opaque2 Modifiers Act Post-Transcriptionally and in a Polar Manner on γ-Zein Gene Expression in Maize Endosperm

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The opaque2 (o2) modifier genes convert the soft endosperm of an o2 mutant to a hard, vitreous phenotype. The primary biochemical change associated with the expression of these genes is a two- to threefold increase in synthesis of the 27-kD γ-zein storage protein. To investigate the mechanism of modifier gene activity, we examined the level of γ-zein mRNA and protein synthesis during the early stages of endosperm development in normal, o2, and modified o2 genotypes. Although the o2 mutation was found to reduce expression of the 27-kD γ-zein genes, the activity of o2 modifier genes dramatically increased the level of both γ-zein protein and mRNAs as early as 16 days after pollination. At this stage, transcription of γ-zein genes is reduced by ~50% in both o2 and modified o2 genotypes compared to wild type. Thus, it appears that the modifiers regulate γ-zein synthesis through a post-transcriptional mechanism. Analysis of transcripts from the two nearly identical genes (A and B) encoding the 27-kD γ-zein protein showed differences in the mRNA ratios in different genotypes. In modified o2 mutants, accumulation of A over B transcript was greatly enhanced during endosperm development. Somatic recombination at this locus was found to reduce the number of B genes in the endosperm, but this could not account for the preferential accumulation of the A transcript. Our results suggest that a product of the o2 modifier genes increases the translation or stability of the A gene mRNA, leading to enhanced synthesis of 27-kD γ-zein protein.

INTRODUCTION

The maize opaque2 (o2) mutation dramatically increases the percent of lysine in the grain (Mertz et al., 1964), but the soft, starchy endosperm of the mutant kernel causes it to be very susceptible to insect pests and mechanical damage (Ortega and Bates, 1983). These problems, as well as the reduced yield and protein content of the seed, were largely responsible for preventing the so-called “high-lysine” maize from gaining wide acceptance. Shortly after the initial characterization of o2 maize, genes were identified that improved the phenotype of the mutant, giving it a normal appearance (Paez et al., 1969). These genes, designated o2 modifiers, were subsequently used by several groups, including plant breeders at the International Maize and Wheat Improvement Center (CIMMYT) (Villegas et al., 1992) and in Pietermaritzburg, South Africa (Geevers and Lake, 1992) to develop maize varieties with normal kernel hardness and protein content, as well as an enhanced percentage of lysine. These modified o2 genotypes are designated Quality Protein Maize (QPM) (Vasal et al., 1980).

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). The number of modifier genes is unknown, although genetic segregation analysis suggests that there are probably not more than two or three loci (Lopes and Larkins, 1991; M. A. Lopes and B. A. Larkins, unpublished data). These genes act in a semidominant fashion and are associated with an increased content of the 27-kD γ-zein storage protein (Geetha et al., 1991). Typically, modified o2 mutants contain two to four times more γ-zein protein than unmodified mutants (Wallace et al., 1990).

The mechanism by which an increase in the 27-kD γ-zein protein converts an opaque kernel to a normal, vitreous phenotype is unknown. It appears that a two- to threefold increase in this protein is sufficient to induce this phenotypic change (Wallace et al., 1990). The γ-zein is cysteine rich and is found primarily in the outer region of protein bodies (Ludevid et al., 1984; Lending and Larkins, 1989). Although it can be extracted in small quantities from developing seeds without a reducing agent, it becomes highly cross-linked by disulfide bonds in maturing seeds and is difficult to solubilize (Wallace et al., 1990). Thus, the mechanism by which γ-zein converts an opaque seed to a vitreous phenotype may involve cross-linkage of the protein through disulfide bridges (Lopes and Larkins, 1991; Paiva et al., 1991).

The genes encoding the 27-kD γ-zein occur in one or two copies, depending on the genetic background (Das et al., 1987; Geetha et al., 1991). The locus is a tandem duplication of 12.5 kb, with the genes oriented in the same 5' to 3' polarity. By
convention, the first coding sequence is designated the A gene
and the second is the B gene. The amino acid sequences of
the proteins encoded by the A and B genes are identical, but
the genes differ in nucleotide sequence and mRNA levels. In
the W22 inbred, transcripts of the B gene are more than twice
as abundant as the A gene (Das et al., 1987). Interestingly,
this locus is somatically unstable, and, depending on the geno-
type, rearrangements occur in which the B gene is deleted
(Das et al., 1990a).

To better understand the mechanisms by which o2 modifier
genes act developmentally, we compared the pattern of γ-zein
protein and mRNA synthesis in several normal and modified
o2 genotypes, including a series of near isogenic materials
Tuxpeno, Tuxpeno o2, and Tuxpeno QPM. Comparison of zein
gene expression in normal, o2, and QPM genotypes between
16 and 22 days after pollination (DAP) revealed that o2 modi-
fiers affect γ-zein synthesis as early as 16 DAP by increasing
the level of the mRNA. The effect of the modifier genes is
manifested primarily through a post-transcriptional mechanism
that principally increases the level of A γ-zein gene mRNA.

