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Expression of A/B zeins in single and double maize endosperm mutants*

J. W. Paulis[1], J. A. Bietz[1], T. P. Bogyo[2], T. C. Nelsen[1], L. L. Darrah[3], and M. S. Zuber[3]

[1] National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL 61604, USA
[2] Washington State University, Pullman, WA 99164-6212, USA
[3] Plant Genetic Research Unit, USDA-ARS, and University of Missouri, Columbia, MO 65211, USA

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Summary. Zeins, the major endosperm proteins in maize (*Zea mays* L.), are deficient in the essential amino acids lysine and tryptophan. Some mutant genes, like *opaque-2* (*o2*) and *floury-2* (*fl2*), reduce the levels of A- and B-zeins, thereby improving maize's nutritional value. Other mutants, such as *amylose-extender* (*ae*), *floury-1* (*fl1*), *soft starch* (*h*), *dull-1* (*du*), *shrunken-1* (*sh1*), *sugary-1* (*su1*), *sugary-2* (*su2*), and *waxy* (*wx*), primarily affect starch composition, but also alter zein composition. We undertook this study to examine the effects of some of these mutant genes on A/B-zein composition and to study the interactions of these genes in double-mutant combinations. Endosperm prolamins were extracted from inbred B37, ten near-isogenic single mutants (*ae*, *du*, *fl1*, *fl2*, *h*, *o2*, *sh1*, *su1*, *su2*, and *wx*), and most double-mutant combinations. Zeins in these extracts were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) into 22–24 peaks. Of the resulting 22 major peaks the areas of 16 (per milligram endosperm) were significantly affected by individual mutant genes relative to the zein composition of the normal inbred. In combination these genes exhibited significant epistatic interactions in regulating the expression of individual A/B zeins. Epistatic interactions were judged to be significant when the amount of a peak in a double mutant differed from the averages for the peak in the two respective single mutants. The *o2* gene, alone and in combination with other mutant genes, significantly decreased the amounts of many individual zeins. The effect of the *o2* gene was the greatest of all the genes examined. Various clustering techniques were used to see if mutant effects could be grouped; among these was principal component analysis, a multivariate statistical technique that analyzes all peak sizes simultaneously. Three-dimensional scatter graphs were constructed based on the first three principal components. For the single mutants, these showed no relationships to gene actions; for the double mutants, however, this technique showed that four single mutants, *o2*, *sh1*, *su1* and *su2*, had the greatest effects on zein composition when combined with each other and with the remaining six single mutants.

Key words: Maize – Zein – Prolamins – Mutants – Genes – HPLC – Quantitation – Epistasis

Introduction

Zeins are the major storage proteins of maize (*Zea mays* L.). They account for about 50% of total endosperm protein in normal genotypes. Zeins, being prolamins, are extractable with aqueous solutions of alcohols. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reveals several unique classes of maize prolamins that differ in molecular size. Some of these prolamins are relatively minor components that are incorporated in their native state through disulfide bonds into polymeric proteins. The most abundant maize prolamins, however, are A- and B-zeins (Wilson 1991), which have molecular weights of 19–24 kDa (Misra et al. 1976).

The isoelectric focusing (IEF) of zeins shows a still greater complexity: typically 15 to 20 components are

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Correspondence to: J. W. Paulis

resolved (Salamini et al. 1985; Wilson 1986). Two-dimensional electrophoresis can still further resolve some IEF bands into 2 or more components (Hagen and Rubenstein 1980; Wall et al. 1984). Recently reversed-phase high-performance liquid chromatography (RP-HPLC) has shown that zeins have a still greater heterogeneity (Paulis and Bietz 1988; Wilson 1991). For example, zeins from hybrid maize resolve into about 24 peaks upon RP-HPLC (Smith and Smith 1988). These prolamins vary between genotypes but are constant within individual lines, so RP-HPLC of zeins permits the accurate identification of maize inbreds and hybrids (Smith and Smith 1987, 1988).

