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Linkages among zein genes determined by isoelectric focusing *

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Summary. Genetic control of the major zein polypeptides in maize (*Zea mays* L.) was studied by isoelectric focusing (IEF) in agarose. Linkage relationships were determined by making a number of crosses, then determining the expression of zein polypeptides in backcross seeds. Chromosome linkages were determined by using the markers *sugary-1* (for chromosome 4), *yellow-8,* and a *waxy* 7–9 translocation (for chromosome 7). Nine zeins were in one linkage group on chromosome 4, six in another linkage group on chromosome 4, and four zeins were in one linkage group on chromosome 7. Some IEF single bands consisted of at least two polypeptides, which were detected by subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis, by aberrant ratios in backcrosses, or by differing recombination percentages. One zein occurred only in homozygous *sugary-1* seeds. Three sets of closely-linked zeins were noted that occurred together almost exclusively in certain inbreds.

Key words: Zein – *Zea mays* L. – Genetic linkages – Isoelectric focusing – Recombination

Introduction

Zeins, the major storage proteins in maize (*Zea mays* L.) endosperm, consist of several classes of proteins that are soluble in alcohol solutions (some only after reduction) and that occur together in protein bodies (Wilson 1987). The most prominent zeins have molecular masses of about 27,000 or 24,000 kDa, make up 50% of the total endosperm protein, are encoded by a mutigene family, and demonstrate considerable heterogeneity among inbreds when separated by isoelectric focusing (IEF), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Soave and Salamini 1984; Wilson 1986), and reversed-phase high-performance liquid chromatography (Smith and Smith 1986). These zeins are readily soluble in solutions of 50%–70% ethanol or isopropanol without reduction of disulfide bonds, and are classified as alpha-zeins on this basis (Esen 1987). Classification based on relative mobility in SDS-PAGE divided alpha-zeins into two groups: A-zein (27 kDa, apparent sizes 21–26 kDa) and B-zein (24 kDa, apparent sizes 18–24 kDa); a 1–10 scale further subdivides A- and B-zeins (Wilson 1986). Two minor high-sulfur zeins, termed C-zein (18 kDa, apparent size about 15 kDa), and D-zein (apparent size about 10 kDa), are also present. When SDS-PAGE is combined with IEF in agarose, 70 different zeins can be detected in extracts from 18 inbred lines (up to 12 in each) (Wilson 1986).

Zein genes are codominant. In triploid endosperm, the amount of any individual zein depends upon the number of genes for that zein which are present (Wilson 1984). Linkages among zein (Zp) genes on chromosomes 4 and 7 have been determined by using IEF in polyacrylamide (Soave et al. 1981; Soave et al. 1982). IEF in agarose gives sharply defined zein bands which are precisely identified by matching against zeins of well-studied inbreds (Wilson 1985a). This makes it possible to study many zeins in a single cross and to compare zeins from one cross with those of another cross. Results of determining zein gene linkage in 11 crosses made among 13 inbreds are reported in this paper. We demonstrate that

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Table 1. Zein polypeptides and mutant markers which were scored in the backcross generation for each of 11 crosses (order taken from Tables 4 and 5) with parental inbred identified

P1[a]	W64A	B57	B57	B57	B57	Oh43	Oh43	M14	WF9	Oh43	R802
P2	A619	Pa91	B84	WF9	*y8*	*y8*	N28	WF9	7–9*wx*	7–9*wx*	7–9*wx*
A Chromosome 4											
A2/60[b]	P1			P2				P2			
B7/17.5				P2				P2			
A3/14, 19	P2				P2						
B9/35	P2				P2		P1				
B6/36	P1	P2	P2	P2			P2				
B8/32	P1	P2	P2	P2	P2[c]	P2[c]	P2				
A1/37				P1							
A3/33.5	P1	P2[d]	P2[d]	P2			P2	P2			
A3/33					P2	P2					
A2/28		P1	P1	P1	P1			P1			
B6/49	P2	P1		P1[c]	P1[c]	P1[c]	P1	P1			
B8/54.5					P2	P2					
su							P1	P1			
A1/30.5							P1	P1			
A2/44.5			P1				P2				
B Chromosome 7											
7–9 *wx*									P2	P2	P2
D/55										P1	P1
B9/22	P2			P2	P2		P1		P1	P1	
B9/10	P2			P2		P1	P1	P2	P1	P1	
B8/38	P1			P1	P1		P2				
y8					P2	P2					
N[e]	240	188	78	120	240	97	222	114	124	124	157

