of 3 min at 95° C, followed by 25 cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min, and extension at 72° C for 1 min. A final extension cycle of 5 min at 72° C was performed before cooling to 4°C.

Cloning and sequencing of gamma-zein genes

PCR products from two modified *o2* genotypes, CMS 450 (Tuxpeno) and Pool 33, were used to clone DNA sequences corresponding to coding and flanking regions of the gamma zein genes A and B. After PCR amplification, the layer of mineral oil was removed with a pipette and the DNA product was extracted twice with phenol/chloroform/isoamyl alcohol, 25:24:1. The DNA was precipitated with cold ethanol, and the pellet was rinsed in 70% ethanol, and briefly dried under vacuum. The DNA pellet was dissolved in 10 µl of 10 mM TRIS-HCl, pH 8.5, 1 mM EDTA (TE). Oligos no. 3 and no. 4 were designed with the restriction sites *Sal* I and *Bam*HI, respectively. Oligos no. 1 and no. 2 were designed without restriction sites at the 3' ends. All recessed ends were filled-in with Klenow polymerase before ligation (Sambrook et al. 1989). After the appropriate restriction enzyme digestions, the PCR product was again extracted in phenol/chloroform/isoamyl alcohol, resuspended in TE, and ligated into the plasmid pUC19 (Maniatis et al. 1992). Transformation of competent *Escherichia coli* cells, strain DH5alpha, with the ligation mixture resulted in many white colonies on LB solid medium containing ampicillin (75 mg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 40 mg/ml). White colonies were picked, lysed with alkali and analyzed by agarose gel electrophoresis. Recombinant colonies were screened for plasmid inserts containing a variable number of *Pst*I sites, as indicated in Fig. 5A. Cloning the flanking sequences of the A and B genes was done in a similar manner, but the PCR product correspond to the coding plus flanking sequences obtained with oligonucleotides no. 3 and no. 2 (Fig. 5A). Identification of alternative alleles was based on screening recombinant colonies for plasmid inserts containing variable numbers of *Pst*I sites and for the presence or absence of the *Eco*RI site in the flanking region. DNA inserts were sequenced by the dideoxynucleotide chain-termination method using a Sequenase kit purchased from United States Biochemical Corporation (Cleveland, Ohio, USA). Sequence analysis was performed with the Microgenie computer program (Beckman, Palo Alto, Calif., USA). Cloned genes from the modified o2 gentotypes Pool 33 (data not shown) and CMS 450 were sequenced and compared with sequences from normal genotypes.

Zein analysis

For each RIL, 20 endosperms were pulverized in a blender and ground to a fine powder with a ball mill. Extraction of zein proteins was as described by Wallace et al. (1990). Protein samples were separated by 7.5 to 18% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) and stained with Coomassie brilliant blue. Gels were scanned with a laser densitometer, and the intensity of the band corresponding to the 27 kDa gamma-zein was measured with the aid of an Image Quant Program (Molecular Dynamics, Sunnyvale, Calif., USA). Absorbance values were used to calculate the relative amounts of gamma-zein among the several genotypes. The concentration was expressed relative to the RIL with the lowest gamma-zein content, the absorbance value of which was arbitrarily set at 1.

Analysis of seed density

Seed density was calculated using the absolute ethanol method (Knicp and Mason 1989).

Results

The approach we used for selecting phenotypes for subsequent genetic mapping of modifier genes is shown in Fig. 1. We were able to search for genomic regions involved in seed modification against a randomized genetic background of unlinked loci by pooling DNA of progeny individuals with parental phenotypes from *o2* by modified *o2* crosses (Michelmore et al. 1991). DNA bulks from fully vitreous and opaque pools were screened for departures from random segregation using RFLP markers located throughout the maize genome (Lopes 1993). Most genomic regions revealed polymorphisms with multiple probes, but only two regions exhibited segregation distortion associated with seed modification, and both were found on chromosome 7. The location of markers that identified RFLPs and endosperm modification in each analyzed population is indicated in Fig. 2. Although the mapping populations were developed with a common modified o2 parent (Pool 33), a modifier locus contributing to the phenotypic variation was identified for each of the two segregating F2 populations. For the population W64Ao2 × Pool 33, a modifier was mapped near the centromere, while for the population Pool 33 × W22o2 a modifier was mapped distally near the telomere of the long arm of chromosome 7. Several probes were informative for the modifier locus near the centromere of chromosome 7, but only umc35 was informative for the modifier near the telomere of 7L.

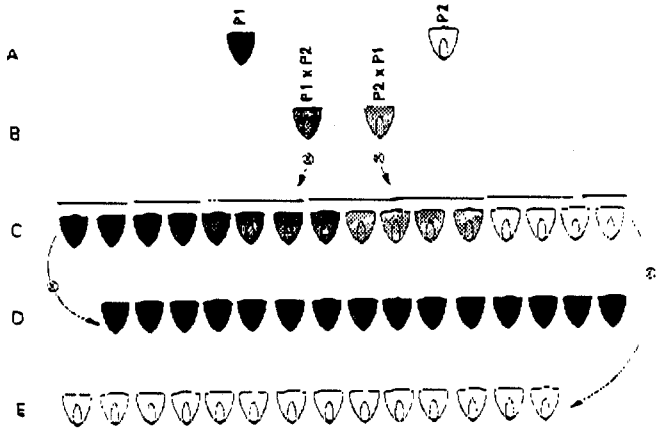


Fig. 1A–E Strategy for mapping *opaque2* (*o2*) modifier genes. The phenotype is depicted of seeds used for bulked segregant analysis (BSA). A The *o2* parent (P1) has soft, starchy seeds that do not transmit light, while the modified *o2* parent (P2) has translucent seeds that are vitreous. B The *o2* modifier genes act in a semidominant fashion, so the reciprocal F1s have intermediate phenotypes. C The F2 consists of progeny with seed phenotype that ranges from completely opaque (*left*) to completely modified (*right*). Leaf tissue was collected from F2 plants originating from seeds displaying parental phenotypes (opaque and modified). D, E F2 plants were selfed and the phenotype of the F3 seeds evaluated. Leaf tissue from F2 plants that bred true for the selected phenotype was used for DNA extraction and bulked segregant analysis