A- and B-zeins, which predominate in normal maize genotypes, are deficient in the essential amino acids lysine and tryptophan (Wilson 1991). Some mutant genes [such as *opaque-2* (*o2*) and *floury-2* (*fl2*)], however, reduce the levels of these zeins (Misra et al. 1975). In such genotypes, proportionately more proteins that contain lysine and tryptophan are synthesized, thus improving nutritional quality. The levels of these essential amino acids are also elevated by several other endosperm mutant genes: *brittle* (*bt*), *sugary-1* (*su1*), *shrunken-1,-2,-4* (*sh1*, *sh2*, *sh4*), *brittle* (*bt2*), and *opaque-7* (*o7*). When the *o2* gene is combined with other endosperm mutant genes in double mutant lines, a synergistic elevation of lysine occurs (Glover and Mertz 1987). The amounts of A- and B-zeins are extremely low in such double mutant genotypes (Tsai et al. 1978; Glover and Mertz 1987).

Total alcohol-soluble proteins [i.e., alcohol-soluble reduced glutelin (ASG) subunits plus A- and B-zeins] have been characterized in *su1*, *floury-2* (*fl2*), *o2*, and normal maize by RP-HPLC (Paulis and Bietz 1986). Subsequently, RP-HPLC was extended to show the quantitative effects of these and other mutant genes upon total alcohol-soluble proteins in ten maize single mutants and in their double mutant combinations (Paulis et al. 1990). In many double mutants, protein compositions differed significantly from the averages of compositions of corresponding single mutants. This showed major new epistatic and synergistic effects of several mutant genes on protein composition. Diallel analysis for all of the mutant peaks of total alcohol-soluble proteins (ASG and A- and B-zeins) indicated the presence of both specific and general combining ability (the average effect of a mutant allele in combination with all other mutants) (Paulis et al. 1990). Unexpectedly, these mutant genes that affected prolamin expression included several genes that primarily affect starch composition or kernel phenotype.

An earlier study (Paulis et al. 1990) considered effects of mutant genes on total amounts of A- and B-zeins (peak area 4). In that study, RP-HPLC conditions were chosen to fractionate alcohol-soluble glutelin subunits and A- and B-zeins simultaneously. Such chromatographic conditions do not optimally separate all of the A- and B-zeins, however. Nevertheless, our earlier study showed that these mutant genes, singly and in combination, affected the amounts of individual A- and B-zeins differently.

By modifying RP-HPLC gradient conditions we were able to resolve A- and B-zeins into 20–24 peaks. In the investigation reported here we performed an optimized RP-HPLC of A- and B-zeins of ten maize endosperm mutant genotypes [*amylose-extender* (*ae*), *dull-1* (*du*), *floury-1, -2* (*fl1*, *fl2*), *soft starch* (*h*), *o2*, *sh1*, *su1*, *su2*, and *waxy* (*wx*)] and of their double mutants. Statistical analyses were used to clarify the quantitative effects of these mutant genes on individual A- and B-zeins.

Materials and methods

Ten near-isogenic endosperm mutant lines of inbred B37 (*ae*, *du*, *fl1*, *fl2*, *h*, *o2*, *sh1*, *su1*, *su2*, and *wx*) and all possible double-mutant combinations (except *ae-wx*, which was not available) were supplied by L. L. Darrah. These were the same mutants used in an earlier study (Paulis et al. 1990). Proteins were prepared from these genotypes as described by Paulis and Bietz (1986), except that ten endosperms/genotype were ground in an Udy cyclone mill through a 0.61-mm screen. Ground endosperms (0.1 g) were then extracted in duplicate for 2 h at room temperature with 2 ml 70% ethanol +0.5% sodium acetate. In addition, the extractant contained 0.2% dithiothreitol (DTT) (for *o2* and *o2* double mutants) or 0.1% DTT (for all other genotypes). For the *o2* mutants, 0.2% DTT seemed to give more reproducible results than did 0.1% DTT. RP-HPLC reproducibility at different concentrations of DTT has been described previously (Paulis and Bietz 1986). Extracts remained at room temperature for 2 days prior to RP-HPLC.

