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# Genetic analysis of *opaque2* modifier gene activity in maize endosperm

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**Abstract** Modifier genes have been described that convert the soft endosperm of *opaque2* mutants to a hard, vitreous phenotype. The mode of action and the components of the genetic system involved in this seed modification are poorly understood. We used genetic and biochemical analyses to investigate the number of *opaque2* modifier genes, their mode of action and their relationship to the biochemical alterations in the modified endosperm. Using two inbred *opaque2* lines, we showed that the activity of *opaque2* modifier genes is influenced by the genetic background. Analysis of segregating progenies and recombinant inbred lines derived from crosses between *opaque2* and modified *opaque2* genotypes indicated two independent loci affecting seed opacity and density. Consistent association between endosperm modification and enhanced accumulation of the gamma-zein storage protein suggested that either this protein is directly involved in the process of seed modification, or else that a modifier gene could be tightly linked to the genes responsible for gamma-zein synthesis.

**Key words** Genetic analysis · Maize · Modifier genes · Recombinant inbreds · *opaque2* · Zeins

## Introduction

The *opaque2* (*o2*) mutation of maize was first described by Jones and Singleton in the early 1920s (Emerson et al. 1935), and the soft texture and opaque phenotype of this mutant has been widely used as a genetic marker for the short arm of chromosome 7. The mutant seeds were subsequently found to contain twice the normal concentration of the essential amino acids lysine and tryptophan (Mertz et al. 1964). The altered proportion of these amino acids in *o2* is due to the reduced amount of zein storage proteins and an increased accumulation of non-zein proteins. Plant breeders have made an enormous effort to improve the nutritional quality of maize with *o2*, but pleiotropic effects associated with the mutation have resulted in seeds with inferior agronomic properties, including slow drydown, low density, low resistance to breakage, and susceptibility to insects and molds. These deficiencies have limited the development of *o2* for commercial use. However, the discovery of modifier genes that cause the formation of a hard, vitreous endosperm (Paez et al. 1969) allowed the development of modified *o2* mutants that have great potential for commercial utilization (reviewed by Bjarnason and Vasal 1992; Gevers and Lake 1992). Modified *o2* genotypes, which resemble normal maize both in kernel phenotype and agronomic performance, are generally called Quality Protein Maize or QPM.

The effects of endosperm modifier genes on *o2* have been the subject of a considerable number of reports during the last 25 years (reviewed by Glover and Mertz 1987). It has been found that modified *o2* mutants contain large amounts of the gamma-zein storage protein (Wallace et al. 1990; Lopes and Larkins 1991) and enhanced levels of gamma-zein mRNA (Geetha et al. 1991). The increase in gamma-zein gene expression appears to be regulated post-transcriptionally (Or et al. 1993). However, there is little information describing the number of modifier genes, their mechanism of action or their mode of inheritance. In certain genetic backgrounds, endosperm modification is unstable and appears to be influenced by environmental conditions, but the factors responsible for the unstable phenotype are poorly understood. Further development and future utilization of QPM germplasm would be accelerated by determining the genetic basis of modifier gene function. In this report we describe the results of genetic and biochemical experiments to study modifier gene number

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and the relationships between modifier gene action and biochemical alterations associated with endosperm modification.

## Materials and methods

### Plant materials

Maize (*Zea mays* L.) stocks were developed at the Purdue University Agronomy Farm, West Lafayette, IN, during the summers of 1988 and 1989, and at the University of Arizona Research Farm, Tucson, AZ, from 1990 to 1992. The inbred lines W64A, W64A*o2* and W22*o2* were obtained from Purdue University. The highly modified QPM population Pool 33 was obtained from the International Maize and Wheat Improvement Center – CIMMYT, Mexico City. Modified *o2* germplasm developed by CIMMYT has been described by Vasal et al. (1980), CIMMYT (1987) and Bjarnason and Vasal (1992). Self-pollinated ears and reciprocal $F_1$ hybrids were produced by hand-pollination. The phenotypic and biochemical characterization of the parental *o2* and modified *o2* materials used in this study have been previously described (Geetha et al. 1991; Lopes and Larkins 1991).

### Seed sampling and analysis of segregating populations

$F_2$ progenies segregating for seed modification were obtained by hand-pollination of $F_1$ plants from the cross Pool 33×W22*o2* and from the reciprocal crosses W64A*o2*×Pool 33 and Pool 33× W64A*o2*. All populations were grown in the same season (summer 1991 in Tucson, AZ) under similar environmental conditions. Twelve well-developed $F_2$ ears were randomly chosen from 45 selfed $F_1$ plants of the crosses W64A*o2*×Pool 33, Pool 33×W64A*o2* and Pool 33×W22*o2*. Seeds from each ear were considered as a progeny and were treated individually after shelling. Progeny seed was examined with a light box and classified according to the degree of light transmission through the endosperm (opacity). Seed modification varies in a dosage-dependent manner depending on the number of modifier genes in the triploid endosperm (Lopes and Larkins 1991). The $F_2$ seeds were assigned to one of four classes based on similarity to the phenotype of the parents and their reciprocal $F_1$ crosses. Seeds similar to the opaque parent (zero doses of modifier genes) were classified as opaque, whereas seeds similar to the modified parent (three doses of modifier genes) were classified as modified. Seeds with intermediate phenotypes were divided into semi-opaque and semi-modified classes (one and two doses of modifier genes, respectively). $F_3$ families were developed by self-pollination of $F_2$ plants to verify if progenies breed true for the selected endosperm phenotype (an indication of homozygosity).

