Planta (2003) 217: 66–74
DOI 10.1007/s00425-002-0971-6

ORIGINAL ARTICLE

Won Seok Kim · Hari B. Krishnan

Allelic variation and differential expression of methionine-rich δ-zeins in maize inbred lines B73 and W23a1

Received: 5 September 2002 / Accepted: 5 December 2002 / Published online: 20 February 2003

Abstract The sulfur-amino-acid-rich δ-zeins of maize (*Zea mays* L.) are represented by 18-kDa and 10-kDa proteins. We have cloned a novel 11-kDa methionine-rich δ-zein from developing endosperm of the inbred line W23a1. The nucleotide sequence of this new δ-zein is identical to the published 10-kDa δ-zein, except for an insertion of 18 nucleotides between +316 and +333 bp from the translation start site. Antibodies raised against the recombinant 18-kDa δ-zein recognized both the 18-kDa and 10-kDa δ-zein from total seed protein extracts of different maize inbred lines. Western blot analysis revealed differences in the levels of the δ-zeins in different inbred lines and some of the inbred lines lacked either the 10-kDa or the 18-kDa δ-zeins. Northern blot analysis revealed temporal differences in the RNA transcript levels of the 11-kDa and 18-kDa δ-zeins between B73 and W23a1. Such differences were not evident on Western blot analysis where similar protein accumulation profiles were seen for both lines. Immunostaining of paraffin sections of developing maize endosperm with the 18-kDa δ-zein antibodies revealed specific labeling of protein bodies found in the first few starchy layers from the aleurone layer. Electron-microscopic observation of thin-sections of B73 and W23a1 endosperm cells confirmed the presence of recently discovered novel, vacuole-like structures in these inbred lines. Immunogold labeling studies revealed that the δ-zeins were localized in the endoplasmic-reticulum-derived protein bodies and showed no preferential gold particle labeling over either the light or electron-dense material found in these protein bodies.

Keywords Amino acid · Methionine-rich protein · Protein body · *Zea* · Zein

Abbreviations *DAP*: days after pollination · *IPTG*: isopropyl β-D-thiogalactopyranoside

H. B. Krishnan (✉)
Plant Genetics Research Unit,
USDA–ARS, 108 W Curtis Hall,
University of Missouri,
Columbia, MO 65211, USA
E-mail: krishnanh@missouri.edu
Fax: +1-573-8847850

W. S. Kim · H. B. Krishnan
Department of Agronomy,
University of Missouri,
Columbia, MO 65211, USA

Introduction

Seed storage proteins have been extensively studied because of their abundance and importance to human and animal nutrition (Shewry et al. 1995; Krishnan and Coe 2001). With the exception of a few cereals, such as rice (*Oryza sativa* L.) and oat (*Avena sativa* L.), the predominant storage proteins of cereals are the alcohol-soluble prolamins (Shewry and Tatham 1999). These proteins are rich in glutamine, leucine, alanine, and proline. Generally, the cereal storage proteins are deficient in lysine (Mertz et al. 1964). The major storage proteins of legumes are the salt-soluble globulins (Nielsen 1996). In contrast to the cereals, the legume globulins are deficient in the sulfur-containing amino acids, methionine and cysteine. As a consequence, considerable effort has been made to improve the overall lysine content of cereals and the sulfur amino acid content of legume seed proteins (Habben and Larkins 1995).

Soybean [*Glycine max* (L.) Merr.] seeds store 40% protein and 20% oil. The high content of seed storage proteins makes soybeans an excellent source of protein for human and animal feed. However, the nutritional quality of soybean proteins is limited by the low sulfur amino acid content in the seed proteins (Nielsen 1996; Krishnan 2000). Since the use of soybean as a protein source has gradually increased in last decade, attempts are being made to improve the overall sulfur amino acid content of soybean seed storage proteins (Nordlee et al. 1996; Muntz et al. 1998).

Maize (*Zea mays* L.) seed storage proteins, zeins, have been grouped into four classes, α- (22-kDa and 19-kDa), *β*- (15-kDa), *γ*- (27-kDa and 16-kDa), and *δ*- (18-kDa and10-kDa) zeins. This grouping is based on sequence homologies and molecular weights of these proteins. The 18-kDa and 10-kDa *δ*-zeins are exceptionally rich in sulfur amino acids (Kirihara et al. 1988a; Chui and Falco 1995; Swarup et al. 1995). The *δ*-zeins contain about 20% methionine. An increase in the levels of the *δ*-zeins in the seed results in improved nutritional quality. For example, maize inbred line BSSS-53, which has a 2-fold increase in the accumulation of the 10-kDa *δ*-zein, shows a 30% increase in the methionine content of the seed (Phillips and McClure 1985). Because of their high methionine content, the *δ*-zeins are ideal candidates for heterologous expression in other plants to improve nutritional quality. We are interested in expressing maize *δ*-zeins in soybeans to improve the sulfur amino acid content of soybean seed. With this objective, we isolated genes encoding maize *δ*-zeins. Here, we report the isolation and characterization of a novel 11-kDa *δ*-zein from the W23a1 maize inbred line.

Materials and methods

Plant material

The B73 and W23al inbred lines of maize (*Zea mays* L.) were grown at the University of Missouri, Bradford Research and Extension Center, near Columbia on Mexico silt loam during the 2001 growing season. Endosperm samples were obtained from ears at different developmental stages and immediately frozen in liquid nitrogen and stored at 80 °C until used.

Reverse transcription–polymerase chain reaction (RT–PCR) and nucleotide sequence analysis

The coding regions corresponding to the 10-kDa and 18-kDa *δ*-zeins were obtained following RT–PCR amplification of total RNA using conserved gene-specific primer pairs. The N- and C-terminal specific primers were 5′-CCAACATATGACCCATATT CCAGGGC-3′and 5′-CCAACGCCGGCGGATCTTACGTCGT GGTTG 3′, which contained an *Nde*I and *Not*I site, respectively. Total RNA (1 μg) was treated with one unit of DNase I (Invitrogen, Grand Island, N.Y., USA) for 15 min at room temperature to remove residual DNA. RT–PCR was carried out according to the procedures recommended by the manufacturer (Qiagen, Valencia, Calif., USA). For sequence analysis, the RT–PCR fragments were cloned into the pGEM-T easy vector (Promega, Madison, Wis., USA) and then sequenced at the University of Missouri DNA Core Facility.

