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Expression of alcohol-soluble endosperm proteins in maize single and double mutants*

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Summary. Many maize (*Zea mays* L.) mutant genes exist. Some affect protein content or composition, while others modify carbohydrates or kernel phenotype. In double-mutant lines, two mutant genes are present. We know little about interactions of such genes, however. We therefore examined a normal maize inbred, B37, 10 near-isogenic single mutants and 46 double mutants to analyze quantitative effects on alcohol-soluble endosperm proteins. Proteins were extracted with 70% ethanol-0.5% sodium acetate-5% mercaptoethanol, and fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). Early peaks were alcohol-soluble glutelin (ASG) subunits, while late peaks contained zein. Results were quantified and statistically analyzed. In many double mutants, protein compositions differed significantly from averages of compositions of corresponding single mutants. For example, a high-methionine, water-insoluble ASG is absent when the opaque-2 (*o2*) gene combines with shrunken-1 (*sh1*) or surgary-1 (*su1*). Another water-insoluble ASG nearly doubled when floury-2 (*fl2*) and *su1* combined. A high-proline, high-histidine, water-soluble ASG nearly doubled in combinations of *fl2* with *o2*, *su1* and sugary-2 (*su2*). Zein was about half its expected value when *o2* combined with amylose-extender (*ae*), floury-1 (*fl1*), soft-starch (*h*), *sh1* and *su1*. Thus, rapid protein extraction and quantitative RP-HPLC showed major new epistatic and synergistic effects of several mutant genes on protein composition. Unexpectedly, these effects often involve genes that primarily affect starch composition or kernel phenotype. Alcohol-soluble proteins often vary in amount, as in *o2* lines. They also differ in nutritional value. Thus, RP-HPLC analysis of these proteins can identify nutritionally superior genotypes, and may help explain the basis of such quality.

Key words: Alcohol-soluble proteins – Maize single mutants – Maize double mutants – Corn

Introduction

Since the early 1960s, maize (*Zea mays* L.) mutants were known that had less zein and more lysine-containing proteins in their endosperm than did normal genotypes. The first high-lysine mutant was opaque-2 (*o2*) (Mertz et al. 1964). Later, other mutants that affect quantity of zein in maize endosperm were discovered (Misra et al. 1972).

Using better analytical techniques, it became clear that maize protein synthesis is influenced by interactions of mutant genes with other genes and by genetic modifier factors. Such studies are facilitated by the development of double-mutant lines, in which two independent mutant genes exist in a near-isogenic background. We must examine the composition of alcohol-soluble endosperm proteins in such genotypes to understand marked changes due to mutant genes.

Various studies have investigated such double-mutant lines. A summary of gene interactions that affect kernel phenotype was compiled by Garwood and Creech (1972). Carbohydrate composition and kernel phenotype change most in many double mutants. For example, *o2* and floury-2 (*fl2*) are epistatic to several other mutant genes associated with wrinkled or shrunken phenotype (Barbosa and Glover 1978a, b).

Double mutants may also vary significantly in protein amount or composition (Glover et al. 1975; Bar-

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bosa and Glover 1978c, d). Misra et al. (1972, 1975a) separated proteins of double mutants of *o2* with other mutant genes into five fractions by the Landry and Moureaux (1970) method. In several genotypes, levels of albumins, globulins and glutelins were higher than in single mutants. Also, double mutants of *o2* with sugary-1 (*su1*), shrunken-1 (*sh1*), shrunken-2 (*sh2*), shrunken-4 (*sh4*), brittle-1 (*bt1*) or brittle-2 (*bt2*) contained very low amounts of prolamins (Misra et al. 1972, 1975a, b, c). Soave et al. (1976) concluded that expression of the *o2* mutant allele depends either on structural or regulatory components specifically present in the background, and on modulated inhibition by the *o2* allele.

Other major genes such as opaque-7 (*o7*) and *fl2* also affect zein synthesis (Misra et al. 1972; Barbosa and Glover 1978d). Di Fonzo et al. (1980) noted differences in the interaction of *o2* with *fl2* and *o7* in regulating amounts of albumins/globulins, zeins and glutelins. There was no additivity between *o2* and *fl2*, but *o2* and *o7* acted additively to repress zein synthesis. Both alleles were active and acted independently. Salamini et al. (1983), in a study of the mucronate (*Mc*) gene that decreases zein and increases methionine content, noted a synergistic interaction with *o2* in repressing zein synthesis. The double mutant *o2;Mc* contained less than 10% as much zein as normal endosperm, while albumins, globulins and glutelins increased more than expected from their levels in the single mutants. The combination of *o2* with starch-modified or starch-deficient mutants produces cumulative and synergistic effects, respectively, in regulating zein synthesis (Misra et al. 1975a, b, c; Tsai et al. 1978). Misra et al. (1975b) and Lee and Tsai (1984) also found nearly complete blockage of zein synthesis in double mutants such as *bt2;o2*. This corresponded with rising ribonuclease levels in the developing endosperm.