[a] P1 – parent 1; P2 – parent 2
[b] First element gives the major and minor size classes as determined by SDS-PAGE, the second element gives the IEF pattern number (Wilson 1986)
[c] Recombination fractions were different for these zeins in these crosses, suggesting that B8/32 and B6/49 might be genetically different from the zeins in the same bands of other inbreds
[d] These zeins did not segregate 1:1 and were found to consist of both A1 and A3 sizes
[e] N–number of seeds assayed. Both backcrosses were assayed when N was greater than 160

zein genes occur in two clusters on chromosome 4 and one cluster on chromosome 7.

Materials and methods

Seed material

Seeds of inbred lines and of lines bearing chromosome markers were obtained from the Illinois Maize Genetics Laboratory and the Maize Genetics Stock Center (MGSC), Urbana/IL. Zein IEF band patterns were determined as described (Wilson 1985a). Crosses were made between inbreds which differed by as many bands as possible. Plants were grown at the Agronomy-Plant Pathology South Farm, University of Illinois, Urbana/IL. Pollinations were made by hand. Mature ears were dried at 40°C–45°C and kernels stored at 5°C.

Crosses among lines

The crosses are listed in Table 1. The two parents are given, along with the zeins (from 1 to 11) which could be scored for each cross. A complete listing of zeins in many inbred lines was reported previously (Wilson 1985a). Inbred lines were crossed to produce F1 seed. The crosses are identified as P1 × P2 or P2 × P1 (maternal parent first). F1 plants were selfed to produce F2 seeds, or were pollinated by a parental inbred to produce backcross seeds. BC × P1 is the designation for seed produced by pollinating an F1 maternal plant with P1 pollen, while BC × P2 indicates the use of P2 pollen. In triploid endosperm, the seed parent contributes two identical sets of genes while the pollen parent contributes one set. A BC × P1 endosperm contains either 0 or 2 doses of each gene segregating in the F1 maternal parent, plus 1 dose of each gene found only in the P1 line (the P1 genes will then be present in 1 or 3 doses). Two levels of zein band intensity are easier to score than the 0, 1, 2, or 3 levels found in F2 seeds. Further, probable errors for linkage calculations are less from backcross data than from F2 data (Immer 1930). Chromosome markers included the gene *surgary-1* (*su*) on the short arm of chromosome 4 and the gene *yellow 8* (*y8*) on the short arm of chromosome 7. Seeds from the backcrosses by the marker parent were separated into normal and mutant types before assay. Both mutants are recessive, so that about half of the backcross seeds exhibited a mutant phenotype.

A second marker for chromosome 7 was the translocation between chromosome 7 and 9 bearing the gene *waxy*, identified as MGSC 4363 (7–9*wx*). The translocations point is near the centromere of both chromosomes. Two inbreds (M14 and W23)

Table 2. Examples of observed phenotypes for 120 backcross seeds of (W64A × A619) × A619. Data are given for 6 out of 55 pairs of zeins

Zeins		Zein dosage																	
		Band A:	0	0	0	0	1	1	1	1	2	2	2	2	3	3	3	3	%	%
		Band B:	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	RC[a]	SD[a]
A	B		No. observed																	
10	14	C[b]	–	–	–	–	–	23	–	33	–	–	–	–	–	31	–	33	55.0	4.5
10	22	C	–	–	–	–	–	56	–	–	–	–	–	–	–	–	–	64	0.0	–
10	38	R	–	–	–	–	–	–	–	56	–	–	–	–	–	64	–	–	0.0	–
32	33.5	C	46	–	18	–	–	–	–	–	13	–	43	–	–	–	–	–	25.8	4.0
33.5	49	R	–	2	–	56	–	–	–	–	–	59	–	3	–	–	–	–	4.2	1.8
35	36	R	–	–	–	–	–	–	57	–	–	–	–	–	63	–	–	–	0.0	–