### Analysis of seed density

Seed density was measured by the absolute ethanol method (Kniepp and Mason 1989). A 500 ml cylinder was placed on an electronic balance and the weight determined. Seeds were poured into the cylinder and the weight recorded. The cylinder was partially filled with absolute ethanol (200 proof, density 0.78612 g/ml at room temperature), agitated to remove trapped air, filled to a pre-determined volume with ethanol and the weight recorded. Calculation of seed density was done as follows: (g seed+ethanol)-g seed=g ethanol; g ethanol/0.78612=ml of ethanol; ml (seed+ethanol)–ml of ethanol=ml seed; g seed/ml seed=seed density. Differences in seed density were judged statistically by analysis of variance and multiple comparison tests (Lopes 1993).

### Development of recombinant inbred lines (RILs)

RILs were developed by inbreeding $F_2$ plants derived from the cross W64A*o2*× Pool 33. The objective was to generate homozygous progenies with a varying dosage of modifier genes. Sixty $F_2$ plants were selected to begin the process, and visual selection for plant and seed phenotype was done for each cycle of inbreeding. Progenies displaying undesirable traits and long maturation cycles were discarded. Seeds from each cycle were examined on a light box and a set of progenies representing the widest possible range of seed phenotypes was chosen for each subsequent cycle of inbreeding. Forty-four near-inbred $F_4$ RILs were chosen for this study. The relationship of seed density with endosperm protein content in these lines was evaluated by the linear regression equation y=b+ax.

### Extraction of seed proteins, SDS-PAGE, and protein measurements

Embryos were hand-dissected from mature kernels of fully developed ears. Twenty endosperms from inbred lines and $F_1$ hybrids were pulverized in a blender and then ground to flour with a ball mill. For analysis of combined $F_3$ progenies, flour from 20 endosperms from each individual $F_3$ progeny was similarly prepared. One gram of meal from each $F_3$ progeny was then combined and homogenized in a blender before sampling for protein analysis. Endosperm flour of 30 $F_3$ progenies with an opaque phenotype was pooled for protein analysis. The same was done for 30 $F_3$ progenies with a modified phenotype. Total zeins and non-zein proteins were isolated according to Wallace et al. (1990). Total endosperm protein was solubilized in 0.0125 M sodium borate (pH 10.0), 1% (w/v) SDS, and 2% (v/v) $\beta$-mercaptoethanol. Samples were extracted overnight with constant shaking at 37°C, and centrifuged for 15 min at 12000 rpm to remove insoluble materials. The supernatant was carefully removed and mixed with absolute ethanol to a final concentration of 70% (v/v). After a 30-min incubation at 37°C with occasional mixing, a precipitate of alcohol-insoluble non-storage proteins (non-zeins) was visible. The sample was centrifuged, the supernatant containing total zeins was transferred to clean microfuge tubes, vacuum dried, and stored at 4°C until used.

SDS-polyacrylamide gradient gels (7.5 to 18% acrylamide, w/v) were prepared according to Laemmli (1970), but the Tris concentrations used in the resolving gel and running buffer were doubled. Protein samples were diluted in SDS-sample buffer (Laemmli 1970) and boiled for 3 min before loading. Gels were run at room temperature at a constant current of 15 mA, stained with Coomassie blue overnight, and destained in 40% (v/v) methanol and 10% (v/v) acetic acid for at least 8 h. Coomassie blue-stained gels were scanned with a laser densitometer and the intensity of the bands was measured for each genotype with the aid of an Image Quant Program (Molecular Dynamics, Sunnyvale, CA). Absorbance values were used to calculate the relative amounts of total zein, gamma-zein and non-zein proteins among the different genotypes. Protein concentration was indexed to a reference genotype that had an absorbance value arbitrarily set at one.

## Results

### Analysis of endosperm modification in segregating progenies

Approximately 14000 $F_2$ seeds from the crosses Pool 33×W22*o2*, W64A*o2*×Pool 33 and Pool 33×W64A*o2* were analyzed for endosperm modification. The number of seeds per phenotypic class for each $F_2$ progeny is shown in Fig. 1. For each cross, most seeds fell into the two intermediate phenotypic classes, with the distribution skewed toward semi-opaque types in crosses that involved W64A*o2* (Fig. 1A) and toward the semi-modified class in crosses that involved W22*o2* (Fig. 1B). A small, but similar number of seeds was in the two extreme classes with