RNA extraction and Northern blot analysis

Total RNAs from B73 and W23a1 endosperms collected at different developmental stages were extracted using Trizol reagent (Invitrogen), following the manufacturer's protocol. RNA concentration was determined by UV spectrophotometry. Equal amounts of total RNA (5 μg) were electrophoresed in a 1.3% agarose–formaldehyde gel, transferred to a Hybond-N+ membrane, and then fixed by UV cross-linking. Radioactive probes were generated by PCR amplification of purified plasmid insert with [^{32}P]dCTP using the Ex Taq kit (PanVera Corporation, Madison, Wis., USA). Prehybridization and hybridization were carried out at 65 °C in 7% SDS, 191 mM Na_2HPO_4, 58 mM NaH_2PO_4, 1% bovine serum albumin (BSA) and 100 μg/ml denatured salmon sperm DNA. Final washes were carried out at 65 °C in 0.5×SSC and 1% SDS. Hybridization signals were visualized by autoradiography with an intensifying screen at –80 °C.

Expression of 18-kDa *δ*-zein in *Escherichia coli*

The coding region of the 18-kDa *δ*-zein was cloned into the *Nde*I and *Not*I sites of expression vector pET 28(a)+ (Calbiochem–Novabiochem, San Diego, Calif., USA). The resulting clone was designated pET28hsp. The predicted fusion protein would contain the full-length mature polypeptide along with an N-terminal fusion of 15 amino acids from the vector, which contains the His tag. Fusion-protein expression and purification followed manufacturer-provided protocols (Qiagen). Affinity-purified His-tag fusion protein was used for production of antibodies in rabbits as described earlier (Krishnan and Okita 1986).

Protein extraction and protein gel blot analysis

B73 and W23a1 endosperms collected at different developmental stages were ground to a fine powder in liquid nitrogen. The tissue powder (200 mg) was extracted with 1 ml of globulin extraction buffer [100 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride]. The slurry was centrifuged for 10 min at 22,150 *g* and the supernatant was discarded. The pellet was extracted with 1 ml of 60% isopropanol containing 5% *β*-mercaptoethanol with constant agitation for 30 min at room temperature. Alcohol-soluble proteins were recovered by centrifugation as above. Zeins from the clear supernatant were precipitated by the addition of three volumes of acetone. The precipitated zeins were recovered by centrifugation for 15 min at 22,150 *g*. The protein pellets were briefly dried under vacuum and resuspended in SDS–sample buffer [2% SDS, 60 mM Tris–HCl (pH 6.8), 5% *β*-mercaptoethanol]. The protein samples were boiled for 5 min at 100 °C, briefly placed on ice, and used for SDS–PAGE and Western blot analysis. Total seed proteins from different maize inbred lines were obtained from the ground seed meal (200 mg) by directly extracting with 1 ml of SDS–sample buffer followed by boiling for 5 min. The samples were centrifuged for 10 min at 22,150 *g* and the supernatants were used for SDS–PAGE analysis. Equal sample volumes (10 μl/lane) were separated on 15% (w/v) acrylamide slab gels (Laemmli 1970). After competition of electrophoresis, the resolved proteins were visualized with Coomassie brilliant blue. Western blot analysis was performed according to Burnett et al. (1981). Nitrocellulose membranes, to which maize proteins were electrophoretically transferred, were incubated with TBS [10 mM Tris–HCl (pH 7.5), 0.5 M NaCl] containing 5% (w/v) non-fat dairy milk for 1 h. Membranes were then incubated with 1:5,000 diluted 18-kDa *δ*-zein polyclonal antibodies in TBS containing 3% (w/v) non-fat dairy milk. Immunoreactive polypeptides were visualized using the horseradish peroxidase color-development procedure provided by the manufacturer (Bio-Rad Laboratories, Richmond, Calif., USA).

Immunocytochemical staining–light microscopy

Developing maize seeds [20 days after pollination (DAP)] were cut into small pieces and immediately fixed in 50% ethyl alcohol, 5% glacial acetic acid and 10% formaldehyde for 24 h at 4 °C. The tissue was dehydrated in a graded ethanol series followed by a graded series of xylene. Following this, the tissue was infiltrated with several changes of paraffin at 60 °C over a 3-day time period and embedded in Paraplast Plus Tissue Embedding Medium (Oxford Labware, St. Louis, Mo., USA). The Paraplast-embedded

tissue was sectioned (5 μm thick) with a microtome and collected on poly-L-lysine-coated slides. Deparaffinized and rehydrated sections were treated with methanol–hydrogen peroxide to inactivate endogenous peroxidase activity. Sections were incubated for 1 h in 5% goat serum, 1% BSA and 0.03% Triton in phosphate buffered saline (PBS). Antibodies raised against the 18 kDa δ-zein were used at 1:1000 dilution in 2% goat serum, 1% BSA, and 0.03% Triton in PBS for 20 min. Following this, sections were treated sequentially with biotinylated linker, streptavidin conjugated to horseradish peroxidase, and substrate–chromogen solution according to the manufacturer's recommendation (DAKO Corporation, Carpinteria, Calif., USA). The sections were counterstained with hematoxylin and examined with bright-field optics.

Immunocytochemical staining–electron microscopy

Protein A–gold immunocytochemical localization was performed essentially as described by Krishnan et al. (1986). The 18-kDa δ-zein antibodies were used at a concentration of 1:1000. Sections were post-stained with 0.5% aqueous uranyl acetate and 0.4% aqueous lead citrate. The sections were examined with a JEOL 1200 EX (Tokyo, Japan) transmission electron microscope at 80 kV.