RP-HPLC of duplicate extracts of mutants was performed on a Waters system including a WISP 710B automatic sample injector, Model 660 solvent programmer, and a Model 450 variable wavelength detector (210 nm; absorbance range 0.1 full scale deflection). A SynChropak C_{18} RP-P (250 × 4.1 mm) column with 300-Å pores was used at 30 °C. Solvents were aqueous solutions of acetonitrile (ACN) +0.1% (v/v) trifluoroacetic acid (Paulis and Bietz 1986). Following injection of 20-µl (for *o2* and its double mutant combinations, to reveal minor peaks better) or 10-µl (for other genotypes) samples, a linear 60-min gradient (47.5% to 57.9% ACN) was followed by a 20-min isocratic elution at 57.9% ACN. The flow rate was 1.0 ml per minute. This gradient, which uses a higher initial ACN concentration than used previously (Paulis et al. 1990), resolves A/B zeins better. Raw chromatographic data were stored in a ModComp computer system for subsequent analysis (Paulis and Bietz 1986; Paulis et al. 1990).

All calculations were based on averaged RP-HPLC data of duplicate extracts expressed as area per milligram mutant endosperm. For the ten single mutants, differences between these peaks and those of B37 were calculated. The statistical significance of these differences was tested by *F*-tests:

$$F_{(1,55)} = \frac{(\bar{\chi}_m - \bar{\chi}_{B37})^2}{s^2_{\bar{\chi}_m} - \bar{\chi}_{B37}}$$

where $\bar{\chi}_m$ is the peak size mean of the mutant, $\bar{\chi}_{B37}$ is the mean of the inbred B37, and s^2 is the mean square calculated from an analysis of variance involving all 55 genotypes of that particular peak. For double mutant combinations, contrasts

were developed as:

$$\text{Contrast} = \bar{\chi}_{ij} - 0.5(\bar{\chi}_i + \bar{\chi}_j)$$

where $\bar{\chi}_{ij}$ is the mean of the peak size of the double mutant, and $\bar{\chi}_i$ and $\bar{\chi}_j$ are means of peak sizes of the corresponding single mutants. The *F*-test was carried out by dividing the square of the value of the contrast by the appropriate mean square for the particular peak multiplied by the coefficients (Steel and Torrie 1980).

Principal component analyses were also performed using both single- and double-mutant peak means as variables. Principal component analysis is a method of analyzing correlation structures. Straight lines (principal components) are drawn in multidimensional space by use of matrix algebra. These lines are described by coefficients calculated simultaneously for all of the original variables in the data set. The first principal component is calculated as the best fit through all variables. The second principal component is then determined by the best fit through the variances unexplained by the first principal component. A third principal component can then be fitted through the variances left unexplained by the first two principal components. The procedure can continue until the number of principal components matches the number of variables, but continuing past three principal components is seldom useful. The relative amount of total variation explained by each principal component can be calculated as a measure of their relative values. The coefficients calculated for the principal components can be examined as a means of identifying subsets of variables with similar characteristics. Each mutant was thus evaluated, developing coordinates using the first three principal components. Three-dimensional scatter plots were then drawn to cluster the mutants.

