Opaque2 Modifiers: What They Are and How They Work

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INTRODUCTION

The discovery in the early 1960s that the *opaque*2 (*o*2) gene increases the percent of lysine in maize endosperm prompted a great deal of optimism that this mutation would provide the basis for dramatically increasing the nutritional value of the grain. However, the euphoria over *o*2 was soon tempered with the realization that the pleiotropic effects of this mutation, a soft endosperm that results in damaged kernels, an increased susceptibility to insects and fungal pests, inferior food processing, and generally reduced yields, were not easily overcome. By the mid-1970s, there were no commercially important *o*2 genotypes grown in the U.S., nor in developing countries.

Not long after the discovery of *o*2, plant breeders began to recognize genes that alter the phenotype of *o*2 mutants, giving them a normal appearance. These genes, designated "*o*2 modifiers", proved to be genetically complex but nevertheless effective in ameliorating the negative features of the opaque phenotype. By systematically introgressing *o*2 modifier genes into *o*2 genetic backgrounds, while simultaneously monitoring the lysine content of the grain, plant breeders were able to develop a new type of *o*2 mutant, Quality Protein Maize. As documented elsewhere in this volume, this new version of *o*2 resolves the long-standing deficiencies of this mutant and offers the potential originally envisioned for "high lysine" corn.

A major focus of our research has been the characterization of the mechanism by which *o*2 modifiers cause the development of a hard, vitreous endosperm. We have also investigated the genetic complexity of the *o*2 modifiers and the molecular mechanism through which they

act. This chapter summarizes the results of our studies comparing patterns of protein synthesis and zein gene expression in normal, *o2* and modified *o2* genotypes, as well as the description of a new mutant we discovered with the properties of a defective *o2* modifier gene.

DISTINCTIVE PATTERNS OF ZEIN SYNTHESIS IN *o2* AND MODIFIED *o2* ENDOSPERM

The endosperm contains approximately 90% of the protein in a maize kernel, and consequently proteins in this tissue dictate the nutritional quality of the grain. In most normal maize genotypes, the storage proteins, or zeins, account for 60% to 70% of the endosperm protein. Since zeins are essentially devoid of lysine and tryptophan (Nelson, 1969; Shotwell and Larkins, 1989), they dilute the contribution of these essential amino acids from the other types of endosperm proteins, which we collectively call non-zeins (Wallace et al., 1990). Differences in the amount of zein and non-zein proteins determine the variation in lysine

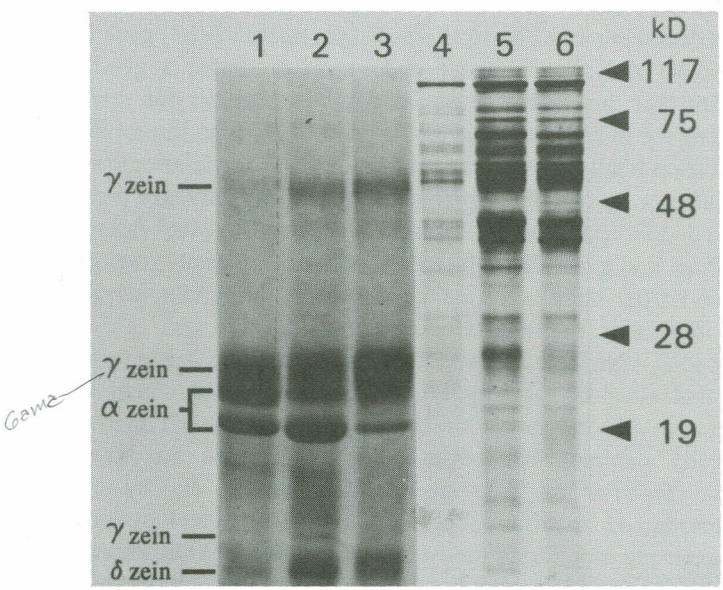

Fig. 1. SDS-PAGE separation of zein and non-zein proteins from Mo17+, *o2*, and modified *o2* maize endosperm. Proteins were extracted with an alkaline pH buffer containing 2-mercaptoethanol (Wallace et al., 1990). Non-zeins were separated from zeins by adjusting the extract to an alcohol concentration of 70%. Each lane represents protein from 1.5 mg of flour. Lane 1, Mo17+, zeins; Lane 2, Mo17*o2*, zeins; Lane 3, Mo17 modified *o2*, zeins; Lane 4, Mo17+, non-zeins; Lane 5, Mo17*o2*, non-zeins; Lane 6, Mo17 modified *o2*, non-zeins. The position and molecular mass of protein markers are shown on the right. Sub-groups of zein proteins are labeled on the left.

content among normal, *o2* and modified *o2* genotypes, while variation in the quantity and composition of zein proteins determines the vitreous phenotype of the kernel.

