Opaque or nonvitreous phenotypes relate to the seed architecture of maize (Zea mays) and are linked to loci that control the accumulation and proper deposition of storage proteins, called zeins, into specialized organelles in the endosperm, called protein bodies. However, in the absence of null mutants of each type of zein (i.e., α, β, γ, and δ), the molecular contribution of these proteins to seed architecture remains unclear. Here, a double null mutant for the δ-zeins, the 22-kD α-zein, the β-zein, and the γ-zein RNA interference (RNAi; designated as z1CRNAi, βRNAi, and γRNAi, respectively) and their combinations have been examined. While the δ-zein double null mutant had negligible effects on protein body formation, the βRNAi and γRNAi alone only cause slight changes. Substantial loss of the 22-kD α-zeins by z1CRNAi resulted in protein body budding structures, indicating that a sufficient amount of the 22-kD zeins is necessary for maintenance of a normal protein body shape. Among different mutant combinations, only the combined βRNAi and γRNAi resulted in drastic morphological changes, while other combinations did not. Overexpression of α-kafirins, the homologues of the maize 22-kD α-zeins in sorghum (Sorghum bicolor), in the β/γRNAi mutant failed to offset the morphological alterations, indicating that β- and γ-zeins have redundant and unique functions in the stabilization of protein bodies. Indeed, opacity of the β/γRNAi mutant was caused by incomplete embedding of the starch granules rather than by reducing the vitreous zone.

In order to enhance their nutritional value, seed crops have been targets of genetic engineering efforts to either produce valuable proteins or alter the amino acid composition of existing proteins (Rademacher et al., 2009). However, what is frequently ignored is the subcellular function that proteins play in the development of the seed. In maize (Zea mays), the endosperm storage proteins constitute a major protein component in the seed. Most of them belong to the prolamin, common in many grass species, and in maize are referred to as zeins. The alcohol-soluble zein fraction extracted by the Osborne method without reducing agent is called zein-1 and consists mainly of the 19-kD (z1A, z1B, and z1D) and 22-kD (z1C) α-zeins (Song and Messing, 2003). The fraction of alcohol-soluble proteins extracted with a disulfide reducing agent (Moureaux and Landry, 1968; Paulis et al., 1969; Landry and Moureaux, 1970) is called zein-2 (Sodek and Wilson, 1971) and is composed of γ-, β-, and δ-zeins (Esen, 1987; Coleman and Larkins, 1998).

α-Zeins with 26 (19-kD) and 16 (22-kD) gene copies in maize inbred B73 constitute 60% to 70%, respectively, of total zeins. γ-Zeins consist of the 50-, 27-, and 16-kD proteins, each encoded by a single gene in B73, and amount to about 20% to 25% of total zeins. The 27- and 16-kD γ-zein genes originated from a common progenitor by allotetraploidization and share high DNA sequence similarity (Xu and Messing, 2008), while the 50-kD γ-zein gene has low similarity to the other two γ-zein genes and its protein is barely detectable by SDS-PAGE (Woo et al., 2001). The 15-kD β-zein protein is encoded by a single gene and its product makes up 5% to 10% of total zeins (Thompson and Larkins, 1994). The 18- and 10-kD δ-zein proteins are also each encoded by a single gene and make up less than 5% of total zeins (Wu et al., 2009). From an evolutionary point of view, the α- and δ-zeins are older and conserved across different subfamilies of the Poaceae (Xu and Messing, 2009).

Zeins are specifically synthesized in the endosperm about 10 d after pollination (DAP) on polyribosomes of the rough endoplasmic reticulum (RER), and the proteins are subsequently translocated into the lumen of the RER, where they assemble into protein bodies (Wolf et al., 1967; Larkins and Dalby, 1975; Burr and Burr, 1976; Lending and Larkins, 1992). Typical protein bodies at 18 to 20 DAP are spherical, discrete, 1 to 2 μm in diameter, and have a highly ordered architecture. α-Zeins and δ-zeins are deposited in the center of the
RESULTS
A Complete Set of Maize Lines Defective in All Four Classes of Zeins

Because the major storage proteins in maize are the alcohol-soluble zeins, null mutants are easily identified by SDS-PAGE (Fig. 1A). The 18-kD δ-zein was usually not well separated, overpowered by large amounts of 19-kD α-zeins, but could be monitored by western-blot analysis (Wu et al., 2009). Among 12 different maize inbred lines and genetic stocks, A654 and SD-purple were null mutants for the 18- and 10-kD δ-zeins (Fig. 1A). Their coding sequences were disrupted by a TTAT INDEL (for insertion and deletion) and transposon insertion, respectively (Wu et al., 2009). In addition to the double null mutant, there were also inbred lines or genetic stocks with single null mutations of the δ-zeins, but none of them exhibited a null mutant for α-, β-, and γ-zeins.