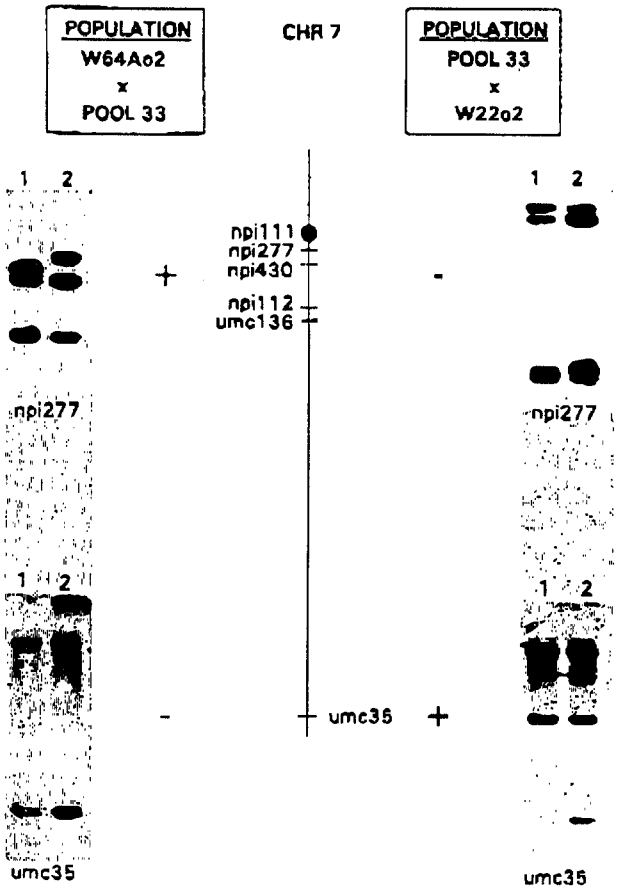


Fig. 2 Mapping of *o2* modifier gene loci on chromosome 7. Restriction fragment length polymorphism (RFLP) probes npi277 and umc35 detected polymorphisms in the bulked DNA pools from plants with seeds showing the parental opaque and vitreous phenotypes. Although npi277/*Eco*RI (as well as npi111, npi430, npi112 and umc136) detected a polymorphism for the W64Ao2 × Pool 33 cross, a polymorphism was not evident with this probe/enzyme combination for the Pool 33 × W22o2 cross. Likewise, umc35/*Hin*dIII detected a polymorphism for the Pool 33 × W22o2 cross that was not detected for the W64Ao2 × Pool 33 cross. Lane 1, DNA bulk of 30 F2 individuals with modified kernels; lane 2, DNA bulk of 30 individuals with opaque kernels. '+' and '−' indicate whether or not the probe/enzyme combination was informative

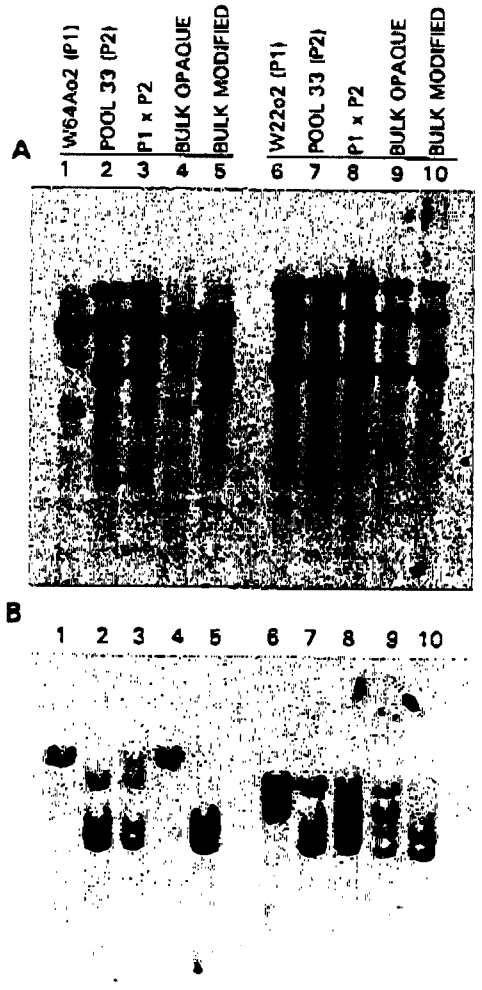


Fig. 3A, B Identification of RFLPs linked to an *o2* modifier gene. BSA analysis was conducted with DNA from 30 *o2* and 30 modified *o2* F2 individuals selected from two populations. A DNA samples from the indicated genotypes were digested with *Eco*RI and prepared for Southern analysis. The blot was probed with the gamma-zein gene sequence described in the Materials and methods. B Same as in A, except that DNA samples were digested with *Sca*I