Figure 1 shows an SDS-PAGE separation of zein and non-zein proteins from equal amounts of flour of the normal, *o2* and modified *o2* versions of the inbred Mo17. In the normal genotype, the predominant zein proteins are the 22-kD and 19-kD alpha-zeins and the 27-kD gamma-zein (Fig.1, lane 1). The O2 gene encodes a transcription factor required for the expression of certain alpha-zein genes (Schmidt, 1993), although it also affects the transcription of other types of zein genes (Or et al., 1993). Thus, in both the *o2* (Fig. 1, lane 2) and modified *o2* mutants (Fig.1, lane 3) there is a marked reduction in alpha-zein content, especially of the 22-kD proteins. The distinctive difference between a starchy *o2* mutant and a modified *o2* mutant is the amount of the 27-kD gamma-zein protein. Although this is not easily quantified from the Commassie blue stained gel in Figure 1, based upon a sensitive immunological assay (ELISA), we generally find between two- and three-times more gamma-zein in modified *o2* mutants (Wallace et al., 1990; Geetha et al., 1991). As a consequence of the enhanced synthesis of the 27-kD gamma-zein, the storage protein content of modified *o2* mutants is higher and hence the percentage of lysine tends to be lower than in soft endosperm *o2* mutants.

Figure 1 also reveals a higher content of non-zein proteins in the *o2* and modified *o2* mutants, compared to the normal genotype (cf. lanes 4, 5 and 6). The increased synthesis of this group of proteins is largely responsible for the higher content of lysine in the *o2* mutants. An analysis of the non-zein proteins increased in *o2* mutants is described in this volume in the chapter by Habben et al.

Zein proteins are synthesized on rough endoplasmic reticulum membranes, and they associate within this organelle into insoluble accretions called protein bodies (Duvick, 1961; Larkins and Hurkman, 1978). We investigated the association of zein proteins during protein body development using antibodies against the various types of zeins (Lending and Larkins, 1989). This study revealed temporal differences in the appearance of zeins, as well as spatial differences in their organization within protein bodies. Figure 2 diagramatically illustrates the way in which protein bodies form in developing endosperm. The smallest and developmentally youngest protein bodies are found in the subaleurone cells and consist of aggregates of beta- and gamma-zeins. These structures subsequently enlarge through the accumulation of alpha-zeins, which coalesce forming locules within the network of beta- and gamma-zeins. In normal genotypes, the protein bodies grow to a

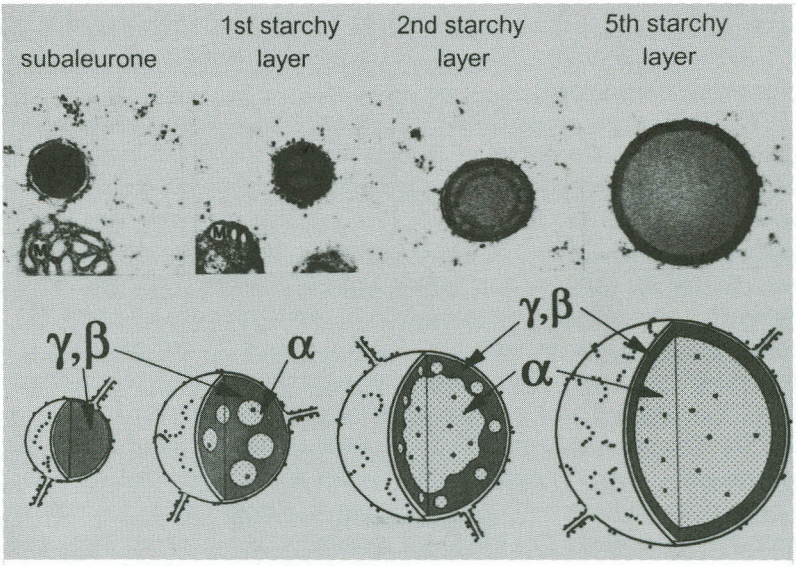

Fig. 2. Electron micrographs and model illustrating protein body development in maize endosperm. The protein body is surrounded by rough endoplasmic reticulum membrane (the dots represent ribosomes). The figures progressing from left to right represent protein bodies in the subaleurone, first, second and fifth starchy endosperm layers. Some beta- and gamma-zeins (heavily stippled) occur in regions that consist of primarily alpha-zein. The positions of alpha-, beta- and gamma-zeins in the protein body are indicated by the arrows.

diameter of one to two microns, and the alpha-zeins and the delta-zein (Esen and Stetler, 1992) fill the center, while the beta- and gamma-zeins are largely, though not completely, distributed toward the periphery.