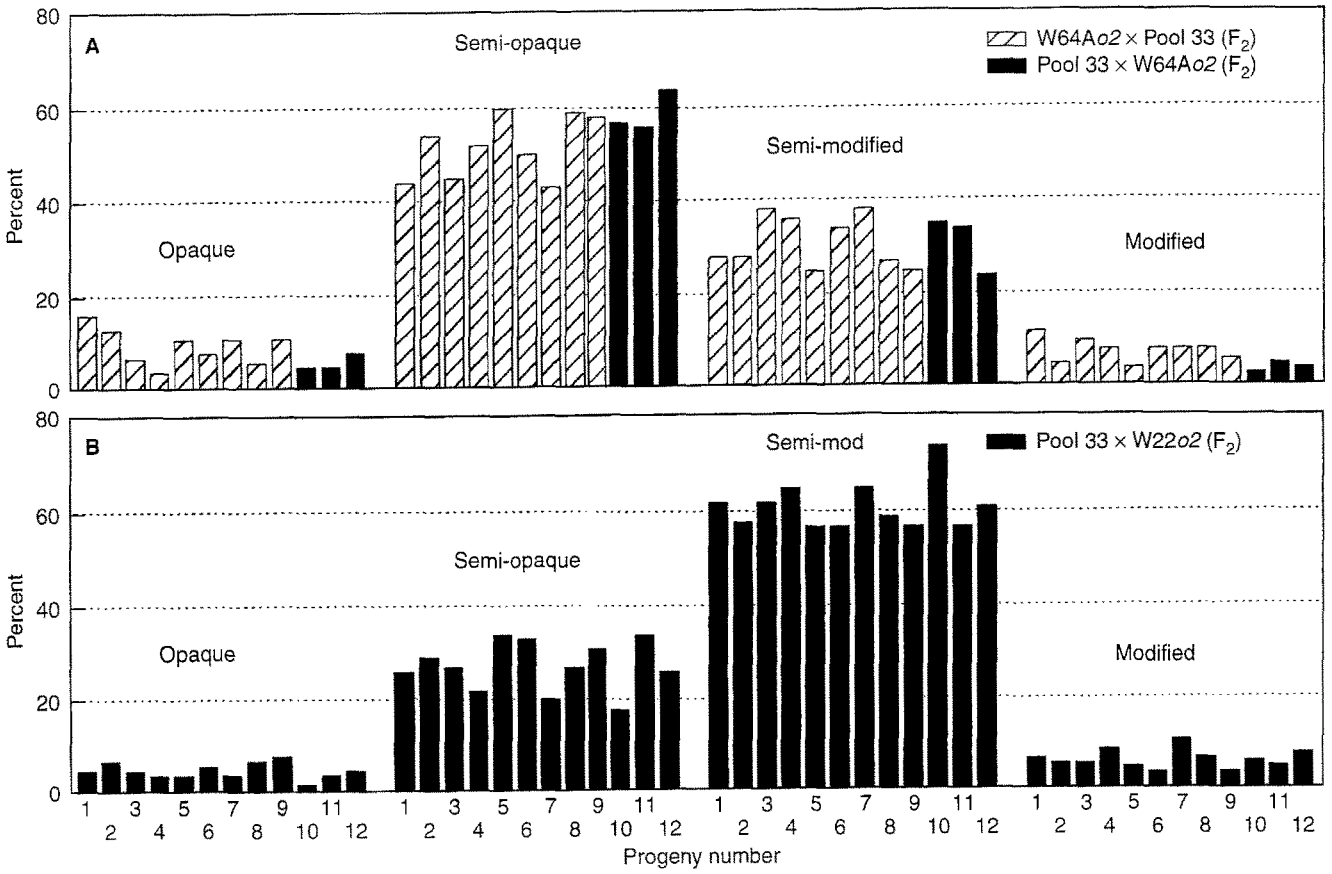


**Fig. 1A, B** Analysis of seed phenotype in $F_2$ progenies from crosses between *o2* and modified *o2* genotypes. **A** Seeds from 12 $F_2$ ears were separated into four phenotypic classes according to the degree of endosperm light transmission. Progeny of individual $F_2$ ears were analyzed separately. *Hatched bars* represent the number of seeds in each class for progenies 1–9 from the cross W64A*o2*×Pool 33; *filled bars* are progenies 10–12 from the reciprocal cross Pool 33×W64A*o2*. **B** Same as **A**, except that all progenies were derived from the cross Pool 33×W22*o2*

**Table 1** Analysis of variance for seed number within phenotypic classes (A) and seed density within phenotypic classes (B) in $F_2$ progenies from the crosses W64A*o2*×Pool 33, Pool 33×W64A*o2*, and Pool 33×W22*o2*

| A | | | B | | |
|---|---|---|---|---|---|
| Source | *df* | F | Source | *df* | F |
| Factor | 3 | 150.12** | Factor | 3 | 42.82** |
| Progeny | 11 | 1.61 NS | Progeny | 11 | 1.76 NS |
| Level | N | Mean[a] | Level | N | Mean[a] |
| Opaque | 12 | 58.17 a | Opaque | 12 | 1.1135 a |
| Semi-Opq. | 12 | 354.33 c | Semi-Opq. | 12 | 1.1789 b |
| Semi-Mod. | 12 | 207.58 b | Semi-Mod. | 12 | 1.2150 c |
| Modified | 12 | 44.83 a | Modified | 12 | 1.2272 c |
| Pooled St. Dev. = 44.20 | | | Pooled St. Dev. = 0.0294 | | |

** Significant at the 0.01 probability level
[a] Means within a column followed by the same letter are not significantly different at $P$=0.05 based on Fisher's protected LSD

phenotypes that resemble the *o2* and modified *o2* parents. Analysis of variance for the data shown in Fig. 1A indicates the number of seeds within each phenotypic class is significantly different ($P$<0.01) (Table 1A). Each of the 12 $F_2$ progenies contributed a similar number of individuals to each phenotypic class, therefore the effect of individual progeny was not significant. Analysis of class means for seed number within progeny was not significantly different between the two extreme phenotypic classes (opaque and modified) (Table 1A), indicating that each $F_2$ progeny contributed a similar number of seeds with parental phenotypes. Cytoplasmic effects did not appear to be involved in endosperm modification, based on the similarity of the $F_2$ progeny in the reciprocal crosses involving Pool 33 and W64A*o2* (Fig. 1A). Similar results were obtained with $F_2$ progenies of the cross Pool 33×W22*o2* (data not shown). The difference in number of seeds in each of the intermediate phenotypic classes (semi-opaque and semi-modified) of crosses involving W64A*o2* (Fig. 1A) and W22*o2* (Fig. 1B) indicates that the background of the *o2* parent can affect the behavior of modifier genes.

