970; Larkins and Hurkman, 1978).

The major focus of the study was to determine whether the 15-kD zein protein would be stable in nonseed tissues of transgenic plants and to check the subcellular site of deposition of this protein in seed and nonseed tissues of dicotyledonous plants. For comparison, we have studied the pattern of accumulation of a β-phaseolin protein, which is a vacuole-targeted seed protein from french bean (Chrispeels, 1991). In this study, we have introduced both the 15-kD zein and the β-phaseolin-coding sequences behind the 35S promoter into tobacco (*Nicotiana tabacum*) and studied the fate of the two proteins in different tissues. Our results demonstrate that the 15-kD zein protein is stable not only in developing seeds but in all nonseed tissues tested, whereas the β-phaseolin protein showed accumulation only in mid- and postmaturation seeds. Furthermore, we have immunolocalized the 15-kD zein protein in novel protein bodies, most likely derived from the ER, in both leaves and seeds of transgenic tobacco.

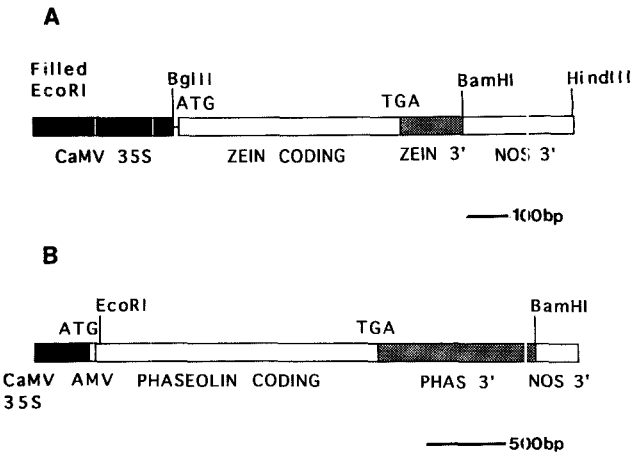

Figure 1. Diagrammatic representation of the 35S zein (pMEZ) and 35S-β-phaseolin (pGEL20) gene constructs. A, pMEZ contains 690 bp of the zein-coding sequence consisting of 20 bp of 5′ untranslated region and 150 bp of 3′ untranslated region inserted between the cauliflower mosaic virus 35S promoter and the NOS 3′ terminator of pMON 316 (Rogers et al., 1987). pMEZ was transferred to *A. tumefaciens* pTiT37ASE by triparental mating. B, pGEL20 is a modified pMON 316 consisting of a 42-bp synthetic translation enhancer sequence (Gallie et al., 1987) followed by the promoterless β-phaseolin gene (3.0 kb; six exons and five introns) truncated 5′ to its ATG. The truncated phaseolin gene (PHAS) was produced by building back toward the 5′ end from its *Eco*RI site to its ATG with a 43-bp synthetic oligonucleotide. pGEL20 was transferred to *A. tumefaciens* pTiT37ASE by triparental mating. AMV, Avian myeloblastosis virus.

MATERIALS AND METHODS

Recombinant DNA Techniques

Standard procedures were used for recombinant DNA manipulations (Maniatis et al., 1982). Plasmid pZ15.1 containing the 15-kD zein cDNA isolated from a corn endosperm cDNA library (Marks and Larkins, 1982) was a gift from Dr. Brian Larkins (University of Arizona, Tucson). The 704-nucleotide *Taq*I-*Bam*HI fragment containing the entire coding region and 150-bp of the 3′ untranslated region was subcloned into the *Cla*I and *Bam*HI sites of pSP73. The gene was recovered as a *Bam*HI-*Bgl*II fragment that was inserted into the *Bam*HI site in the polylinker of pMON316 (Rogers et al., 1987) in the sense orientation relative to the 35S cauliflower mosaic virus promoter and NOS 3′ transcription terminator. The resulting plasmid was named pMEZ (Fig. 1A). pGEL20 is a modification of pMON316 and contains an insertion of a 42-bp synthetic translation enhancer sequence (Gallie et al., 1987), followed by 3.0 kb of the β-phaseolin gene (with six exons and five introns) truncated from its 5′ end to its ATG site (Reichert, 1989) behind the 35S promoter (Fig. 1B).

Plant Transformation and Regeneration

Plasmid pMEZ and pGEL2 were mobilized from *Escherichia coli* DH5a into the *Agrobacterium tumefaciens* receptor strain pTiT37ASE by triparental mating as described by Rogers et al. (1987). *Nicotiana tabacum* cv Xanthi plants were transformed using the leaf disc transformation procedure (Horsch et al., 1985). Transformants were selected and regenerated on Murashige and Skoog medium containing 100 mg of kanamycin/L. Shoots appeared 4 to 6 weeks after inoculation. These shoots were rooted on the same medium without the hormones and then transferred to the soil.

Western Analysis

Different tissues were extracted in PBS and centrifuged, and the supernatant was used for protein determination and analysis of β-phaseolin and the tobacco seed storage proteins. The pellet from the centrifugation was incubated in 70% EtOH, 1% mercaptoethanol at 65°C for 30 min to extract the 15-kD zein protein. For western analysis, a known amount of the PBS-soluble fraction or EtOH-extractable fraction from a known amount of protein extract (μg) was subjected to an SDS-PAGE system according to the method of Laemmli (1970) using 14% slab gels. Fractionated proteins were then blotted onto nitrocellulose electrophoretically in 25 mM Tris, 192 mM Gly, 5% methanol (pH 8.2). The nitrocellulose was blocked overnight with 1% BSA in Tris-buffered saline containing 0.05% Tween 20 and was then incubated in the same solution with the appropriate antibody. The anti-15-kD zein antibody was kindly provided by Dr. Brian Larkins (Lending et al., 1988). The protein bands reacting to the antibodies were made

visible using an alkaline phosphatase-linked second antibody and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate according to the manufacturer's instructions (Promega).

Isolation of RNA and Its Analysis

Total RNA was isolated from tobacco tissues and corn seeds using the lithium chloride precipitation method (de Vries, 1982). The RNA was fractionated on 1% agarose-formaldehyde gels, transferred to nitrocellulose filters, and probed with ^{32}P-labeled fragments of the β-phaseolin or 15-kD zein gene as indicated in the figure legends. Hybridization was carried out in 50% formamide at 42°C using standard conditions (Maniatis et al., 1982). The filters were washed three times with 2× SSC, 0.1% SDS at 42°C for 15 min, followed by two washes with 0.1× SSC, 0.1% SDS at 42°C for 20 min, and exposed to x-ray film.