Results

Identification of sequences that encode an 11-kDa methionine rich δ-zein

Previous studies have shown that the methionine rich δ-zeins are composed of an 18-kDa and a 10-kDa protein. The methionine rich δ-zeins are located on chromosomes 9 and 6 and are encoded by one or two copies in the genome (Kirihara et al. 1988b; Benner et al. 1989; Swarup et al. 1995). The N- and C-terminals of the18-kDa and 10-kDa δ-zeins are highly conserved. The differences in the sizes are due to an insertion of a methionine-rich 53-amino-acid repeat sequence in the middle region of the 18-kDa δ-zein (Woo et al. 2001). To isolate 10-kDa and 18-kDa δ-zein genes at the same time, we designed oligonucleotides from the conserved N- and C-terminal regions (see Materials and methods). By utilizing total RNA isolated from 20-DAP maize endosperm, two RT–PCR products were obtained. Inbred B73 revealed two RT–PCR products of 635 and 453 bp, while the sizes of RT–PCR products from inbred W23a1 were 635 and 471 bp, respectively. All of these PCR products were individually cloned into pGEM-T easy vector and then sequenced. The determined sequences were identical to published 10- and 18-kDa δ-zeins. However, the 10-kDa δ-zein from inbred W23al differs from the published 10-kDa δ-zeins classes by insertion of six amino acids (CRMLLP) near the carboxy terminus of the protein (Fig. 1). This novel protein has 32 methionine residues and 7 cysteine residues, and these 2 sulfur amino acids account for 25% of the total amino acid of the protein. However, the general composition of the encoded polypeptide and the regular distribution of Met-Met-X-Pro motifs found in the central region are similar to 10-kDa δ-zein from other maize inbred lines (Kirihara et al. 1988a).

Expression of methionine rich δ-zein in *E. coli*

To express the methionine rich δ-zein, we cloned the coding region of the 18-kDa δ-zein into the *E. coli* expression vector pET 28(a)+. SDS–PAGE analysis of the total proteins from *E. coli* cells harboring this plasmid grown in the presence of isopropyl β-D-thiogalactopyranoside (IPTG) revealed the accumulation of a prominent 18-kDa protein (Fig. 2). This protein was not detected when *E. coli* cells were grown in the absence of IPTG (Fig. 2). The *E. coli*-expressed protein was purified by chromatography on a nickel–nitrilotriacetic acid (Ni–NTA) metal-affinity column (Qiagen). The purified protein, which appears homogeneous on an SDS–PAGE gel (Fig. 2), was used as an antigen to raise polyclonal antibodies in rabbits.

Analysis of δ-zein RNA levels during development of inbred B73 and W23a1 seed

For comparison of δ-zein gene transcription levels, Northern blot analysis was performed using total endosperm RNA extracted from B73 and W23a1 kernels at 8, 10, 15, 20, 25, and 30 DAP. The expression pattern of 18-kDa δ-zein in inbred W23a1 exhibited a different developmental pattern relative to inbred B73 (Fig. 3). In inbred B73, the 18-kDa δ-zein transcript level gradually increased throughout seed development, while in inbred W23a1, it peaked at 10–15 DAP (Fig. 3). During the early developmental stages, the expression level of the 18-kDa δ-zein in inbred W23a1 was substantially higher than that of inbred B73 (Fig. 3). In addition, the 18-kDa δ-zein transcript level in inbred W23a1 endosperm decreased rapidly after 15 DAP relative to the levels found in inbred B73 endosperm (Fig. 3). Even though the 18-kDa δ-zein transcript level decreased after 25 DAP in inbred B73, the decline was not as sharp as in inbred W23a1. The RNA accumulation patterns for the 10-kDa and 11-kDa δ-zeins in inbreds B73 and W23a1 showed similar trends during the seed development, except at 10 DAP where in inbred W23a1 the 11-kDa δ-zein transcript was present at considerably higher amounts (Fig. 3). In inbreds B73 and W23a1, the abundances of the 10- and the 11-kDa δ-zein transcripts were several-fold greater than that of the 18-kDa δ-zein (Fig. 3).

Accumulation of δ-zein protein during seed development

We isolated zeins from endosperm at different developmental stages from inbred lines B73 and W23a1 and analyzed them by SDS–PAGE (Fig. 4). The accumulation of the major zeins was seen around 10 DAP and showed a gradual increase throughout seed development (Fig. 4). The antibodies raised against the recombinant 18-kDa δ-zein were used to detect the accumulation of δ-zeins during seed development. Western blot analysis revealed that the antibodies, in addition to reacting