Since qualitative analysis of seed phenotype by light transmission is subjective, we also measured seed density as a quantitative index of seed modification. Figure 2A shows the seed density for the progenies described in Fig. 1A. The average seed density increased continuously from the opaque to the modified class, and the analysis of variance indicated the difference between classes is significant ($P$<0.01) (Table 1B). As with opacity, the 12 $F_2$ progenies contributed a similar number of individuals to each

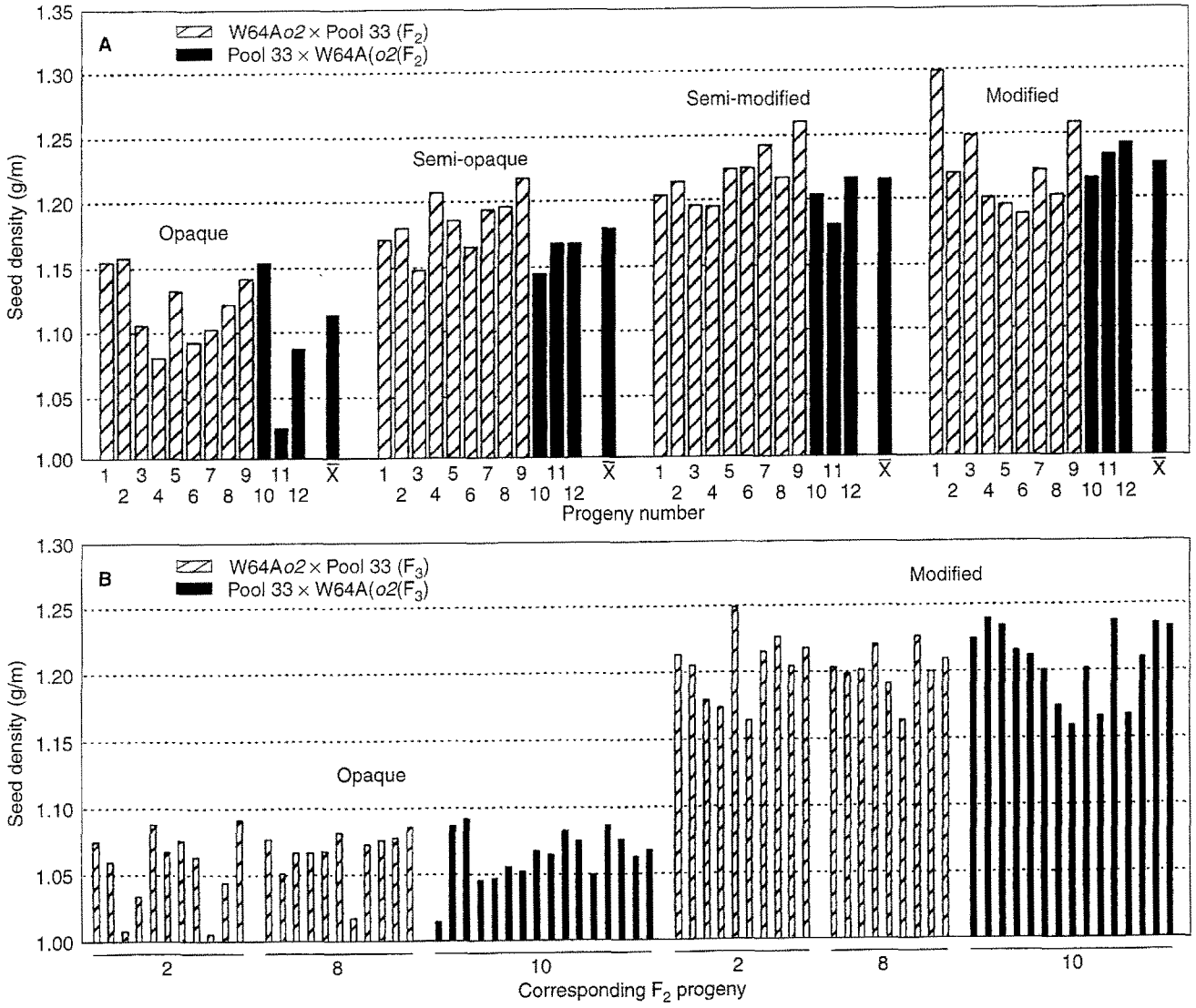


**Fig. 2A, B** Analysis of seed density in $F_2$ and $F_3$ segregating progenies. **A** The density of seeds in each phenotypic class was measured for the progenies described in Fig. 1A. *Hatched bars* are progenies 1–9 from the cross W64A*o2*×Pool 33, *filled bars* are progenies 10–12 from the reciprocal cross Pool 33×W64A*o2*. **B** Seeds with parental phenotypes (opaque and modified) were chosen from $F_2$ progenies 2, 8 and 10, planted and selfed, and the corresponding $F_3$ families were analyzed for seed density

density class, indicating that the effect of individual progeny was not significant. Like seed opacity, cytoplasmic effects did not appear to influence seed density. Class means of seed density are not significantly different for seeds designated as modified and semi-modified (Table 1B), indicating that the difference in light transmission between these two classes is not detected by measuring density.