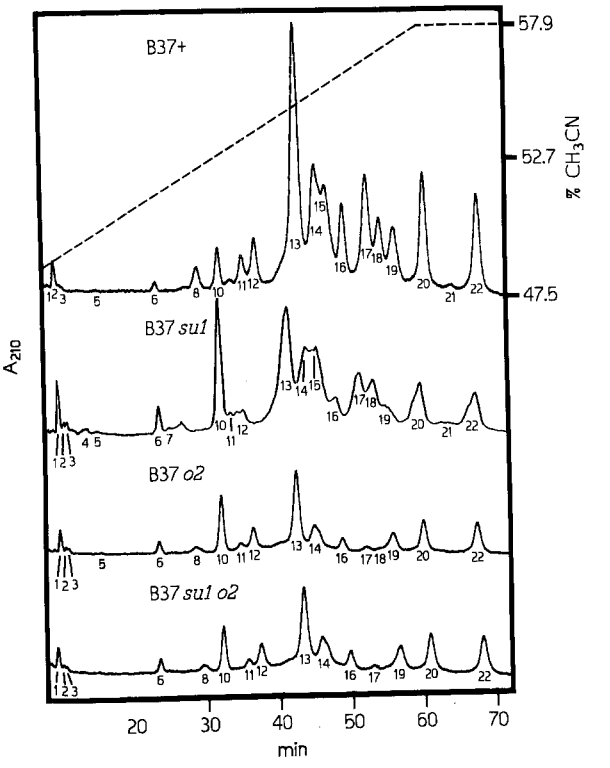

Fig. 1. Reversed-phase high-performance liquid chromatography RP-HPLC) of A/B zeins from B37 maize endosperm genotypes. All of the chromatograms are plotted at the same absorbance scale

Results and discussion

RP-HPLC of mutant maize alcohol extracts

Under the gradient conditions indicated with a higher initial ACN concentration than used previously (Paulis et al. 1990), alcohol-soluble glutelins are not retained by the reversed-phase column and elute in the initial solvent peak. Previously, these proteins eluted after 30–40 min (see Fig. 1 in Paulis et al. 1990). A- and B-zeins, previously grouped in 'multiple peak area 4' (Paulis and Bietz 1986), are now more completely resolved using the 47.5–57.9% ACN gradient. Typical chromatograms of A/B zeins from B37 normal, single-mutant, and double-mutant lines are shown in Fig. 1. Twenty-two major peaks (which varied significantly with genotype) occur (peak 9, not numbered, is a minor component found in only some mutants). In these chromatograms, plotted so that each represents the same amount of endosperm, the levels of A/B zeins decreased (both visibly and as demonstrated by integration) in *sul*, and even more in *o2* and *sul*; *o2* double-mutant genotypes. Peak 15 is totally absent in *o2* and *sul*; *o2*. Thus, although total elution profiles of all of the mutant genotypes resemble that of inbred B37, both qualitative and quantitative differences exist in the regulation of A- and B-zeins by the *sul* and *o2* genes.

Similarly, RP-HPLC was used to fractionate zeins of 55 maize single- and double-mutant genotypes. Between 22 and 24 distinct peaks resulted, each representing at least one unique protein. Of these peaks, 8 varied little with genotype and were excluded from further analysis. Chromatographic data were quantified, and data normalized to represent common injection volumes.

Effects of individual mutant genes on zein composition

To determine the effects of mutant genes on zein composition, the amounts of individual A/B zeins in near-isogenic single-mutant lines, as determined by RP-HPLC, were compared to those found in the parental inbred B37. Most mutant genes significantly affect the expression of some, but not all, individual zeins. In general, early-eluting peaks (1–12) seem to be less affected than later eluting ones. Peak 13 is significantly changed (usually reduced) by all of the mutant genes except *fl1* and *sul*. Similarly, peak 16 is affected by all of the genes except *fl1* and peak 22 is affected by all except *su2*. Generally, lower amounts of individual zeins are present in the near-isogenic single-mutant lines than in B37. Late-eluting peaks were generally lower in the single-mutant lines, except for *fl1* and *su2*.