[a] RC – recombination %, SD – standard deviation (Bailey 1961)
[b] C – coupling phase; R – repulsion phase

bearing the translocation were crossed to produce a vigorous hybrid. F1 plants were used as pollen parents for crosses to normal inbred lines WF9, Oh43, and R802. The seeds thus produced, all with normal phenotype, were planted and self-pollinated. Waxy seeds were identified by a brown color when treated with an I_2-KI solution. After IEF, putative crossover samples were re-examined to be sure they had been correctly classified as waxy or normal. Residues from starchy endosperms were granular, while residues from waxy endosperms were gummy. Zein genes not on chromosome 7 are expected to segregate for 0, 1, 2, and 3 doses of each zein, independent of endosperm type. Zein with genes on chromosome 7 will segregate with normal or waxy endosperm character (except for crossovers), depending upon which parent provides the zein genes, and will occur in 0 or 3 doses.

Scoring and statistics

Zeins in backcross seeds which occurred in only one parent were scored for 0 or 2 doses (when absent from the pollen parent), or for 1 versus 3 doses (when present in the pollen parent). The results were tabulated with Lotus 1-2-3 (Lotus Development Corp.); then all combinations of pairs of zeins were tested for linkage. The recombination frequencies (or percent crossoves) were determined by the method of maximum likelihood (Bailey 1961). The 11 scorable zeins for this cross make 55 pairs which must be compared, so sample calculations for only a few pairs are presented in Table 2. Standard deviations ranged from 1.4% to 4.6% for samples of 120 backcross seeds. Pairs which gave apparent recombination frequencies less than 0.41 were considered to be linked ($P > 0.05$, or $P > 0.01$ for frequencies less than 0.38). After the calculations were made, the original gels were reexamined if infrequent or unusual crossover events occurred. Some results were omitted when ambiguities were found, usually because the zein bands were faint or nearly overlapped other bands. A total of 283 pairs of items (zein bands plus genetic markers) was examined in the 11 crosses used; 201 appeared to be linked, and reasonable calculations were made for 110 pairs for chromosome 4 and 22 pairs for chromosome 7. Because of the large number of items, only a summary of the data is presented.

Electrophoresis

Endosperm was removed as a powder from single seeds by using a small power drill. As little as 15 mg can be sampled, so seeds may be saved and planted. The powder was weighed and 5 µl per mg of 55% isopropanol containing 2% mercaptoethanol (ME) was added to the powder. After overnight steeping at room temperature, samples were centrifuged and 1.5–2 µl of the supernatants (15–20 µg of zein) were applied directly to the agarose gels. Up to 50 samples could be applied to each gel. Every gel contained N28 zein as a standard. Also, two or three samples of P1, P2, and F1 were placed among backcross samples on each gel. IEF was done in 1% agarose with pH 5–8 or 6–8 Ampholine, 5 M urea, and 2 m*M* dithiothreitol. Gels were stained with Coomassie Blue R, dried, and stored at room temperature. SDS-PAGE was performed in slab gels containing 15.2% acrylamide and Tris-borate buffer (Wilson 1985a, 1986). SDS sample buffers contained 2% ME.

Zeins are designated by a nomenclature developed for serial analyses by IEF in agarose and SDS-PAGE (Wilson 1985b, 1986). For example, A2/60 in Table 1 means a zein of the true (A) 27 kDa class (apparent size 22 kDa in some systems), which moves to position 2 on a 1–10 scale for the major zeins. The number "60" corresponds to the IEF position (Wilson 1985a). B7/17.5 is a zein of the true (B) 24 kDa class (apparent size 19 kDa in some systems) and at position 17.5 by IEF. D/55, a zein of the 10 kDa size class, and B5/55, a zein in the 24 kDa class, migrate to the same position (55) on IEF. Numbers were changed for a few bands as more comparisons were made during these experiments. For example, zein B7/17.5 was originally named B7/18 (Wilson 1986), but it can sometimes be distinguished from A3/18 by IEF alone, as shown in Fig. 1. However, these zeins are too close to score in genetic experiments. The zein found only in homozygous *su* endosperms has been renumbered from A1/28.5 to A1/30.5. IEF was used throughout, and zeins were usually identified by IEF number alone. The SDS-Page size class and the parent inbred line are given where appropriate.