In the absence of natural null mutants, we constructed RNAi transgenes for the other zein genes. The 15-kD β-zein gene exists as a single copy in the maize genome and exhibits little similarity to other zein genes. The 27- and 16-kD γ-zein genes share high DNA sequence similarity; therefore, we used the β-zein and 27-kD γ-zein full-length coding sequences for their RNAi construction to create a single and a double knockdown mutant, respectively (Fig. 1, B and C). The third copy of this class, the 50-kD γ-zein (data not shown).

A knockdown mutant of α-zein genes with a zICRNAi construct against the 22-kD zein genes had already been generated (Segal et al., 2003), and when examined by SDS-PAGE, it showed the expected effect (Fig. 1D). The opaque2 (o2) mutant (Fig. 1E), which is a null mutant of a transcription factor of the 22-kD α-zein genes (Schmidt et al., 1992; Song et al., 2001), was also reinvestigated in this work to compare it with the specific zICRNAi mutant. In summary, we collected a set of null and knockdown mutants that reduce or eliminate the accumulation of each of the different storage protein classes from either this or previous work. Although we realize that normal lines, the o2 allele, the δ-zein mutants, and the RNAi constructs are not in an isogenic background, we suggest that various alleles other than the ones investigated here are unlikely to impact protein body formation to a noticeable degree. Therefore, we proceeded with the examination of their subcellular function singly or in combination with the mutant collection.

Analysis of the New Set of Transgenic Seeds and Their Crosses

A triple mutant for the 15-kD β-zein and the 27- and 16-kD γ-zeins was generated by crosses of the βRNAi and γRNAi events. At 18 DAP, 40 kernels were collected from hybrids, 20 for protein and electron microscopy analysis and the other 20 for real-time PCR. The 40 embryos were dissected to extract genomic DNA for PCR amplification. As expected, both of the RNAi constructs showed 1:1 segregation ratios in the analysis of the 40 embryos by PCR (data not shown). Furthermore, there was a perfect correlation between
genotype and phenotype (Fig. 2, A and B), exhibiting progeny with both the RNAi constructs, progeny lacking accumulation of the 15-kD $\beta$-zein and the 27- and 16-kD $\gamma$-zeins, progeny showing normal protein accumulation, and single RNAi events lacking only one of the corresponding proteins. Furthermore, as shown in Figure 1B, the accumulation of $\alpha$- and $\delta$-zeins was not affected by the knockdown of $\beta$- or $\gamma$-zeins, either in combination or as a single knockdown (Fig. 2B).

Further quantitative analysis was achieved by real-time PCR of 20 kernels from the same progeny. As shown in Figure 2C, the mRNA levels of the 27- and 16-kD $\gamma$-zein and the 15-kD $\beta$-zein genes were reduced to a negligible level compared with normal endosperm, illustrating the efficiency and specificity of RNAi targeting. However, there was no compensatory effect on the 50-kD $\gamma$-zein gene (data not shown), which was expressed at normal levels in the RNAi mutants. Therefore, $\gamma$- and $\beta$-zeins are not required for the normal accumulation of $\alpha$- and $\delta$-zeins in maize endosperm. This was rather unexpected because in heterologous systems like tobacco ($Nicotiana tabacum$), $\alpha$- and $\delta$-zeins could never accumulate at high levels unless they were coexpressed with the 27-kD $\gamma$- or $\beta$-zein (Coleman et al., 1996; Bagga et al., 1997).

**Subcellular Analysis of the Natural and RNAi Mutant Lines**

To investigate the specific role of each class of zeins in protein body formation, 18-DAP immature endosperms of each mutant line were processed for transmission electron microscopy. In nontransgenic Hi-II hybrids, protein bodies were spherical and discrete with a distinct membrane (Fig. 3A). In inbred A654, a natural haplotype where both $\delta$-zeins were mutated, protein bodies gave almost indistinguishable shapes compared with normal endosperm (Fig. 3B). In contrast, the $\beta$RNAi and $\gamma$RNAi mutant lines exhibited slightly altered protein body formation (Fig. 3, C and D). However, more underdeveloped protein bodies were seen in the two RNAi mutants.