RESULTS

o2 Modifiers Enhance the γ-Zein Protein Level Early in
Development

The enhanced production of γ-zein protein in modified o2 mu-
tants is detectable early in development, as illustrated in Figure
1A, which shows an SDS-PAGE comparison of zein proteins
from 18-DAP kernels of three normal genotypes, Tuxpeno (lane
1), A188 (lane 3), and W64A (lane 5) and the modified o2 mu-
tants Pool 33 QPM (lane 2) and CMS450 QPM (lane 4). In the
normal genotypes, the predominant proteins are α-zeins, cor-
responding to the 19- and 22-kD bands, although β-, γ- and
δ-zeins are also present at this stage. There is some variation
in the proportion of the 19- and 22-kD components between
the normal genotypes; however, combined they account for
~70% of the total zein protein. In the modified o2 genotypes
(Figure 1A, lanes 2 and 4), there is relatively little α-zein as
a consequence of the defective Opaque2 (O2) transcription
factor (Hartings et al., 1989; Schmidt et al., 1990). At 16 DAP,
the most prevalent storage protein is the 27-kD γ-zein, which
accounts for ~95% of the total zein. As shown in Figure 1B,
based on laser densitometry, there is ~1.5 to 3 times more
27-kD γ-zein in Pool 33 QPM and CMS450 QPM than in any
of the normal genotypes. In both of the modified o2 genotypes,
the β-zein protein is not detectable by Coomassie blue staining,
and the 10-kD δ-zein is also not apparent.

The increased synthesis of the 27-kD γ-zein protein in the
modified o2 seeds correlates with a higher level of mRNAs
encoding this protein. As shown in Figure 2, when we com-
pared the steady state levels of the 27-kD γ-zein transcripts
at 18 DAP in these five genotypes, both of the modified o2
materials, Pool 33 QPM (lane 2) and CMS 450 QPM (lane 4),

Figure 1. SDS-PAGE Analysis of Zeins from Developing Kernels of
Tuxpeno, Pool 33 QPM, A188, CMS450 QPM, and W64A.

(A) Zeins were extracted from kernels 18 DAP, as described in Methods,
and separated on a 12.5% SDS–polyacrylamide gel by electrophore-
sis. Proteins were extracted from equal amounts of kernel flour; samples
corresponding to 0.75 mg of flour were loaded onto the gel. Lane 1,
Tuxpeno; lane 2, Pool 33 QPM; lane 3, A188; lane 4, CMS450 QPM;
lane 5, W64A. Molecular masses of the protein standards are indicated
on the left and the type of zein is indicated on the right of the gel.

(B) Relative amount of 27-kD γ-zein in endosperm 18 DAP from sam-
ples shown in (A). The Coomassie blue–stained gel was scanned with
a laser densitometer, and the absorbance values were used to esti-
mate the relative amount of 27-kD γ-zein. The values were normalized
against the lowest amount of γ-zein, which was the W64A sample.

contained 1.5 to 2.5 times more transcript than the normal geno-
types. W64A (Figure 2, lane 5) contained the smallest amount
of γ-zein transcripts among the normal genotypes. This may
be a consequence of W64A having only a single 27-kD γ-zein
gen, whereas A188 and Tuxpeno as well as Pool 33 QPM and
CMS450 QPM contain two genes (Das and Messing, 1987;
Geetha et al., 1991).

The specific increase in γ-zein protein and RNA transcripts
appears to be a consistent effect of o2 modifiers, regardless
of the genetic background. However, because of the variation
in γ-zein gene number and the genetic heterogeneity of maize
lines, we decided to further investigate the activity of modifier genes in three near-isogenic materials: Tuxpeno, Tuxpeno o2, and the modified Tuxpeno o2 (QPM). Figure 3A shows an SDS-PAGE separation of zein proteins from endosperms from these three genotypes: 16, 18, and 22 DAP. In the wild type, the most abundant proteins at all three developmental stages (Figure 3A, lanes 1, 4, and 7) are α-zeins, but the β- and γ-zeins are also detectable at these stages. In Tuxpeno o2 (Figure 3A, lanes 2, 5, and 8), the major storage protein is the 27-kD γ-zein protein. It is found in small amounts at 16 DAP, but it accumulates significantly between 16 and 22 DAP. The 19-kD α-zein, which is detectable only after 18 DAP, is greatly reduced in this mutant at all developmental stages, and the 22-kD α-zeins and the 14-kD β-zein are not observed by Coomassie blue staining. The zein composition of Tuxpeno QPM resembles that of Tuxpeno o2, but shows some quantitative differences. The activity of the o2 modifier genes results in levels of the 27-kD γ-zein protein that are higher than in the wild type or unmodified

Figure 2. Analysis of the Level of γ-Zein Transcripts in Developing Kernels of Tuxpeno, Pool 33 QPM, A188, CMS450 QPM, and W64A.

(A) RNA gel blot analysis showing the relative levels of 27-kD γ-zein at 18 DAP. Total RNA was extracted as described in Methods, and 15-μg samples were loaded in each lane. After gel blotting, the membrane was hybridized to a radioactive fragment of the 27-kD γ-zein coding sequence. Lane 1, Tuxpeno; lane 2, Pool 33 QPM; lane 3, A188; lane 4, CMS450 QPM; lane 5, W64A.

(B) RNA bands shown in (A) were excised, and the radioactivity was measured by liquid scintillation spectroscopy. The results are representative of three different measurements.