Results

Zein gene linkage for W64A × A619

Results from the cross between W64A as P1 and A619 as P2 are presented as an example of the procedure (Fig. 1). Ten zeins were scored in BC × P1 and 11 zeins in BC × P2. Bands 10, 14, and 22 occur in A619 (in the same parent or in coupling phase). Thus, when W64A was the pollen parent (samples 1–6), these zeins occurred in 0 or 2 doses. When A619 was the pollen parent (samples 7–12),

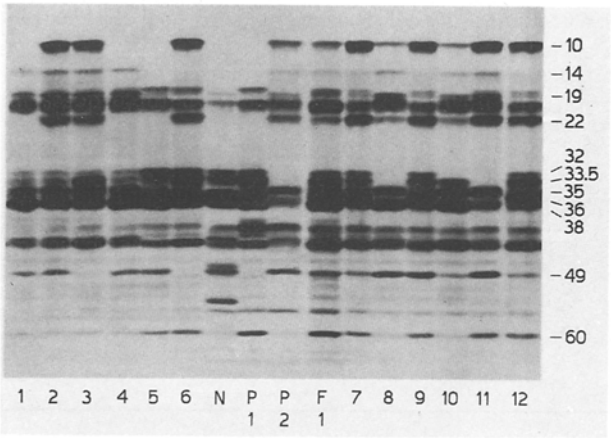

Fig. 1. IEF of zeins from single seeds of the cross W64A × A619. N is N28, a standard inbred, P1 is W64A, P2 is A619, and F1 is P1 × P2. *Lanes 1–6:* BC × P1; *lanes 7–12:* BC × P2. Scorable bands are numbered according to IEF designations

Table 3. Zein gene linkages. Recombination frequencies calculated for backcrosses of W64A × A619

	Recombination %						
A Linkages on chromosome 4							
Zeins	14	19	35	36	32	33.5	49
A2/60/W64A	7	7	7	7	7	30	33
A3/14/A619		0	0	0	0	23	26
A3/19/A619			0	0	0	23	26
B9/35/A619				0	0	23	26
B6/36/W64A					0	23	26
B8/32/W64A						23	26
A3/33.5/W64A							4
B9/49/A619							
B Linkages on chromosomes 7							
Zeins		10		38			
B9/22/A619		0		0			
B9/10/A619				0			
B9/38/W64A							

they occurred in 1 or 3 doses. All four possible combinations of doses of bands 10 and 14 occurred in about equal numbers (Table 2, BC × P2 only), which showed that they are not linked.

Bands 10 and 22 occurred together with equal dosages, showing strong linkage. Because they are from the same parent, the genes must be close together on the same chromosome. Zeins B9/35/A619 and B6/36/W64A (repulsion phase) migrated very close to each other on IEF gels. Each sample was examined visually, and in some cases by a scanning densitometer. In every sample of backcross or F2 seed, the total dosage for bands 35 plus 36 appeared to be three. In BC × P2 (Table 2), a single symmetrical band occurred at position 35 in 63 samples, while a peak with a maximum at 36 and a shoulder at 35 occurred in 57 samples, indicating 2 doses of 36 and 1 of 35. In BC × P1, about half of the seeds showed a symmetrical band 36 and half a peak with a maximum at 35 and a shoulder at 36. These 2 zeins showed less than 1 crossover per 240 seeds. Because they come from different parents, we cannot determine whether they are alleles at the same locus or if they are at two loci very close together.

Zein B9/38, a darkly staining zein in W64A, is scored somewhat differently. Most inbreds contain a band at position 38, but the amount of protein may be high or low. Tests to data show no electrophoretic differences among zeins at position 38. Some backcrosses did shown distinct differences in amounts of zein at position 38 and were scored. Zeins 10 and 38, from different parents (repulsion phase), occurred in 1 and 3 or 3 and 1 doses in BC × P2, but could not be scored in BC × P1. The two combinations were found in about equal numbers of seeds (Table 2), suggesting strong linkage. Intensity variations are discussed below. Zeins B8/32 and A3/33.5 are in coupling phase, and were in equal dosages in 9 of 12 backcross seeds (Fig. 1). Complete analysis showed 25% crossing over (Table 2). Bands 33.5 and 49 showed only 4.2% crossing over. Zeins B7/17.5/W64A, A3/18/A619, and A3/19/A619 were so close that only band 19 was scored for this cross. B7/17.5 can be distinguished from A3/18 when the parents are compared (Fig. 1), but reliable scoring of backcrosses was not possible. Band 19, which was scored, may not be apparent on Fig. 1.