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**Figure 1.** Zein accumulation in normal and RNAi mutant seeds detected by SDS-PAGE and PCR. A, SDS-PAGE of 12 different maize inbred lines and genetic varieties. Lane numbers refer to different materials: 1, BSSS53; 2, B73; 3, B37; 4, Mo17; 5, W64A; 6, W22; 7, P1-ww-112; 8, A69Y; 9, ILLIZE; 10, A188; 11, SD-purple; 12, A654. Bands for 27-kD $\gamma$, 22-kD $\alpha$, 19-kD $\alpha$, 16-kD $\gamma$, 15-kD $\beta$, and 10-kD $\delta$ are well separated. Several lines are missing the 10-kD $\delta$-zein. B, The $\beta$RNAi mutant. The top panel shows the construct (see “Materials and Methods”). The middle panel shows PCR assay of genomic DNA from different transgenic lines. K1, K4, and K8 represent the progeny inheriting the RNAi event. Two nontransgenic kernels, C1 and C2, serve as controls. The bottom panel shows the corresponding SDS-PAGE. In lanes K1, K4, and K8, the 15-kD band is missing. C, The $\gamma$RNAi mutant. Analysis is the same as in B. K3 and K4 represent the progeny inheriting the RNAi event. D, SDS-PAGE for a $\alpha$CRNAi seed is shown, where the 22-kD zein band is reduced (arrow). E, SDS-PAGE for W64A $\alpha2$ and normal W64A seeds is shown. In the $\alpha2$ mutant, bands for 22-kD $\alpha$-zein, 15-kD $\beta$-zein, and 10-kD $\delta$-zein are reduced (arrows). Total zein loaded in each lane was equal to 300 $\mu$g of dry seed meal (A) and 500 $\mu$g of fresh endosperm at 18 DAP (B–D). Protein markers from top to bottom are 25, 20, 15, and 10 kD. M, Protein marker; F and R, primer GFPF and T35S-Hin digest (see “Materials and Methods”). [See online article for color version of this figure.]
than in normal seed, consistent with the reduction in total zein.

These altered shapes of protein bodies differed from the classical o2 mutant, where protein body membranes were still spherical but their sizes were dramatically reduced (Fig. 3E). On the other hand, changes in the z1C RNAi mutant differed significantly from the natural o2 mutant line. As indicated by arrows, most of the mature protein bodies produced protuberances, as if they were budding small protein bodies (Fig. 3F). In normal endosperm, protein bodies were initiated in the lumen of RER and then extruded from the RER when they grew. However, the size reduction seen in the o2 mutant did not occur, indicating that additional zein genes, like the 15-kD β-zein (Cord Neto et al., 1995) and nonstorage protein genes (Lohmer et al., 1991; Hunter et al., 2002), were coordinated by O2 transcriptional regulation. If protein bodies were allowed to expand with reduced quantities of α-zeins, a regular round spherical protein body structure was aborted, giving α-zeins an indispensable structural role in protein body formation. This comparison of subcellular structures illustrates that an RNAi approach is critical for the analysis of the functional role of storage proteins that could not be achieved with previously reported mutants.

Specific Protein Body Distortion in βRNAi and γRNAi Combined Mutant Lines

Given the specificity of each RNAi and null mutant, one can now study the possible redundant roles between different subgroups of zeins by combining the mutants through conventional crosses (Fig. 4A). The combination of the βRNAi and γRNAi did not prevent the accumulation of other zeins, as shown by SDS-PAGE (Fig. 2B). To combine the two RNAs with the natural δ-zein null mutant, they were backcrossed with A654 for two generations and the δ-zein null alleles were screened by PCR assay (Fig. 4B; see “Materials and Methods”).

The combination of the δ-zein null mutant with either the βRNAi or γRNAi transgene (Fig. 5, A and B) did not differ much in protein body morphology from their parental lines (Fig. 3, B–D), indicating no additive effect by δ-zeins. However, progeny with both βRNAi and γRNAi transgenes produced an irregular shape of protein bodies, particularly at their periphery (Fig. 5C). The protein body membranes seemed to be unevenly contracted or potentially had a vesiculation defect. At a higher resolution (Supplemental Fig. S1), it appeared that the protein body membrane became loose, as if hydrophobic repulsion forces arose in their peripheral areas. The presence of both βRNAi and γRNAi transgenes caused all the protein bodies to lose their normal shape to a degree not seen with single RNAi transgenes, indicating that γ- and β-zeins have a redundant and specific role in stabilizing the formation of protein bodies.

Increased α-Prolamins in the Presence of βRNAi and γRNAi Transgenes

While combining the βRNAi and γRNAi had a synergistic effect, the combination of the δ-zein null mutant with either of them did not. Since the combination of the βRNAi and γRNAi had a slightly greater reduction in total zein than that of the δ-zein null
mutant and γRNAi, one might wonder whether this slight difference could be critical. We had available a transgenic plant that could increase the accumulation of total storage proteins through expression of the related α-kafrins, homologues to the maize 22-kD α-zeins in sorghum (Sorghum bicolor; Song et al., 2004). In maize, 19-kD α-zeins accumulated to a higher degree than the 22-kD α-zeins. By introduction of 10 copies of 22-kD α-kafrin genes, the ratio between the 22- and 19-kD α-prolamins rose to nearly 1:1 (Fig. 4C). Nevertheless, electron microscopy showed that introduction of kafrins resulted in normal protein body morphology (data not shown), indicating that the 22-kD α-kafrins were compatible with zeins in protein body formation. Increased accumulation of the total “zeins” (mixture of zeins and kafrins), however, did not suppress the formation of the amorphous protein bodies in the simultaneous presence of βRNAi and γRNAi transgenes (Fig. 5D). Overexpression of α-“zein” could not compensate for the loss of β- and γ-zeins, indicating that Cys-rich zeins have other roles than a storage function.