The effect of the *o2* mutation on protein body formation is illustrated in Figure 3A. This micrograph shows adjoining cells in an Spm-mutable *o2* mutant (Schmidt et al., 1990). When the transposon excises from *o2*, it leaves behind a functional gene resulting in a cell with a normal phenotype. In this cell, the protein bodies are large and contain significant amounts of the light-staining alpha-zeins. However, in the *o2* mutant cell the protein bodies are small and dark-staining, indicating the reduced content of alpha-zeins and higher proportion of beta- and gamma-zeins.

Figure 3B shows an electron micrograph of developing endosperm from a modified *o2* mutant. Since modified *o2* mutants contain significantly more gamma-zein than their starchy *o2* counterparts, one would predict somewhat larger protein bodies with more of the dark-staining protein. Protein bodies in modified *o2* mutants have a variable amount of dark-staining, and in general their size is similar to that in starchy *o2* mutants. Because modified *o2* mutants contain two- to three-

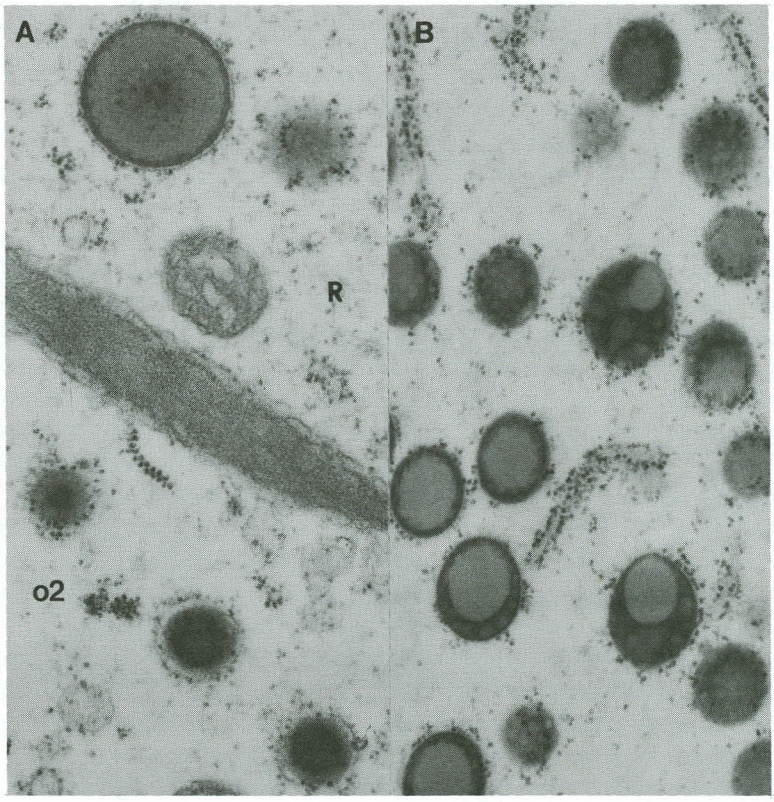

Fig. 3. Electron micrographs of developing maize endosperm from kernels of an Spm-mutable *o2* (*o2-m20*; Schmidt et al., 1990) and modified *o2* mutant (Pool 33 QPM). A. Sectored endosperm, *o2* (*o2*) and revertant (R) cells. B. Modified endosperm. Bar = 1 micron.

times more gamma-zein, they probably contain a larger number of protein bodies than their starchy *o2* counterparts.

o2 MODIFIERS REGULATE gamma-ZEIN mRNA ACCUMULATION

Because the principle effect of *o2* modifiers is on the regulation of gamma-zein synthesis, we have made an extensive analysis of the gamma-zein genes and their expression in normal, *o2* and modified *o2* genotypes. The 27-kD gamma-zein protein is encoded by one (Ra) or two (A and B) genes. The A and B genes are each found on a 12.5 kb tandem DNA duplication near the centromere of chromosome 7. A spontaneous rearrangement of this locus can occur somatically (Das et al., 1990), giving rise to the Ra allele. Because of the close proximity of

