opaque-15, a maize mutation with properties of a defective opaque-2 modifier

(γ -zein/Quality Protein Maize/storage proteins)

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ABSTRACT An opaque mutation was identified that reduces γ -zein synthesis in maize endosperm. The mutation, opaque-15, causes a 2- to 3-fold reduction in γ -zein mRNA and protein synthesis and reduces the proportion of the 27-kDa γ -zein A gene transcript. Although the protein bodies in opaque-15 are similar in size and morphology compared to wild type, there are fewer of them in developing endosperm cells. The opaque-15 mutation maps near the telomere of chromosome 7L, coincident with an opaque-2 modifier locus. Based on its phenotype, opaque-15 appears to be a mutation of an opaque-2 modifier gene.

Mutations that reduce the synthesis of maize seed storage proteins, zeins, typically result in endosperm with a soft, starchy phenotype. Some of these mutations, notably opaque-2 (*o2*) and floury-2 (*fl2*), increase the synthesis of other endosperm proteins (1, 2), resulting in a higher lysine content (3, 4). Many years of effort were devoted to introgressing *o2* into agronomically important maize genotypes to create high-lysine corn. However, the soft, starchy endosperm of the mutant kernel caused it to be susceptible to mechanical damage and insect pests and ultimately prevented wide utilization of *o2* (5).

In recent years, the introgression of genes known as *o2* modifiers has solved many of the problems associated with the soft phenotype of *o2* mutants (6). The modifiers increase seed density, vitreousness, and γ -zein content of *o2* mutants (7). Activity of the modifier genes is associated with a 2- to 3-fold increase in the synthesis of the 27-kDa γ -zein mRNA and protein (8, 9) and appears to involve posttranscriptional regulation of γ -zein gene expression (9). Several breeding programs have used the modifier genes to develop vitreous genotypes with a high lysine content that are referred to as Quality Protein Maize (6). Although *o2* modification behaves as a complex quantitative trait (10), recent genetic evidence suggests that as few as two genes may be involved (11). In addition, two loci involved in *o2* modification have been identified by restriction fragment length polymorphism (RFLP) mapping (11); one locus maps near the centromere of chromosome 7, and the second maps near the telomere on the long arm of chromosome 7.

To isolate *o2* modifier genes and determine their mode of action, we screened a group of mutant seeds for reduced levels of the 27-kDa γ -zein protein. Here we report an opaque mutation, designated opaque-15 (*o15*), that significantly reduces γ -zein mRNA and protein without significantly affecting the accumulation of other zeins. The mutation maps near an *o2* modifier and, like an *o2* modifier, alters the ratio of mRNAs encoded by the A and B γ -zein genes. Understanding the biochemical basis of *o15* will help clarify the regulation of γ -zein gene expression and the factors responsible for development of vitreous endosperm in maize.

MATERIALS AND METHODS

Maize (*Zea mays* L.) kernels of the ethyl methanesulfonate-induced opaque mutant MGN-25:969-5 (*o15*) were obtained from M. G. Neuffer (University of Missouri, Columbia). The inbred lines W22+, W64A+, W64Ao2, W64Ao6 (opaque-6), and W64A *fl2* were from Purdue University (West Lafayette, IN). All inbreds and crosses were grown at the University of Arizona (Tucson).

Restriction endonucleases were purchased from Bethesda Research Laboratories/Life Technologies (Gaithersburg, MD). [α - 32 P]dATP and [α - 32 P]dCTP were obtained from DuPont/NEN. Digoxigenin-11-dUTP was obtained from Boehringer Mannheim. Nylon membranes were obtained from either Schleicher & Schuell or Micron Separations (Westboro, MA). Goat-anti-rabbit/10-nm gold was obtained from EY Laboratories.

Although the terms vitreous and opaque describe mature kernel phenotypes, developing kernels that are wild type (i.e., *O15*) will be called vitreous, whereas mutant kernels (i.e., *o15*) will be called opaque.