Quantitative and Spatial Effects of Kernel Phenotypes

On a whole kernel basis, endosperm is hard (vitreous) in the peripheral region and soft (starchy) in the central region. Natural mutants (e.g. o2 and o7) resulting in the reduction of α-zeins were well recognized because of the opacity or nonvitreous appearance of seeds. However, as we pointed out in the case of transacting factors like O2, this phenotype could also be due to the loss or reduced levels of nonstorage proteins. Therefore, we examined the kernel phenotype of the different RNAi mutant lines alone and in crosses. Consistent with a relatively normal protein body phenotype and their small proportion to the total zein pool, A654 and the βRNAi kernels were vitreous either alone (Fig. 6B) or combined (data not shown). The γCRNAi kernels showed a similar opaque phenotype as the o2 mutant. For lines containing the γRNAi transgene, the opaque phenotype was rather variable. In the T1 generation, the opacity of the kernels from the two independent ears was not apparent (data not shown). In T2 and T3 generations, most of the ears showed opacity of kernels (Fig. 6C). In contrast to the o2 mutant and the γCRNAi seeds (Fig. 7, A and F), opacity was restricted to the crown area (Figs. 6C and 7D). When the γRNAi transgenic plant was back-crossed with A654, kernels bearing the RNAi construct could easily be sorted with the light box. Thirty opaque kernels were tested, all of them being RNAi positive (data not shown), indicating variable penetrance of opacity in different genetic backgrounds. However, among the 30 opaque kernels, the null and intact alleles of δ-zein genes showed normal 1:1 segregation, indicating that the small amount of δ-zeins did not significantly contribute to kernel phenotype. In the presence of both βRNAi and γRNAi transgenes, opacity became stronger. All the crosses and subsequently their selfed progeny presented much stronger opacity than the γRNAi seeds, and the opacity apparently spread out to a larger area or even the entire kernel (Fig. 6, D and E). As in the case of the γRNAi opaque seeds, the presence of both βRNAi and γRNAi transgenes often produced vitreous patches on opaque background (Figs. 6, C–E, and 7, D and E).

Because only the combination of βRNAi and γRNAi transgenes resulted in irregular protein bodies, it seemed that the reduction in expression of γ- and β-zeins also needed to reach a certain threshold level to produce a nonvitreous appearance of the kernel. To confirm the quantitative effect on opaque phenotypes, 20 randomly chosen vitreous and opaque seeds from a cross of the βRNAi and γRNAi transgenes were grown for genotyping by PCR. Fifteen vitreous and 17 opaque seeds germinated successfully. As shown in Figure 6F, all opaque seeds contained the γRNAi transgene and 10 of them had in addition the βRNAi transgene. Except for one, all vitreous seeds had just the βRNAi transgene and γRNAi, one might wonder whether this slight difference could be critical. We had available a transgenic plant that could increase the accumulation of total storage proteins through expression of the related α-kafrins, homologues to the maize 22-kD α-zeins in sorghum (Sorghum bicolor; Song et al., 2004). In maize, 19-kD α-zeins accumulated to a higher degree than the 22-kD α-zeins. By introduction of 10 copies of 22-kD α-kafrin genes, the ratio between the 22- and 19-kD α-prolamins rose to nearly 1:1 (Fig. 4C). Nevertheless, electron microscopy showed that introduction of kafrins resulted in normal protein body morphology (data not shown), indicating that the 22-kD α-kafrins were compatible with zeins in protein body formation. Increased accumulation of the total “zeins” (mixture of zeins and kafrins), however, did not suppress the formation of the amorphous protein bodies in the simultaneous presence of βRNAi and γRNAi transgenes (Fig. 5D). Overexpression of α-“zein” could not compensate for the loss of β- and γ-zeins, indicating that Cys-rich zeins have other roles than a storage function.

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transgene or no transgene at all. We also chose six vitreous and seven seeds with strong opacity for protein analysis by SDS-PAGE (Fig. 6G). Consistent with their phenotypes, all vitreous seeds had a normal total zein accumulation pattern, except one with the 15-kD α-zein knocked down, while all seeds with strong opacity contained both βRNAi and γRNAi transgenes except for one, which contained only the γRNAi transgene (Fig. 6G), consistent with a quantitative opaque phenotype.