Previous studies have not, however, adequately studied differences in compositions or amounts of maize protein classes or of individual zeins. An improved analytical method, reversed-phase high-performance liquid chromatography (RP-HPLC), now makes such analyses possible. Paulis and Bietz (1986) used RP-HPLC to quantitate alcohol-soluble proteins from normal, *o2* and *su1* genotypes. This rapid technique indicated protein quality and endosperm type. Three early eluting peaks contained water-insoluble and -soluble alcohol-soluble glutelin subunits (wiASG and wsASG). Zein eluted last and had multiple components. The *o2* gene significantly reduced zein and slightly reduced a high-methionine wiASG, while increasing other glutelin subunits. The *su1* gene reduced zein to a lesser degree, but increased the other three protein peaks.

Because of these initial studies, the ability to better quantify RP-HPLC data, and the availability of additional double-mutant lines, we further explored effects of single mutants on the expression of zein and alcohol-soluble glutelin subunits, and we investigated epistatic and synergistic effects of these genes in combination. We thus performed RP-HPLC of alcohol-soluble proteins of ten maize endosperm mutants [amylose-extender (*ae*), dull (*du*), floury-1 (*fl1*), *fl2*, soft starch (*h*), *o2*, *sh1*, *su1*, *su2*, and waxy (*wx*)] and all possible double-mutant combinations (except *ae;wx*, which was not available).

Materials and methods

Maize genotypes

We obtained ten near-isogenic mutant strains of inbred line B37 (*ae, du, fl1, fl2, h, o2, sh1, su1, su2* and *wx*), all possible double-mutant combinations (except *ae;wx*), and the normal counterpart from Darrah and Zuber. All mutant seed had been self-pollinated by hand and grown using 120–160 lb nitrogen/acre from 1976 to 1982 in Missouri or Puerto Rico. These mutants are described by Coe and Neuffer (1976). Mutants were all backcrossed at least six times to the inbred B37, so about 99% of all alleles should be near-isogenic (i.e., from B37) except for the mutant genes.

Sample preparation

Endosperms were ground in a Udy cyclone mill through a 0.02-in. screen. Total alcohol-soluble proteins (zein, wsASG and wiASG) were simultaneously extracted at room temperature from ten single B37 mutants and from all double mutants (except *ae;wx*). Samples were shaken with 70% (v/v) ethanol-0.5% sodium acetate-5% (v/v) mercaptoethanol (ME) at a meal-to-solvent ratio of 0.1 g per 2 ml (Paulis and Bietz 1986).

RP-HPLC

RP-HPLC was done as described previously (Paulis and Bietz 1986), except for use of a Waters Model 660 solvent programmer and Model 450 variable wavelength detector (210 nm; 0.4 absorbance units full scale). We performed duplicate analyses of all single- and double-mutant samples on separate 20-µl aliquots of extracts, to provide a measure of sampling error for statistical tests. A fixed experimental model was used with phenotype as a fixed source of variation. A B37 extract was assayed every ten samples as a standard to monitor any change in elution. The residual variance was low (0.483). Chromatogram peak areas were integrated with the assistance of a computer program (MANCPC) in which start and stop times and baseline positions are operator defined. At 210 nm, relative areas (volt × seconds) are assumed proportional to amounts of protein.

As before (Paulis and Bietz 1986), three major early-eluting peaks and one major late-eluting group of peaks resulted upon RP-HPLC of alcohol-soluble maize proteins (Fig. 1). Amino acid analysis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to identify these fractions (Paulis and Bietz 1986; Landry et al. 1983) and compare them to those of Landry and Moureaux (1970) (sequence D), as used by Misra et al. (1975a) to compare single mutants to *o2* double mutants. Peak 1 is mainly a high-methionine 15-K wiASG subunit [part of Landry-Moureaux (1970) fraction III]. Peak 2 (including associated shoulder 2c) contained the high-proline, high-histidine wsASG (part of Landry-Moureaux fractions III and IV). Peak 3 contained a wiASG subunit (part of Landry-Moureaux fraction III). Peak area 4 contained primarily zein (Landry-Moureaux fraction II), plus a minor 10-K high-methionine wiASG subunit (part of Landry-Moureaux fraction III).