Analysis of Developing Endosperm. Six self-pollinated ears of MGN-25:969-5, heterozygous for the opaque mutation (*O15o15*), were harvested at the following developmental stages: 16, 20, 24, 28, 32, and 36 days after pollination (DAP). Heterozygous plants were necessary for the production of opaque kernels, because *o15* is lethal when homozygous. Forty kernels were harvested from each ear and cut in half. One half of the kernel was frozen in liquid nitrogen, lyophilized, and used for protein analysis to determine the normal or mutant phenotype. The other half was used for RNA or anatomical analysis.

Zein and nonzein proteins were isolated by the method of Wallace *et al.* (8) and separated by SDS/PAGE (12.5% acrylamide). After electrophoresis, gels were stained with Coomassie brilliant blue R250. For protein quantification, flour was pooled from 10 lyophilized half kernels from two ears at each stage. The relative amount of γ -zein was estimated by ELISA as described by Wallace *et al.* (8) and modified by Moro *et al.* (12).

For RNA analysis, 10 vitreous and opaque half kernels were pooled from two segregating ears at 20, 24, 28, and 32 DAP. Methods of RNA isolation, blotting, hybridization, and determination of the A/B γ -zein RNA ratios were as described (9).

DNA Isolation and Bulk-Segregant Analysis. Crosses were made between MGN-25:969-5 (heterozygous for *o15*) and the inbred W22+. The F₁ progeny were selfed and the homozygous opaque F₂ kernels were identified on a light box; the homozygous vitreous and heterozygous vitreous F₂ individuals were genotyped after selfing and F₃ segregation analysis. DNA was isolated from lyophilized leaf tissue or endosperm flour similar to the method of Saghai-Marooof *et al.* (13). DNA was

pooled from ≈ 20 F₂ homozygous individuals for each phenotype (vitreous and opaque). Approximately 10 μ g of DNA was digested to completion with the appropriate enzyme, separated in a 0.9% (wt/vol) agarose gel, and transferred to nylon membrane as described (14). Labeling of probes, prehybridization, hybridization, and chemiluminescent detection were as described (15, 16).

Anatomical and Immunocytochemical Studies. Half kernels were fixed and processed for microscopic examination and immunolocalization of α -zein and γ -zein following, with minor modifications, the detailed protocols of Lending and Larkins (17). Light micrographs were taken of endosperm samples embedded in Spurr's resin, whereas electron micrographs were taken of LR White resin embedded endosperm. For immunocytochemistry, antibody dilutions were 1:1000 α -zein, 1:500 γ -zein, and 1:75 goat anti-rabbit/10-nm gold. Immunocytochemical controls included omitting primary antiserum or substituting primary antiserum with preimmune rabbit serum.

Both 20 and 28 DAP kernels were sampled. For 20 DAP material, at least two kernels of each phenotype, vitreous and opaque, from at least three ears were examined. A total of 30 sections from 12 kernels on three segregating ears (20 DAP) were used to estimate the protein body number per cross-sectional area. Eight 50 μ m² squares (located 65–100 μ m from the aleurone) were randomly selected for each section. The mean number of protein bodies per 50 μ m² area ($\pm$ SEM) was calculated for each phenotype.

RESULTS

We have previously shown that the *o2* modifiers coordinately increase the γ -zein content and vitreousness of the endosperm in *o2* maize genotypes (7–9). Therefore, to search for mutants of *o2* modifiers we screened an ethyl methanesulfonate-generated population of opaque kernels for seeds with reduced amounts of γ -zein. Three candidates were found that showed a significant reduction in the 27-kDa γ -zein protein. Fig. 1 shows the proteins from one of these mutants, *o15*, compared with three well-known opaque mutations. The vitreous kernels, W64A+ and O15, contain high concentrations of the 19-

FIG. 1. SDS/PAGE separation of zein and nonzein proteins. (A) Zein proteins from 0.5 mg of endosperm flour. Molecular masses on the left (kDa) correspond to the zein classification on the right. (B) Nonzein proteins from 1.0 mg of endosperm flour. Molecular mass standards (kDa) are indicated on the left. The *o2*, *o6*, and *fl2* mutations are in the W64A(+) background; *o15* and O15 are in MGN-25:969-5.