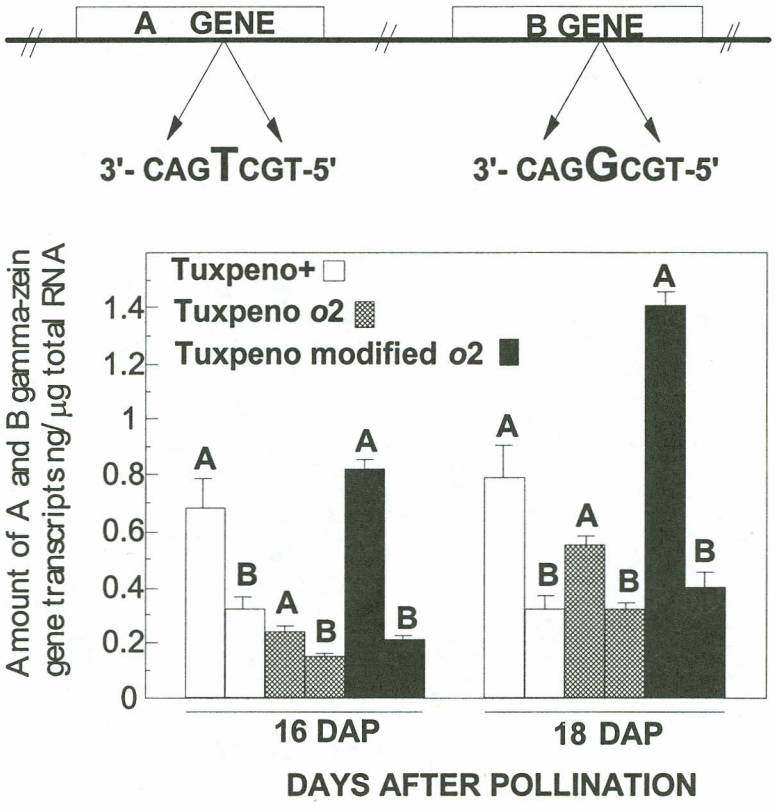

Fig. 4. Measurement of A and B 27-kD gamma-zein transcripts in developing endosperm of Tuxpeno+, Tuxpeno o2 and modified Tuxpeno o2. A and B transcripts were measured using oligonucleotide primers that distinguish the A and B RNAs by a single nucleotide (Or et al., 1993). Lines on top of the bars represent the standard deviations of the measurements. (From Or et al., 1993, by permission of ASPP)

the A and B genes, they behave as a single locus in genetic crosses. The A and B genes encode identical proteins, but the coding sequences can be distinguished by restriction enzyme site polymorphisms (Das and Messing, 1987; Lopes et al., 1995). Our analysis of the gamma-zein locus in a large number of modified *o2* genotypes has uniformly shown the presence of A and B gamma-zein genes (Lopes et al., 1995). Furthermore, crosses between modified *o2* and starchy *o2* genotypes with an Ra locus produced no vitreous progeny containing the Ra gene. Based on this limited analysis, it appears that a gamma-zein locus with A and B genes is required to develop the modified *o2* phenotype.

Figure 4 illustrates the expression of the A and B genes at early stages of endosperm development in Tuxpeno, Tuxpeno *o2* and modified Tuxpeno *o2*. To distinguish the A and B transcripts, we used a sensitive PCR-based assay with oligonucleotide primers that differentiate A and B RNAs by a single nucleotide (Or et al., 1993). At 16 days after pollination (DAP), the ratio of A to B transcripts in the normal genotype was approximately 2:1, while in *o2* the ratio was somewhat less than 2:1. In Tuxpeno modified *o2* there was more total gamma-zein mRNA (A + B) than in the *o2* or the wild type, and the ratio of A to B transcripts exceeded 3:1. By 18 DAP, there was a much higher rate of gamma-zein RNA accumulation in Tuxpeno modified *o2* than in the other two genotypes and the ratio of A to B RNAs continued to exceed 3:1. These results suggest that, while the *o2* modifiers generally increase the level of gamma-zein RNAs, by some mechanism they preferentially increase accumulation of the A transcript.