## Inheritance of endosperm modifier genes

To test whether variation of seed phenotype in reciprocal crosses between *o2* and modified *o2* genotypes indicates a semidominant or additive mode of inheritance, $F_2$ seeds with extreme phenotypes (opaque and modified) (Fig. 1A) were planted and selfed. All $F_3$ families derived from $F_2$ modified seeds had the expected parental endosperm phenotype. However, a few families expected to have an opaque phenotype segregated for seed modification (data not shown). This is probably a consequence of the difficulty in separating $F_2$ seeds that are completely opaque from those with a limited degree of modification. Seed density measured for three $F_3$ progenies is shown in Fig. 2B. All seeds displaying a vitreous phenotype have a consistently higher seed density than those with an opaque phenotype, indicating that $F_2$ individuals with parental phenotypes are homozygous at modifier gene loci.

The mean seed number per phenotypic class is shown in Table 1A. The 12 progenies analyzed have on average 6.7% of their seeds with a modified phenotype, and 8.7% of their seeds with an opaque phenotype. These percentages are suggestive of two independent loci controlling endosperm modification, implying that modifier genes act in an additive manner (Lopes and Larkins 1991). In such a case, the segregating progeny is expected to have each pa-

**Table 2** Distribution of seeds per phenotypic class in populations segregating for modifier genes

| Classes | Opaque | Semi-opq. | Semi-mod. | Modified |
|---|---|---|---|---|
| Populations | W64*o2* × Pool 33 and Pool 33 × W64A*o2* | | | |
| Observed $F_2$s | 689 | 4252 | 2491 | 538 |
| Ratio | 1.39 | 8.53 | 5.00 | 1.08 |
| Populations | Pool 33 × W22*o2* | | | |
| Observed $F_2$s | 306 | 1612 | 3703 | 373 |
| Ratio | 0.83 | 4.30 | 9.88 | 0.99 |

rental type represented in a proportion of 1/16 of the total (6.25%). Assuming additivity, the existence of one or three modifier genes, instead of two, would lead to proportions of opaque and modified $F_2$ individuals greatly deviant from the observed values (1/4 and 1/64, instead of 1/16).

Even though we can predict the genotype of individuals with extreme phenotypes (AAABBB, vitreous; and aaabbb, opaque), it is difficult to predict the genotypic boundaries of intermediate phenotypic classes that have 1–5 active modifier alleles. This is especially true when modifier genes contribute unequally to the phenotype. In fact, the ratios of opaque: semi-opaque: semi-modified: modified seeds in the $F_2$ progenies analyzed (Table 2) indicate an unequal contribution of modifier genes. Since the modified *o2* parent is common for all crosses, it appears that the modifiers respond differently within the background of the two *o2* parents (W64A*o2* and W22*o2*). Alternatively, the *o2* parents could differ in alleles at minor modifier loci, while having non-functional alleles at the two major modifier loci present in Pool 33.

## Effects of endosperm modification on storage protein accumulation

To investigate whether endosperm modification is correlated with increased accumulation of the gamma-zein storage protein, we analyzed zeins from Pool 33, W64A*o2*, their reciprocal crosses, the $F_3$ pools and W64A by SDS PAGE (Fig. 3A). Quantification of the 27-kDa gamma-zein band by laser densitometry is shown in Fig. 3B. Changes in modifier gene dosage are positively correlated with an increased content of this protein in the reciprocal crosses (Fig. 3A, lanes 1–4). Modified and opaque $F_3$ seed pools (Fig. 3A, lanes 5 and 6) derived from $F_2$ plants homozygous at modifier gene loci (Fig. 2B) show a pattern of gamma-zein content similar to that of the modified and opaque parents (Fig. 3A, lanes 1 and 4). These results confirm that segregating progenies that breed true for seed modification also have a gamma-zein protein content that is positively correlated with the degree of endosperm modification. Also, comparison of lanes 1 and 5 indicates transgressive segregation for gamma-zein production, reinforcing the hypothesis that the inbred W64A*o2* has functional alleles at minor modifier loci.