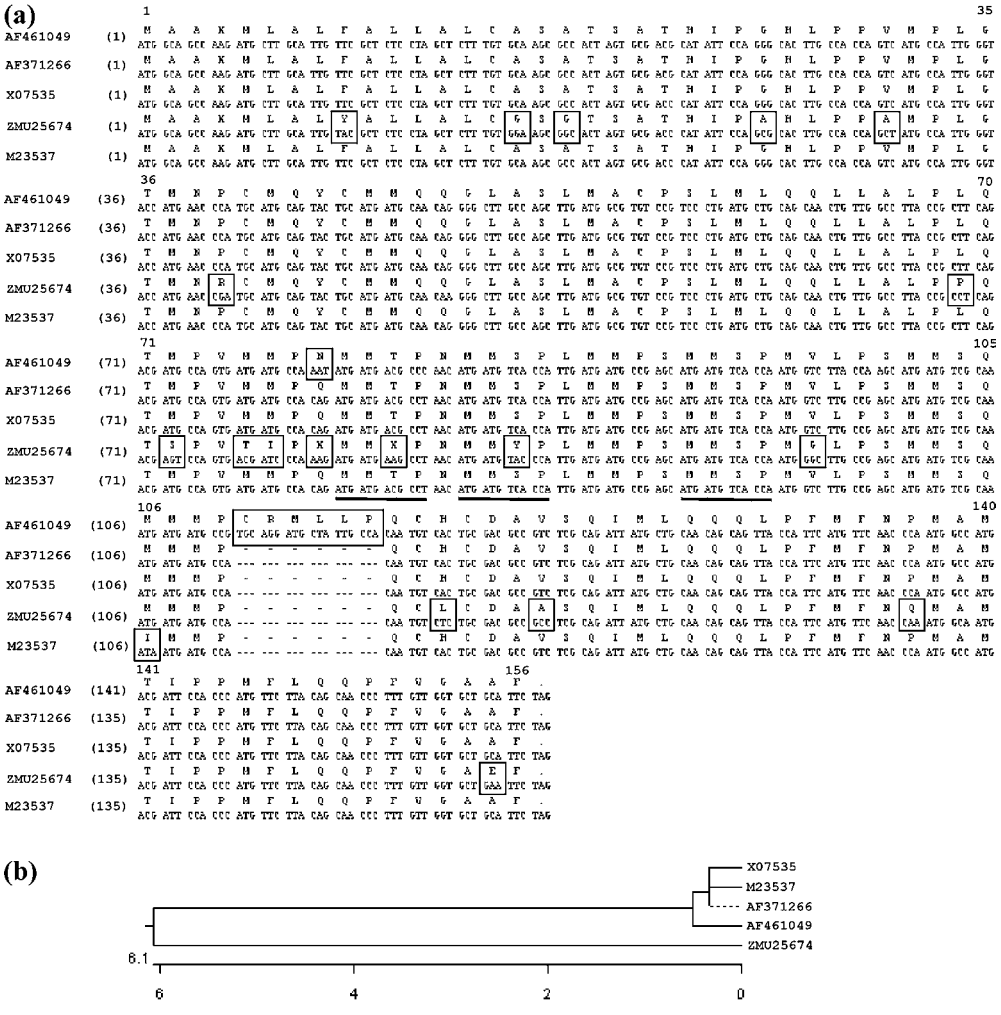


Fig. 1a, b Nucleotide and amino acid sequence alignments of 10-kDa δ-zein classes from maize (*Zea mays*) inbred lines. **a** The 11-kDa δ-zein sequence from inbred W23a1 differs from the published 10-kDa δ-zein classes by insertion of six amino acid residues between +110 and +115 (CRMLLP) from the translation start site. The Met-Met-X-Pro repeat motif that is found in the central region of δ-zein classes is *underlined*. Differences in the amino acids among the δ-zeins are shown in the *boxes*. GenBank accession numbers and varieties are as follows: AF461049, W23a1; AF371266, B73; X07535, W23; ZMU25674, Zhong dan 120; M23573, BSSS-53. **b** Dendrogram of the alignment shown in **a**. The scale at the bottom represents the branch distance as the number of changes in character state between neighbors

against the 18-kDa δ-zein, also strongly cross-reacted against the 10-kDa and 11-kDa δ-zeins (Fig. 4). In both the B73 and W23a1 inbreds, accumulation of the 18-kDa δ-zein could be detected at 10 DAP and showed a gradual increase during seed development (Fig. 4). Even though the RNA transcript encoding the 18-kDa δ-zein in W23a1 declined during the seed development

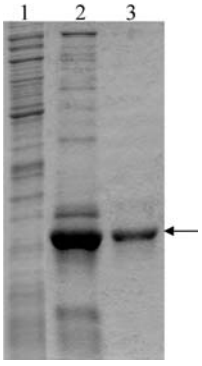


Fig. 2 Expression of 18-kDa δ-zein in *Escherichia coli*. The coding region of the18-kDa δ-zein was cloned in-frame into an expression vector pET28(a). Protein expression was achieved by the addition of 1 mM IPTG to *E. coli* cells. Proteins were resolved on a 12% SDS gel and visualized by staining with Coomassie Brilliant Blue. *Lanes*: *1* total protein from induced cultures containing the pET28(a), *2* total protein from induced *E. coli* containing the 18-kDa δ-zein coding region, *3* purified His-tagged 18-kDa δ-zein. The *arrow* points to the affinity-purified recombinant 18-kDa δ-zein

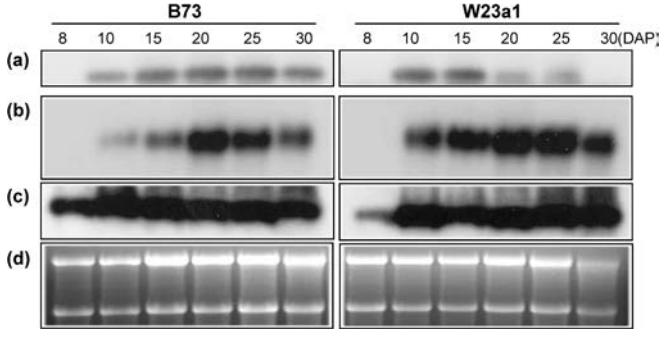


Fig. 3a–d Differential expression of the10-kDa and 18-kDa δ-zein transcripts in inbred maize lines B73 and W23a1. Five micrograms of total RNA was fractionated on a denaturing formaldehyde agarose gel and blotted onto Hybond-N+ membrane. The membranes were hybridized to probes specific to the 18-kDa δ-zeins (**a**), the 10-kDa δ-zeins (**b**), and 18S rRNA (**c**). The ethidium bromide-stained gel picture shows uniform loading of RNA samples (**d**)