Figure 3. Accumulation of Zeins in Developing Kernels of Tuxpeno, Tuxpeno o2, and Modified Tuxpeno o2.

(A) Zeins were extracted from kernels 16, 18, and 22 DAP and separated, as described in Figure 1. Lanes 1, 4, and 7, Tuxpeno; lanes 2, 5, and 8, Tuxpeno o2; lanes 3, 6, and 9, modified Tuxpeno o2. Molecular masses of protein standards are indicated on the left side and the types of zein on the right side of the gel.

(B) Relative content of the 27-kD γ-zein in developing endosperm. The Coomassie blue-stained gel shown in (A) was scanned with a laser densitometer, and the relative amount of γ-zein estimated, as described in Figure 1. Stippled bars, Tuxpeno; cross-hatched bars, Tuxpeno o2; solid bars, modified Tuxpeno o2. The values shown were normalized against the smallest amount of γ-zein, which was that in Tuxpeno o2 at 16 DAP. The results are representative of three separate measurements.

To better estimate the relative amounts of γ-zein in these lines, the Coomassie blue-stained gel was scanned by laser densitometry. The data in Figure 3B illustrate that there is significant accumulation of the 27-kD γ-zein in all three genotypes, although it is found at the lowest concentration in Tuxpeno o2. By 16 DAP, Tuxpeno QPM contains five times more γ-zein protein than the unmodified o2 mutant, and it has twice the amount of γ-zein found in the normal genotype. The differences in the ratios of γ-zeins between these genotypes decreased somewhat at 18 and 22 DAP, but became more pronounced at later
Figure 4. Analysis of the Steady State Levels of Zein Gene Transcripts in Developing Kernels of Tuxpeno, Tuxpeno 02, and Modified Tuxpeno 02.

Total RNA was isolated from kernels 16, 18, and 22 DAP as described in Methods. The RNA was immobilized on a nylon membrane using a dot blot apparatus and hybridized to radioactive cDNA sequences corresponding to a 22-kD α-zein, the 14-kD β-zein, the 27-kD γ-zein, and the 10-kD δ-zein. The amounts of RNA applied to the filters are as follows: 0.3 μg for the α- and β-zein blots; 1.5 μg for the γ-zein blots; and 4.0 μg for the δ-zein blots. The type of probe is indicated for each figure. Radioactivity was measured with a Betascope; the values are the average of three separate measurements. Stippled bars, Tuxpeno; cross-hatched bars, Tuxpeno 02; solid bars, modified Tuxpeno 02. Lines on top of the bars represent the standard deviations. 

(A) Level of 22-kD α-zein transcripts. 
(B) Level of 14-kD β-zein transcripts. 
(C) Level of 27-kD γ-zein transcripts. 
(D) Level of 10-kD δ-zein transcripts.

stages of development (data not shown). Nevertheless, it is clear from this analysis that the effect of the modifier genes on the accumulation of the 27-kD γ-zein protein is apparent as early as 16 DAP.

o2 Modifiers Compensate for the Reduction in γ-Zein mRNA Caused by the o2 Mutation

To assess the degree to which changes in zein protein synthesis are reflected at the mRNA level, RNA from kernels of the isogenic Tuxpeno series was hybridized with cDNA probes corresponding to α-, β-, γ- and δ-zeins. Figure 4 shows the developmental accumulation of transcripts encoding these proteins between 16, 18, and 22 DAP. For this analysis, we used a cDNA clone corresponding to the subfamily of 22-kD α-zeins that is regulated by o2 (Kodrzycki et al., 1989). Figure 4A shows that in the normal genotype, there is continuous accumulation of 22-kD α-zein transcripts between 16 and 22 DAP; however, these RNAs were dramatically reduced in both the o2 and Tuxpeno QPM mutants. The levels of β-zein (Figure 4B) and δ-zein (Figure 4D) transcripts are also significantly reduced in Tuxpeno o2 and Tuxpeno QPM compared to normal. By 22 DAP, the level of β-zein transcript is reduced more than fourfold compared to the wild type, whereas the δ-zein transcript is reduced approximately sixfold. The β-zein RNA occurs at similar concentrations in Tuxpeno o2 and Tuxpeno QPM at all three stages. Although there are similar concentrations of δ-zein RNA in the mutants at 16 DAP, in Tuxpeno QPM the level of this RNA appears to decline between 16 and 22 DAP.

The level of the 27-kD γ-zein mRNA increased in all three genotypes between 16 and 22 DAP (Figure 4C), but the kinetics of accumulation were quite different. At 16 DAP, the concentration of γ-zein transcripts in Tuxpeno normal and Tuxpeno QPM was 2.7 times higher than in Tuxpeno o2. Between 16 and 18 DAP, there was a slight increase in the amount of γ-zein transcripts in the normal genotype, but the concentration nearly doubled in Tuxpeno o2 and Tuxpeno QPM during this period. By 22 DAP, the level of transcripts was similar between the Tuxpeno normal and o2 genotypes, but both were 30% less than in Tuxpeno QPM. Thus, the o2 mutation causes a reduction in the level of γ-zein mRNA, but this is offset through the activity of the modifier genes.