Different Mechanisms Underlying Kernel Phenotypes

Usually, opaque and floury mutants had largely reduced vitreous regions compared with the normal ones when dried kernels were decapped (Fig. 7, G and H). In the zβCRNAi seed, the vitreous region was also much thinner than in normal seed (Fig. 7, I and J). Yet, the combination of βRNAi and γRNAi transgenes seemed to produce an opaque phenotype by a different mechanism. When the seed crowns of the βRNAi and γRNAi mutant lines and their crosses were removed, the width of the vitreous region was unchanged (Fig. 7, K–M). While the section of the βRNAi seed looked no different than a normal seed (Fig. 7, I and K), the vitreous region of the γRNAi seed began to turn starchy (Fig. 7L) and the penetration of starch became stronger in the presence of both βRNAi and γRNAi transgenes (Fig. 7M). It seemed that the floury portion, supposed to be restricted to the central region, had been exposed outside in the peripheral vitreous region. The penetration was not evenly distributed (Fig. 7, L and M). This observation would explain how vitreous patches were occasionally formed on opaque kernels (Figs. 6, C–E, and 7, D and E). When the kernels were cut along the longitudinal axis, we found that the crown area had the thinnest vitreous region (Fig. 7N). One could envision that this region was more susceptible to the penetration of starch granules (Fig. 7P and Q), consistent with the opacity to first emerge in the crown. The spread of the vitreous region was more extensive in the γRNAi seed than in the presence of both βRNAi and γRNAi transgenes, consistent with their intact kernel phenotype (Fig. 7, D and E).

**DISCUSSION**

Need of Homologous Expression Systems to Validate Gene Function

Because maize transformation was not routine until recently, zein gene expression has been studied in transgene or no transgene at all. We also chose six vitreous and seven seeds with strong opacity for protein analysis by SDS-PAGE (Fig. 6G). Consistent with their phenotypes, all vitreous seeds had a normal total zein accumulation pattern, except one with the 15-kD α-zein knocked down, while all seeds with strong opacity contained both βRNAi and γRNAi transgenes except for one, which contained only the γRNAi transgene (Fig. 6G), consistent with a quantitative opaque phenotype.
heterologous systems like *Escherichia coli*, yeast (*Saccharomyces cerevisiae*), *Xenopus laevis*, *Petunia hybrida*, and tobacco (Larkins et al., 1979; Langridge et al., 1984; Norrander et al., 1985; Ueng et al., 1988; Ohtani et al., 1991). Expression of zeins in heterologous systems has been thought of as a test case for genetic engineering of nutritionally improved seeds. But when an attempt was made to express single *α* - or *δ*-zein gene copies in tobacco endosperm, zein proteins failed to accumulate unless coexpressed with *γ* - or *β*-zein (Coleman et al., 1996, 2004; Bagga et al., 1997; Hinchliffe and Kemp, 2002). These findings indicated that *α*-zein or *δ*-zein in tobacco was prone to degradation and that coexpression of Cys-rich *γ*- or *β*-zein, which could initiate protein body formation alone, stabilized *α*- or *δ*-zein by sequestering them into protein bodies. Therefore, it has been proposed that such an additive system could provide a model to study the higher structure of protein-protein interactions that might take place in maize seeds (Kim et al., 2002). However, here we have shown that in maize seeds, *α*- and *δ*-zeins could accumulate to normal levels, even if both *γ*- and *β*-zeins were nearly eliminated (Figs. 1, B and C, and 2B). Therefore, heterologous systems were not valid models because of the absence of maize-specific non-storage proteins in tobacco that were needed for protein body formation. As an example, the protein body membrane protein FL1 appears to facilitate the correct spatial deposition of the 22-kD *α*-zeins (Holding et al., 2007), indicating that the normal development of protein bodies requires not only sufficient expression of zeins but also specific nonzein “helpers.” An alternative explanation for why *α*-zeins can accumulate in maize without *β*- and *γ*-zeins would be that zeins as insoluble accretions cannot be processed through ER-associated degradation pathways. On the other hand, in tobacco, the low amount of total zein may not amount to the same load on its secretory pathway, thus resulting in regular protein degradation.