A locus encoding the gamma-zein storage protein maps near the centromere of chromosome 7L (Benner et al. 1989). To test for linkage between this locus and the *o2* modifier, the bulked DNA samples were analyzed with a 27-kDa gamma-zein gene probe. The results presented in Fig. 3 show there is close linkage between the *o2* modifier locus and the gamma-zein gene. Only weak linkage with the gamma-zein locus was detected for the population Pool 33 × W22o2, as inferred from the slight variation in the DNA pattern between the two bulks (Fig. 3A and B, cf. lanes 6–10). Most of the progeny in the modified *o2* bulk had the gamma-zein locus from the Pool 33 parent, although several individuals contained the locus from the W22o2 parent (data not shown). In contrast to the results from the W22o2 cross, complete linkage between the gamma-zein locus and the *o2* modifier was found for the W64o2 × Pool 33 cross, as indicated by the RFLPs detected between the bulks (Fig. 3A and B, lanes 1–5). For this population, each individual in the bulks was analyzed separately, and no recombination was detected between the gamma-zein locus and the modifier locus (data not shown). This is an indication that the genomic region recognized by the gamma-zein probe is in linkage disequilibrium in both bulks. The probability of a bulk of 30 individuals having an RFLP that is not present in a segregating bulk of the same size, is extremely small when the locus is unlinked to the targeted region (Michelmore et al. 1991). Therefore, it appears that for population W64Ao2 × Pool 33,

gamma-zein genes lie at or near a locus associated with endosperm modification.

To locate more precisely this modifier gene locus for the W64Ao2 × Pool 33 population, RFLP markers on either side of the centromere were tested (Fig. 4). The marker *O2*-Xho identifies the *O2* gene, which is located on the short arm of chromosome 7, approximately 25 cM from the centromere. The markers nip389 and npi283 are located on the long arm of chromosome 7, approximately 30 cM from the centromere (Maize Genetics Cooperation Newsletter, 1993). Analysis of the opaque and vitreous bulks with these probes showed recombination between the modifier locus and distal regions of the chromosome, as indicated by DNA bands of different hybridization intensity in the DNA

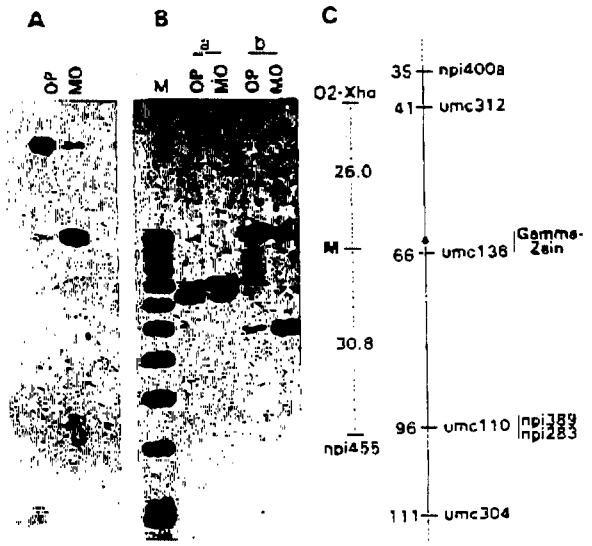


Fig. 4A–C A modifier gene maps near the gamma-zein locus in population W64Ao2 × Pool 33. For all Southern blots, lanes identified as OP and MO correspond to DNA from bulks of opaque and modified individuals, respectively. RFLP markers bounding the centromeric region of chromosome 7 were used to locate the exact map position of the modifier locus identified for the population W64Ao2 × Pool 33 (Fig. 3). A Bulked DNA digested with *Hin*dIII and probed with *O2*-Xho. B Bulked DNA digested with *Hin*dIII (a) and *Eco*RI (b) and probed with npi389. Lane M is the 1 kb ladder (BRL). The uppermost band is the 12 kb marker. C Map distances among RFLP markers are from the integrated maize RFLP map (Maize Genetics Cooperation Newsletter, 1993). Map distances between the markers *O2*-Xho and npi455 and the modifier gene M are indicated by *dotted lines* (left). The *black circle* represents the approximate position of the centromere

Table 1 Recombination frequency[a] between the *opaque2* (*o2*) modifier locus (M) and the restriction fragment length polymorphism (RFLP) markers *O2*-Xho and npi455

Segregating loci		Observed genotypes				% Recombination	SE[b]
A	B	AB	aB	Ab	ab		
M	*O2*-Xho	22	6	7	21	26.0	7.3
M	npi455	21	7	9	19	30.8	7.6

[a] Recombination percentages calculated according to the maximum-likelihood method described by Kramer and Burnham (1947)
[b] Standard error