Table 1. Analysis of variance for RP-HPLC peak areas

Source of variation	*df*	Mean squares				
		Peak 1	Peak 2	Peak 2C	Peak 3	Peak 4
Genotypes	54	1.36**	22.17**	16.91**	38.31**	186.38**
Diallel entries vs *sh2; sul*	1	0.06	14.61**	10.30**	74.16**	237.20**
Among-diallel entries	53	1.38**	22.32**	17.04**	37.64**	185.42**
General combining ability[a]	9	2.50**	54.80**	87.48**	165.30**	794.90**
Specific combining ability[a]	44	1.16**	15.94**	4.14**	13.30**	69.52**
Error	61	0.60	0.91	0.63	2.41	7.18
Coefficient of variation (%)		32.3	10.8	22.1	28.2	3.4
Coefficient of determination (%)		66.8	95.6	96.0	93.4	95.8

[a] Sums of squares for general and specific combining ability do not add to the sums of squares for among-diallel entries because of inclusion of estimated values for *ae; wx* when performing the diallel analysis. Sums of squares for all other sources were calculated without the estimated values

** Significant at the 0.01 level of probability

Statistical analyses

Single mutants were treated as parents and double mutants as crosses for application of Griffing's (1956) Model I (fixed parents), Method 2 (parents and one set of crosses) diallel analysis of variance to estimate general and specific combining ability effects. Diallel mating systems are explained in detail by Cockerham (1963).

The statistical model for Model I, Method 2 is

$$X_{ij}=\mu+g_i+g_j+s_{ij}+e_{ijk}$$

where X_{ij} is the value for cross ij, μ is the population mean, g_i is the general effect for parent i, g_j is the general effect for parent j, s_{ij} is the specific effect for the cross ij and e_{ijk} is the random error term associated with the cross ij for the k^{th} observation.

Analysis of variance was performed for RP-HPLC peaks 1, 2, 2C, 3 and 4 (Table 1). The missing double mutant, *ae;wx*, was estimated using the procedure outlined by Eckhardt (1952); one degree of freedom was subtracted from the specific combining ability degrees of freedom. Several duplicate enteries of *sh2;su1* were analyzed, and the contrast between the diallel entries and *sh2;su1* was partitioned out of the entry source of variation. The error term in the analysis was based on differences among duplicate laboratory analyses (runs) of entries.

Preliminary diallel analysis (Table 1) indicated the presence ($P<0.01$) of both a general effect (i.e., the average effect of a single mutant over all combinations with the other nine mutants) and specific effects (in this case, deviations from the average effect in specific combinations). This analysis was appropriate, since (although originally developed for testing combining ability in maize) it is a specialized analysis for designs with a factorial arrangement of treatments, where treatments are all possible combinations of levels of main effects.

In our results, quantities of protein fractions are expressed as relative peak area units/mg endosperm. For single mutants, effects were calculated as peak size/mg mutant endosperm minus the corresponding area/mass ratio for the normal allele. The significance of these differences was tested by *F*-tests, using statistical methods developed for testing contrasts or, in this case, for mutant effect (Steel and Torrie 1980).

For double mutants, comparisons (contrasts) were between peak areas/mg endosperm and the average area/mass ratio for the two corresponding single mutants. These contrasts were tested by an appropriate *F*-test. Results listed below for double mutants specify area/mass ratios, percent differences of areas of double mutants compared to averages of the two corresponding single mutants and significant differences.

Results and discussion

Figure 1 shows typical RP-HPLC chromatograms of proteins from B37, two single mutant lines and their double mutant. Zein (peak area 4) decreases in *o2* mutants. This decrease is extreme in the *o2;sh1* double mutant.

In some mutant lines, apparent changes in protein content may be due to altered starch synthesis, which suggests that we should compare protein on a per kernel basis. Tsai et al. (1978) reported zein on a mg per endosperm basis for single and *o2* double maize mutants. Some B37 mutant lines were grown during different years and at different locations, however, and endosperm sizes may vary with environment. Thus, protein amounts (below) were reported per mg endosperm.