Table 1. Effect of single mutants on average area per milligram normal B37+endosperm

Average peak sizes	Peak 1	Peak 2	Peak 3	Peak 6	Peak 8	Peak 10	Peak 12	Peak 13	Peak 14	Peak 15	Peak 16	Peak 17	Peak 18	Peak 19	Peak 20	Peak 22
ae	56	10	7	22	17	139	36	290	162	138	49	100	72	95	99	111
du	54	8	10	11	10	91	33	316	138	135	54	85	62	81	97	95
fl1	19	4	5	15	50	87	96	818	564	384	131	296	227	112	196	175
fl2	32	5	8	11	21	101	56	399	295	166	70	9	95	78	105	94
h	19	3	4	11	31	93	84	583	359	277	105	253	157	97	182	151
o2	21	4	4	15	14	82	37	189	79	0	23	9	3	46	58	66
sh1	44	8	8	28	19	132	64	466	272	321	76	185	161	64	111	114
su1	39	6	8	36	19	203	42	418	204	246	40	123	118	37	110	108
su2	12	1	1	17	72	64	103	775	531	412	157	0	210	121	244	221
wx	53	14	15	17	30	111	50	414	267	239	66	179	149	80	110	115
B37+	24	3	3	9	35	62	79	676	328	228	135	235	128	124	215	202
Differences of peak sizes (mutant-B37)																
ae-B37+	32*	7*	4*	13*	−18*	77*	−44*	−385*	−166*	−90*	−86*	−134*	−56*	−29*	−116*	−91*
du-B37+	30*	6	7*	2*	−25	29	−46*	−360*	−190*	−93*	−81*	−149*	−65*	−43*	−118*	−107*
fl1-B37+	−5	1	2	6	15	24	16	142*	236*	155*	−4	62*	99*	−12	−19	−27*
fl2-B37+	8*	3	5*	2*	−14	39	−23*	−277*	−33	−62	−65*	−226*	−32*	−46*	−110*	−108*
h-B37+	−5	1	2	2	−4	30	4	−92*	31	48	−30*	18	29*	−27*	−32*	−51*
o2-B37+	−3	1	1	6	−21	20	−43*	−486*	−249*	−228*	−112*	−225*	−124*	−78*	−157*	−136*
sh1-B37+	20*	6	5*	19*	−16*	70*	−16	−210*	−56	92*	−59*	−50*	33*	−59*	−104*	−88*
su1-B37+	15*	3	6*	27*	−17*	141*	−38*	−257*	−124*	18	−95*	−112*	−9	−87*	−105*	−94*
su2-B37+	−12*	−2	−2	8	37	1	24*	99*	202*	183*	22*	−235*	83*	−3	30	19
wx-B37+	28*	11*	12*	8*	−5	48	−29*	−262*	−61	11	−69*	−56*	22*	−44	−105*	−87*
Error MS[a]	15	8	3	37	71	624	84	1699	1210	1188	118	506	171	120	239	176

* Indicates that an area of a single mutant differs significantly ($P < 0.05$) from the normal case (B37+)

[a] Error mean square, indicating unexplained variation in the data

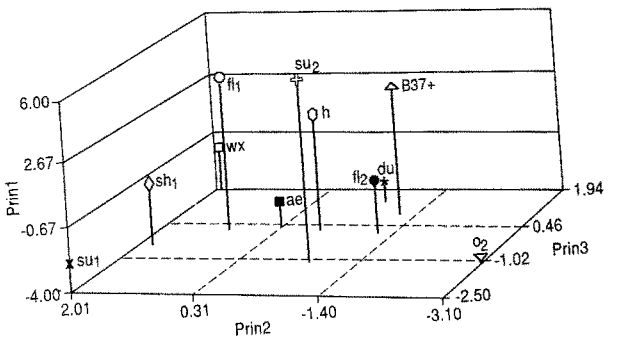

Fig. 2. A three-dimensional principal coordinates chart of the ten single mutant RP-HPLC peaks

Table 1 lists actual peak sizes for all of the single mutants and B37 and the differences between the mutants and B37 for each peak. The significance of these differences was determined by an F test with 1 and 55 degrees of freedom, where the mean square of each peak was obtained from an analysis of variance over all replications and genotypes. Table 1 also shows differences significant at the 5% level; many of these were also significant at the 1% and 0.1% levels.