The γ-Zein A Gene Transcript Preferentially Accumulates in the Modified o2 Mutant

The 27-kD γ-zein is encoded by two nearly identical genes (A and B) that differ in their level of expression. In the inbred W22, there is approximately three times more B than A transcript (Das and Messing, 1987). To determine whether o2 modifiers differentially affect the expression of the A and B genes, we used a sensitive and specific assay to compare the ratio of the two transcripts and calculate their concentration (Kuppvswamy
et al., 1991). Total RNA from kernels 18 DAP was used as a template for reverse transcription, and a DNA amplification reaction was used to produce a 794-bp product representing both the A and B transcripts. The purified DNA product was then used for a DNA synthesis reaction, utilizing a primer that is identical for the A and B transcripts but for which the following 3' nucleotide is different. For comparison, A and B RNAs transcribed in vitro were mixed in different ratios and analyzed.

We initially analyzed the W22 inbred as well as the wild-type Tuxpeno and Pool 33 QPM. The results in Figure 5 show that with RNA from W22, the A to B transcript ratio was ~1:5, which compares favorably with the results of Das and Messing (1987), who found a ratio of 1:3 using oligonucleotide probes on RNA dot blots. Surprisingly, in Tuxpeno the ratio of A to B transcript was ~2:1, whereas in Pool 33 QPM, it was 3:4:1. Thus, the polarity of A and B expression is reversed in W22 compared to the normal or o2 genotypes developed at CIMMYT.

Figure 5. Quantitative Analysis of RNAs Corresponding to the A and B 27-kD γ-Zein Genes.

The tandemly repeated A and B γ-zein genes differ by a single nucleotide at position 449 of the coding region. The autoradiograms show the detection of the A and B transcripts in total RNA purified from kernels 18 DAP. A reverse transcriptase product containing A and B cDNAs was amplified by DNA amplification and used in a SNaPE reaction in which extension of an identical primer with either 32P-dATP or 32P-dCTP allowed detection of products corresponding to the A and B genes, which differ by a single nucleotide (T versus G) in this region. The autoradiogram shows the assay of the A and B transcripts in nine separate reactions. Radioactivity was measured with a Betascope, and the values indicated below each autoradiogram show the average measurement from the nine reactions. The ratio of the A to B transcripts for W22, Tuxpeno, and Pool 33 QPM is shown on the right of the figure.

Figure 6. Measurement of A and B 27-kD γ-Zein Transcripts in Developing Endosperms of Tuxpeno, Tuxpeno o2, and Modified Tuxpeno o2.

Detection of A and B RNAs and calculation of the A-to-B ratio is as described in Figure 5. The total amount of A and B RNAs was calculated based on the A-to-B ratio and the hybridization of the γ-zein transcripts in the total RNA. To estimate the concentration of mRNAs, γ-zein transcripts were made in vitro, and the hybridization of 1.0, 2.5, 5.0, and 10 ng amounts was compared with that of RNA extracted from developing endosperms. Stippled bars, Tuxpeno; cross-hatched bars, Tuxpeno o2; solid bars, modified Tuxpeno o2. Lines on top of the bars represent the standard deviations.

When we analyzed the ratio of A to B γ-zein transcripts from kernels of Tuxpeno, Tuxpeno o2, and Tuxpeno QPM 16 and 18 DAP, we also found significantly more A than B RNA. As shown in Figure 6, in Tuxpeno the ratio of A to B transcripts was 2:1 and 2.4:1, respectively, at these developmental stages. In Tuxpeno o2, the ratios were 1.6:1 and 1.7:1. These data indicate that the reduction in 27-kD γ-zein mRNAs caused by the o2 mutation affects both the A and B genes, but the effect on the A transcript is more pronounced. At these stages, the A-to-B ratio in Tuxpeno QPM was between 3.9:1 and 3.5:1, indicating a dramatic increase in the amount of mRNA for the A gene. By comparing the hybridization of γ-zein transcripts in the total RNA with that of a synthetic γ-zein transcript and using the A-to-B ratio, we were able to calculate the concentration of each transcript at 16 and 18 DAP. This analysis showed that the dramatic increase of γ-zein RNAs occurring between 16 and 18 DAP in Tuxpeno QPM involves mainly the A gene. In fact, at 18 DAP, the level of B transcripts in these three genotypes was similar.

Enhanced Accumulation of the A Transcript Cannot Be Explained by Rearrangement of the γ-Zein Locus

The locus containing the 27-kD γ-zein genes is somatically unstable, and this can lead to a rearrangement in which the B
Gene is lost from the chromosome (Das et al., 1990a). Since our previous work showed that this rearrangement can also occur in QPM genotypes (Geetha et al., 1991), we decided to investigate whether loss of the B gene might explain the enhanced proportion of A transcripts in QPM genotypes. Because of restriction site polymorphisms in the sequences flanking the A and B genes, it is possible to distinguish the standard from the rearranged locus containing the A gene (rA) with an EcoRI digestion of the DNA. The data in Figure 7 show the organization of the 27-kD γ-zein locus in DNA of 18-DAP kernels from several normal and QPM genotypes. The γ-zein locus in A188 is relatively unstable; it is evident that by 18 DAP there have been a significant number of events leading to the deletion of the B gene (Figure 7, lanes 1a and 1b). Based upon the ratio of the two DNA bands, we estimate that there was 30% recombination at the locus. In W22, the locus is stable, and there is no evidence of somatic rearrangements (Figure 7, lanes 2a and 2b). Although there is some evidence of somatic instability of the γ-zein locus in CMS450 QPM (Figure 7, lane 3b) and Tuxpeno (Figure 7, lane 5b), by 18 DAP, it appears to have occurred only 5 to 15% of the time. Significantly, there was no evidence for rearrangement of the γ-zein locus in DNA from Pool 33 QPM or Tuxpeno QPM 18 DAP. Consequently, the preferential accumulation of A transcripts in Tuxpeno QPM cannot be explained on the basis of loss of the B gene.