Poly(A)$^+$ RNA was isolated by subjecting the total RNA to poly(U)-Sephadex chromatography (Murray et al., 1981). The RNA was translated in the wheat germ system (Promega) using [^{35}S]Met (NEN) as the tracer amino acid. To isolate the 15-kD zein precursor from the rest of the translation products, the total translation products were made 70% with respect to EtOH and centrifuged, and the supernatant was dried for further analysis on SDS-PAGE. The fractionated translation products were made visible by fluorography and autoradiography (Laskey and Mills, 1975).

EM

Small pieces of leaf and seed were fixed in 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer for 2 h and then postfixed in 1% aqueous osmium tetroxide for 1 h. The samples were dehydrated in EtOH and embedded in Spurr's resin at 70°C. Silver sections on copper grids were then stained in uranyl acetate and Reynold's lead citrate. The grids were then examined in a Hitachi H7000 transmission electron microscope.

Immunoelectron Microscopy

Small pieces of leaf and seed were fixed for 2 h on ice in 4% paraformaldehyde and 0.6% glutaraldehyde in 0.033 M sodium/potassium phosphate buffer (pH 7.3) containing 0.1 M Suc. The tissue was washed in three changes of buffer containing 7% Suc and kept in the last change overnight at 4°C. The tissue was then dehydrated in EtOH and embedded in Spurr's resin at 55°C. The following steps were done at room temperature. Silver sections on nickel grids were first incubated in a blocking solution of 10 mM Tris-saline containing 1% BSA (Sigma), 0.05% Tween 20 (Sigma), and 15 mM NaCl. This buffer mixture was used in all of the remaining steps. The grids were then drained and incubated with rabbit anti-zein/anti-phaseolin/anti-BiP70 antibody diluted 1:100 to 1:500 in buffer for 45 min to 4 h. Controls were incubated in nonimmune rabbit IgG (Sigma). They were then washed in the buffer and placed in 5 nm of gold-labeled, goat anti-rabbit IgG (Sigma) diluted 1:50 in buffer for 45 min. The grids were washed in the Tris-saline containing Tween 20 and NaCl, followed by double-distilled water. The grids were then examined either unstained or lightly poststained in uranyl acetate and lead citrate.

RESULTS

Analysis of 15-kD Zein Transcripts in Transgenic Tobacco

RNA from leaves and seeds (at mid-maturation) from tobacco plants transformed with pMEZ (Fig. 1A) were subjected to northern analyses along with RNA from corn kernels (30 DAP), using the 15-kD zein-coding sequence as a probe. The blot was also probed with a rRNA gene probe to check for RNA loads. The hybridization patterns (Fig. 2A) were identical for leaves and seeds, both qualitatively and quantitatively. In both cases, two hybridizing bands of equal intensity were observed, one band comigrating with the 1-kb transcript in corn and the other band migrating as a 1.2-kb species. (The faster migrating hybridizing band on the corn RNA lane is due to nonspecific hybridization with the rRNA gene probe.) Since pMEZ contains the transcrip-

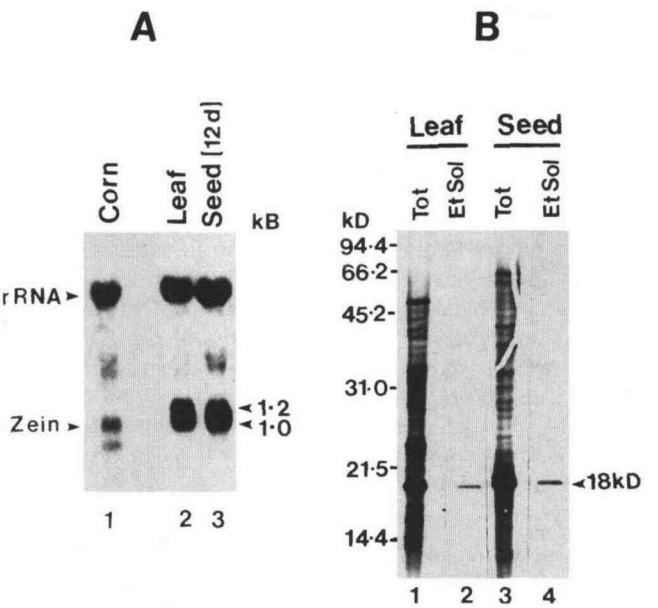

Figure 2. Analysis of the 15-kD zein transcripts and the primary translation products in seeds and leaves of transgenic tobacco. A, Fifteen micrograms of total leaf and developing seed (12 DAP) RNA from a tobacco plant transformed with pMEZ (lanes 2 and 3) and 2.5 μg of total corn endosperm RNA (lane 1) were subjected to northern analysis using a 690-bp *Bgl*II/*Bam*HI fragment of the 15-kD zein gene as a probe. The blot was also hybridized with a 28S rRNA gene probe as an indicator of RNA loads. The sizes of the hybridizing bands were calculated based on the position of RNA standards of known molecular mass included in the gel. B, The mRNA samples from leaf and developing seed (11 DAP) were translated in vitro using the wheat germ system. An aliquot of the translation products was made 70% with respect to EtOH and centrifuged, and the supernatant was dried. The EtOH-soluble fraction (Et Sol; lanes 2 and 4) and total translation products (Tot; lanes 1 and 3) were analyzed by SDS-PAGE, followed by autoradiography. The molecular mass positions indicated are based on the positions of standard molecular mass markers.

tion termination sites for both the 15-kD zein gene and the NOS gene, the two different-sized transcripts might be a result of the utilization of both termination sites by the transcription machinery.

Based on the levels of hybridization and the amount of total RNA loaded, it appears that the transcript level in the transgenic tissue is 2- to 5-fold higher than in corn seeds. (The corn seed lane [lane 1] was loaded with 2.5 μg of total seed RNA, whereas the tobacco seed and leaf lanes [lanes 2 and 3] were loaded with 15 μg of the total RNA.) This difference in transcript level between corn seeds and the tissues from the transformant could be a reflection of differences in promoter strength, number of functional genes integrated into the genome, or transcript stability.