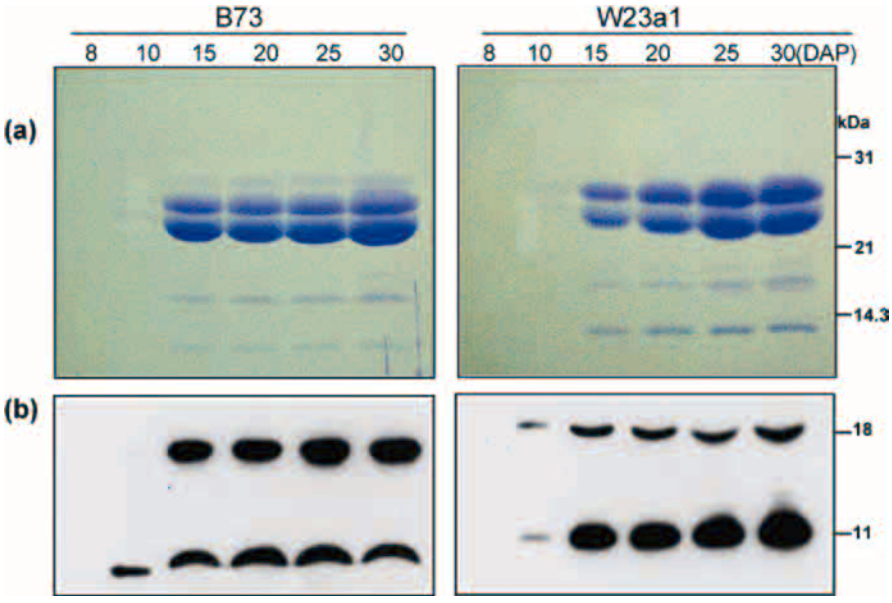


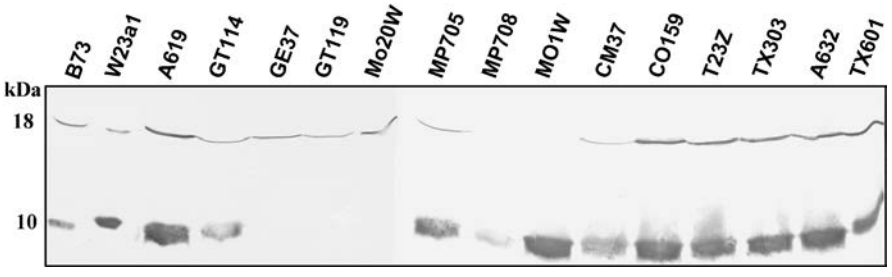
Fig. 4a, b Western blot analysis of δ-zein accumulation. Alcohol-soluble proteins from maize inbred lines B73 and W23a1 were isolated at different days after pollination. The zeins were resolved on 15% SDS–polyacrylamide gels and either stained with Coomassie Brilliant Blue (**a**) or electrophoretically transferred to nitrocellulose membrane for immunodetection analysis with δ-class zein-specific antibodies (**b**). The numbers on the *right* of the figure indicate the molecular weight of proteins in kDa

(Fig. 3), Western blot analysis revealed no such decline (Fig. 4). Accumulation of the 10-kDa and 11-kDa δ-zeins could be seen around 8–10 DAP and reached a maximum at 20–25 DAP (Fig. 4).

Some maize inbred lines lack either the 10-kDa or the 18-kDa δ-zein

To examine if there is variation in the amounts of δ-zeins in different maize inbred lines, we performed Western blot analysis. Most maize inbred lines accumulated both the 10-kDa and the 18-kDa δzeins (Fig. 5). However, the relative concentrations of the δ-zeins were variable among the maize inbred lines. Interestingly, we were unable to detect the 10-kDa δ-zein in inbreds GE37, GT119, or Mo20 W (Fig. 5). Similarly, inbreds MP708 and Mo1 W did not accumulate the 18-kDa δ-zein (Fig. 5).

Fig. 5 Western blot analysis of δ-zeins in different maize inbred lines. Total seed proteins were extracted from dry seeds and resolved on 15% SDS–acrylamide gels followed by transfer to a nitrocellulose membrane. The membrane was probed with 18-kDa δ-zein antibodies. Note that some inbred lines lack either the 18 kDa or the 10-kDa δ-zein



Localization of the methionine-rich δ-zein in endosperm cells

To examine the distribution of δ-zein in the endosperm cells, thick sections of paraffin-embedded 20-DAP W23a1 and B73 seeds were subjected to immunohistochemical analysis. Light-microscopy examination of basic-fuchsin-stained sections revealed that both W23a1 and B73 endosperm cells contained prominent starch grains and protein bodies (Fig. 6a, d). Numerous protein bodies were found in the starchy endosperm layer beneath the aleurone layer. The concentration of protein bodies gradually decreased from the outer to inner starchy endosperm cells. Immunostaining of paraffin sections with 18-kDa δ-zein antibody revealed intense labeling over the protein bodies found in first few starchy layers from the aleurone layer (Fig. 6b, e). The labeling intensity was greater in inbred W23a1 cells than in B73. The observed differences in the labeling intensity could also be due to comparison of different sections taken from different areas of developing endosperm. In general, the immunostaining was weaker in the endosperm cells farther from the aleurone layer. Immunostaining of the paraffin sections with pre-immune serum gave no specific reaction over the protein bodies. Only a very low background labeling was detected (Fig. 6c, f).