The 22 peak areas for each single mutant were used as variables in a principal components analysis. More than 90% of the total variation was explained by the first three principal components. For each single mutant, a total for each principal component was calculated using peak areas for that mutant and coefficients derived from the analysis. A three-dimensional scatter plot (Fig. 2) of the totals indicates the overall similarities and differences of single mutants with respect to the distribution of the 22 peak areas. The *o2* and *su1* mutants each appear to be independent of the other mutants. The *fl2* and *du* mutants were similar to one another with B37+ loosely connected to both of them. A second general subgroup was the *ae*, *su2*, and *h* mutants. The *sh1*, *fl*, and *wx* mutants might be considered to be a loosely connected subgroup. The *fl2* and *su2* mutant genes are both on chromosome 4, but showed few similarities in this analysis. The *wx* and *sh1* mutant genes are both on chromosome 9, and did show some similarities. In general, the *o2* mutant had the greatest effect on the zein composition of the mutant zeins examined. It decreased amounts of many individual zeins the most, especially late eluting ones, and completely suppressed peak 15. The *su2* mutant suppressed peak 17, while the *fl2* and *o2* mutants greatly reduced peak 17.

Zein expression in double-mutant combinations

We next examined how mutant genes in combination regulate the expression of A/B zeins. Areas for all of the peaks were recorded and represented in diallel tables. An example, for peak 15, is shown in Table 2. Diallel analyses, performed as reported previously (Paulis et al. 1990), showed significant specific effects. These indicated that certain combinations of mutants deviated

Table 2. Average areas of peak 15 (area per milligram endosperm) in single and double combinations (a) and Contrasts of peak 15 area means of double mutants compared to average areas of the two single mutants[a] (b)

a										
ae	*ae*	*du*	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
du	138	184	169	320	72	0	195	213	112	0
fl1		135	224	336	103	0	242	302	394	384
fl2			384	301	370	0	386	353	457	298
h				166	324	0	253	476	385	289
o2					277	0	331	353	222	307
sh1						0	0	0	0	0
su1							321	350	289	350
su2								246	363	263
wx									412	406
										239
b										
ae		*du*	*fl1*	*fl2*	*h*	*02*	*sh1*	*su1*	*su2*	*wx*
du			(92)	168	(135)	(69)			(163)	
fl1				186	(103)	(68)		111	120	197
fl2					81	(185)	225		75	
h					103	(83)		270	96	86
o2						(138)		92	84	
sh1							(160)	(123)	(206)	(120)
su1								67	(77)	70
su2										286

[a] Only significant ($P < 0.05$) positive (or negative in parentheses) values are shown

from the general effects of the individual single mutants. This suggests that the effect of the double mutants on A/B zein expression differs from what one would expect assuming an additive relationship of the two effects; thus, epistasis, or epistatic deviation. To estimate the direction and amount of this interaction, contrasts were calculated as described in the Materials and methods section. For Peak 15, significant interactions are also shown in Table 2. Positive values show that the peak areas were larger for the double mutants than the average of the two single mutants, and negative values (in parentheses) indicate reductions in peak sizes from what would be expected if mutant gene effects were additive. Similar tables were constructed for all 16 peaks; Table 3 summarizes the results. In this table, only contrasts significant at the 5% level and the direction of deviations are shown; many deviations were significant at the 1% or 0.1% levels.

Table 3 shows that most double-mutant genotypes containing *o2* have significantly fewer late-eluting peaks (13 or higher). Double mutants containing *fl2* showed the opposite effect and had increased numbers of these peaks. Every double mutant containing *o2* lacked peak 15 (shown in Table 3 as a significant negative deviation

Table 3. Significant epistatic effects of double mutants[a]