**Roles of *γ*-Zeins and *β*-Zein in Protein Body Formation**

Protein bodies have a highly ordered architecture. Their formation in the normal seed proceeds via temporally coordinated transcription and proper spatial compartmentalization of the various types of zeins. Within the subaleurone cell layer, protein bodies are the smallest and contain little or no *α*- and *δ*-zeins, while *γ* - and *β*-zeins can be detected throughout, indicating that these Cys-rich zeins prime the organization of protein bodies while *α* - and *δ*-zeins enlarge their size (Lending and Larkins, 1989). An interesting aspect of this study was the specific effect of different mutant lines and their crosses on protein body morphology. To appreciate these effects, we needed to consider that *β* / *γ*-zeins and *α* / *δ*-zeins differed in their solubility and cross-linking abilities. *β*-Zein and *γ*-zeins were linked as polymers by disulfide bonds. Without reducing agent, the 27-kD *γ*-zein would be largely lost in the process of extraction of total zeins (Tsai, 1980). When protein bodies were isolated from endosperm with buffer containing a reducing agent,

![Figure 6](http://www.plantphysiol.org)
most of them were irregular (Ludevid et al., 1984), indicating that disulfide bonds are important in maintaining normal protein body shape. Moreover, \( \alpha \)- and \( \gamma \)-zeins differed in their affinity to water, a property determined by their spatial amino acid arrangement (Momany et al., 2006). \( \alpha \)-Zeins were very hydrophobic, while \( \gamma \)-zeins could dissolve in water in the presence of a reducing agent (Paulus and Wall, 1977). In a survey of the solubility of different domains of the 27-kD \( \gamma \)-zein, it was found that the N-terminal region was more hydrophobic while the C-terminal end had stronger affinity to water (Ems-McClung and Hainline, 1998). Given the fact that the \( \alpha \)-zeins were located in the central area of the protein body and \( \gamma \)-zeins were deposited in the peripheral region (Ludevid et al., 1984; Lending and Larkins, 1989), one could envision that the most stable organization of the components in the protein body was that the \( \alpha \)-zeins and the inner side of the RER membrane interact with the N-terminal and C-terminal ends of the \( \gamma \)-zeins, respectively. Also from an evolutionary point of view, the \( \gamma \)-zeins were not only the oldest prolamins but were thought to have originated from the water-soluble storage proteins, the globulins, by tandem gene duplication (Xu and Messing, 2009). This evolutionary path illustrated how gene copying and divergence created the novel function of an “osmoregulator” through chimeric domains, probably from unequal crossing over. A selective advantage was the water balance and nitrogen storage during desiccation of the seed.

Mechanisms of Vitreous and Opaque Phenotypes

To better understand the mutant phenotype of the maize kernel, we also had to consider the immature and mature stages. A major change was the water content of the seed. While gene expression was studied with immature seed tissue, phenotype was based on the mature seed. Although the combination of \( \beta \)RNAi and \( \gamma \)RNAi transgenes gave rise to irregular protein bodies, even mature seeds did not produce a kernel phenotype before seeds were dried. The opaque phenotype became visible after water had evaporated from seeds. The same was true for most opaque mutants, indicating two requirements for opaque and vitreous properties, protein accumulation during development and seed desiccation. During development, endosperm cells filled with bigger and lighter-staining starch granules interspersed with much smaller and darker-staining protein bodies (Supplemental Fig. S2). In normal endosperm cells, starch granules seemed to be interwoven with a proteinaceous matrix made of protein bodies (Supplemental Fig. S3A; Gibbon et al., 2003). However, at the whole kernel level, light and scanning electron microscopy results showed that

Figure 7. Kernel opacity of the RNAi mutants. A to F, Translucency of intact kernels on a light box. A, W64A and W64A o2. B, BA normal kernels. C, The \( \beta \)RNAi mutant. D, The \( \gamma \)RNAi mutant. E, The \( \beta \)RNAi and \( \gamma \)RNAi combination. F, z/CRNAi. G to Q, Latitudinal and longitudinal sections of kernels. Vitreous region was largely reduced in W64A o2 (H) and z/CRNAi (I). In the \( \gamma \)RNAi mutant (L and P), the starch granules began to penetrate outside. The crown of the \( \gamma \)RNAi mutant seed was opaque (P), while the normal BA kernels were vitreous (N; arrow). Most of the seed trunk of the \( \gamma \)RNAi mutant still remained vitreous (P; arrowhead). The penetration was reinforced in the combined mutant of the \( \beta \)RNAi and \( \gamma \)RNAi (M and Q), with no reduction of vitreous width (M and Q; arrows). Still, vitreous patches could be seen (Q; arrowhead). G to M show latitudinal sections of W64A (G), W64A o2 (H), BA normal type (I), the z/CRNAi mutant (J), the \( \beta \)RNAi mutant (K), the \( \gamma \)RNAi mutant (L), and the \( \beta \)RNAi and \( \gamma \)RNAi combination (M).