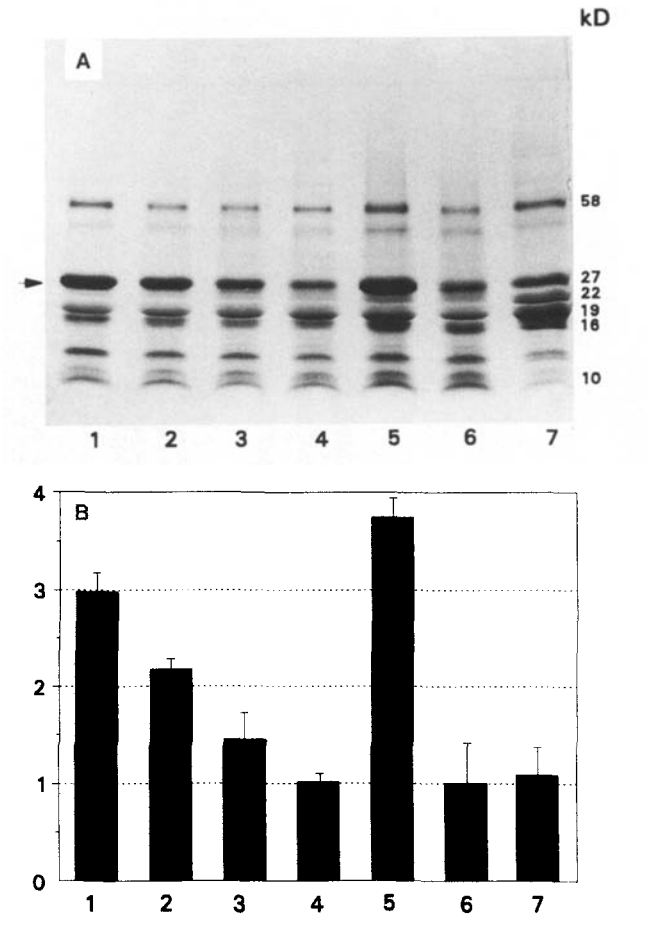


**Fig. 3A, B** SDS-PAGE analysis of zein proteins from modified *o2* and *o2* maize. **A** Protein samples were extracted from equal amounts of endosperm, and protein extracts corresponding to 2 mg of flour were solubilized in sample buffer (Laemmli 1970) and separated by a 7.5% to 18% gradient gel SDS-PAGE. Gels were stained with Coomassie-blue. *Lane 1*, Pool 33; *lane 2*, Pool 33×W64A*o2*; *lane 3*, W64A*o2*×Pool 33; *lane 4*, W64A*o2*; *lane 5*, pool of $F_3$ seeds with modified phenotype; *lane 6*, pool of $F_3$ individuals with opaque phenotype; *lane 7*, W64A+. **B** Quantification of the 27-kDa gamma-zein by laser densitometry. Duplicate Coomassie blue-stained gels were scanned with a laser densitometer, and absorbance values corresponding to the 27-kDa band were used to calculate the relative amounts of gamma-zein among the several genotypes. Concentrations were indexed to the value of the inbred W64A*o2*, which was arbitrarily set at one. *Bars 1–7* correspond to lanes identified in **A**

## RILs indicate the effect of modifier genes on endosperm-protein composition

RILs were generated to fix the degree of modification in the progenies derived from the crosses of W64A*o2* with Pool 33. To maintain a broad range of seed modification, visual selection was carried out before each cycle of inbreeding. RILs with extreme phenotypes (modified and opaque) are expected to have the parental modifier loci (endosperm genotypes AAABBB and aaabbb, respectively). Also, considering independent segregation, selfing with selection for intermediate phenotypes can lead to the fixation of intermediate combinations of modifier alleles.