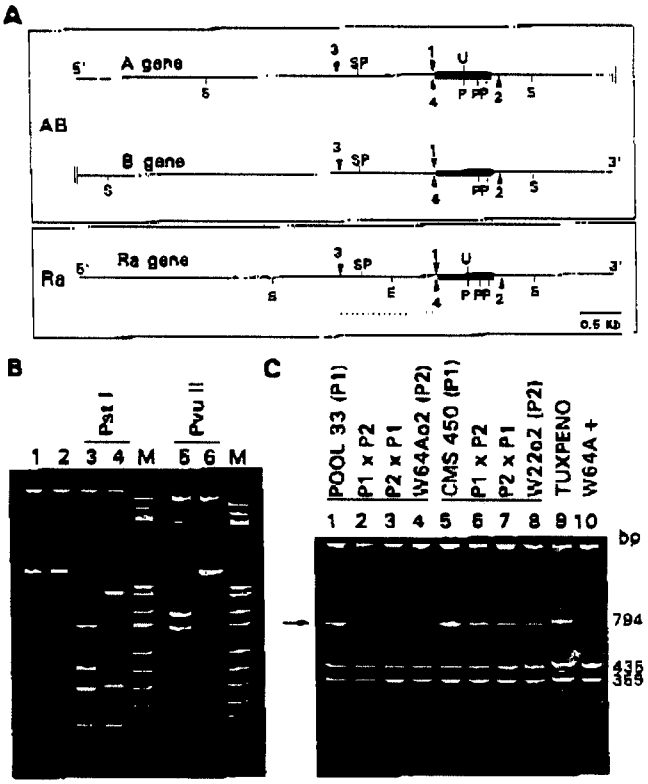


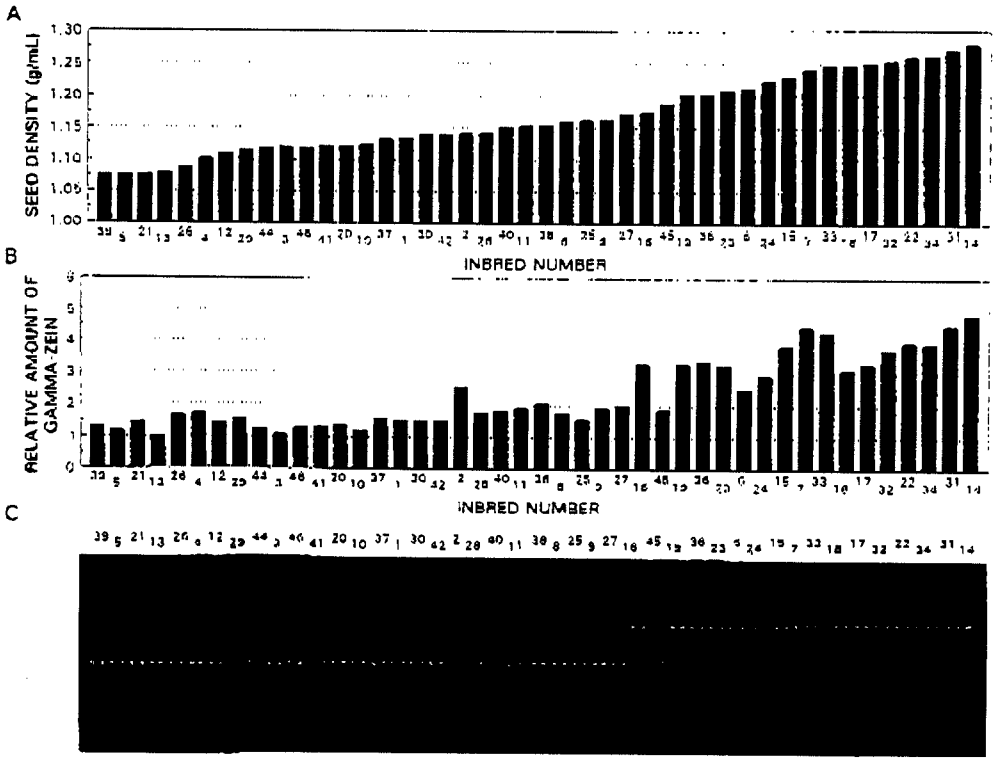
Fig. 5A–C Amplification, cloning and analysis of gamma-zein sequences. A The gamma-zein locus has been shown to have two alternative configurations (Das et al. 1990). Configuration AB has two tandemly repeated, highly homologous genes, designated A and B. Configuration Ra is a rearranged version of AB, in which the downstream B gene is lost. *Rectangles* represent sequences bound by the ATG and TGA codons. Oligonucleotides (indicated by *arrows*) were designed for polymerase chain reaction (PCR) amplification of coding (oligos no. 1 and no. 2) and flanking (oligos no. 3 and no. 4) sequences from the gamma-zein locus. Restriction enzyme sites are as follows: *S*, *Sal*I; E, *Eco*RI; U, *Pvu*II; P, *Pst*I; and SP, *Spe*I. In configuration B, variation in *Pst*I, *Pvu*II, and *Eco*RI restriction sites at the locations indicated in the map allow identification of PCR-amplified sequences corresponding to each allele at the locus. B To show that PCR-amplified DNA products can be used to analyze allelic variation at this locus, PCR products were obtained from a modified o2 genotype and cloned into pUC19. Clones corresponding to the A and B genes were reamplified and sequences were distinguished by digestion with *Pst*I and *Pvu*II and separation in a 6% polyacrylamide gel in 1 × TAE buffer (Sambrook et al. 1989). Lane 1, DNA from gene A; lane 2, DNA from gene B; lane 3, DNA from gene A digested with *Pst*I; lane 4, DNA from gene B digested with *Pst*I; lane 5, DNA from gene A digested with *Pvu*II; lane 6, DNA from gene B digested with *Pvu*II; lanes M, molecular weight markers (lambda DNA cut with *Hin*dIII, *Bam*HI and *Eco*RI, plus pUC18 digested with *Msp*I). C Analyses of allelic composition at the gamma-zein locus. DNA was obtained from endosperm flour of each indicated genotype, and the coding region of gamma-zein genes was amplified as described. DNA was digested with *Pvu*II and separated in a 6% polyacrylamide gel in 1 × TAE buffer. The *arrow* indicates the 794 bp and band corresponding to the B gene product not digested by *Pvu*II

blots of each bulk (Fig. 4). Probing DNA of individuals from each bulk with flanking markers allowed more precise localization of the modifier gene locus (Kramer and Burnham 1947; Soave et al. 1982). The data in Table 1 show that the calculated map distance between the modifier gene locus and *O2*-Xho was 26 cM and between the modifier gene locus and npi455 was 30.8 cM. This linkage further supports the hypothesis that a modifier gene and the gamma-zein genes are located in the same region, near the centromere of chromosome 7 (Fig. 4C).