N to Q show longitudinal sections of BA normal type (N), the \( \beta \)RNAi mutant (O), the \( \gamma \)RNAi mutant (P), and the \( \beta \)RNAi and \( \gamma \)RNAi combination (Q). [See online article for color version of this figure.]
outer cell layers accumulated protein bodies at higher density than central endosperm cells, which are mainly dominated by starch granules (Supplemental Figs. S2 and S3, A and B). During the process of seed desiccation, cells and RER membranes were broken down due to the osmotic pressure created from the withdrawal of water. Zeins originally surrounded by RER membranes began to be exposed. The peripheral region of the kernel with more protein bodies and fewer starch granules formed a vitreous region (Supplemental Fig. S3, A and C), while the central region with more starch granules and fewer protein bodies formed the starchy region (Supplemental Fig. S3, B and D).

In o2 and zICRNAi mutants, the lower protein levels resulted in a reduced vitreous zone that gave rise to an opaque phenotype (Fig. 7, H and J). However, the opaque phenotype caused by loss of β- and γ-zeins was totally different (Fig. 7M). This difference could be explained by two possible mechanisms. One was differential partitioning of γ- and α-zeins. Although protein bodies in vitreous regions contained more α-zeins than those in the starchy regions (Dombrink-Kurtzman and Bietz, 1993), levels of γ- and β-zeins were never higher than the 22-kD α-zeins in total zein accumulation (Thompson and Larkins, 1994). This would explain why the width of the vitreous regions was not as much affected in the γRNAi mutant as it was in the zICRNAi mutant (Fig. 7, M and J).

The other reason was the specific properties of the Cys-rich zeins. It has been proposed that the α-zeins provided the “bricks” and the γ- and β-zeins the “cement” for the seed (Chandrashekar and Mazhar, 1999). This was consistent with the fact that α-zeins were deposited in the central area of protein bodies and existed as monomers while β- and γ-zeins were located at the periphery of protein bodies and formed polymers linked by disulfide bonds. When RER membranes broke down during desiccation, exposed zeins mixed with the other content of the cytoplasm, thereby interacting directly with starch granules. The cement acted like glue, which could then interconnect starch granules tightly in the peripheral vitreous region of the kernel (Fig. 8A). When the bricks were removed, starch granules were no longer embraced with a proteinaceous matrix (Fig. 8, B and C). When Cys-rich zeins were very low in the presence of both βRNAi and γRNAi transgenes, the net holding of starch granules within the peripheral region of the seed broke down, leaving the “underglued” starch granules loose (Fig. 8D). This mechanism would be consistent with a gradual reduction of the β- and γ-zeins throughout the seed and the patchy vitreous phenotype in the crown of the seed (Figs. 6, C–E, and 7, H–J).

**Figure 8.** Scanning electron micrographs of the peripheral regions of decapped wild-type and mutant dry kernels. A, BA. B, W64A o2. C, The zICRNAi mutant. D, The βRNAi and γRNAi combination. The starch granules (arrows) and the proteinaceous matrix mixed with broken protein bodies (arrowheads) are indicated. Bars at left = 100 μm; bars at right = 10 μm.
D and E). Indeed, the different physical properties of prolamin genes from gene duplications exemplify mechanisms by which important agricultural traits can emerge.

MATERIALS AND METHODS

Genetic Stocks

Maize (Zea mays) inbreds BSSS53, B73, B37, Mo17, W64A, W22, A69V, ILLIZE, A186, and A654 and genetic varieties SD-purple and p1-ww-1112 were from our own collection. The variety here referred to as p1-ww-1112 carries the p1-ww-1112 null allele (Athma and Peterson, 1991).

Plasmid Construction, Plant Transformation, and Total Zein Extraction

All primers for RNAi construction are listed in Supplemental Table S1. The RNAi transcripts were driven by the 27-kD \( \gamma \)-zein promoter amplified from maize inbred line B73 with the primer pair P27-EcoRI and P27-XmaI; the inverted 15-kD \( \beta \)-zein and 27-kD \( \gamma \)-zein coding sequences were amplified by two pairs of primers, 15kD-Xma1/15kD-BspE1 and 15kD-BglII/15kD-Xba1, respectively. The inverted 27-kD \( \gamma \)-zein and 15-kD \( \beta \)-zein genes were separated by the GFP-coding sequence in order to form a loop in the RNAi transcripts. It was amplified from the plasmid pEGFP (Clontech) with the primer pair GFPF and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. It was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. It was amplified from the plasmid pEGFP (Clontech) with the primer pair GFPF and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1 and GFP-BglII. T35S was amplified from the plasmid PTF102 with the primer pair T35S-Xba1 and T35S-BspE1.