For further analysis, RNA from leaf and seed tissues was translated in vitro in the wheat germ system, and the primary translation product for the 15-kD zein was isolated from the total translation products as a 70% EtOH-soluble fraction. SDS-PAGE analysis of the total translation products and the EtOH-soluble fraction showed that, although major differences were detected in the total translation products between leaf and seed, in both cases an 18-kD protein was found in the EtOH-soluble fraction (Fig. 2B). The control sample with no RNA did not show the 18-kD band (data not shown). The 15-kD zein protein with its signal peptide migrated as an 18-kD band on SDS-PAGE (Larkins and Hurkman, 1978), thus suggesting that the 18-kD band in our experiment was indeed the primary translation product for the 15-kD zein in leaves and seeds of transgenic plants. These results suggest that the primary translation products from the introduced gene in both leaves and seeds are probably identical. Moreover, these results also suggest that the two 15-kD zein transcripts in transgenic tobacco contain the same protein-coding sequence but probably different lengths of the 3′ untranslated regions. Taken together these results suggest that the 15-kD zein gene, when driven by the 35S promoter, is transcribed efficiently in seeds and vegetative tissues of transgenic tobacco and the transcript level in these transgenic plants is higher than in corn seeds.

Steady-State Accumulation Pattern of the 15-kD Zein and β-Phaseolin Protein in Different Organs of Transgenic Tobacco

To determine the fate of the 15-kD zein and β-phaseolin protein in seeds and nonseed tissues, we analyzed different organs of transgenic tobacco containing pMEZ or pGEL20, for the steady-state levels of the 15-kD zein and β-phaseolin proteins. In all organs tested, levels of the 15-kD zein protein were higher than those in mature seeds (Fig. 3A). However, we cannot comment on the levels in absolute terms, since the immunoreactive band was diffused in all nonseed tissues and in immature seeds. By mixing leaf extracts from nontransformed plants with mature seed extract from transformed plants (Fig. 3A, lane 5), we found that the diffused band of the 15-kD zein protein in leaves was due to the presence of some compound in the EtOH-soluble fraction that retards the migration of the 15-kD zein protein in SDS-PAGE. Analysis of the different tissues from

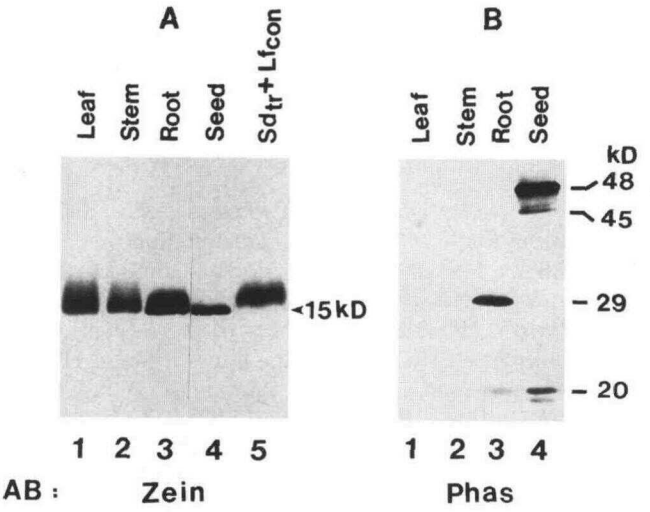

Figure 3. Steady-state accumulation pattern of the 15-kD zein protein and β-phaseolin in different organs of transgenic tobacco. A, Seventy percent EtOH-soluble protein (equivalent to 50 μg of the PBS-soluble fraction) from different organs of a pMEZ-transformed tobacco plant was subjected to SDS-PAGE, transferred to nitrocellulose, and followed by immunoblot analysis using the 15-kD zein antibody. Lanes 1 to 4, Samples from different organs (as indicated); lane 5, a sample of the 70% EtoH-soluble fraction of transformed mature seed (Sd_{tr}) was combined with an equivalent sample from untransformed leaf (Lf_{con}) before electrophoresis. AB, Antibody used. B, Fifty micrograms of the PBS-soluble fraction from different organs of a pGEL20-transformed tobacco plant were subjected to SDS-PAGE, followed by immunoblot analysis using the β-phaseolin antibody. Lanes 1 to 4, Samples from different organs as indicated. The molecular masses pertinent to each panel are indicated.

a pGEL20 transformed plant showed that the authentic-size β-phaseolin proteins (the 48-kD glycosylated form and the 45-kD nonglycosylated form) accumulated only in mature seeds (Fig. 3B). As reported earlier (Sengupta-Gopalan et al., 1985), in addition to the authentic-size β-phaseolin protein, the seeds also showed the accumulation of small molecular mass immunoreactive products, probably representing specific cleavage products. Whereas the leaf and stem showed very low levels of small molecular mass immunoreactive bands, the roots showed accumulation of a major immunoreactive band of 29 kD (Fig. 3B). Tissues from nontransformed plants, when tested with both antibodies, did not show any significant immunoreactive bands (data not shown). Taken together, these results suggest that the β-phaseolin protein is unstable in nonseed tissues, whereas the 15-kD zein is stable in all tissues tested.

Analysis of Developing Seeds for the Accumulation of the 15-kD Zein and β-Phaseolin Transcript and Protein

We extended our studies of protein stability to developing seeds. Seed protein genes are known to be transcriptionally regulated during seed development, being induced sometime during mid-maturation (Sengupta-Gopalan et al., 1985). However, since induction in transcription is followed by the synthesis and accumulation of the seed protein in protein bodies, it is difficult to study

seed protein accumulation pattern independently of transcriptional regulation in the host plant.

One way to resolve this would be to study the pattern of accumulation of the seed protein in seeds in which the corresponding gene is being driven by a constitutive promoter. The patterns of accumulation of the 15-kD zein and the β-phaseolin transcript and protein were determined by analyzing seeds at different stages of development following crossing between pMEZ-transformed and pGEL20-transformed (Fig. 1B) tobacco plants. As expected of a constitutively driven gene, the levels of the 15-kD zein gene transcript and the β-phaseolin gene transcript were identical in seeds at all developmental stages (Fig. 4). The difference in the hybridization signal between the β-phaseolin and the 15-kD zein transcripts does not reflect the relative levels of the two transcripts, since the two probes used in the hybridization did not have the same specific activity. Western analysis of proteins from the same seeds at different developmental stages, however, showed that, whereas the 15-kD zein protein accumulated to high levels as early as 4 DAP, the β-phaseolin protein did not start accumulating until after 12 DAP (Fig. 5, B and C). Coomassie blue staining of SDS-PAGE of seed proteins (Fig. 5A) showed that the most abundant proteins, which probably represent tobacco seed storage proteins (marked by asterisks), did not start to accumulate until after 14 DAP. The 15-kD zein protein band and the authentic-size β-phaseolin protein appear to be at their maximum levels between 14 and 18 DAP. The cleavage products of β-phaseolin start appearing at 14 DAP and reach steady-state levels by 18 DAP coincident with the steady-state accumulation of the tobacco seed storage proteins. The results presented imply that the β-phaseolin protein that is made during the early stages of seed development is not stable and does not accumulate, whereas the 15-kD zein protein is stable at all stages of seed development.