To investigate further the relationship between the *o2* modifier and the gamma-zein locus, we analyzed the structure of the gamma-zein genes in modified and non-modified *o2* mutants. Some maize lines have two nearly identical gamma-zein genes, A and B, located on tandemly repeated DNA sequences of approximately 12 kb (Fig. 5A) (Geraghty 1985). This allelic composition is typical of the inbred lines W22, W23, and A188 (Das and Messing 1987). However, other maize genotypes have only one gamma-zein gene (Prat et al. 1985), which has been shown to originate from recombination between the tandemly repeated A and B genes (Das et al. 1990). Following this event, only the proximal gene, Ra, remains (Fig. 5A).

Oligonucleotides were used to amplify the coding and 5′ flanking sequences of the gamma-zein genes, as shown in Fig. 5A. RFLPs in the targeted regions allowed identification of DNA products corresponding to the A and B genes. Figure 5B shows *Pst*I-digested PCR products corresponding to gamma-zein coding sequences of the modified o2 genotype CMS450. DNAs corresponding to the A and B genes were amplified from sequences cloned into the plasmid pUC19 (Fig. 5B, lanes 1 and 2). The A gene has three sites for *Pst*I (Fig. 5A). Therefore, *Pst*I-digested DNA yielded four bands (Fig. 5B, lane 3). Since the B gene has two sites for *Pst*I, digestion yielded only three DNA bands (Fig. 5B, lane 4). Similar analysis of A and B genes with *Pvu*II digestion (Fig. 5B, lanes 5 and 6, respectively) also distinguished allelic variation at the gamma-zein locus. Figure 5C shows *Pvu*II-digested DNA products amplified from gamma-zein coding sequences of o2, modified o2, reciprocal F1 crosses and normal genotypes. The A and B genes were amplified by oligos no. 1 and no. 2, as shown in Fig. 5A. Digested DNA products from Pool 33, CMS 450, W22o2, and Tuxpeno (Fig. 5C) showed that these genotypes have A and

Fig. 6A–C Allelic composition at the gamma-zein locus correlates with seed physical properties and amount of gamma-zein protein accumulated in the endosperm. Recombinant inbred lines were derived from the cross W64Ao2 × Pool 33, as described in the text. Relationship between seed modification (density) (**A**) and relative amount of gamma-zein (**B**) in recombinant inbred lines was assessed by regression analysis (Lopes 1993). The coefficient of determination (R^2) between the two traits was 0.82 ($P < 0.01$). **C** Allelic composition at the gamma-zein locus of each individual line was determined as indicated in Fig. 5A. Gamma-zein 5′ flanking sequences were amplified from genomic DNA after extraction from endosperm flour, then digested with *Eco*RI, and separated in a 6% polyacrylamide gel. Samples were loaded according to the ascending order of seed density. The upper band corresponds to DNA sequences amplified from the 5′ regions of the A and B genes. The lower band corresponds to a 593 bp plus a 534 bp DNA fragment resulting from digestion of the 1127 bp sequence amplified from the Ra gene



B genes at the gamma-zein locus. The inbred line W64A has only the Ra gene, as indicated by complete digestion of the amplified DNA by *Pvu*II (Fig. 5C, lanes 4 and 10). Since W64Ao2 and Pool 33 have different configurations at the gamma-zein locus, there is gene dosage-dependent variation in the 794 bp fragment detected by this analysis (Fig. 5C, lanes 1–4). In contrast, reciprocal crosses between CM450 (or Pool 33) and W22o2 did not show variation in the banding pattern, because both genotypes have A and B genes at the gamma-zein locus (Fig. 5C, lanes 5–8).

The RFLPs at the gamma-zein locus allowed us to characterize the gamma-zein genes in a large number of modified *o2* genotypes. Of 33 genetic backgrounds analyzed, all had the A and B genes at the gamma-zein locus (data not shown). Since our data suggested linkage between the AB gamma-zein locus and formation of vitreous endosperm in *o2*, we analyzed the effect of selection for seed modification on the gamma-zein locus and gamma-zein protein content. This was done by developing recombinant inbred lines from the W64Ao2 × Pool 33 cross with a wide range of seed modification. The parental genotypes have the AB (Pool 33) and the Ra (W64Ao2) configuration at the gamma-zein locus (Fig. 5A). Near-inbred F4 lines with seed density ranging from 1.07 to 1.28 g/ml (Fig. 6A) were analyzed for gamma-zein content (Fig. 6B) and the relationship between the two traits was evaluated