![Diagram of γ-Zein Locus Organization](image)

**Figure 7.** Organization of the γ-Zein Locus in Endosperms of A188, W22, CMS450 QPM, Pool 33 QPM, Tuxpeno, and Modified Tuxpeno o2 18 DAP.

DNA was purified from developing endosperm and used for DNA amplification of a 1050-bp DNA fragment corresponding to the 5' noncoding sequence of the A, B, and rA γ-zein genes. Between 100 and 150 ng of the product was digested with EcoRI, which distinguishes a polymorphism between the A and rA genes. Equal amounts of undigested (a) and digested (b) DNA of each maize genotype were separated by gel electrophoresis and hybridized to a radioactive probe of the 1050-bp fragment. Radioactivity was measured with a Betascope to calculate the percentage of recombination. The positions of the rA and A and B gene sequences are indicated on the left. Lanes 1, A188; lanes 2, W22; lanes 3, CMS450 QPM; lanes 4, Pool 33 QPM; lanes 5, Tuxpeno; lanes 6, modified Tuxpeno o2.

**DISCUSSION**

Zein gene expression initiates around 12 DAP, following the syncytial stage of endosperm development, and for most gene families, transcription increases through 20 DAP (Kodzyczyk et al., 1989). During this period, there is a dramatic increase in all types of zein mRNAs and proteins. It is evident from the results presented in Figures 1 through 4 that o2 modifier genes become active during this early period of development. We found that there are similar levels of γ-zein protein in W64A o2 and Pool 34 QPM at 14 DAP, and by 18 DAP, the protein level in Pool 34 QPM is nearly double that in W64A o2 (Geetha et al., 1991). In Tuxpeno QPM, synthesis of γ-zein protein and mRNA is significantly enhanced by 16 DAP compared to the
normal or o2 genotypes. Thus, effects of the modifier genes on zein synthesis become detectable between 14 and 16 DAP. We previously reported that in reciprocal crosses of W64A o2 and Pool 34 QPM, the accumulation of y-zein protein and mRNA was dependent on the dosage of modifier genes in the triploid endosperm (Geetha et al., 1991). However, because W64A contains only the A gene and Pool 34 contains both A and B genes, there was also a difference in the number of y-zein coding sequences in the parents and F1 progeny. Consequently, the differences in y-zein protein and mRNA levels could also be explained on the basis of differences in the copy number of the 27-kD y-zein coding sequences. The data in Figures 1 through 4 show that although the amount of y-zein protein and mRNA is partially a function of the number of coding sequences, the activity of the modifiers significantly enhances the amount of protein and mRNA produced. There is less y-zein protein and mRNA in 18-DAP seeds of W64A, which contains only the A gene (Figure 1A, lane 5; Figure 2A, lane 5), than in the wild-type A188 or Tuxpeno, both of which contain the A and B genes (Figure 1A, lanes 1 and 3; Figure 2, lanes 1 and 3). This difference is also reflected in the final amount of protein in mature kernels (Wallace et al., 1990; Geetha et al., 1991). In Pool 33 QPM and CMS450 QPM, both of which contain A and B genes, there is more y-zein protein (Figure 1A, lanes 2 and 4) and transcript (Figure 2A, lanes 2 and 4) than in either Tuxpeno or A188. Thus, the amount of 27-kD y-zein protein and mRNA in QPM genotypes is significantly enhanced by the o2 modifier genes.

The results of our study support a model in which o2 modifiers act post-transcriptionally to increase the level of y-zein mRNA, thereby leading to increased synthesis of the protein. Between 16 and 18 DAP, there is more rapid accumulation of y-zein transcripts in Tuxpeno QPM compared to Tuxpeno o2 (Figure 3), yet the rate of y-zein transcription in these genotypes is nearly identical (Figure 8). The run-on transcription data should represent an accurate measure of gene expression, because there are both negative and positive controls. The 22-kD a-zein genes are not transcribed in either Tuxpeno o2 or Tuxpeno QPM because of the defective o2 protein, whereas transcription of sucrose synthase in both genotypes is the same as in Tuxpeno. We also measured the transcription of these genes at 18 DAP in Tuxpeno QPM (data not shown) and found results comparable with those at 16 DAP. These levels of gene transcription are also similar to our earlier measurements in W64A normal and o2 (Kodrzycki et al., 1989).