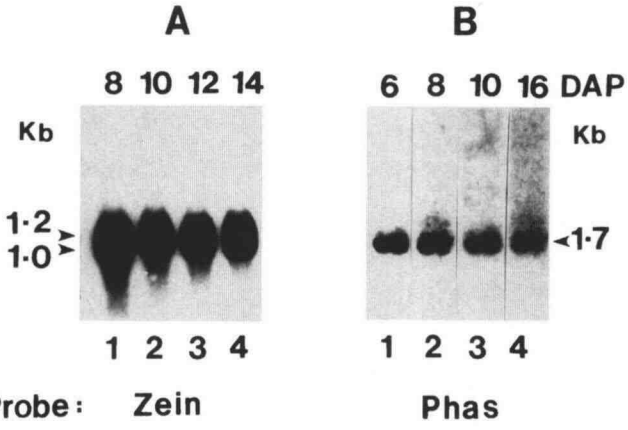

Figure 4. Analysis of developing transgenic seeds for steady-state levels of the 15-kD zein and β-phaseolin (Phas) transcripts. Fifteen micrograms of total RNA isolated from developing seeds at different times following pollination (DAP as indicated) were subjected to northern analyses using the *Bgl*II-*Bam*HI 690-bp fragment of the 15-kD zein cDNA clone (pMEZ; A) or 3.0-kb *Eco*RI-*Bam*HI genomic fragment of β-phaseolin gene (pGEL2; B) as probes. The sizes of the hybridizing bands were calculated based on the position of RNA standards of known sizes included in the gel.

Localization of the 15-kD Zein Protein in Leaf Cells of Transgenic Tobacco

To understand the basis for the stability of the 15-kD zein protein in the leaves of transgenic tobacco, we examined the location of the 15-kD zein protein in the leaves of transformed tobacco plants by immunocytochemistry combined with EM. The 15-kD zein protein appears to be localized in unique rosette-shaped protein bodies in leaf cells, and the localization appears to be uniform throughout (Fig. 6A). Examination of several leaf sections of control and transformed plants showed that these organelles were found only in pMEZ-transformed plants (Fig. 7, A, C, and D) and were not present in untransformed plants (Fig. 7B) or plants transformed with the β-phaseolin gene (pGEL20) (data not shown). Furthermore, these protein bodies were found in all cell types in the leaf, including the mesophyll and vascular cells. These protein bodies consisted of a central compartment with small compartments radiating out as though the latter were budding out of the main compartment (Fig. 7, A and C). The protein bodies were lined by a dark staining matrix and had a central light staining chamber very similar to the protein bodies in corn endosperm (Lending et al., 1988). In some sections, the whole cluster of protein bodies appeared to be lined by the RER (Fig. 7A, arrows), suggesting that they are situated in the lumen of the ER. However, because of the large size of the aggregates, the ER membranes sometimes appeared to be distorted.

Attempts were made to immunostain leaf sections of pMEZ transformants with a corn BiP70 antibody (Zhang and Boston, 1992) to confirm the ER origin of these specialized protein bodies. BiP70 is an ER-resident chaperone (Fontes et. al., 1991). In addition to staining the ER membrane, the BiP70 antibody showed nonspecific binding all over the cell (data not shown). Since β-phaseolin did not show significant levels of accumulation in nonseed tissue, no attempts were made to localize it at a subcellular level.

Localization of the 15-kD Zein Protein in Developing and Mature Seeds of Transgenic Tobacco

In an earlier paper, Hoffman et al. (1987) reported that, in transgenic tobacco in which the 15-kD zein gene is driven by the seed-specific β-phaseolin gene promoter, the zein protein was found to accumulate in vacuolar protein bodies in mature seeds. To determine whether the same pattern would hold in our system with the 15-kD zein gene being driven by the constitutive 35S promoter, both immature (Fig. 6B and Fig. 8) and mature seeds (Fig. 9) were analyzed by immunocytochemistry and EM. The same type of protein bodies as seen in the leaves (Fig. 6A) were seen in both immature (Fig. 6B and Fig. 8) and mature seeds (Fig. 9), and the zein was localized in these protein bodies. These protein inclusions in the seeds, as in the leaves, were surrounded by the ER membrane (Fig. 6B and Fig. 8),