To determine if the increase in gamma-zein mRNA in the modified *o2* mutant was transcriptionally regulated, we performed a series of nuclear run-on transcription experiments (Or et al., 1993). These studies demonstrated that transcription of the gamma-zein genes (A + B) in Tuxpeno *o2* and modified *o2* was half the wild type level at both 16 DAP and 18 DAP. Thus, the increase in gamma-zein mRNA accumulation in modified *o2* mutants must be regulated post-transcriptionally. Since the principle difference between the A and B gamma-zein RNAs is in the 3' non-coding sequence, we hypothesize that the products of the *o2* modifier genes interact with these sequences and promote the accumulation of the A gene transcript (Or et al., 1993).

GENETIC ANALYSIS OF *o2* MODIFIER GENES

The *o2* modifiers are a genetically complex system (Vasal et al., 1980). This is partially a consequence of the triploid nature of the endosperm, but it is also reflected in the incomplete and unstable expression of the modifier genes and their variable penetrance in different genetic backgrounds (Belousov, 1987). While the number of modifier genes is unknown, genetic segregation analysis suggests there are probably two major loci (Lopes and Larkins, 1995; Lopes et al., 1995). Using RFLPs and a bulk segregation analysis of crosses between *o2* and modified *o2* genotypes, we identified two loci associated with formation of vitreous endosperm. One locus was tightly linked with the gamma-zein coding sequences near the centromere of chromosome 7, while the other was near the telomere of the long arm of chromosome 7 (Lopes et al., 1995). Details of the genetic characterization of these loci appear in this volume in the chapter by Lopes et al.

CHARACTERIZATION OF A MUTANT *o2* MODIFIER

Since an increase of gamma-zein synthesis in a starchy *o2* mutant can create a vitreous phenotype, we postulated that a mutation reducing gamma-zein synthesis would lead to a starchy endosperm. Upon screening a large number of starchy endosperm mutants provided to us by Dr. M.J. Neuffer (University of Missouri, Columbia), we identified a

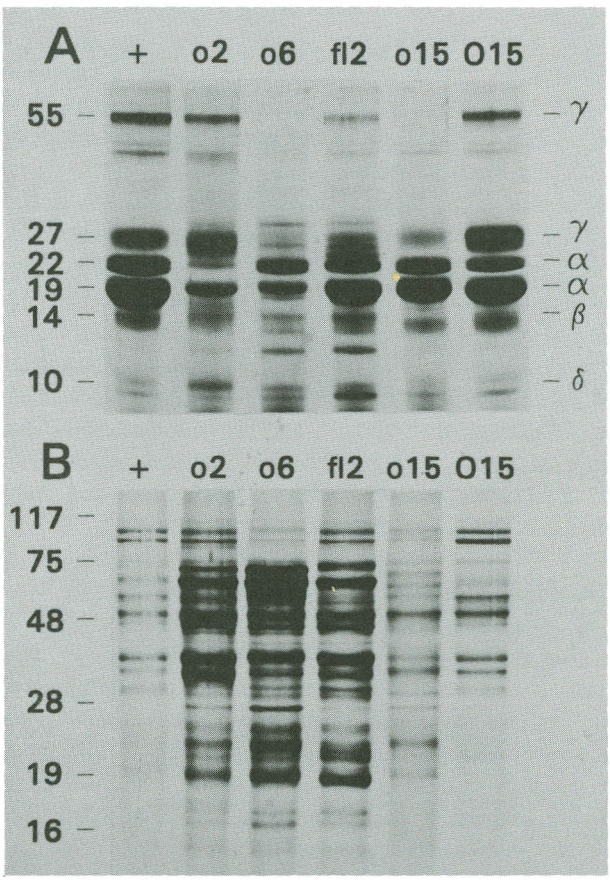

Fig. 5. SDS-PAGE of zein and non-zein proteins from normal and opaque mutant genotypes. (A) Zein proteins from 0.5 mg of endosperm flour. Molecular masses on the left (kD) correspond to the zein classification on the right. (B) Non-zein proteins from 1.0 mg of endosperm flour. Molecular mass standards (kD) on the left. The *o2*, *o6*, and *fl2* mutations are in the W64A+ background; *o15* and O15 are in MGN-25:969-5. (From Dannenhoffer et al., 1995, by permission of N.A.S. Press)