Measurement of the ratio of A to B y-zein transcripts showed that most of the increased y-zein RNA in the modified o2 mutants comes from the A gene (Figure 6). The ratio of A to B RNAs increased dramatically in Tuxpeno QPM between 16 and 18 DAP, with most of the increase resulting from accumulation of A transcript rather than a decrease in B transcript. Indeed, by 18 DAP, there was relatively little difference in the amount of B RNA in the three Tuxpeno genotypes. We have not found an explanation for the preferential accumulation of the A gene RNA, and we do not know why the polarity for expression of the A and B genes is inverted in the Tuxpeno genotypes compared to W22. Loss of the B gene through somatic recombination (Das et al., 1990a) could account for depletion of the B transcript during endosperm development. However, our analysis of the frequency with which the y-zein locus was rearranged in the endosperm at 18 DAP demonstrated that although some somatic recombination occurred, the frequency with which the B gene was lost in Tuxpeno and Tuxpeno QPM could not account for the developmental increase in the A transcript or the change in polarity of expression compared with W22. At 18 DAP, the frequency of rearrangement of the y-zein locus in endosperm of A188, Tuxpeno, and CMS450 was 30, 15, and 5%, respectively. No rearrangement was detected for Pool 33 QPM or Tuxpeno QPM at this stage. If we assume that the locus in W22 is stable, has a similar pattern of transcription as in Tuxpeno, and has a B-to-A transcript ratio of 2:1 (Das and Messing, 1987), then to obtain the A-to-B ratio found in Tuxpeno as a consequence of rearrangement, the B gene would have to be lost ~60% of the time. The frequency with which this occurred was far less than this in all of the genotypes we analyzed.

Figure 6. Comparison of Rates of y-Zein Gene Transcription in Endosperms of Tuxpeno, Tuxpeno o2, and Modified Tuxpeno o2 16 DAP. Nuclei isolated from developing endosperm were added to an in vitro transcription reaction, as described in Methods. DNA sequences corresponding to the 22-kD a-zein, the 14-kD b-zein, the 27-kD y-zein, the 10-kD 6-zein, and the two sucrose synthetase genes Shl and Sus were immobilized on a nitrocellulose membrane, and the radioactive products from the run-on transcription reaction were used as probes. Radioactivity was measured with a betascope, and the values for the different zeins and Shl were normalized to that of Sus. A, 22-kD a-zein; B, 14-kD b-zein; G, 27-kD y-zein; D, 10-kD 6-zein; Shl, Shrunken-7. Stippled bars, Tuxpeno; cross-hatched bars, Tuxpeno o2; solid bars, modified Tuxpeno o2. Lines on top of the bars represent the standard deviations.
We cannot rule out the possibility that the modifier genes establish a difference in the polarity of transcription and that this is partially responsible for the differences in the steady state levels of A and B RNAs. With the run-on transcription assay, it was not possible to distinguish expression of the A and B genes, so we do not know how much each gene contributed to the transcription products. Differences in the level of transcription of the A and B genes could establish a ratio of A and B RNAs that is enhanced post-transcriptionally. Nevertheless, it appears that mRNA accumulation plays the primary role in the dramatic increase in γ-zein transcripts in modified o2 mutants.

Analysis of the mRNAs encoded by the A and B genes has not provided any obvious clues that suggest reasons for differences in their translation or stability. We have sequenced the A and B genes from Pool 33 QPM and CMS450 QPM and found only minor differences in 1.2 kb of 5' flanking sequence or the coding regions of either the A or B genes (M.A. Lopes, K. Takasaki, T.G. Helentjaris, and B.A. Larkins, unpublished data). Both sets of sequences are nearly identical with those of the A and B genes in W22, A188, and W64A (Geraghty, 1985; Das et al., 1991). There are some differences in the 3' noncoding sequences of these genes. There is a 7-bp gap between 24 and 30 nucleotides after the stop codon in the A genes of Pool 33 QPM and CMS450 QPM compared to W22. This gap is not present in the B genes of these three genotypes. Otherwise, there is only one nucleotide difference in the first 0.1 kb of the 3' noncoding sequence. Comparison of the following 370 nucleotides of 3' noncoding sequence showed no difference between the A gene of Pool 33 QPM and W22. Das et al. (1991) identified a 37-bp region just beyond 125 bp past the stop codon where there are multiple sequence differences between the A and B genes. This region is near the polyadenylation signal (Wu et al., 1993), and perhaps sequence differences in this region provide the basis for differential accumulation of A and B mRNAs.