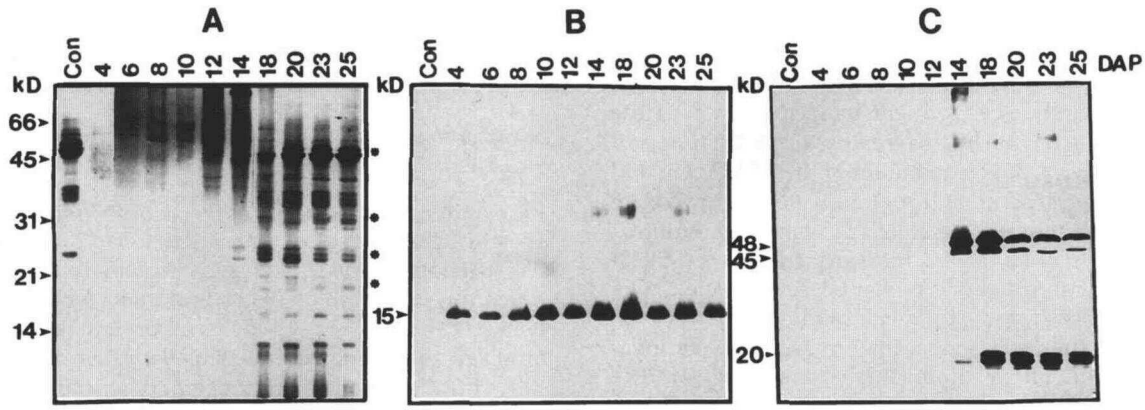

Figure 5. Analysis of developing transgenic seeds for the accumulation of tobacco seed storage protein, zein, and β-phaseolin. Transgenic tobacco containing the β-phaseolin gene construct (pGEL20) were pollinated with pollen from transgenic tobacco containing the 15-kD zein gene construct (pMEZ), and seeds at different times following pollination (as indicated) were harvested. Fifty micrograms of PBS-soluble protein extract (A and C) or 70% EtoH-soluble protein equivalent to 50 μg of PBS-soluble extract (B) were subjected to SDS-PAGE and stained with Coomassie blue (A), subjected to immunoblot analysis using the 15-kD zein antibody (B), or the phaseolin antibody (C). The lane labeled Con contains protein from mature seeds from untransformed tobacco. The molecular masses pertinent to each panel are indicated on the left. The asterisks mark the positions of the major seed storage proteins of tobacco.

suggesting their derivation from the ER. The vacuoles in younger seeds showed no trace of zein (Fig. 8A). Even in mature seeds, the 15-kD zein protein was not localized in the well-formed vacuolar protein bodies (Fig. 9A). In very rare cases, the ER-derived protein bodies containing the 15-kD zein appeared to be engulfed in the matrix of the vacuolar protein bodies (Fig. 9B).

DISCUSSION

The data presented in this paper clearly demonstrate that the constitutive expression of the 15-kD zein gene in transgenic tobacco results in the accumulation of the protein in all tissues analyzed. Furthermore, our data also show that the 15-kD zein protein accumulates in novel protein bodies

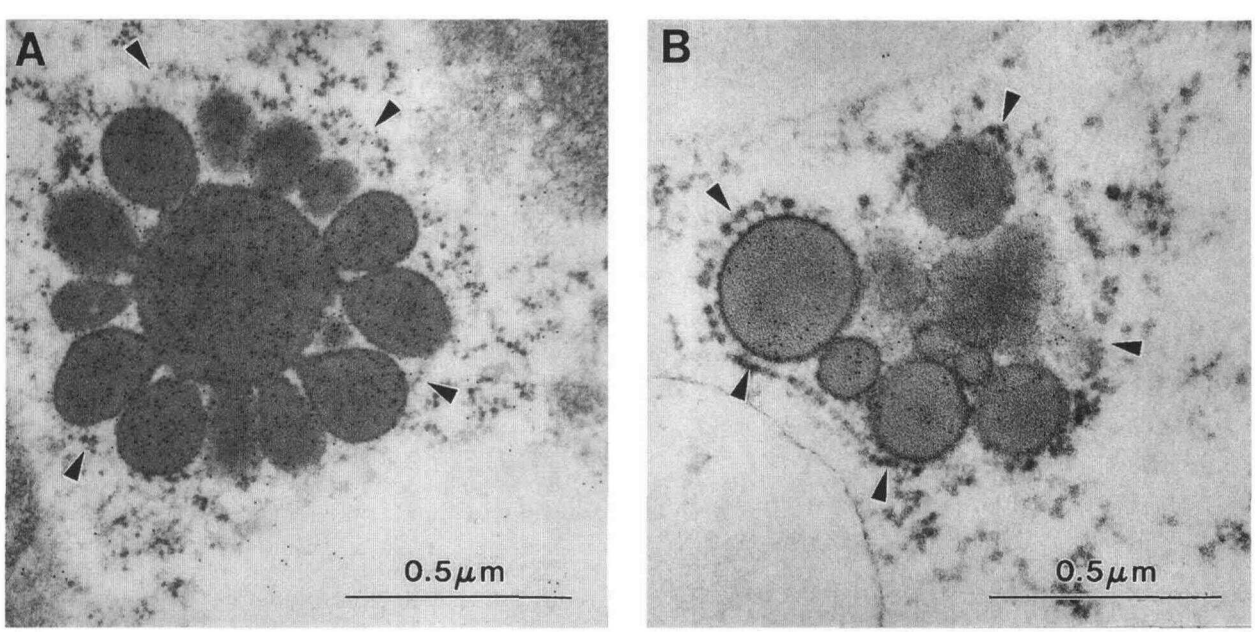

Figure 6. Subcellular localization of the 15-kD zein protein in leaves and young seeds of pMEZ-transformed tobacco plants. The tissue was fixed in 4% paraformaldehyde and 0.65% glutaraldehyde and embedded in Spurr's resin. Silver sections on nickel grids were incubated in the rabbit anti-zein antibody followed by incubation in gold-labeled, goat anti-rabbit IgG. The grids were washed and lightly stained in uranyl acetate and lead citrate. A, Leaf section labeled with anti-zein antibody (1:100) for 70 min. Magnification ×60,000. B, Section of 12-d-old seed labeled with anti-zein antibody (1:200) for 45 min. Magnification ×60,000. The arrowheads point to the ER.