This approach is justified, however, since Paulis and Bietz (1986) showed similar elution profiles for *o2*, *su1* and *fl2* lines from different locations and grown during different years. Similarly, Smith and Smith (1986), studying alcohol-soluble maize inbred proteins from different environments, found variation of 2% or less within inbreds. Thus, environment has minimal effect on RP-HPLC profile, and results can be reliably expressed as area/mg endosperm without bias from endosperm size.

Comparison of single mutants with B37 (+)

Protein compositions of single mutants may vary significantly from that of the parental inbred (Table 2). All single mutants had decreased amounts of one or more peaks. Only in *fl2* were all four peaks decreased signifi-

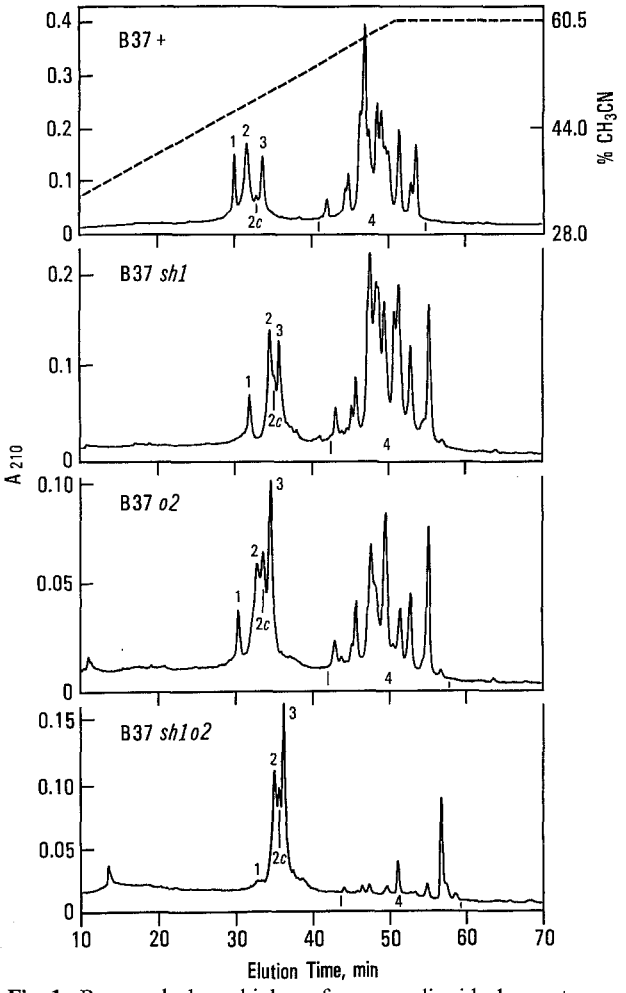

Fig. 1. Reversed-phase high-performance liquid chromatography (RP-HPLC) of proteins extracted with 70% ethanol-0.5% sodium acetate-5% 2-mercaptoethanol from normal maize inbred (B37), two B37 single mutants (*sh1* and *o2*) and the B37 double mutant *sh1;o2*

cantly; Soave et al. (1976) also found decreased zein in *fl2*. Mutants *ae, fl2, o2, sh1* and *wx* significantly ($P<0.001$) reduce the size of peak 1 (wiASG). Peak 2 (wsASG) is significantly ($P<0.001$) reduced in the *fl2, o2, sh1* and *su1* mutants. Peak 3 (wiASG) decreases significantly ($P<0.001$) in *fl1* and *fl2*. Zein (peak area 4) decreases greatly in *o2*, and to a lesser extent in *ae* and *fl2* mutants.

Comparison of double with single mutants

Peak 1. In double mutants of *du* with *fl1, fl2, sh1*, and *su1* (Table 3), peak 1 (a high-methionine 15-K wiASG subunit) decreased ($P<0.05$) to ca. 48% of the average of the two corresponding single mutants. In the *su1;o2* and *shl;o2* double mutants, peak 1 disappeared completely. In *ae;su2*, peak 1 increased ($P<0.05$) 76% compared to the average of the single mutants.