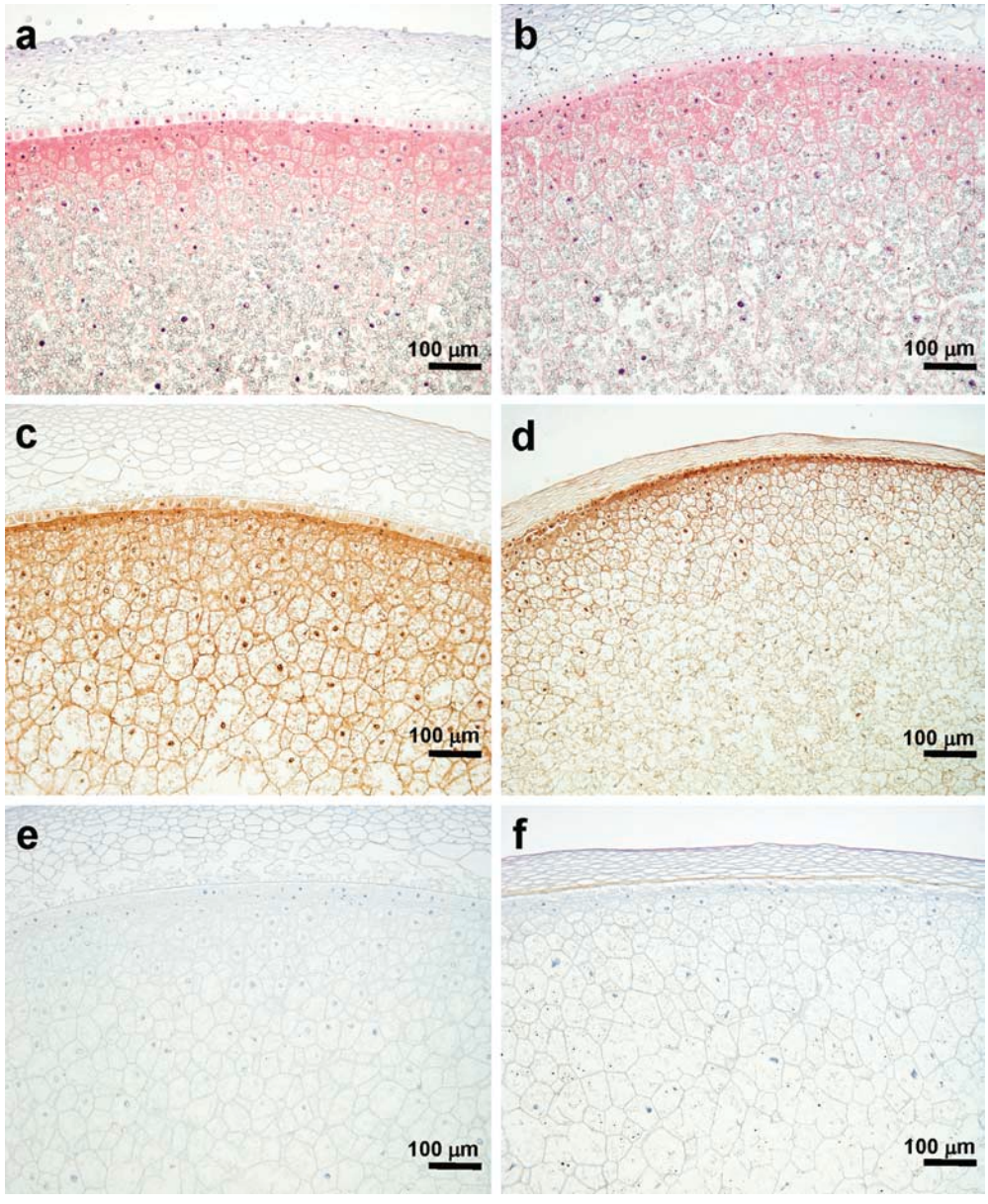


Fig. 6a–f Immunohistochemical localization of δ-zeins. Twenty days after pollination, W23a1 (**a–c**) and B73 (**d–f**) seeds were embedded in paraffin either stained with basic fuchsin (**a, d**) or processed for immunocytochemical localization. Paraffin-embedded sections were treated with 18-kDa δ-zein antibodies (**b, e**) or preimmune serum (**c, f**). Note the brown color indicative of the specific labeling of δ-zeins on the protein bodies in the subaleurone and starchy endosperm cells (**b, e**) and absence of similar reaction when treated with pre-immune serum (**c, f**)

Electron-microscope observation of 20-DAP W23a1 and B73 endosperm cells revealed numerous protein bodies that varied in size from 0.3 to 0.8 μm in diameter (Fig. 7a, b). In addition to the round protein bodies, occasionally, large irregularly shaped protein bodies were also seen in the endosperm cells (Fig. 7a, b). These protein bodies stained much darker than the round protein bodies. Ribosomes, which surrounded the round protein bodies, were absent on these irregularly shaped protein bodies (Fig. 7a, b). Thin sections of W23a1 and B73 endosperm cells incubated with 18-kDa δ-zein antibody and protein A–gold particles revealed specific labeling over most parts of the protein bodies (Fig. 7c, d). No preferential labeling over the light- and dark-staining regions of the protein bodies was evident. Replacing the 18-kDa δ-zein antibodies with pre-immune serum resulted in negligible background labeling over the protein bodies (Fig. 7e, f).

Discussion

The δ-zeins of the maize inbred line W23a1 are made up of 18-kDa and 11-kDa proteins. The nucleotide and amino acid sequences of the 18-kDa δ-zein are identical to the previously published sequence from inbred Mo17. Comparisons of the amino acid sequences of the 11-kDa δ-zein sequences with the previously published 10-kDa δ-zein sequences revealed remarkable sequence polymorphism (Fig. 1). These polypeptides are identical to each other except that W23a1 has an insertion of six amino acids (CRMLLP), which are missing in other