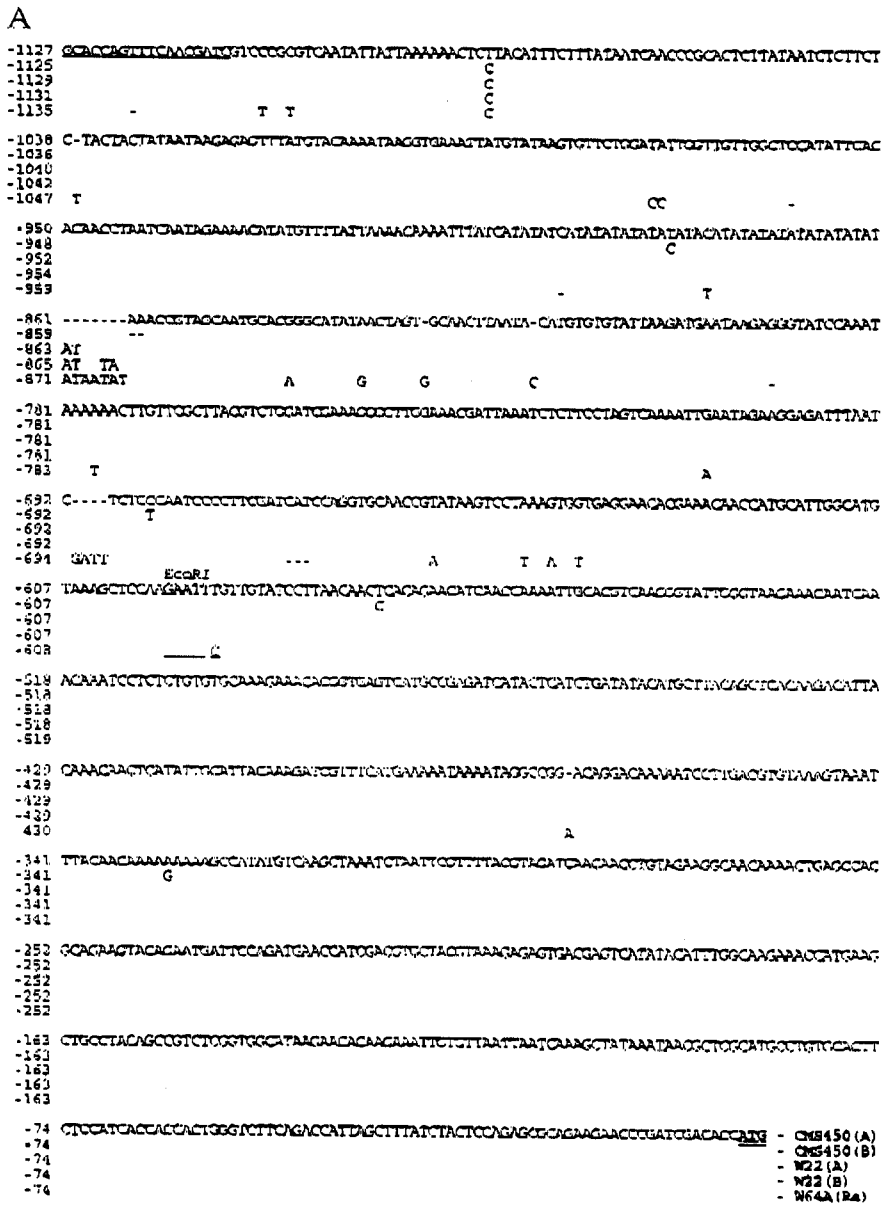
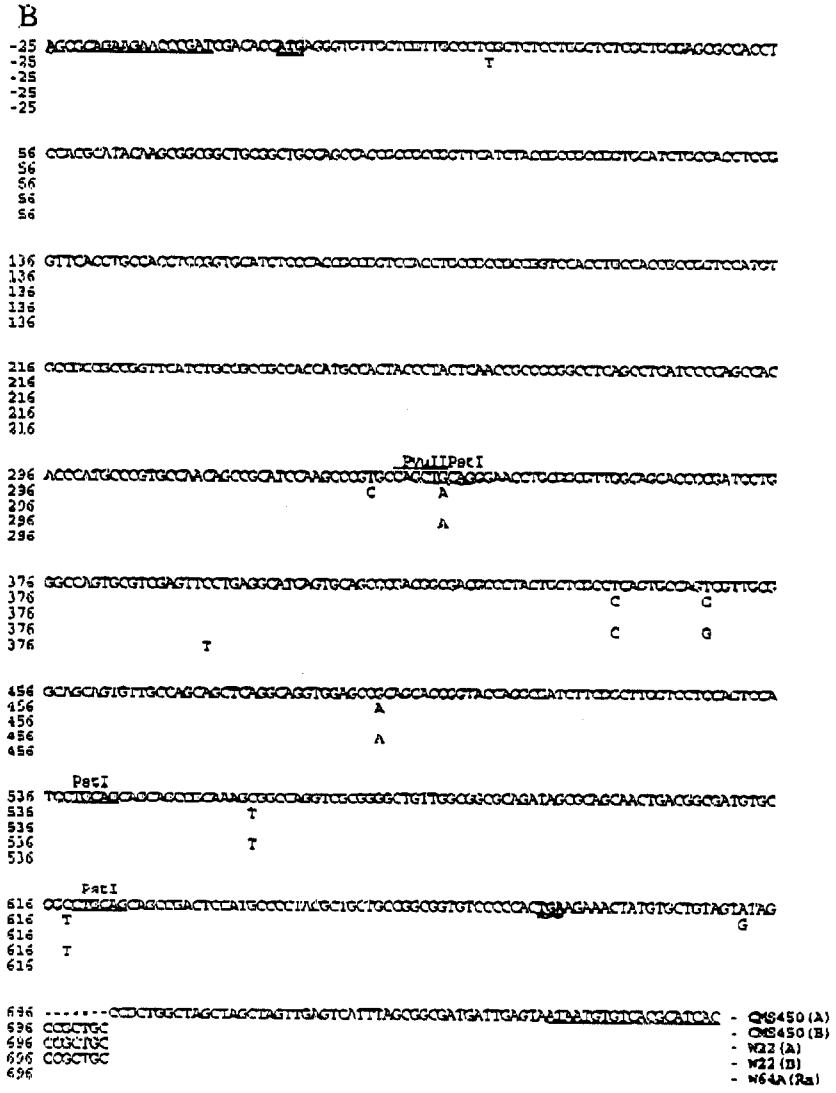


Fig. 7 Continued



by regression analysis (data not shown). Gamma-zein content was positively correlated with seed density ($R^2 = 0.82$, $P < 0.01$), consistent with the previously noted association between these two traits (Lopes and Larkins 1991).