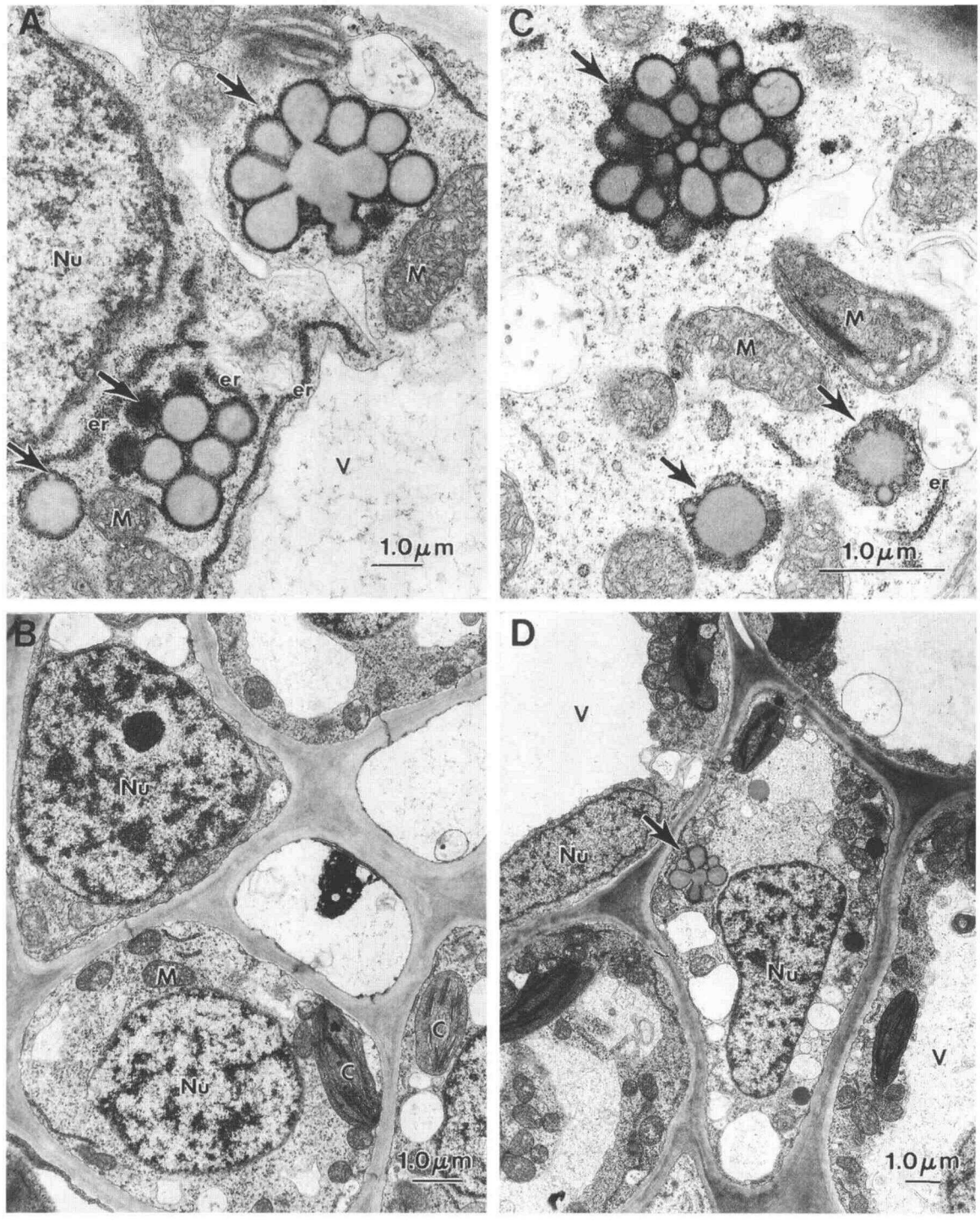

Figure 7. Analysis of the leaf ultrastructure of the 35S zein gene transformants. Leaf sections from transformants containing the 35S zein construct (A, C, and D) and from the control (B) were subjected to routine fixation and embedded in Spurr's resin. Silver sections on copper grids were then stained in uranyl acetate and lead citrate. A and C are close-ups of the novel protein bodies found in the transformants but not in the control. The arrows point to the protein bodies. The different cellular components are labeled: Nu, nucleus; M, mitochondria; C, chloroplast; er, ER; V, vacuole.

that appear to occur within the lumen of the ER. The presence of ribosomes ringing the 15-kD zein-containing protein bodies is strongly suggestive of their derivation from the ER. Moreover, these protein bodies resemble those found in corn endosperm (Lending et al., 1988). The protein bodies in both corn endosperm and the pMEZ-transformed plants appear to be well defined, spherical bodies with a light staining central region and dark stain-