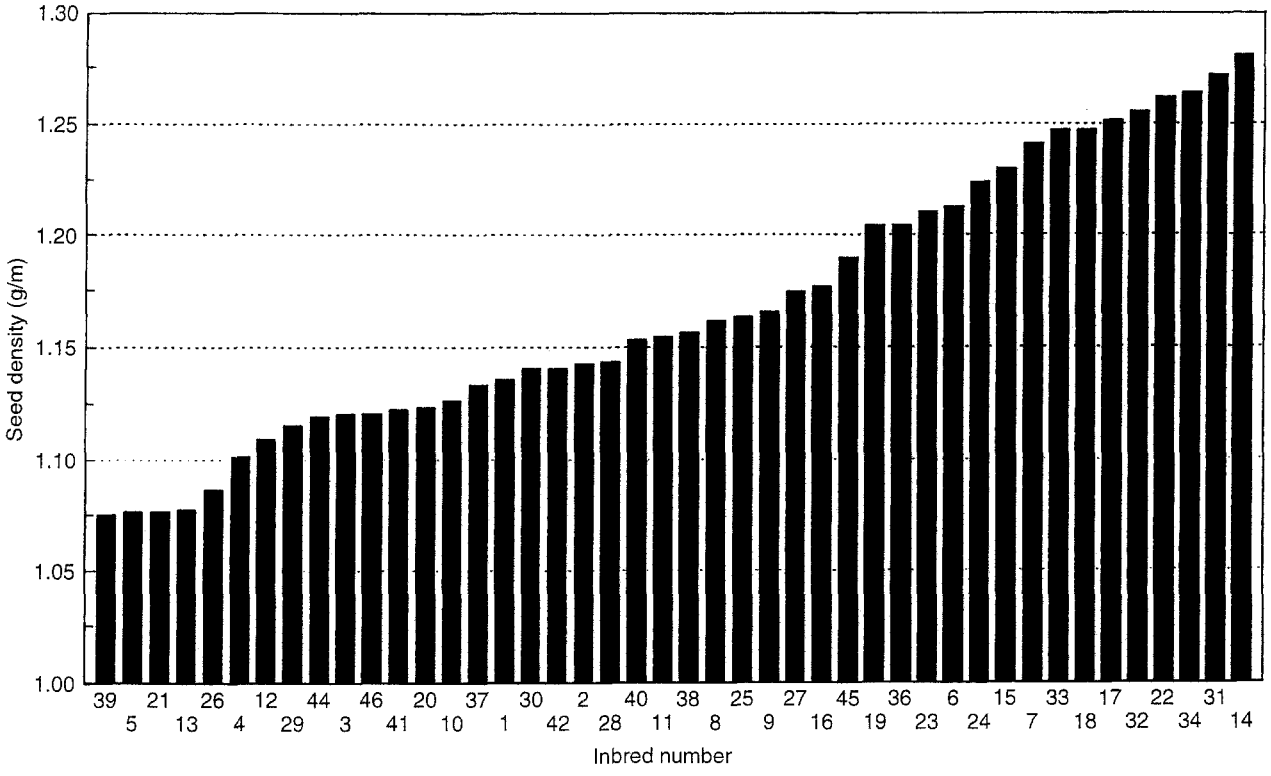


**Fig. 4** Seed density of recombinant inbred lines developed from the cross W64A*o2*×Pool 33

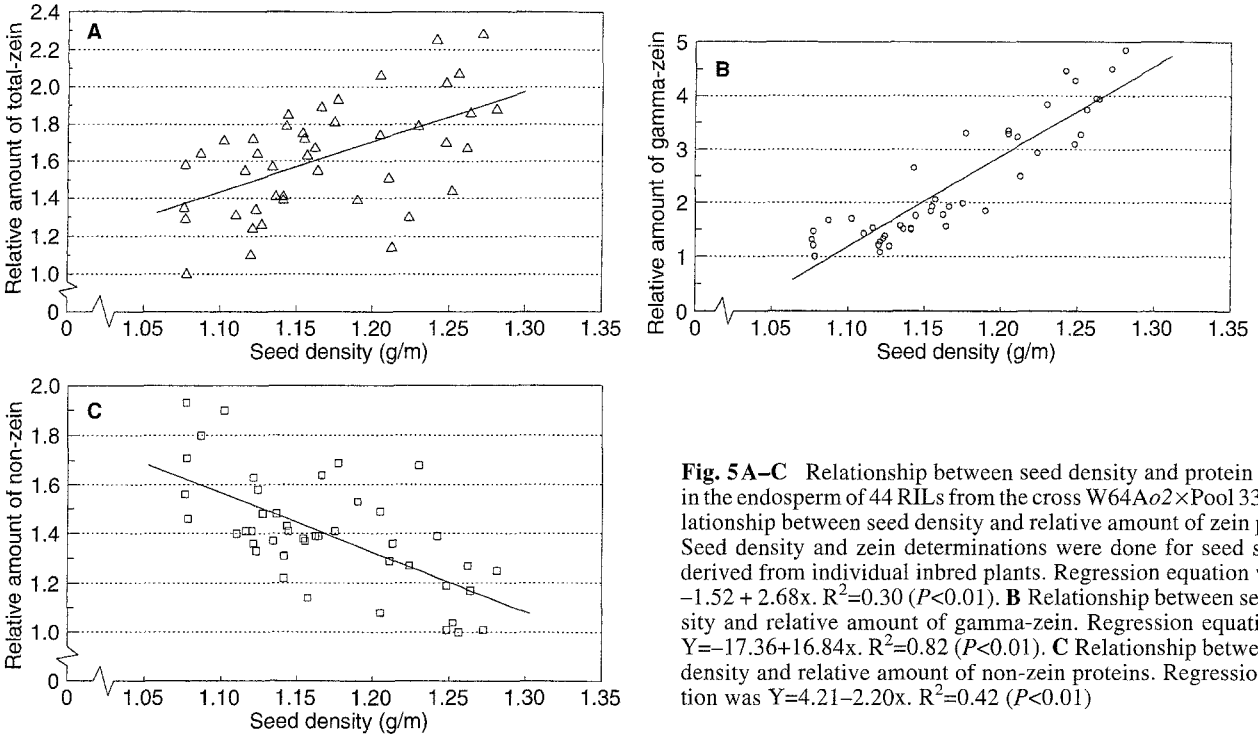


**Fig. 5A–C** Relationship between seed density and protein content in the endosperm of 44 RILs from the cross W64A*o2*×Pool 33. **A** Relationship between seed density and relative amount of zein protein. Seed density and zein determinations were done for seed samples derived from individual inbred plants. Regression equation was Y= −1.52 + 2.68x. $R^2$=0.30 ($P$<0.01). **B** Relationship between seed density and relative amount of gamma-zein. Regression equation was Y=−17.36+16.84x. $R^2$=0.82 ($P$<0.01). **C** Relationship between seed density and relative amount of non-zein proteins. Regression equation was Y=4.21−2.20x. $R^2$=0.42 ($P$<0.01)

The 44 near-inbred (F4) RILs analyzed have a broad range of kernel density (Fig. 4). Figure 5A shows the relationship between seed density and the relative amount of total storage proteins (zeins) in the endosperm. Seed density and total zein are positively correlated with an $R^2$ value of 0.30 ($P$<0.01). However, seed density is much better correlated with gamma-zein accumulation in the endosperm ($R^2$=0.82, $P$<0.01) (Fig. 5B). Figure 5C shows that seed density is inversely correlated with accumulation of non-zein proteins ($R^2$=0.42, $P$<0.01).

## Discussion

Endosperm modification in *o2* maize genotypes results from the action of a system of genetic modifiers that were accumulated by backcross and recurrent selection methods (Vasal et al. 1980; Bjarnason and Vasal 1992; Gevers and Lake 1992). After repeated cycles of phenotypic selection, chemical analyses and recombination, remarkable improvements in kernel hardness, kernel density, and overall agronomic performance of *o2* were obtained. Furthermore, the enhanced nutritional quality associated with this mutation was maintained. Despite the success of this approach, very little is known about the genetic changes underlying the process of seed modification.

A gradual increase in the proportion of hard to soft endosperm is consistently observed in reciprocal $F_1$ hybrids developed by crossing *o2* and modified *o2* genotypes. In addition, nearly continuous phenotypic variation ranging from completely opaque to completely modified seeds is found in single ears of $F_2$ progenies segregating for endosperm modification (Lopes and Larkins 1991; Fig. 1). Selfing of $F_2$ individuals with an extreme (modified and opaque) phenotype yielded an $F_3$ with the selected phenotype (Fig. 2B), confirming that modifier genes function in an additive fashion. Also, recovery of similar amounts of parental types at a proportion that approximates 1/16 of the total progeny (Table 2) indicates the involvement of two genes with the trait. Because the existence of one or three modifier genes, instead of two, would lead to proportions of opaque and modified $F_2$ individuals that greatly deviate from the observed values, the evidence for two genes is especially strong.