and 22-kDa α -zeins, but these proteins are reduced in the *o2*, *o6*, and *fl2* kernels (Fig. 1A). Although α -zein synthesis is not significantly affected in *o15*, there is a large reduction in the 55- and 27-kDa γ -zein proteins. The nonzein proteins are increased in *o2*, *o6*, and *fl2* compared to their wild-type counterparts (Fig. 1B). Since there is not a pronounced increase in the nonzein proteins of *o15*, it is unlikely to be a high-lysine mutant.

o15 acts as a single, recessive gene and kernels that are homozygous for *o15* do not germinate. Therefore, to study the development of the mutant endosperm, we analyzed kernels from heterozygous ears. Distinctive differences in zein synthesis of the opaque and vitreous kernels were first detected at 20 DAP. Fig. 2A shows an SDS/PAGE analysis of zeins from vitreous and opaque kernels at 20, 24, 28, 32, and 36 DAP. Based on Coomassie blue staining, the γ -zein proteins are significantly reduced in the opaque kernels at each developmental stage. The reduction of γ -zein was quantitatively determined by ELISA with γ -zein antibodies. In developing kernels, γ -zein is approximately 2- to 3-fold less abundant in opaque versus vitreous endosperm (Fig. 2B).

To determine the effects of *o15* on zein gene expression, we compared the transcript levels between vitreous and opaque kernels from 20 to 32 DAP (Fig. 3). Although there was some variation in the relative amount of the 19-kDa (Fig. 3A) and 22-kDa (Fig. 3B) α -zein transcripts throughout development, the total amount of α -zein RNA was similar between vitreous and opaque endosperm. With the exception of 20 DAP, there was also little variation in the relative amount of β -zein transcripts between vitreous and opaque endosperm (Fig. 3C).

FIG. 2. Analysis of zeins from developing wild-type and *o15* kernels. (A) SDS/PAGE separation of zeins; proteins from 0.5 mg of endosperm flour of vitreous (lanes V) and opaque (lanes O) kernels from 20 to 36 DAP. (B) Quantitative estimate of γ -zein content by ELISA. Relative amount of γ -zein is based on the zero protein ELISA control. Data are presented as means $\pm$ SEM of three replicates for vitreous (hatched bars) and *o15* (solid bars) kernels.

FIG. 3. Comparison of zein RNA levels in wild-type and *o15* kernels. RNA was extracted from developing vitreous and opaque endosperm between 20 and 32 DAP and hybridized with cDNA probes corresponding to the 19-kDa α -zein (A), 22-kDa α -zein (B), 14-kDa β -zein (C), and 27-kDa γ -zein (D). Radioactivity was measured with a betascope blot analyzer (Betagen, Waltham, MA). Data are presented as means $\pm$ SEM of three replicates for vitreous (hatched bars) and *o15* (solid bars) kernels.

However, the level of the 27-kDa γ -zein was reduced by 50–60% in *o15* endosperm throughout development (Fig. 3D).

The γ -zein locus contains either one gene (A) or two genes (A and B) depending on the maize genotype (18, 19). The parental background of *o15* contains both A and B γ -zein genes. Transcripts of these genes were distinguished based on nucleotide differences using a PCR-based assay (9). In vitreous kernels (O15) at 24 and 28 DAP, the A/B transcript ratios were 2.5 and 2.2, respectively; whereas in the *o15* mutant the A/B ratios were 1.3 and 1.1, respectively (data not shown). Thus, the mutation appears to cause a preferential reduction in the level of the A gene transcript.

We used a combination of bulk-segregant analysis and RFLP mapping to determine the chromosomal location of the *o15* mutation. Since this mutation affects γ -zein synthesis/accumulation, we tested several RFLP probes near the γ -zein locus (near the centromere on chromosome 7), but none of these revealed any polymorphisms. We also used several probes diagnostic for *o2* modifier genes (11). One of these probes, *umc35*, identified several RFLPs with different restriction endonucleases that placed *o15* near an *o2* modifier on the distal end of chromosome 7L (Fig. 4).