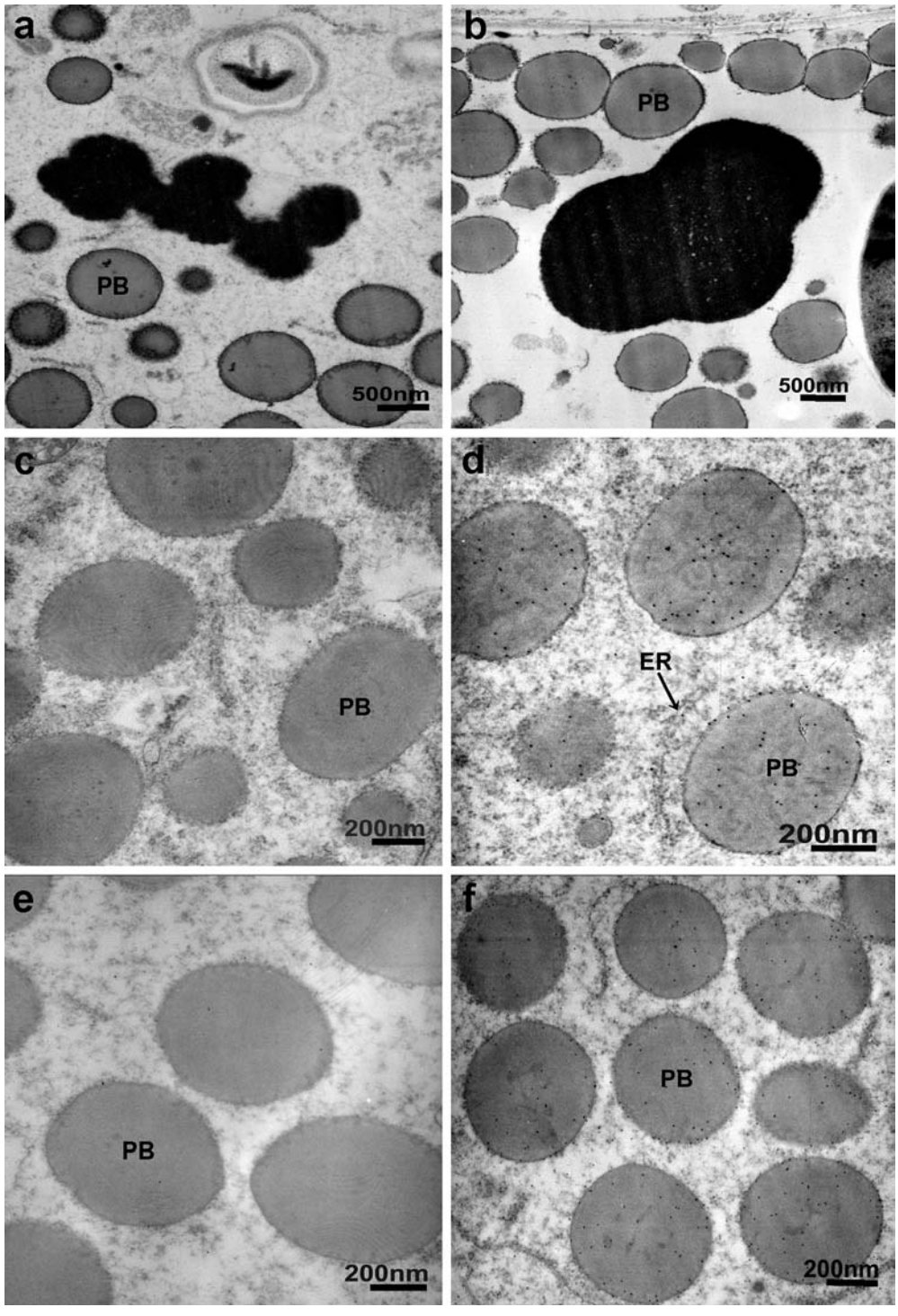


Fig. 7a–f Electron micrographs of maize endosperm. Dark-staining irregular-shaped protein bodies amidst spherical protein bodies are seen in the endosperm cells (**a, b**). Note that, unlike the spherical protein bodies, the irregular-shaped protein bodies do not contain ribosomes on their outer surface. Thin sections of maize endosperm cells when incubated with 18-kDa δ-zein antibodies and protein A–gold particles revealed deposition of gold particles on the protein bodies (**c, d**). Incubation of the thin sections with pre-immune serum resulted in negligible labeling over the protein bodies (**e, f**). *PB* Protein bodies, *ER* endoplasmic reticulum. **a, c, e** W23a1; **b, d, f** B73

inbreds (Fig. 1). Similarly, we noted that the amino acid sequences of the 18-kDa δ-zeins from inbreds W23a1 and B73 have also diverged. In the case of B73, the 18-kDa δ-zein contained five fewer methionine residues than the W23a1 18-kDa δ-zein (data not shown). A recent study investigating the expression of maize seed storage-protein genes utilizing endosperm expressed sequence tag (EST) libraries also identified similar sequence polymorphism in the alleles encoding the18 kDa δ-zeins of inbreds B73 and Mo17 (Woo et al. 2001). These findings, which reveal variability in the amino acid sequences in both the 10-kDa and 18-kDa δ-zeins among the maize inbred lines, support the suggestion of Woo et al. (2001) that there was low evolutionary pressure on the δ-zein genes to maintain the primary protein structure.

The expression of the 10-kDa δ-zein in developing inbred B73 was recently investigated by RNA in situ

hybridization (Woo et al. 2001). Expression of the 10-kDa δ-zein was detected in two small regions of the adgerminal and abgerminal endosperm (Woo et al. 2001). Moreover, no 10-kDa δ-zein RNA transcripts were detected in 10-DAP endosperm. In contrast, Northern blot analyses clearly showed that RNA transcripts encoding the 10-kDa were present at 10 DAP. This discrepancy could be attributed to differences in the sensitivity of the techniques used to detect the RNA transcripts. Northern blot analysis also showed that the relative RNA levels for the 10-kDa and 11-kDa δ-zeins were severalfold higher than that of the 18-kDa zein. The abundance of 10-kDa δ-zein RNA could be attributed to increased transcription of 10-kDa zein genes or to differences in the mRNA stability between these classes of zeins (Kirihara et al. 1988b). Our study also shows that the RNA transcripts encoding the 18-kDa δ-zein were differentially expressed in inbreds B73 and W23a1. In W23a1, the 18-kDa zein mRNA level peaks at 15 DAP and thereafter declines sharply, while in B73 the mRNA peaks at 20 DAP and shows only a gradual decline at later stages of seed development. In contrast, the transcripts encoding the 10-kDa zeins in inbreds B73 and W23a1 showed very similar accumulation patterns during seed development.

We were unable to detect the accumulation of the 10-kDa δ-zein in GE37, GT119, or Mo20 W. Similarly, an earlier study also failed to detect the accumulation of the 10-kDa δ-zein in Mo17, A654, Co64, C103, or SD Purple (Swarup et al. 1995). The molecular basis for this was investigated in inbred Mo17 (Schickler et al. 1993). It was found that Mo17 accumulated low levels of mRNA encoding the 10-kDa δ-zein and the developmental time frame of mRNA accumulation was also restricted (Schickler et al. 1993). Post-transcriptional regulation also plays an important role in determining the expression levels of the δ-zeins (Cruz-Alvarez et al. 1991). It is not known if similar mechanisms are also responsible for the absence of 10-kDa δ-zein in GE37, GT119 and Mo20 W. Several wild maize germplasms, including teosinte lines, accumulate relatively higher amounts of the 18-kDa δ-zein than most inbred lines. The relatively high accumulation of the 10-kDa and 18-kDa δ-zeins resulted in a high methionine content in these wild maize germplasms (Swarup et al. 1995). It has been proposed that in the absence of selectable markers for the high methionine trait, such a trait was eliminated during initial domestication and breeding of maize (Swarup et al. 1995). Based on the sequence polymorphism in the zeins among maize inbred lines, it was proposed that there was little or no selection for alleles during domestication (Woo et al. 2001).