mutant (*o*15) that showed a marked reduction in gamma-zein synthesis (Dannehoffer et al., 1995). Figure 5 compares the zein and non-zein proteins in normal and *o*15 endosperm with that in W64A+ and several starchy endosperm mutants (*o*2, *o*6, and *fl*2) in this inbred. The *o*6 mutation corresponds to a defect in proline synthesis (Gavazzi et al., 1975) and causes a generalized reduction in storage protein synthesis, while *fl*2 appears to correspond to a defective alpha-zein protein whose signal peptide is not cleaved (Lopes et al., 1994; Coleman et al., 1995). As a consequence of the *fl*2 mutation, there is also a general reduction in all four types of zeins. The diagnostic feature of the *o*15 mutation is the marked reduction in the 27-kD gamma-zein protein, as well as its aggregated form that migrates at 55-kD. A comparison of the non-zein proteins from equal amounts of flour from these six genotypes indicates that *o*15 does not cause a pronounced increase in the non-zein proteins, hence it is probably not a high lysine mutant, like *o*2 and *fl*2.

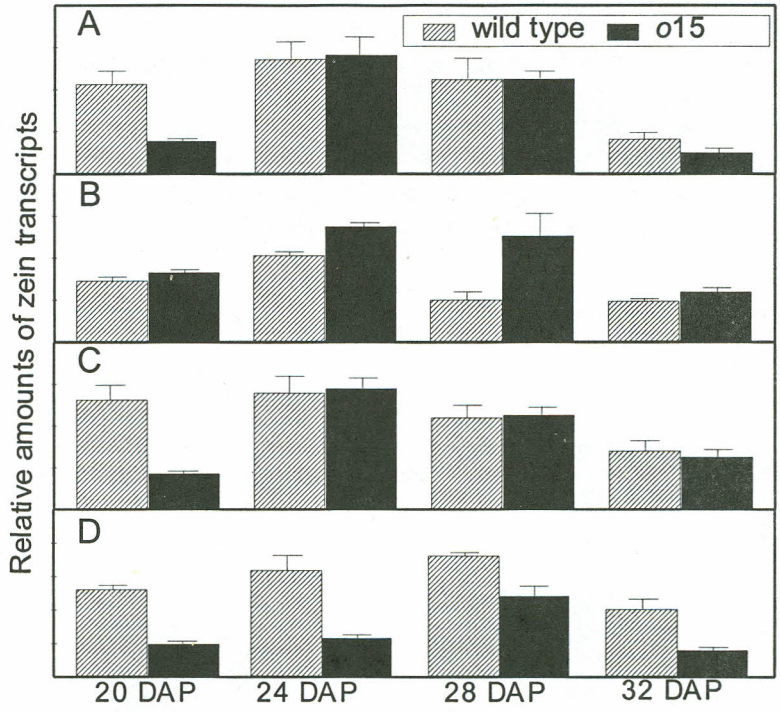

Fig. 6. Comparison of zein RNA levels in wild-type and *o*15 kernels. RNA was extracted from developing normal and mutant endosperm between 20 DAP and 32 DAP and hybridized with cDNA probes corresponding to the 19-kD alpha-zein (A), 22-kD alpha-zein (B), 14-kD beta-zein (C), and 27-kD gamma-zein (D). Data are presented as means plus standard errors of the mean. (From Dannenhoffer et al., 1995, by permission of N.A.S.Press)

The effect of *o*15 on zein protein synthesis is reflected at the mRNA level. Figure 6 illustrates the level of 19-kD and 22-kD alpha-zeins (Fig. 6, panels, A and B), the 14-kD beta-zein (Fig. 6, panel C) and the 27-kD gamma-zein RNAs (Fig. 6, panel D) in *o*15 and its wild type counterpart. There was some variation in alpha-zein RNAs in the normal and mutant genotypes between 20 and 32 DAP, and the level of 22-kD RNA may be somewhat higher in the *o*15 mutant. However, there is relatively little difference in beta-zein RNA between the two genotypes at all stages of development. Differences in the level of gamma-zein RNA are apparent throughout endosperm development, with about a 50% average decrease in *o*15. Thus, except for the slight increase in 22-kD alpha-zein mRNA, the primary effect of the *o*15 mutation is on gamma-zein mRNA accumulation.