Several zeins between positions 38 and 49 (Fig. 1) usually could not be scored. Zeins near position 41 are C-zeins (15 kDa zeins or high-methionine zeins) (Wilson 1986, 1987). C-zeins tend to precipitate and make irregular bands on agarose gels, and so were not scored. Zeins A1/43.5, B9/44, and A2/44.5 (Wilson 1986) could not be scored here or in other crosses where two or more of these polypeptides were present.

Data from the cross W64A × A619 are summarized in Table 3; chromosome assignments are from experiments described below. Eight zeins were assigned to the linkage group on chromosome 4. Six zeins were in one group, with five of them showing no crossovers in the 240 seeds examined. Two zeins occurred in a linkage group 23 to 26 crossover units away. Both chromosome 4 linkage groups contained A- and B-zeins. Three zeins were linked on chromosome 7, with no crossovers detected. All three chromosome 7 zeins were B-zeins.

Linkage tests for chromosome 4 and 7

Sugary-1 (*su*) is a common marker at position 71 on the short arm of chromosome 4 (Sheridan 1982) and is linked to several zein genes (Soave et al. 1982). Total zein content is reduced in *su* endosperm (Paulis et al. 1978). Zein banding on IEF was much less intense for extracts from

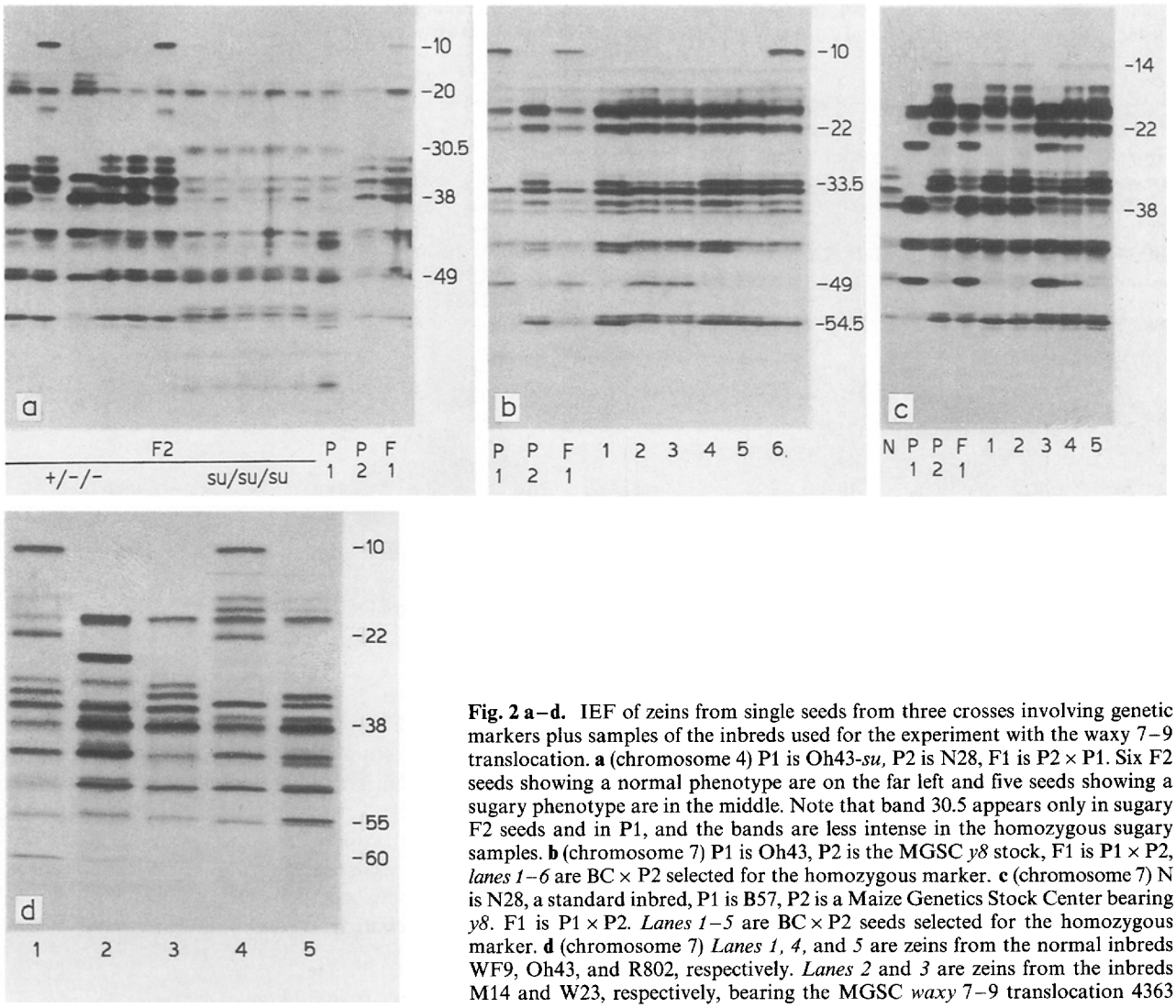

Fig. 2 a–d. IEF of zeins from single seeds from three crosses involving genetic markers plus samples of the inbreds used for the experiment with the waxy 7–9 translocation. **a** (chromosome 4) P1 is Oh43-*su*, P2 is N28, F1 is P2 × P1. Six F2 seeds showing a normal phenotype are on the far left and five seeds showing a sugary phenotype are in the middle. Note that band 30.5 appears only in sugary F2 seeds and in P1, and the bands are less intense in the homozygous sugary samples. **b** (chromosome 7) P1 is Oh43, P2 is the MGSC *y8* stock, F1 is P1 × P2, *lanes 1–6* are BC × P2 selected for the homozygous marker. **c** (chromosome 7) N is N28, a standard inbred, P1 is B57, P2 is a Maize Genetics Stock Center bearing *y8*. F1 is P1 × P2. *Lanes 1–5* are BC × P2 seeds selected for the homozygous marker. **d** (chromosome 7) *Lanes 1, 4,* and *5* are zeins from the normal inbreds WF9, Oh43, and R802, respectively. *Lanes 2* and *3* are zeins from the inbreds M14 and W23, respectively, bearing the MGSC *waxy* 7–9 translocation 4363