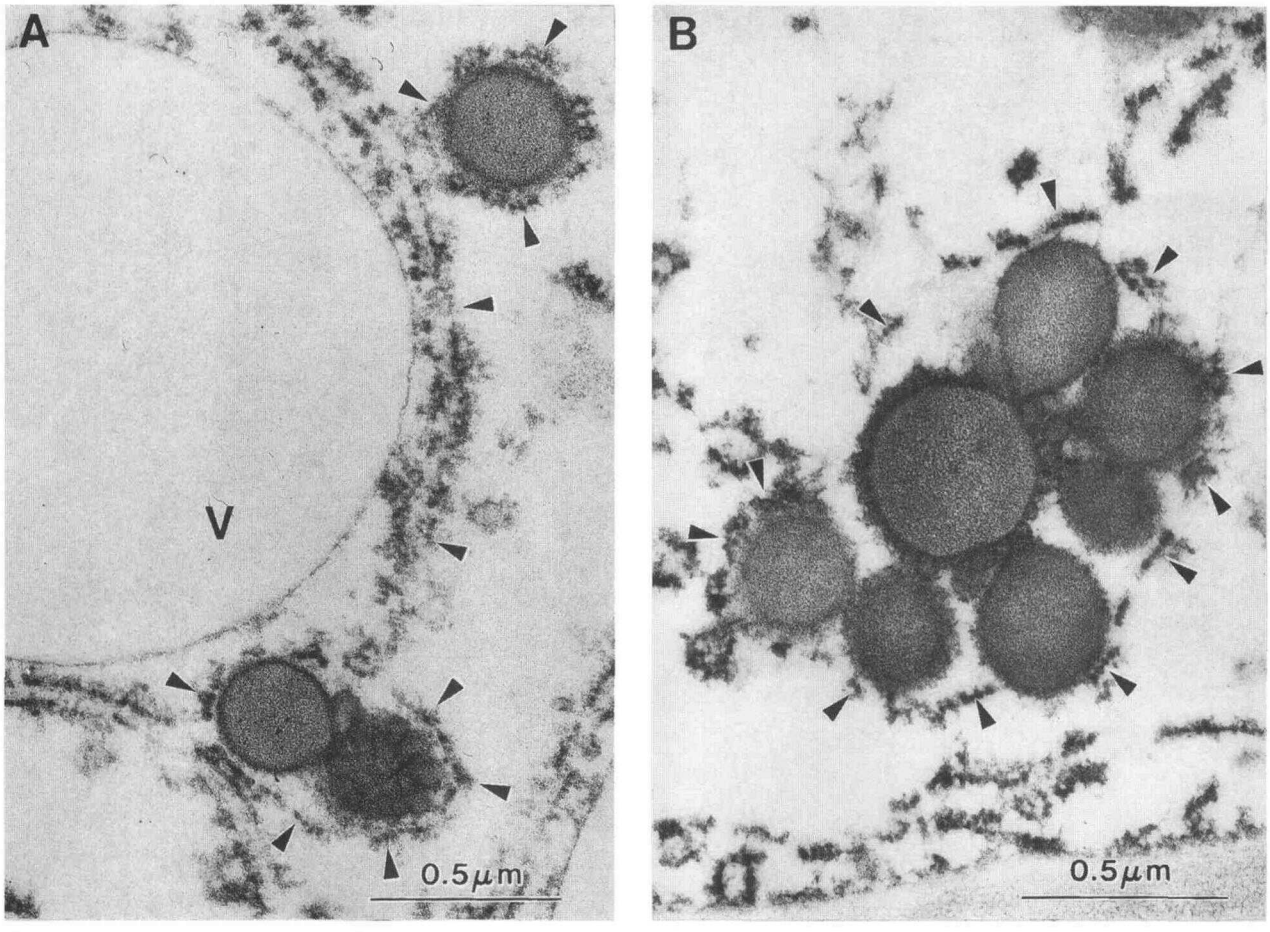

Figure 8. Zein protein biogenesis in young seeds of pMEZ transformed tobacco. Seed tissue fixed in glutaraldehyde and stained with lead citrate and uranyl acetate. A, RER (indicated by arrowheads) is shown intimately associated with the protein bodies (×50,000). The section has also been stained with anti-zein antibody (1:200) for 45 min. The vacuole is indicated by V. B, A section demonstrating the sequential nature of protein body formation (×54,000). The arrowheads point to the ER.

ing periphery. The protein bodies in the transgenic tobacco, however, are more complex and more branched, although it is not clear whether the lateral branches are due to a budding process or a result of fusion. Protein body biogenesis in corn endosperm is not well understood. However, analysis of the accumulation pattern of the different zeins in the protein bodies in corn endosperm has led to the theory that the synthesis of these protein bodies is a result of interaction among the different zeins (Lending and Larkins, 1989). The formation of distinct protein bodies with just the 15-kD zein in nonseed tissues is consistent with the "self-assembly" hypothesis and further suggests that intramolecular interaction is sufficient for the biogenesis of these ER-derived protein bodies. The retention of the 15-kD zein in the ER, even though it does not contain the well-defined ER retention signal (Pederson et al., 1986), would suggest that there is an alternate mechanism by which the protein is prevented from exiting the ER. There is a suggestion that the zein proteins are associated with an ER-resident protein, BiP70 (a cognate of the 70-kD heat-shock protein) in corn endosperm (Boston et al., 1991). This association may be the mechanism by which the zeins are retained in the ER of corn endosperm.

Such a mechanism might be applicable to the situation with the 15-kD zein in seed and nonseed tissues of transgenic tobacco. Of course, there is still the possibility that the signal peptide on the zein proteins is the sole determinant for directing the protein into the lumen of "protein body ER." In a recent report, Li et al. (1993a) showed that rice endosperm cells display two distinct ER: cisternal ER and protein body ER. Whereas the former is involved in the secretory pathway, the latter delimits the ER-derived protein bodies. The signal peptide on the protein determines to which kind of ER the protein should be targeted (Li et al., 1993a). Whatever the mechanism for the retention of zein in the ER in transgenic tobacco, our data clearly establish that the signals involved in the insertion and retention of the 15-kD zein in the ER are shared by both monocots and dicots. There is still the possibility that the zein protein has no specific signal for retention in the ER and that the zein

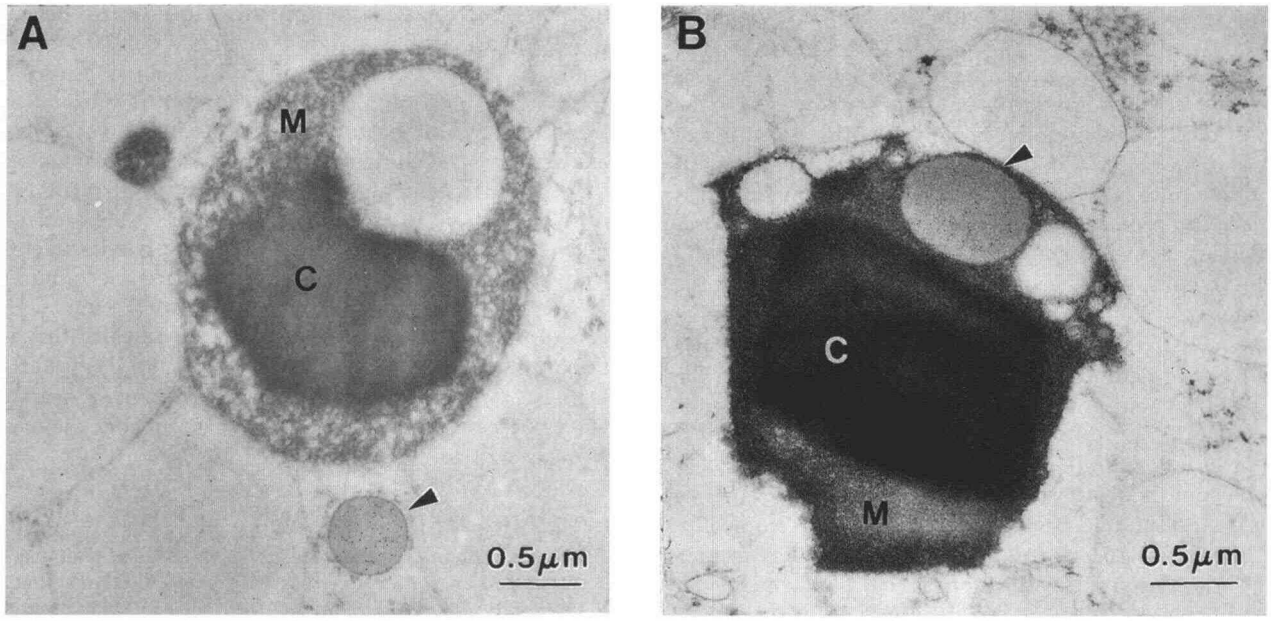

Figure 9. Immunostaining of mature seed sections (pMEZ to transformant) with anti-zein antibody. Tissue sections were prepared as described for Figure 5 (anti-zein antibody was used at a dilution of 1:200). A, The section reveals a mature vacuole-derived protein body next to a zein protein body (marked by arrow) (×22,000). B, The section reveals a mature vacuole-derived body containing a zein protein body (marked by arrow) in its matrix (×24,000). The crystalline region is marked "C" and the amorphous matrix is marked "M."

protein body formation is just a consequence of the high-ionic-strength environment of the ER, inducing aggregation of the very hydrophobic zein protein.