On theoretical grounds, a modified *o2* individual with two independent semi-dominant modifier genes would have six active modifier alleles in the triploid endosperm. Active modifier alleles (A and B) are taken here as effectors of modification, in contrast to the inactive counterparts (a and b) that do not contribute to the trait. Reciprocal crosses of modified *o2* with *o2* should result in $F_1$ hybrids with four and two modifier alleles in the endosperm, depending on how the cross is made. The dosage variation in the number of modifier alleles in the endosperm, considering both parents and their progeny (from six to zero), would explain the previously reported stepwise variation in the seed phenotype of these crosses (Lopes and Larkins 1991).

In extending the two-gene model, any $F_1$ cross between modified *o2* and *o2* should generate $F_2$ progeny with a continuous pattern of segregation for seed modification. This is due to triploidy and additivity, factors that generate complex patterns in the endosperm of segregating seeds. In addition to having individuals with zero to six modifier alleles, $F_2$ progenies may vary in phenotype depending on a differential contribution of modifier genes to the phenotype and/or epistatic relationships between the genes involved. For instance, individuals with three modifier alleles could have different phenotypes, depending on their genotypes (AAAbbb, aaaBBB, AaaBBb, AAaBbb). Due to difficulty in precisely evaluating small variations in seed phenotype, assessment of these effects will be possible only after molecular characterization of the modifier genes.

The action of modifier genes appears to be affected by genetic background. Figure 1 shows the proportion of seeds with different degrees of endosperm modification for crosses of the same modified parent with two different inbred lines. Although the modifier genes are contributed from the same source, the proportions of intermediate phenotypes (semi-opaque and semi-modified) are significantly different, depending on the *o2* inbred line. Environmental effects cannot explain this result, since all progenies were obtained during the same season under similar conditions. Therefore, it appears that the effect modifier genes have on the phenotype depends on the genetic background in which they act. Observation of background effects on *o2* modification is consistent with the fact that modifiers tend be more effective in flint versus dent maize (Vasal et al. 1980; personal observation). Also, part of this variation could result from differences in populations of functional alleles at minor modifier loci, although the number and contributions of these loci to seed modification is difficult to assess.

Storage proteins have long been thought to be implicated in the definition of the physical properties of the endosperm (Lopes and Larkins 1993). Several opaque mutations that affect endosperm texture also cause alterations in storage protein accumulation (Schmidt 1993). We have shown that increased accumulation of the 27-kDa gamma-zein storage protein is always associated with endosperm modification (Wallace et al. 1990; Lopes and Larkins 1991; Geetha et al. 1991). As the mode of action of storage protein genes is additive, similar to modifier genes, it is plausible to imagine their involvement with this trait.

Since the relationship between gamma-zein accumulation and endosperm modification cannot be fully characterized by the relationships seen in the parental and $F_1$ generations, we analyzed the content of this protein in $F_2$ and $F_3$ segregating progenies. Analysis of total storage proteins in *o2*, modified *o2* and their reciprocal crosses indicated that the degree of modification and the increased deposition of gamma-zein are dosage-dependent and directly correlated (Fig. 3). Pooled $F_3$ individuals with a modified phenotype accumulate approximately 3.5-times more gamma-zein than pooled individuals with an opaque phenotype. Except for the 27-kDa polypeptide, the pattern of zein accumulation in both parents and their progeny is similar to that observed for individuals displaying the *o2* phenotype. However, only analysis of pooled $F_3$ progenies homozygous at modifier gene loci provided definitive proof of linkage between these two traits (Fig. 3). Since each pool was composed of 30 individuals derived from a random sample of $F_2$'s homozygous at modifier gene loci, it is unlikely that the association between endosperm modification and high gamma-zein accumulation was established by chance. This protein could be either directly involved in the process of seed modification, or a modifier gene could be

tightly linked to genes responsible for its increased deposition, thus explaining the consistent linkage between these traits.

Although useful to this study, segregating progenies have limited value for investigating modifier gene action. The transient nature of segregating progenies and the inability to identify, with precision, intermediate genotypic combinations from generation to generation makes it difficult to use them for long-term studies. One solution to this problem was the development of RILs from progenies segregating for modifier genes. By selecting a wide range of endosperm phenotypes during each cycle of inbreeding it was possible to develop inbreds with potentially all homozygous combinations of modifier genes. Unlike transient $F_2$ or $F_3$ progenies, these RILs are genetically fixed. These stocks may provide more efficient ways to identify candidate modifier genes and to study their individual effects. Also, transposon tagging of modifier genes could be a less difficult task with these genetic stocks.

Physical and biochemical analyses of seeds from the RILs confirmed the association between seed modification and the accumulation of endosperm proteins (Fig. 5). These analyses showed that modifier gene action is positively correlated with storage protein accumulation, and the magnitude of the correlation coefficients indicated that gamma-zein content, rather than total storage protein content, is better correlated with endosperm modification. Also, a negative correlation between endosperm modification and the accumulation of the lysine-rich non-zein proteins (Fig. 5C) indicated that modification of the *o2* mutant may lead to a decrease in the nutritional quality of the seed. Several reports have shown that slight decreases in the contents of lysine and tryptophan are consistently associated with endosperm modification (Ortega and Bates 1984; Bjarnason and Vasal 1992). However, the relatively low $R^2$ value (42%) calculated from the regression of non-zein content on seed density (Fig. 5C) indicates that it might be possible to select lines with reasonable levels of seed modification and high non-zein content. This knowledge may provide a tool for more efficient selection and development of improved modified *o2* cultivars.

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