The O2 gene encodes a basic domain/leucine zipper (bZIP) transcriptional factor that binds the promoter of 22-kD α-zein genes (Hartings et al., 1988; Schmidt et al. 1990) and is necessary for their expression (Kodrzycki et al., 1989; Ueda et al., 1992). Although the reduced transcription of the 22-kD α-zein genes in QPM genotypes is not unexpected, the lower rates of the β-, γ- and δ-zein gene transcription in Tuxpeno o2 and Tuxpeno opaque-2 are not readily explained based on what is known about the O2 protein. Rates of transcription for these other zein genes range from 20 to 45% that in wild-type Tuxpeno and correlate with significantly reduced steady state levels of mRNAs; yet, transcription of sucrose synthase is nearly the same in all three genotypes. Ueda et al. (1992) showed that the 27-kD γ-zein promoter contains a motif similar to the O2 binding site. However, the O2 protein will not bind this sequence unless it is converted to the O2 target sequence. Thus, at least for the γ-zein genes, it appears that transcription does not directly involve the O2 protein. Recently, Pysh et al. (1993) isolated a second bZIP protein that appears to interfere with O2 binding, suggesting that multiple bZIP proteins are involved with transcription of zein genes. Because transcription factors of this type frequently function as heterodimers (Halazonetis et al., 1988), it is possible that the O2 protein may normally be part of a heterodimer that transcribes the β-, γ- and δ-zein genes. In the absence of this protein, other regulatory proteins could transcribe these genes, albeit at a lower efficiency. Higher affinity of a heterodimer than a homodimer with the target DNA sequence has been shown for the transcriptional activators Fos and Jun (Able and Maniatis, 1989), and this has been suggested to be true of the O2 protein (Schmidt, 1993).

Post-transcriptional regulation appears to play an important role in controlling the synthesis of seed storage proteins. In barley (Sorensen et al., 1989) and maize (Kodrzycki et al., 1989), transcription of some prolamin genes is higher, or in some cases lower, than would be predicted based on steady state levels of mRNA and the final amount of protein in the mature seed. Among the factors affecting the steady state levels of storage protein mRNAs are nitrogen and sulfur availability as well as amino acid biosynthesis (Shewry et al., 1983; Sorensen et al., 1989; Singletary et al., 1990). Zein levels are dramatically reduced in the o6 mutant, which is defective in proline biosynthesis (Motto et al., 1989). In vitro culture of developing o2 kernels with appropriate amino acids appears to restore synthesis of the 22-kD α-zein proteins (Balconi et al., 1993). In legume seeds, the availability of sulfur-containing amino acids plays an important role in regulating the synthesis of the 7S and 11S globulins by controlling mRNA stability (Chandler et al., 1983; Holowach et al., 1984; Beach et al., 1985). Considering the unusual amino acid composition of the γ-zein protein, it is conceivable that the modifiers could be involved in amino acid biosynthesis. The 27-kD γ-zein contains 25% proline and 7% cysteine (Prat et al., 1985). But if the products of the modifier genes were involved in proline or sulfur amino acid metabolism, we might expect that there would also be effects on some of the other zein proteins. The α-, β-, and δ-zeins contain ~10% proline (Shotwell and Larkins, 1989), and the β- and δ-zeins contain 11 and 24% sulfur amino acids, respectively. If the modifiers were to enhance the biosynthesis of either proline or cysteine, we might expect to see increased synthesis of these other proteins as well, and this is not the case (Figures 3 and 4). Because the o2 modifiers have such a specific effect on the accumulation of γ-zein protein and RNAs, we believe that it is more likely that the products of these genes interact with γ-zein mRNA transcripts and enhance their transport from the nucleus or increase their stability and translation.

METHODS

Plant Materials and Chemicals

The inbred lines W64A, A188, and W22 were obtained from Purdue University (West Lafayette, IN), whereas Pool 33 QPM, CMS450 QPM, and the near-isogenic lines Tuxpeno, Tuxpeno opaque-2 (o2), and modified Tuxpeno o2 (Quality Protein Maize [QPM]) were obtained from

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the International Maize and Wheat Improvement Center (CIMMYT) and the National Maize and Sorghum Improvement Center (CNPMS/SETA Lagoas, Brazil). All inbred stocks were maintained by self-pollination. Plants were grown at the University of Arizona West Campus Agricultural Center during the summers of 1991 and 1992. Developing seeds were harvested at appropriate stages, frozen in liquid nitrogen, and stored at -80°C until use.

Enzymes were purchased from Bethesda Research Laboratories, Life Technologies, Inc. and Boehringer Mannheim. α-32P-dATP, α-32P-dCTP, and α-32P-UTP were obtained from New England Nuclear Research Products (Boston, MA). Nylon membranes and nitrocellulose were purchased from Schleicher & Schuell.

**Extraction of Zeins and SDS-PAGE**

Developing seeds were collected at 16, 18, and 22 days after pollination (DAP) and lyophilized prior to protein extraction. Total zeins were isolated according to the method of Wallace et al. (1990). Protein from samples equivalent to 0.75 mg of kernel flour were separated electrophoretically on a 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R 250 (Laemmli, 1970). The stained gels were scanned with a laser densitometer, and the intensity of the bands was measured with the aid of an Image Quant Program (Molecular Dynamics, Sunnyvale, CA).

**Isolation of DNA from Developing Kernels**

Approximately 2.5 g of developing endosperm was ground with a mortar and pestle using liquid nitrogen. Approximately 50 mg of flour was suspended in 0.75 mL of buffer containing 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, 20 mM EDTA, and 1% n-lauroyl sarcosine (Das et al., 1990b). An equal volume of phenol was added, and samples were spun in an Eppendorf centrifuge at 16,000g for 5 min. The aqueous phase was extracted twice with phenol/chloroform/isooamyl alcohol (25:24:1) and once with chloroform/isooamyl alcohol (24:1). The volume was adjusted to 0.9 mL with suspension buffer, and the DNA was precipitated with 0.45 mL of cold isopropanol at -20°C for 10 min.