As noted earlier, *o*2 modifiers increase the amount of gamma-zein mRNA and cause a preferential increase in the level of the A transcript (Or et al., 1993). When we measured the level of A and B gamma-zein RNAs in *o*15 (Fig. 7), we found that the ratio changed from 2:1 in the wild type to 1:1 in the mutant, just as one would predict for a mutation in an *o*2 modifier. Furthermore, we were able to map the *o*15 mutation near the telomere of 7L with the same RFLP marker that identified the *o*2 modifier locus in this region (Dannenhoffer et al., 1995). These data are consistent with the hypothesis that *o*15 corresponds to a defective *o*2 modifier. It follows that the second *o*2 modifier locus in *o*15 must carry

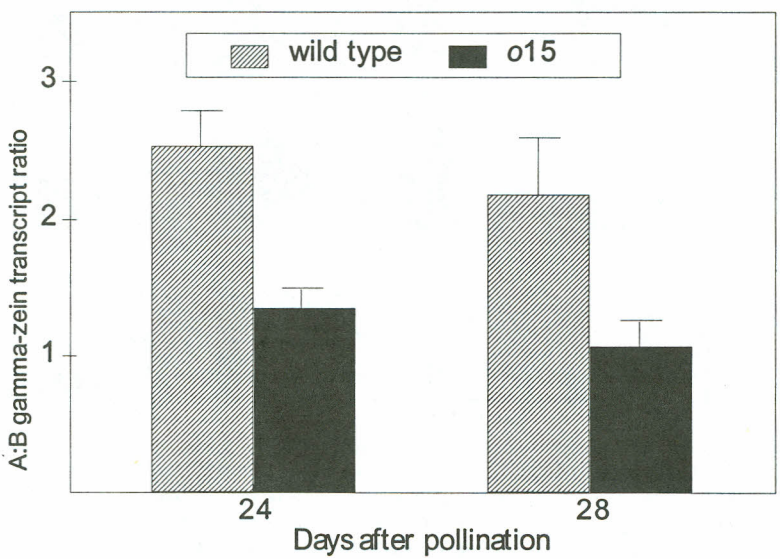

Fig. 7. Comparison of the A and B gene 27-kD gamma-zein transcript ratios in developing endosperm of wild-type and *o*15 kernels.

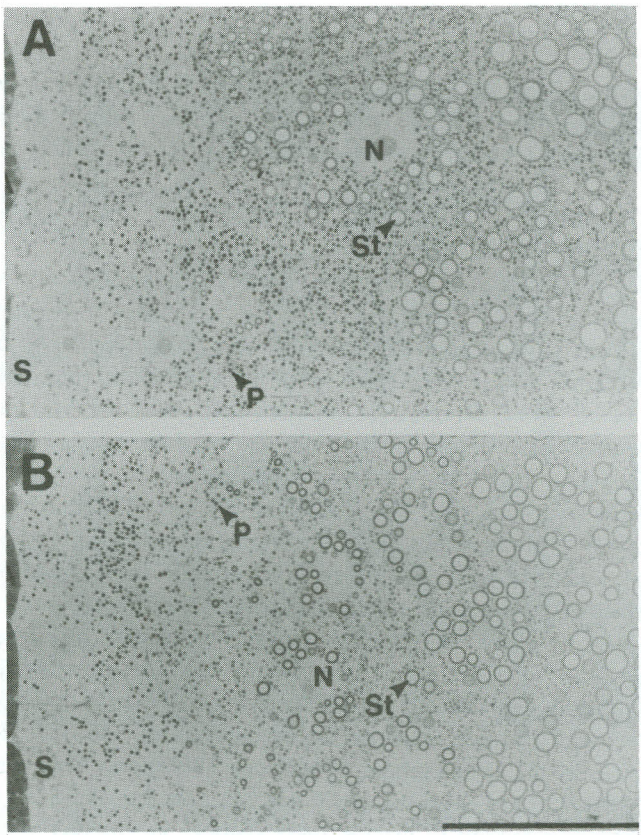

Fig. 8. Light micrographs of developing normal and *o*15 mutant endosperms. The normal (A) and *o*15 (B) endosperm (20 DAP) was sectioned to include the aleurone, subaleurone, and several starchy endosperm layers. A, aleurone; N, nucleus; P, protein body; S, subaleurone; St, starch grain. Cells, protein bodies, and starch grains within the cells nearest the aleurone are the smallest and least developed and become larger toward the internal portion of the endosperm. (Bar = 50 microns) (From Dannenhoffer et al., 1995, by permission of N.A.S. Press)

a weak or null allele.