It is interesting to note that Hoffman et al. (1987) did not report the presence of the specialized ER-derived protein bodies in seeds of transgenic tobacco containing the 15-kD zein gene behind the β-phaseolin gene promoter. The 15-kD zein protein in their system appeared to be localized in the crystalloid component of the storage vacuole. In transgenic tobacco with the 35S promoter driving the 15-kD zein gene, the protein was found only in the novel protein bodies and not in the vacuoles. Even in mature seeds with well-defined vacuolar protein bodies, the zein was found only in the specialized ER-derived protein bodies and not in the vacuolar protein bodies. In very rare cases, the 15-kD zein-containing protein bodies was found embedded in the matrix of the vacuolar protein bodies (Fig. 9B). The mechanism by which these protein bodies from the ER are lodged into the vacuolar protein bodies is not clear, but it could occur in a manner similar to autophagy of wheat prolamines (Levanony et al., 1992).

Can the discrepancy between our results and those of Hoffman et al. (1987) be attributed to differences in the nature of the promoter driving the 15-kD zein gene in transgenic tobacco? Although the two promoters do differ in their temporal and spatial patterns of expression, it is difficult to envision how that could determine the final destination of the protein. One possible explanation for this discrepancy could simply be that there are differences in the timing of expression of the two promoters. It is likely that the 35S promoter quits functioning in seeds by mid-maturation and there is no new synthesis of the 15-kD zein in older seeds. In support of this theory is the report that the 19- and 23-kD zeins were not detected in the developing embryos of transgenic tobacco seeds when the genes were driven by the 35S promoter (Schernthaner, 1988). This might also account for the relatively lower level of the 15-kD zein in the mature seeds of pMEZ-transformed plants (Figs. 3 and 5). The β-phaseolin promoter, on the other hand, is induced at mid-maturation along with the tobacco seed storage protein genes. The slow accumulation of the 15-kD zein in the latter case probably prevents its aggregation in the ER and allows it to exit the ER. Its accumulation in the vacuole-derived protein bodies, however, would imply that the 15-kD zein has a vacuolar targeting signal. It is also possible that the 15-kD zein synthesized in mid-maturing seeds is carried to the vacuole-derived protein bodies by the tobacco seed storage proteins. The most definitive way, however, to resolve this issue of differential targeting between the two systems would be to do parallel analyses of the two kinds of transformants.

The stability of the 15-kD zein protein in the vegetative tissues of transgenic tobacco would imply that the protein is not accessible to proteases. In the same context, the instability of the β-phaseolin protein in vegetative tissues of tobacco (Fig. 3B) and alfalfa (Bagga et al., 1992) could probably be ascribed to the fact that it is exposed to proteases in all tissues except mature seeds. The differences in behavior between the two proteins with regards to protein degradation could be attributed to the fact that the 15-kD zein is retained in the ER, whereas the β-phaseolin protein

is not retained and is shunted into the vacuoles. Vacuoles are known to have very powerful proteases (Boller and Kende, 1979), and proteins targeted to vacuoles are exposed to a highly degradative environment.

In support of this hypothesis is the fact that retention of the vacuolar pea vicilin protein in the ER when an ER retention signal is engineered on to the protein stabilizes the modified protein 100-fold over unmodified vicilin protein (Wandelt et al., 1992). The stability of the β-phaseolin protein in mature seeds is probably due to the fact that the protein is now shunted to differentiated protein bodies that do not have active proteases. The pattern of β-phaseolin protein accumulation in developing seeds transcribing the β-phaseolin gene in a constitutive manner attests to this proposal. Although translatable mRNA for β-phaseolin is present in seeds at all stages of development, the protein starts accumulating at a stage just preceding the onset of tobacco seed storage protein accumulation. It would appear, then, that the maturation of vacuoles into protein bodies in seeds at mid-maturation is what is responsible for the protection of β-phaseolin protein in mature seeds. The accumulation of a 29-kD immunoreactive protein in roots of transgenic tobacco suggests that the 29-kD protein is the first proteolytic product in vacuoles that is broken down further.

In our discussion of the stability/instability of the protein in transgenic plants, we cannot, however, ignore the fact that under physiological conditions the zein protein is much less soluble than phaseolin and aggregation of the insoluble zein may make it less available for proteolytic degradation. We also cannot rule out the possibility that proteases specific for native zeins are not present in dicotyledonous plants. The 15-kD zein remains intact even during germination of the transgenic tobacco seeds (our unpublished data), supporting the possibility that zein-specific proteases are absent even in germinating tobacco seeds. It is interesting to note that the 19- and 23-kD zein genes, when driven by the 35S promoter, also showed significant accumulation of the proteins in an undegraded form in all tissues except the embryos of transgenic tobacco (Schernthaner, 1988). The 19-kD zein gene, however, when driven by the β-phaseolin gene promoter, showed high levels of the transcript but no accumulation of the corresponding protein in the seeds (Ohtani et al., 1990). It appears that there is no consistent pattern for the accumulation of the different zeins in transgenic dicotyledonous plants.

Based on the expression studies of the 19-kD zein gene in transgenic petunia, Ueng et al. (1988) concluded that the regulatory sequences responsible for the developmental regulation of the gene in corn are not recognized in the dicot system. It has also been suggested that the introns from monocot genes are not spliced correctly in dicot plants (Keith and Chua, 1986). Our results, however, show that the 3′ termination sequence of the 15-kD zein gene is recognized as efficiently as the NOS gene terminator in tobacco.

Although no systematic analysis has been made in this study to quantitate the amount of 15-kD zein accumulating in transgenic tobacco, an approximate estimate based on the Coomassie blue staining of SDS-PAGE fractionated total soluble proteins and the 15-kD zein in leaves and seeds (data not shown) suggests that the 15-kD zein forms from 1 to 2% of the total soluble protein. Based on the analysis of R_1 seedlings (kanamycin resistant versus sensitive), we also estimate that most of the transgenic plants used in our study had one copy of the integrated gene. Furthermore, plants obtained by crossing two independent transformants showed that the amount of accumulated 15-kD zein protein is twice that in individual parents. Thus, the approach of introducing multiple copies of the functional 15-kD zein genes into a plant could significantly increase the amount of 15-kD zein protein in seeds and leaves of transgenic dicot plants and thus alter the overall amino acid composition.

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