Mutant combination	Peak number 1	2	3	6	8	10	12	13	14	15	16	17	18	19	20	22
ae-du	+		+					+	+		+	+	+	+	+	+
ae-fl1	+	+	+				−	−	−	−	−	−	−	−	−	−
ae-fl2	−	−	−		+	−	+	+	+	+	+	+	+	+	+	+
ae-h	+	+	+	+		+	−	−	−	−	−	−	−	−	−	−
ae-o2	−							−	−	−	−	−	−	−	−	−
ae-sh1											+					+
ae-su1				+		+	+	+		+	−					+
ae-su2			+		−		+	−	+	−	−	−	−			−
du-fl1	+							−	−		−	−	−			
du-fl2	−		−		+		+	+	+	+	+	+	+	+	+	
du-h	+						−	−	+	−	−	−	−	−	−	
du-o2	−		−							−	−					
du-sh1				+		+	+					−			+	+
du-su1	−		−	+	+		+	+	+	+	+	+	+	+	+	+
du-su2	−		−		+		+	+	+	+	+	+	+	+	+	−
du-wx				+	+		+	+	+	+	+	+	+	+	+	−
fl1-fl2	−		−					+			+	+		+	+	+
fl1-h					+					+	+	+	+			
fl1-o2					−		−	+	−	−	−	−	−	−	−	−
fl1-sh1	−				+	+	+	+	+	+	+	+	+	+	+	+
fl1-su1	−			+			+	+	+		+		+	+	+	+
fl1-su2			−		−	−				+	+		+		−	−
fl1-wx							−				−	+			−	
fl2-h	−		−				+	+	+	+	+	+	+	+	+	+
fl2-o2	−		−				+		−	−			−		+	+
fl2-sh1	−	+	−		+	−	+	+	+		+	−		+		+
fl2-su1			−	+	+	+	+	+	+	+	+	−	+	+	+	+
fl2-su2	−		−				+	+	+	+	+	+	+		+	
fl2-wx			−					+		+	+	+	+			−
h-o2				+		+		−	−	−	−	−	−		−	
h-sh1								+			+		+			
h-su1	+			+		+	+	+	+	+	+	−	+			+
h-su2	−				+		+	+	+	+	+	+	+	+	+	+
h-wx	−		+								−		+			
o2-sh1	−		−		−		−	−	−	−	−	−	−	−	−	−
o2-su1						−				−	+	+	−	+		
o2-su2	+				−		−	−	−	−	−		−	−	−	−
o2-wx			−	+						−	+	−	−	+		+
sh1-su1		+	−		+	+	+	+	+	+	+		+	+	+	+
sh1-su2	+	+	+		−	+		−	−	−	−	+	−	−	−	−
sh1-wx				+		+	+	+	+	+	+		+		+	+
su1-su2	−		−		−		+	+			+	+	+		+	
su1-wx				+		+	−	+	+		+		+		+	+
su2-wx	−	−	−					+	+	+	+	+	+	+	+	+

[a] + and − indicate statistically significant ($P > 0.05$) positive or negative contrasts, respectively

from the averages). Of the 44 mutant combinations, 42 showed significant increases or decreases for peak 16; peak 2 had the smallest number of significant contrasts (7 out of 44). Full interpretation of these data should lead to an improved understanding of the specific regulatory actions of mutant genes on the complex loci coding for A and B zeins.

In search of clustering of effects principal component analysis was next done for all double mutants containing the major mutant genes *o2*, *sh1*, *su1*, and *su2*, considering all A/B zein peaks simultaneously. The first three principal components accounted for more than 83% of the total variation. Plotting these coordinates for each genotype yielded a three-dimensional scattergram for the 36 double mutants (Fig. 3). This scattergram shows that double mutants containing *o2* tend to cluster together. As noted above, all genotypes containing *o2* lack peak 15 and generally have low amounts of A/B zeins. One *o2* double mutant, *o2-sh1*, completely lacks peaks 8, 15, 16, 17, 18, and 19.

The scattergram in Fig. 3 shows that double mutants containing *su1* also tend to cluster, as do those containing *su2* (with the exception of the *ae-su2* mutant). Double mutants containing *sh1* formed a looser cluster.

Other clusters of mutant genes (Fig. 3) also seem to be associated with specific regulatory effects on A/B zeins. In the double mutants containing *su2* peaks 2 and 3 are either small or absent, while peaks 13 and 15 are quite high. Double mutants containing the *sh1* gene characteristically have intermediate amounts of A/B zeins; peak 17 is missing from two double mutants (*fl2-sh1* and *sh1-su1*) containing this gene.