RNAi Silencing Efficiency

A total of 40 kernels from a cross of \( g \)-RNAi and \( y \)-RNAi were analyzed. Half of the immature kernels were used for protein extraction and electron microscopy observation, and their embryos were used to extract genomic DNA individually for genotyping by PCR, as described above. Kernels with RNAi events and homozygous alleles for \( dza18-A654 \) and \( dza10-A654 \) were screened by PCR. The primer pair for the RNAi was described above. Since \( dza18 \) and \( dza10 \) are very conserved in DNA sequence, a common primer pair could be designed to amplify the two genes simultaneously. Compared with the functional alleles, \( dza18-A654 \) and \( dza10-A654 \) alleles had an insertion in the INDEL TTAT and a 10-kb transposon M601, respectively (Wu et al., 2009). Therefore, a common primer pair, \( dza18-10F \) and \( dza18-10R \), was designed to specifically amplify the two functional alleles, with the forward one at the TTAT site and the reverse one at the stop codon site, flanking the 10-kb transposon. The homozygous alleles for \( dza18-A654 \) and \( dza10-A654 \) were screened for the absence of the normal allele using the primer pair. Expression of the 22-kD sorghum (Sorghum bicolor) kafirins in the \( b \)-RNAi and \( y \)-RNAi combination was achieved by pollinating the combination with a homozygous kafirin transgenic plant. Immature kernels were collected at 18 DAP, and analysis was conducted as described above.

Light, Transmission, and Scanning Electron Microscopy

Previously published methods were used on immature kernels from BA, A654, W64A \( a2 \), and a series of mutant combinations of 18 DAP with some modifications (Burr and Burr, 1976; Lending and Larkins, 1992). A couple of 2-mm-thick sections were sliced perpendicular to the aleurone layer in order to include the pericarp, aleurone, and 10 to 20 cell layers of the endosperm. All these slices were fixed in 5\% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2\% Suc in a 2-mL tube. Fixation was kept at 4°C overnight and for another 3 h at room temperature. The tissues were rinsed for 2 to 3 h with several changes of 0.1 M sodium cacodylate buffer containing decreasing amounts of Suc. They were then postfixed in buffered 1\% osmium tetroxide at 4°C overnight followed by dehydration in a graded series of acetone washings and embedded in epon resin.

For light microscopy, the dehydrated samples were embedded in epon resin. The 1-\( \mu \)m-thick sections were cut with a glass knife and picked up on a glass slide. The sections were stained with methylene blue. Sections were also used to extract genomic DNA individually for genotyping by PCR, as described above. Kernels with RNAi events and homozygous alleles had an insertion by the INDEL TTAT and a 10-kb transposon Dzs10 and Dzs18, respectively (Wu et al., 2009). Therefore, a common primer pair Dzs10-A654 and Dzs18-A654 were screened for the absence of the normal allele using the primer pair. The combination of Different RNAi Events

The combination of the \( b \)-RNAi and \( y \)-RNAi was created by crossing and screened as described above. The combination of the \( \delta \)-zein double null mutant with the \( b \)-RNAi or \( y \)-RNAi mutant was accomplished by back-cross of the two RNAi mutants with A654 for two generations. After two generations, a number of immature kernels at 18 DAP were collected. The endosperms were used for protein extraction and electron microscopy observation, and their embryos were used to extract genomic DNA individually for genotyping by PCR, as described above. Kernels with RNAi events and homozygous alleles for \( dza18-A654 \) and \( dza10-A654 \) were screened by PCR. The primer pair for the RNAi was described above. Since \( dza18 \) and \( dza10 \) are very conserved in DNA sequence, a common primer pair could be designed to amplify the two genes simultaneously. Compared with the functional alleles, \( dza18-A654 \) and \( dza10-A654 \) alleles had an insertion in the INDEL TTAT and a 10-kb transposon M601, respectively (Wu et al., 2009). Therefore, a common primer pair, \( dza18-10F \) and \( dza18-10R \), was designed to specifically amplify the two functional alleles, with the forward one at the TTAT site and the reverse one at the stop codon site, flanking the 10-kb transposon. The homozygous alleles for \( dza18-A654 \) and \( dza10-A654 \) were screened for the absence of the normal allele using the primer pair. Expression of the 22-kD sorghum (Sorghum bicolor) kafirins in the \( b \)-RNAi and \( y \)-RNAi combination was achieved by pollinating the combination with a homozygous kafirin transgenic plant. Immature kernels were collected at 18 DAP, and analysis was conducted as described above.

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Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transmission electron micrograph of the \( b \)-RNAi and \( y \)-RNAi combination at a higher resolution.

Supplemental Figure S2. Light microscopy of the BA endosperm cells at 18 DAP.

Supplemental Figure S3. Scanning electron micrographs of the peripheral and central regions of BA endosperm from 18-DAP and mature dry kernels.

Supplemental Table S1. List of primers.

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