To examine allelic composition at the gamma-zein locus, the gamma-zein genes were amplified from each RIL and assayed for the AB or Ra configuration. Since only the Ra gene has an *Eco*RI site in the 5′ flanking region (Fig. 5A), digestion of PCR amplified products allowed identification of genotypes that contain either the tandemly repeated A and B genes, or the Ra gene. This analysis showed that all lines selected for low or intermediate levels of seed modification (density) are homozygous for the Ra locus (Fig. 6C). *Eco*RI digestion of DNA sequences corresponding to the Ra gene generated fragments of 593 and 534 bp. Due to the similarity in size, only a single band is resolved by gel

Fig. 7 A Comparison of nucleotide sequences of the 5′ flanking region of gamma-zein alleles from several genotypes. Numbering is relative to the first nucleotide of the initiation codon. Only nucleotide differences between sequences are indicated. *Dashes* represent deletions, and the *underlined* sequence at the 5′ end corresponds to the upstream oligonucleotide used to prime PCR amplification. The indicated *Eco*RI site identifies the Ra allele. Names of the genotypes from which the sequences were obtained are listed below, followed by the allele designation. Sequences from CMS 450 (A and B genes) are from this work; sequences from W22 (A and B genes) are from Geraghty (1985), sequence from W64A (Ra gene) is from Prat et al. (1985). B Comparison of the nucleotide sequences of the coding region of gamma-zein alleles from several genotypes. Numbering is relative to the first nucleotide of the initiation codon. Only nucleotide differences between these sequences are indicated. The *underlined* sequences at the 5′ and 3′ ends of the sequences correspond to the oligonucleotides used to prime PCR amplification. Also, the *Pst*I and *Pvu*II sites, diagnostic for the A and B alleles, are indicated. Names of the genotypes from which the sequences were obtained are listed below, followed by the allele designation. Sources of the sequences are the same as indicated in A

electrophoresis. The only exception to this pattern was line no. 2, where the DNA was only partially digested. This is an indication that this line is heterozygous at the gamma-zein locus. All inbreds with seed density greater than 1.2 g/ml have at least 2.5-times the amount of gamma-zein found in the least modified line, and these lines always had the AB configuration at the gamma-zein locus. Similar results were obtained for recombinant inbred lines developed from a different cross (W64Ao2 × CMS450) (data not shown; Lopes 1993).

One possible explanation for the increased synthesis of gamma-zein in modified *o2* mutants is that a modifier corresponds to an additional copy of the gene, as suggested by the results from the W64Ao2 × Pool 33 cross (Fig. 6). However, there could be also be unique *cis*-acting modifications of gamma-zein genes in QPM genotypes that enhance gamma-zein gene expression. To investigate the structure of the A and B gamma-zein genes from modified *o2* genotypes, the sequences from CMS450 and Pool 33 were amplified by PCR, cloned and sequenced. Figure 7A shows a comparison of 5′ flanking sequences of the A and B genes from CMS450 with those of the lines W22 (A and B genes; Geragthy 1985) and W64A (Ra gene) (Reina et al. 1990). Only minor differences were found between the A and B gene sequences of these genotypes, and Pool 33 (data not shown). The only significant variations in the 5′ flanking regions, in addition to single base changes, were small deletions in a long AT-rich sequence located between nucleotides −977 and −851. Sequences of the A and B genes from the modified o2 genotypes are very similar to those of the W22 genes. A larger number of base changes were detected for the Ra gene of W64A when compared to the A and B genes of CMS450 and W22. It is interesting to note that these changes are mostly concentrated in the upstream part of the promoter between nucleotides −1100 and −600. Very few base changes could be found in the promoter region near the coding sequence, where the prolamine box, CAAT and TATA boxes are located. Figure 7B shows a comparison of the coding and 3′ noncoding sequences of these genes. Relatively few nucleotide differences were found between these sequences, with the most significant variation in the 3′ noncoding region. One major difference between the A and B genes is the deletion of a 7 bp stretch in the 3′ untranslated region of the A gene from CMS450 and Ra gene from W64A. This deletion is not found in the A and B genes of W22.

Discussion

Endosperm modification in *o2* mutants is a difficult trait to study due to the complex patterns of phenotypic variation observed in segregating progenies of o2 by modified *o2* crosses (Belousov 1987; Lopes and Larkins 1991). The non-availability of near isogenic modified *o2* lines that have been genetically well characterized, as well as the difficulty of scoring intermediate phenotypes, are among the major limitations to genetic and biochemical studies of endosperm modification. Furthermore, the effect of *o2* modifier genes on seed phenotype can only be easily assessed when individuals are homozygous recessive at the *O2* locus. The need for two genetic systems (*o2* and *o2* modifiers) for expression of the modified phenotype poses a serious limitation to the use of more traditional mapping strategies, such as BA translocations (Beckett 1978), for assigning modifier genes to linkage groups or chromosome arms. Transfer of these two gene systems to elite inbred lines by backcross selection is a difficult task and perhaps the major limitation for the introgression of these genes into commercially competitive hybrids. Consequently, identification of RFLP probes linked to modifier gene loci, or isolation of the genes themselves, would greatly facilitate the process of developing better QPM germplasm.

Progenies developed from the W64Ao2 × Pool 33 and Pool 33 × W2o2 crosses gave rise to approximately one-sixteenth of the F2 seeds with the parental opaque or vitreous phenotypes (Lopes and Larkins 1995). The remainder of the F2 seeds had phenotypes ranging from semi-opaque to semi-vitreous, depending on the *o2* parent. These results suggest there are likely to be two independently segregating, semidominant loci that control variation for endosperm modification in each of these crosses. Given the proposed nature of gene action, examination of *o2* modifiers seemed particularly well-suited to the bulked segregant analysis procedure of Michelmore et al. (1991). Accordingly, we tested more than 90 RFLP markers selected from across the genome with multiple restriction enzymes to search for genomic regions associated with the formation of vitreous endosperm in an *o2* background.