**Isolation of RNA from Developing Kernels**

For RNA isolation, ~2.5 g of kernels 16, 18, or 22 DAP was ground in a mortar and pestle with liquid nitrogen. The meal was suspended in 4.5 mL of buffer containing 0.3 M Tris-HCl, pH 7.5, 5 M guanidine HCl, 10 mM EDTA, and 8% 2-mercaptoethanol and spun in an Eppendorf centrifuge at 16,000g for 5 min. Seven volumes of 4 M LiCl were added to the supernatant, and the samples were placed at 4°C for 1 hr. After centrifugation for 10 min, the RNA pellet was washed with 3 M LiCl and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS. RNA samples were extracted twice with phenol/chloroform/isooamyl alcohol (25:24:1) and precipitated with 2.5 volumes of ethanol and 2.5 M ammonium acetate at -20°C. RNA was resuspended in diethylpyrocarbonate-treated water and stored at -20°C. The RNA concentration was calculated based on absorbance at 260 nm; 24 A260 = 1 mg/mL.

**Radioactive Labeling of DNA Probes**

The cDNA clones corresponding to the 22-kD α-zein, 14-kD β-zein, 27-kD γ-zein, and 10-kD δ-zein were described previously (Kodrzycki et al., 1989). Clones corresponding to a maize ribosomal gene and sucrose synthase (Shrunken1 [Shn] and Sus) genes were obtained from B. Hunter, University of Arizona, and L. C. Hannah, University of Florida, Gainesville, respectively. DNA fragments from the coding regions were prepared by DNA amplification of genomic DNA or from cDNA fragments cloned into the pT7/T3 α-18 or pT7/T3 α-19 vector (Bethesda Research Laboratories). The DNA fragments were radiolabeled using a random primer kit (Ambion, Inc., Austin, TX).

**In Vitro Synthesis of γ-Zein Transcripts**

Transcripts of the A and B 27-kD γ-zein genes were synthesized in vitro from 0.8-kb DNA fragments (beginning 25 bp upstream of the ATG codon and extending 97 bp downstream from the γ-zein coding region) that were excised from pUC19 and cloned into the same sites in the pT3/T7 α-18 vector. Transcription reactions were done using an in vitro transcription kit (Megascript; Ambion Inc.).

**DNA and RNA Blotting and Hybridization Analyses**

RNA samples were suspended in glyoxal mix (1 mL glyoxal, 10 mM sodium phosphate, pH 7, 80% formamide) H2O (9:1) solution and incubated at 55°C for 15 min. For RNA blots, samples were separated on 1% agarose gel and transferred to a Nytran membrane with 10 mM NaOH. For dot blots, RNA was applied to Nytran membranes in a Minifold TM filtration manifold SRC-96 dot blot apparatus (Schleicher & Schuell). After UV cross-linking, the membranes were baked at 80°C under vacuum for 1 hr.

For DNA analyses, DNA was separated on a 1% agarose gel, denatured for 45 min with 0.5 M NaOH and 1.5 M NaCl, and neutralized for 1 hr in 1.0 M ammonium acetate and 0.02 M NaOH. DNA was transferred onto a Nytran membrane for 16 hr with the neutralization solution. Hybridization was at 42°C for DNA gel blots and 47°C for RNA gel blots. Membranes were prehybridized for 4 hr in hybridization solution containing 7 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M NaH2PO4, and 0.001 M EDTA), 50% formamide, 5 × Denhardt’s solution (1 × Denhardt’s solution is 0.02% Ficoll [w/v], 0.02% PVP [w/v], and 0.02% BSA [w/v]), 0.3% SDS, and 100 μg/mL sheared salmon sperm DNA. Hybridization was for 16 hr in fresh hybridization solution containing radioactive denatured probe (1 to 2 × 106 cpm/mL). The membranes were washed twice with 2 × SSC, 0.1% SDS (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature for 5 min and once with 0.2 × SSC, 0.1% SDS at hybridization temperature for 45 min. For RNA blots, duplicate membranes were hybridized with a maize 26S RNA gene and sucrose synthase to establish that equal amounts of RNA were applied to the blot. Radioactivity was measured with the betaoscope blot analyzer (Betagen, Waltham, MA) or by liquid scintillation spectroscopy in Biosafe NA (Research Products Inc., Mount Prospect, IL).

**Measurement of A and B γ-Zein Gene-Specific Transcripts**

A common 794-bp fragment of the A and B γ-zein genes beginning 25 bp upstream of the ATG codon and extending 97 bp downstream of the ATG codon was amplified by use of the DNA amplification primers AGGCAGAGAAGACCGCTG (5’ primer) and GTTATATGCGTACAGTAT (3’ primer). Total RNA was isolated from developing kernels and 1 to 3 ng was reverse transcribed in a buffer containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 5 mM MgCl2, 1 mM deoxynucleotide...
trophoretic separation of the products on 12% polyacrylamide gels, 

In order to accurately measure the amount of these two RNAs, 60th 

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Nuclear Run-On Transcription Assays of Developing Endosperm 

Developing maize kernels were harvested from ears at 16 DAP and 

and 1 ng/4 ng) were used to determine the ability of this 

Mixtures of A and B-γ-zein in vitro transcripts at two concentrations 

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