Table 2. Effect of single mutants of B37 on composition of alcohol-soluble proteins[a]

Genotype	Peak 1	Peak 2	Peak 3	Combined Peak area 4
ae	18***	111	52	506*
du	45	97	25*	575
fl1	30	90	23***	819
fl2	17***	41***	23***	554*
h	24*	92	35	880
o2	10***	72***	56	258***
sh1	16***	68***	31*	743
su1	25	75***	35	608
su2	23*	89	30*	873
wx	12***	88	31*	747
+	42	108	52	721

[a] Protein compositions are expressed as average peak areas/mg endosperm

*, *** Indicate that average RP-HPLC peak area of the mutant endosperm differs at the 5% or 0.1% levels respectively, of significance from that of B37 (+)

Peak 2. Peak 2, containing high-proline, high-histidine wsASG, varies greatly and significantly ($P<0.001$) in many double mutants (Table 4). In most cases (e.g. *su1;fl1* and *su1;sh1*), the peak is significantly ($P<0.001$) larger (49%) than the average of the corresponding single mutants. In *fl2* double mutants with *ae, du, o2, su1* and *su2*, peak 2 increases ($P<0.001$) by an average of 77% compared to the average of the single mutants. The *fl2* gene also interacts less significantly with *h* and *wx* to increase peak 2. In part, these increases may represent decreased zein synthesis: Soave et al. (1978) found that *fl2* homogeneously inhibited all zeins. In the double mutant *ae;h*, peak 2 decreases by 37%. Less significant ($P<0.01$ or 0.05) increases (*o2;du, su1;h, su2;du, wx;du* and *wx;su2*) and decreases (*sh1;h*) in peak 2 occurred in other double mutants.

Peak 3. Amounts of wiASG in peak 3 are positively ($P<0.01$) affected in *su1;du* and *su1;fl2* (Table 5). The double mutants *fl2;o2, h;o2, sh1;h, su1;fl1, su2;du* and *wx;fl1* combine to a lesser extent ($P<0.05$) to increase peak 3. These significant effects mostly differ from those for high-methionine wiASG (peak 1).

Peak 4. The amount of protein in peak area 4 (zein) (Table 6) is significantly ($P<0.001$) increased in *fl2;du* and *fl2;su1*. It is less significantly ($P<0.01$ or 0.05) increased in *fl2;ae, su1;fl1, su1;sh1, wx;du, wx;fl2, wx;sh1* and *wx;su1*. Zein content is significantly ($P<0.001$) decreased in *h;ae, o2;fl1, o2;h* and *o2;sh1;* this synergistic effect is especially prominent in *o2;sh1*. Zeins decrease to a lesser extent ($P<0.01$ or 0.05) in *h;du* and *su1;o2*.

Significant quantitative differences also exist for zeins in *o2, fl2* and *ae* single mutants (Table 2), suggesting that

Table 3. Comparison of single mutants (on the diagonal) with double mutants (off the diagonal) for peak area 1 (high-methionine wiASG)

Mutant	(a) Average peak area/mg endosperm[a]									
	ae	*du*	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae	18	20	16	33	11	7	20	24	36*	
du		45	20*	14*	20	15	13*	17*	35	28
fl1			30	27	17	8	25	26	29	19
fl2				17	29	14	16	37	33	22
h					24	10	35	33	23	22
o2						10	0	0	12	7
sh1							16	24	14	18
su1								25	23	23
su2									23	24
wx										12
	(b) Contrasts of double mutants vs average of single mutants (% increase or decrease of expected value)									
		du	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae		−37	−33	88	−48	−50	17	12	76	
du			−47	−55	−42	−45	−57	−51	3	−2
fl1				5	−37	−60	9	−5	9	−10
fl2					42	4	3	76	65	52
h						−41	75	35	−2	22
o2							−100	−100	−27	−36
sh1								17	−28	29
su1									−4	24
su2										37

[a] Peak areas were measured by absorbance at 210 nm
* Indicates that area size of the double mutant is significantly different from the average of the two single mutants at the 5% level of significance

Table 4. Comparison of single mutants (on the diagonal) with double mutants (off the diagonal) for peak area 2 (high-proline and -histidine wsASG)

Mutant	(a) Average peak area/mg endosperm[a]									
	ae	*du*	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae	111	104	88	120***	64***	75	98	99	116	
du		97	101	111***	86	111**	73	97	120**	120**
fl1			90	83	86	78	80	121***	89	96
fl2				41	93*	110***	35	116***	112***	100**
h					92	98	112**	109*	101	82
o2						72	77	91	82	82
sh1							68	108***	82	81
su1								75	89	81
su2									89	111*
wx										88
	(b) Contrasts of double mutants vs average of single mutants (% increase or decrease of expected value)									
		du	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae		0	−12	58	−37	−18	9	6	16	
du			8	61	−9	31	−12	13	29	30
fl1				27	−5	−4	1	47	− 1	8
fl2					40	95	−36	100	72	55
h						20	40	31	12	−9
o2							10	24	2	2
sh1								51	4	4
su1									9	−1
su2										25