The formation of protein bodies in maize endosperm has been thoroughly studied (Ludevid et al. 1984; Lending et al. 1988; Lending and Larkins 1989). These studies have established that the different classes of zeins accumulate in distinct regions within the protein bodies. The central region of the protein bodies is occupied by the α-zeins and the peripheral region mostly contains the β- and γ-zeins (Lending and Larkins 1989). However, the location and distribution of the δ-zein in developing endosperm has not been previously reported. Here, we report that most of the δ-zeins are localized primarily in the subaleurone and the first few layers of the starchy endosperm. Even though the antibodies we used in this study were raised against the 18-kDa δ-zein, it recognized both the 10-kDa and the 18-kDa δ-zeins. This cross-reaction is to be expected given the fact that both the 10-kDa and the 18-kDa δ-zeins show extensive sequence identity. Antibodies raised against the region that is least conserved between the 10-kDa and 18-kDa δ-zeins have been shown to exhibit high specificity (Woo et al. 2001). Use of such specific antibodies may be necessary to distinguish the location of the 10-kDa and 18-kDa δ-zeins within the protein bodies. Nevertheless, the δ-zeins appear to be distributed throughout the protein bodies. It is now well established that the γ-zeins plays an important role in protein-body assembly. Currently, we do not know the role, if any, of the δ-zeins in protein-body formation in maize endosperm.

Earlier attempts to overexpress zeins in *E. coli* have had only limited success. For example, Norrander et al. (1985) inserted the coding region of the zein in different M13mp phage vectors and introduced them into *E. coli* JM109. The expression of the phage-encoded proteins in *E. coli* resulted in very low levels of zein expression that could be detected by immunoblot analysis. These authors speculated that zein protein may be unstable in *E. coli* or that the zein proteins may be harmful to *E. coli* (Norrander et al. 1985). In contrast, we were able to successfully express the 18-kDa δ-zein in *E. coli*. This may be because the amino acid composition of δ-zein is quite different than the other classes of zeins. However, it appears that this may not the case since large peptides of the 27-kDa γ-zein, 19-kDa α-zein, and 22-kDa α-zein have recently been successfully expressed in *E. coli* as His-tag fusion or glutathione-*S*-transferase fusion products (Woo et al. 2001). The ability to overexpress the zeins in *E. coli* should facilitate studies investigating the structure of proteins that have incorporated specific amino acid changes to improve the overall protein quality of zeins.

Acknowledgements The authors gratefully thank Dr. Michael McMullen for growing the maize plants in the field and Drs. Larry Darrah and Jack Gardiner for critical review of this manuscript.

References

Benner MS, Phillips RL, Kirihara JA, Messing JW (1989) Genetic analysis of methionine-rich storage protein accumulation in maize. Theor Appl Genet 78:761–767

Burnett WN (1981) Western blotting: electrophoretic transfer of proteins from SDS–polyacrylamide gel to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195-203

Chui CF, Falco SC (1995) A new methionine-rich seed storage protein from maize. Plant Physiol 107:291

Cruz-Alvarez M, Kirihara JA, Messing JW (1991) Post-transcriptional regulation of methionine content in maize kernels. Mol Gen Genet 225:331–339

Habben JE, Larkins BA (1995) Improving protein quality in seeds. In: Kige J, Galili G (eds) Seed development and germination. Dekker, New York, pp 791–810

Kirihara JA, Petri JB, Messing JW (1988a) Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize. Gene 71:359–370

Kirihara JA, Hunsperger JP, Mahoney WC, Messing JW (1988b) Differential expression of a methionine-rich storage protein gene in maize. Mol Gen Genet 211:477–484

Krishnan HB (2000) Biochemistry and molecular biology of soybean seed storage proteins. J New Seeds 2:1–25

Krishnan HB, Coe EH (2001) Seed storage proteins. In: Brenner S, Miller JH (eds) Encyclopedia of genetics. Academic Press, San Diego, pp 1782–1787

Krishnan HB, Okita TW (1986) Structural relationship among the rice glutelin polypeptides. Plant Physiol 81:748–753

Krishnan HB, Franceschi VR, Okita TW (1986) Immunocytochemical studies on the role of the Golgi complex in protein body formation in rice seeds. Planta 169:471–480

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lending CR, Larkins BA (1989) Changes in the zein composition of protein bodies during maize endosperm development. Plant Cell 1:1011–1023

Lending CR, Kriz AL, Larkins BA, Bracker CE (1988) Structure of maize protein bodies and immunocytochemical localization of zeins. Protoplasma 143:51–62

Ludevid MD, Torrent M, Martinez-Izquierdo JA, Puigdomenech P, Palau J (1984) Subcellular localization of glutelin-2 in maize (*Zea mays* L.) endosperm. Plant Mol Biol 3:227–234

Mertz ET, Bates LS, Nelson OE (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145:279–280

Muntz K, Christov V, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O, Wustenhagen T (1998) Genetic engineering for high methionine grain legumes. Nahrung 42:125–127

Nielsen NC (1996) Soybean seed composition. In: Verma DPS, Shoemaker RC (eds) Soybean: genetics, molecular biology and biotechnology. CAB, Tucson, pp 127–163

Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RH (1996) Identification of a Brazil-nut allergen in transgenic soybeans. N Engl J Med 334:688–692

Norrander JM, Vieira J, Rubenstein I, Messing J (1985) Manipulation and expression of the maize zein storage proteins in *Escherichia coli*. J Biotechnol 2:157–175

Phillips RL McClure BA (1985) Elevated protein-bound methionine in seeds of a maize line resistant to lysine plus threonine. Cereal Chem 62:213–218

Schickler H, Benner MS, Messing JW (1993) Repression of the high-methionine zein gene in the maize inbred line Mo17. Plant J 3:221–229

Shewry PR, Tatham AS (1999) The characteristics, structures and evolutionary relationships of prolamins. In: Shewry P, Casey R (eds) Seed proteins. Kluwer, Norwel, MA, pp 11–33

Shewry PR, Napier JA, Tatham AS (1995) Seed storage proteins: structures and biosynthesis. Plant Cell 7:945–956

Swarup S, Timmermans MCP, Chaudhuri S, Messing JW (1995) Determinants of the high-methionine trait in wild and exotic germplasm may have escaped selection during early cultivation of maize. Plant J 8:359–368

Woo YM, Hu DW, Larkins BA, Jung R (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. Plant Cell 13:2297–2317