There is a remarkable positive correlation between the amount of starch synthesized by a mutant and the amount of zein in the endosperm (Glover and Mertz 1987). In some mutant lines these apparent changes in protein content due to altered starch synthesis suggest that we should compare amounts of protein per kernel. Since some B37 mutant lines were grown in different years and locations and endosperm sizes vary with

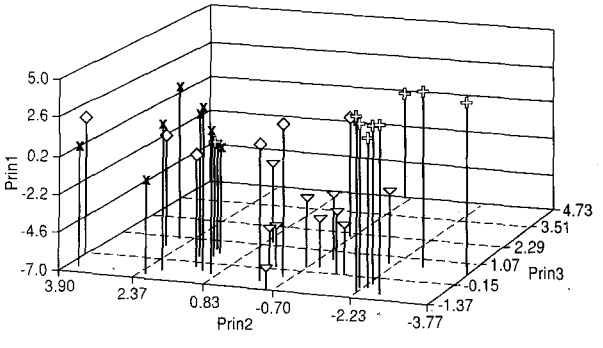

Fig. 3. A three-dimensional principal coordinates chart of the four major double-mutant groups, each with combinations of all other mutants. ▽Group *o2*, ◇ *sh1*, ✕ *su1*, ✙ *su2*

environment, protein amounts have been reported per milligram dried mature endosperm. Paulis et al. (1990) reported that environment has a minimum effect on zein RP-HPLC profiles.

The *o2* gene, alone and in combination with other starch mutants, reduces A/B-zeins (mg/endosperm) more than any other mutant (Tsai et al. 1978). Isoelectric focusing revealed that this decrease in zeins results in selective decreases in some subunits (Soave et al. 1975). RP-HPLC has shown that the amounts of individual A/B zeins can be related to mutant genes by principal component analysis. If only starch were changing, all of the zeins would change by the same amount. A/B zeins cluster for the *o2*, *sh*, *su1*, and *su2* genes in double mutants (Fig. 3). Principal component analysis of A/B zeins separated by RP-HPLC shows that *su1*, *su2*, *sh*, and *o2* in double mutant combinations act as primary mutant genes affecting individual zein synthesis. An explanation of the precise mechanisms of action awaits further studies, however.

A/B zein separations by combinations of methods based on size, charge, and surface hydrophobicity reveal far greater heterogeneity and complexity than is apparent from any single method (Paulis and Bietz 1988; Wilson 1991). Presumably similar heterogeneity exists in the single- and double-mutant genotypes investigated here, and more A/B zeins exist than are resolved by RP-HPLC. This may partially explain the apparently complex regulation of A/B zeins (actually zein-containing peaks) by the mutants (Table 3) and why a few HPLC peaks (which may contain multiple A/B zeins) do not vary significantly among mutant genotypes.

Our results clearly show major epistatic interactions among mutant genes regulating the production of A/B zeins in maize. As noted for alcohol-soluble glutelin subunits (Paulis et al. 1990), these effects, surprisingly, often involve double-mutant combinations of genes primarily known to modify carbohydrate accumulation or composition. Clearly, the regulation of expression of complex zein loci, and of individual genes, is highly intricate; such effects may also often be secondary or indirect. Misra et al. (1975) also noted epistatic effects on zein accumulation in *o2* double mutants. Such mutant combinations may be important because they have improved nutritional value due to their lower contents of A/B zeins, which are deficient in lysine and tryptophan (Mertz 1986).

In conclusion, we have shown that the quantitative capabilities of RP-HPLC add a new dimension to the analysis of the genetic regulation and relationships of maize A/B zeins. Individual zeins and overall zein composition may be regulated by various mutant gene loci, singly or in combination. Such knowledge may be useful in breeding genotypes with improved nutritional value and desirable kernel characteristics.

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