To investigate the effects of reduced gamma-zein synthesis on protein body formation in *o*15, we compared the structure of normal and mutant endosperm. Figure 8 shows light micrographs of the wild type and *o*15 endosperm at 20 DAP. At this level of magnification it can be seen that protein bodies are prominent and near maximum size (about 1 micron) in the second starchy endosperm layer of both normal and mutant kernels. Nevertheless, there appears to be a larger number of protein bodies in endosperm cells of the normal genotype. When we determined the number of protein bodies per cross-sectional area, we found about 2.5-times more protein bodies in normal than mutant

endosperm. These data suggest the reduction in gamma-zein does not necessarily lead to smaller protein bodies. Rather, it may be the reduced number of protein bodies in *o*15 that causes the starchy phenotype.

RELATIONSHIP BETWEEN PROTEIN BODIES AND ENDOSPERM HARDNESS

The mechanism(s) responsible for the formation of vitreous endosperm in maize have been difficult to define. A number of investigators have attempted to explain this phenotype by comparing differences in protein composition of the starchy and vitreous endosperm (Paiva et al., 1991; Dombrink-Kurtzman and Bietz, 1993). However, these studies have produced only correlative data, much of which is inconclusive. Based on what we have learned by studying opaque mutants, it is clear that the size, shape and number of protein bodies can individually or collectively affect the formation of vitreous endosperm. It is generally thought that the reduced size of protein bodies in *o*2 mutants causes the opaque phenotype. Yet, modified *o*2 mutants have small protein bodies and develop vitreous endosperm. Furthermore, protein bodies in *o*15 are near normal size, but this mutant develops a starchy phenotype. While it is possible that gamma-zein, by virtue of its ability to form disulfide linkages (Lopes and Larkins, 1990), could play a direct role in the development of vitreous endosperm, this phenotype could also be related to protein body number. To date, there has been no systematic comparison of protein body number in vitreous and starchy genotypes, but such a study could be instructive. Although our analysis of protein bodies in *o*15 and modified *o*2 mutants is incomplete, the results imply that gamma-zein plays a key role in the initiation of protein body formation. Genotypes with reduced levels of gamma-zein would be predicted to have soft endosperms and fewer protein bodies.

High levels of alpha-zein have also been found to correlate with a hard, vitreous endosperm (Moro et al., 1994). Since alpha-zeins are confined to the center of the protein body, it is difficult to imagine that this relationship is based on interactions between alpha-zeins and other endosperm proteins. Rather, the quantity of alpha-zein more likely provides an index of the number of protein bodies.

Figure 9 illustrates our hypothesis regarding the relationship between protein body size and number and the development of vitreous

Fig. 9. Model of the relationship of protein body size and number and the development of vitreous endosperm. When the endosperm desiccates, the interaction of protein bodies (black spheres) with other protein bodies and/or the cytoplasmic matrix (CM) is dependent on the size and number of protein bodies. Large protein bodies or many protein bodies results in endosperm through which light can be transmitted.

DEVELOPING
MATURE
Wild type
opaque2
modified opaque2
opaque15
S
cm

endosperm. As the endosperm begins to desiccate, the membranes surrounding protein bodies disintegrate, allowing the protein bodies to interact with one another, as well as with proteins in the dehydrating cytoplasmic matrix. Disulfide linkages could form between gamma-zein proteins on the surface of protein bodies, or between gamma-zeins and non-reduced disulfide groups in the cytoplasmic proteins. With a larger number of protein bodies, there is greater potential for the formation of a continuous proteinaceous matrix around the starch grains, which would promote transmission of light through the fully desiccated endosperm. There is less potential for this to occur in *o*2 or *o*15 mutants that have a small number of protein bodies, because of air spaces that form around the starch grains. The discontinuities created by the air spaces would disrupt light transmission.

WHAT ARE *o*2 MODIFIERS AND HOW DO THEY WORK?

Based on our research, we are able to propose the following hypothesis regarding the number of *o*2 modifier genes and their mechanism of action. There appear to be two co-dominant, unlinked modifier genes, whose activity increases synthesis of the 27-kD gamma-zein protein through a post-transcriptional mechanism involving the 3' non-coding sequence of the mRNA . One of the modifier genes is near the telomere of chromosome 7L. By increasing the synthesis of gamma-zein, the *o*2 modifiers promote the formation of protein bodies, which ultimately leads to the formation of a more vitreous endosperm. Further characterization of the *o*15 gene product, or the gene itself, should lead to a better resolution of the molecular mechanism through which the *o*2 modifier genes increase the synthesis of gamma-zein.

ACKNOWLEDGMENTS

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