Opaque mutations have generally been found to affect protein body structure and size. To investigate the phenotypic

FIG. 4. Bulk-segregant analysis and RFLP mapping of *o15*. Ten micrograms of DNA from each bulk was digested with *Hind*III (A) or *Xba* I (B) and probed with *umc35*. Lane 1, F₂ homozygous vitreous bulk; lane 2, F₂ homozygous opaque bulk. RFLPs are distinguished in the pooled DNA populations by *umc35* with both restriction enzymes.

effects of reduced γ -zein synthesis on protein body formation, we compared the structure of vitreous (O15) and opaque (*o15*) endosperm. At the light microscopic level (20 DAP), the protein bodies are prominent and near maximum size ($\approx 1 \mu\text{m}$) by the second starchy layer in both vitreous (Fig. 5A) and opaque (Fig. 5B) kernels; however, the vitreous kernel appears to have more protein bodies than the opaque kernel. The difference in protein body number is most obvious in the second through fifth starchy layers. We subsequently determined the number of protein bodies per cross-sectional area in the endosperm. Opaque endosperm had ≈ 2.5 times fewer protein bodies (5.5 ± 0.6 per $50 \mu\text{m}^2$) than vitreous endosperm (14.3 ± 1.8 per $50 \mu\text{m}^2$). A reduction in protein body number in *o15* was also evident at 28 DAP (data not shown).

FIG. 5. Light micrographs of wild-type and *o15* kernels. Vitreous (A) and opaque (B) endosperm (20 DAP) was sectioned to include the aleurone, subaleurone, and several starchy layers. A, aleurone; N, nuclei; P, protein bodies; S, subaleurone; St, starch grains. Cells and the protein bodies and starch grains within the cells nearest the aleurone are the smallest and least developed, becoming larger toward the internal portion of the endosperm. (Bar = $50 \mu\text{m}$.)

To examine the organization and developmental accumulation of zein proteins in *o15*, we compared the protein bodies in the first few cell layers of vitreous and opaque kernels by electron microscopy. The protein bodies of the vitreous kernels are larger than those of the opaque kernel in the subaleurone (Fig. 6 *A* and *B*) and first starchy endosperm layers. Endosperms of both genotypes are characterized by protein bodies with internal locules of lightly stained material within a darkly stained region, with the larger protein bodies having larger internal locules. By the second starchy layer, the size difference between the protein bodies of vitreous and opaque kernels is reduced, and, by the fifth starchy layer, the largest protein bodies of the two phenotypes are similar in size ($\approx 1 \mu\text{m}$ in diameter) and appearance (Fig. 6 *C* and *D*).

Localization of α -zein and γ -zein within the protein bodies of vitreous and opaque kernels is similar. Within the subaleurone layer, the α -zein generally appears to be located within the lightly stained locules of the protein bodies (Fig. 6 *E* and *F*). The larger protein bodies of the fifth starchy layer are densely labeled by α -zein antibodies (Fig. 6 *G* and *H*); the colloidal gold is mostly within the central lightly stained area, whereas the peripheral darkly stained area is less heavily labeled. Conversely, the γ -zein antibody generally labels the darkly stained areas of the protein bodies. The subaleurone (Fig. 6 *I* and *J*) protein bodies are more densely labeled with gold than those of the fifth starchy layer (Fig. 6 *K* and *L*). No significant labeling occurred on control material (data not shown).

DISCUSSION

The phenotype of maize endosperm is influenced by the structure and composition of the protein bodies, which are composed of four structurally distinct proteins—the α -, β -, γ -, and δ -zeins. Mutations that reduce α -zein synthesis, like *o2* and *fl2*, result in small protein bodies (19, 20) and are associated with the formation of a soft, starchy endosperm. Mutations that reduce either β - or γ -zein synthesis have not previously been described, and the roles these sulfur-rich proteins play in the process of protein body formation are poorly understood.