[a] Peak areas were measured by absorbance at 210 nm
*, **, *** Indicate that area size of the double mutant is significantly different from the aveage of the two single mutants at the 5%, 1%, or 0.1% level of significance, respectively

Table 5. Comparison of single mutants (on the diagonal) with double mutants (off the diagonal) for peak area 3 (wiASG)

Mutant	(a) Average peak area/mg endosperm[a]									
	ae	*du*	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae	52	39	36	31	32	54	42	38	13	
du		25	41	16	32	51	29	54**	48*	45
fl1			23	38	37	43	32	51*	40	46*
fl2				23	43	59*	20	58**	43	18
h					35	66*	54*	30	29	32
o2						56	55	40	36	61
sh1							31	39	29	35
su1								35	31	44
su2									30	33
wx										31
	(b) Contrasts of double mutants vs average of single mutants (% increase or decrease of expected value)									
		du	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae		1	−4	−17	−26	0	1	−13	−68	
du			71	−33	7	26	4	80	45	61
fl1				65	28	9	19	76	51	70
fl2					48	49	−26	100	62	−33
h						45	64	−14	−11	−3
o2							26	−12	−16	40
sh1								18	−5	13
su1									−5	33
su2										8

[a] Peak areas were measured by absorbance at 210 nm
*, ** Indicate that area size of the double mutant is significantly different from the average of the two single mutants at the 5% or 1% level of significance, respectively

Table 6. Comparison of single mutants (on the diagonal) with double mutants (off the diagonal) for combined peaks in area 4 (Zeins)

Mutant	(a) Average peak area/mg endosperm[a]									
	ae	*du*	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae	506	614	608	770**	402***	199*	704	620	659	
du		575	702	904***	570*	347	702	547	833	852*
fl1			819	878*	791	247***	714	909**	873	776
fl2				554	853	391	688	856***	827	863**
h					880	292***	806	848	930	879
o2						258	69***	193**	499	394
sh1							743	856*	790	954**
su1								608	810	844*
su2									873	937
wx										747
	(b) Contrasts of double mutants vs average of single mutants (% increase or decrease of expected value)									
		du	*fl1*	*fl2*	*h*	*o2*	*sh1*	*su1*	*su2*	*wx*
ae		14	−8	45	−42	−48	13	11	−4	
du			1	60	−22	−17	7	−8	15	29
fl1				28	−7	−54	−9	27	3	−1
fl2					19	− 4	6	47	16	33
h						−49	−1	14	6	8
o2							−86	−55	−12	−22
sh1								27	−2	28
su1									9	25
su2										16

[a] Peak areas were measured by absorbance at 210 nm
*, **, *** Indicate that area size of the double mutant is significantly different from the average of the two single mutants at the 5%, 1% or 0.1% level of significance, respectively

these genes may regulate different loci. We have confirmed this by showing different quantitative effects of mutant genes, singly and in combination, on individual zein polypeptides. We will describe these studies in detail in a separate paper.

Misra et al. (1975a) and Tsai et al. (1978) also noted that *sh1* and *su1*, in combination with *o2*, led to a major decrease in zein. The combinations with *o2* in this study did not cause as marked a decrease in zein per mg endosperm compared to some *o2* double mutants (*sh2;o2, sh4;o2, bt;o2,* and *bt2;o2*), which all have smaller endosperms than their respective single mutants (Misra et al. 1975a; Tsai et al. 1978).

Our results clearly show major synergistic and epistatic interactions among mutant genes in maize in regulating accumulation of alcohol-soluble endosperm proteins. Often, these effects somewhat surprisingly involve double mutants of genes primarily thought to modify carbohydrate accumulation or composition. Misra et al. (1975a) noted a similar effect on zein in *o2* double mutants. Such lines have improved nutritional value because of their lower contents of zeins which are deficient in lysine and tryptophan (Mertz 1986).

RP-HPLC, combined with a rapid technique to extract all alcohol-soluble endosperm storage proteins simultaneously, is especially valuable in measuring quantitative genetic relationships in maize mutants. HPLC achieves high-resolution separations of these polypeptides of interest, while for the first time permitting simple and accurate measurement of their amounts. However, a precise biochemical explanation for these observations awaits further studies.

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