Only two genomic regions were detected by polymorphic marker/enzyme combinations that exhibited a distortion from random segregation, associated with endosperm modification. One locus mapped near the centromere and one locus mapped near the telomere of chromosome 7L. For the W64Ao2 × Pool 33 cross, an *o2* modifier was unambiguously mapped near the gamma-zein locus (Fig. 4). Furthermore, analysis of RILs developed from this cross showed a consistent segregation of the Ra gamma-zein locus of the W64Ao2 parent with the opaque phenotype and the AB locus of the Pool 33 parent with the vitreous phenotype. Since the only criterion used to develop the RILs was evaluation of the degree of seed modification, based upon assessment of light transmission, we conclude that seed modification is linked with a difference in the allelic composition of the gamma-zein locus.

The development of vitreous endosperm in *o2* mutants has been shown consistently to be correlated with an increase in gamma-zein protein accumulation (Wallace et al. 1990; Lopes and Larkins 1991; Geetha

et al. 1991). Comparison of storage protein accumulation in *o2*, modified *o2*, and their reciprocal F1 hybrids showed that gamma-zein increases in a dosage-dependent manner, depending upon the contribution of the genome of the modified parent (Lopes and Larkins 1991; Geetha et al. 1991). Segregating progeny that breed true for seed modification also accumulate gamma-zein protein in an amount that is positively correlated with the degree of endosperm modification (Lopes and Larkins 1995). The data of Fig. 6 show that a threefold increase in gamma-zein protein is required for conversion of the starchy endosperm of *o2* to a vitreous phenotype. Considered together with these data, the RFLP mapping data would strongly suggest that not only is the gamma-zein protein the primary effector of modification but that the gamma-zein gene itself is a strong candidate for one of those genes contributing to variation associated with the vitreous phenotype.

A second modifier near the telomere of 7L was mapped with the Pool 33 × W22*o2* cross, and then only with a single marker, umc35. Numerous other probes were tested in this region, but this was the only informative (i.e. polymorphic) marker. Additional evidence that a modifier for vitreous endosperm lies in this region comes from the recent characterization of a novel mutant, *o15*, which shows a specific reduction in gamma-zein synthesis (Dannenhoffer et al. 1995). The mutant endosperm contains approximately 60% less gamma-zein protein and mRNA than the wild type. Furthermore, this mutation results in a preferential reduction in the A gamma-zein mRNA, as would be predicted for an *o2* modifier mutant (Or et al. 1993). As is also true for the Pool 33 × W22*o2* cross, umc35 proved to be the only RFLP marker near the telomere of 7L which was diagnostic for this mutation using bulked segregation analysis. Given this correlation and the relationship between gamma-zein levels and endosperm modification, natural variation among non-mutant alleles of *o15* would have to be considered to be strong candidates for the modifier gene found in this region.

Despite the strong genetic inference that two modifier genes exist (Lopes and Larkins 1995), two apparent conundrums remain to be clarified. The first concerns the fact that while two modifier loci were identified, only one was evidently contributing to the variation seen in either of the two crosses analyzed. It should be remembered that comparative marker analysis is only able to identify those loci that contribute to the variation observed for a particular trait in the segregating progeny of two parental materials. This procedure does not identify all the genes that contribute to the phenotype, only those that are functionally different between the two parents. Hence, it is not surprising that the gamma-zein gene was not identified as a modifier locus in the cross of Pool 33 × W22*o2*, since both of the these parents contain essentially the same type of gamma-zein (AB) locus. Perhaps this situation may also exist for the modifier gene near the telomere of 7L in the W64A*o2* × Pool 33 cross. The modifier genes in the two parental lines may be functionally equivalent, and no variation for the trait can be attributed to their segregation. Nevertheless, the genetic analyses of both crosses implied at least two independently segregating modifiers, suggesting that at least one other modifer gene was not detected by this approach and remains to be identified.

This hypothesis is consistent with the second anomalous observation: while there is strong evidence that the AB gamma-zein locus is required for the modified phenotype, this locus by itself is not sufficient for modification. Rather, it is dependent upon other elements of the modification system. Several *o2* inbreds have been identified that possess the appropriate AB gamma-zein arrangement, yet express an opaque endosperm (Fig. 5 and Or et al. 1993). This hypothesis is also in agreement with results of the DNA sequence comparison of the A and B gamma-zein genes from modified and unmodified *o2* genotypes, which did not reveal any major nuclotide differences in their promoters, coding, or 3′ noncoding regions (Fig. 7). Of course we cannot rule out the possibility that some of the minor nucleotide differences between these genes could have important effects on their expression, but a more attractive explanation is that the potentiation of this difference between Ra and AB loci requires the action of an additional locus, which varies in expression among maize germplasm. We have recently identified a second opaque mutant with dramatically reduced levels of gamma-zein protein. This mutation could be an allele of *o15* or an additional modifier gene, as postulated from these observations. We are currently conducting an allelism test for these two mutants and are comparing their effects on gamma-zein gene expression in an effort to identify remaining modifier genes.

Analysis of gamma-zein gene expression in wild-type, *o2*, and modified *o2* genotypes suggested that the products of *o2* modifier genes act via a post-transcriptional mechanism to increase the accumulation of the gamma-zein mRNAs (Or et al. 1993). Although both A and B transcripts are increased, the modifiers preferentially enhance the concentration of the A RNA. There are only eight random nucleotide differences in the 5′ noncoding and coding regions between the A and B RNAs (Fig. 7); thus the products of the modifier genes are likely to recognize a sequence of conformational differences in the 3′ noncoding regions. Based on these data, we hypothesize that the *o2* modifier gene near the telomere of chromosome 7L encodes a trans-acting factor that affects gamma-zein RNA stability. Further characterization of the factors interacting with the gamma-zein RNAs may lead to the isolation of the modifier gene products and provide an explanation of their mechanism of action.

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