Although mutations that reduce γ -zein synthesis have not been previously identified, genes that dramatically increase the synthesis of these proteins are known. The so called *o2* modifier genes cause a 2- to 3-fold increase in the amount of γ -zein protein and mRNA in the endosperm (8, 9). As a consequence, they convert the soft, starchy endosperm of the mutant to a hard, vitreous phenotype. The molecular mechanism(s) by which *o2* modifier genes increase γ -zein synthesis is poorly understood. The increased accumulation of γ -zein mRNA occurs through a posttranscriptional mechanism that favors the A γ -zein gene transcript (9). As a consequence, the ratio of A to B γ -zein RNAs is increased from $\approx 2:1$ in wild-type and *o2* endosperm to 3.5:1 in modified *o2* endosperm. Since the sequences of the A and B RNAs are nearly identical except for the 3' noncoding region, it appears that the products of the modifier genes regulate mRNA translation or stability by interacting with this region of the mRNA.

FIG. 6. Protein body ultrastructure and immunolocalization of α - and γ -zein. Development of protein bodies was compared in cells of the subaleurone and fifth starchy layers from vitreous and opaque kernels. (*A*, *E*, and *I*) Vitreous, subaleurone. (*B*, *F*, and *J*) Opaque, subaleurone. (*C*, *G*, and *K*) Vitreous, fifth starchy layer. (*D*, *H*, and *L*) Opaque, fifth starchy layer. (*A*–*D*) Uranyl acetate- and lead citrate-stained sections. (*E*–*H*) Immunolocalization of α -zein. (*I*–*L*) Immunolocalization of γ -zein. (Bar = 1 μm .)

Several properties of *o15* suggest it is a mutant of an *o2* modifier. The mutation maps coincidentally with an *o2* modifier near the distal end of 7L. Like an *o2* modifier, *o15* specifically affects γ -zein mRNA and protein synthesis. The *o2* modifiers preferentially increase the accumulation of the A γ -zein gene transcript (9), whereas in *o15* there is a preferential reduction of the A gene RNA. We have no data regarding γ -zein gene transcription in *o15*, but since the promoters of the A and B genes are nearly identical (18), it is difficult to explain the altered A to B RNA ratio on the basis of transcription. An allelism test between *o15* and modified *o2* would ultimately confirm (or deny) that *o15* is a mutant of a modifier gene; however, the presence of the *o2* gene in the modified materials complicates the test. We have developed recombinant inbred lines that contain the modifier genes but lack the *o2* gene for this purpose.

γ -Zein has been postulated to play an important role in protein body initiation as well as in the development of vitreous endosperm (7). Protein bodies first develop as a network of disulfide-linked β - and γ -zein, to which there is a sequential accumulation of α -zeins that causes the protein bodies to enlarge (17). In mutants that reduce α -zein synthesis, such as *o2* and *fl2*, the protein bodies do not enlarge to the size of those in wild type (19, 20). If γ -zein and β -zein nucleate protein bodies and α -zeins enlarge them, one might predict a change in γ -zein would not result in a change in protein body size but perhaps in composition or number. Increasing the synthesis of γ -zein through the activity of the *o2* modifier genes does not increase the protein body size; rather, the protein bodies remain small as in *o2* (19). It is unknown whether the number of protein bodies differs in modified *o2* mutants compared to *o2* mutants. The reduction in γ -zein synthesis in *o15* does not change the size of the protein bodies; however, there is a decrease in the number of protein bodies compared to wild-type endosperm. The morphology of the protein bodies does not appear to greatly change with either an increase or a decrease in γ -zein, but a detailed developmental and spatial study of zein content within individual protein bodies is needed to address possible compositional differences. Although γ -zein content may directly influence the formation of protein bodies, and therefore the number of protein bodies, the mechanism by which an increase in γ -zein ultimately leads to a vitreous endosperm is not yet understood.

o15 seeds do not germinate when planted in soil or on moist filter paper. However, we have recently found that if the seeds are imbibed in sterile water and germinated on MS medium (21), the embryos develop normally. This indicates that the inability to germinate is a consequence of a nutritional deficiency. Since γ -zein is the first storage protein hydrolyzed during germination (22, 23), this suggests that the amino acids provided by this protein are essential for early development of the seedling.

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