sugary (*su/su/su*) than from normal endosperms (with one, two, or three doses of *Su*), although identical extraction conditions were used (Fig. 2a). The decreased intensity makes scoring seeds with sugary phenotypes difficult. Table 1 (part A) lists zein bands which appear linked to *su* in backcross seeds from Oh43*su* × N28 and M14*su* × WF9. Zeins not linked to *su* are listed in part B. The recombination percentages for some of these zeins are listed in Table 4 (below), in the column under "*su*". Other zeins were too faint to calculate reliable percentages. Bands 10 and 38, two major zeins in normal kernels not linked to *su*, were strongly decreased in the sugary kernels (Fig. 2a). In all normal phenotype seeds, whether F2 or backcross, dosage levels of zeins from the *su* parent were as would be expected if the normal version of the inbred were used. F1 seed in Fig. 2a was from the cross N28 × Oh43-*su*, but single doses of genes for zeins 10 and 22 produced more intense bands than three doses in Oh43-*su*. These bands were still more intense in the reciprocal F1 (not shown).

Zein-band A1/30.5 (Fig. 2a) occurred only in seeds homozygous for *su*. The gene for this zein would be present in two-thirds of normal phenotype F2 seeds and in all normal phenotype backcross seeds when the *su* parent was the pollen parent. Zein 30.5 was not detected in any seeds with a normal phenotype. All other zeins in all other inbred lines showed dosage effects in which the zein band intensity depended upon the number of genes present. The gene for zein A1/30.5 may be closely linked to the *su* gene. This zein occurred in two different dosages in *su* seeds, the control of which seemed linked to *su* on chromosome 4. Further studies are underway to elucidate the genetics of the unusual relationships between *su* and zein synthesis.

Table 4. Zein gene linkages on chromosome 4. Crossover percentages were determined for up to five crosses. Average values are given in the lower left, with the number of crosses (if not one) in parentheses. Ranges are given in the upper right

Zeins	60	17.5	14[a]	35	36	37	32[b]	32y[b]	33.5	33	28	49[c]	54.5	*su*	30.5[d]
	Recombination %														
A2/60	–	5.8	6.7	6.7	4.2–6.7	5.8	4.2–6.7	–	21–30	–	24–29	31–33	–	–	21.9
B7/17.5	6	–	–	–	3.3	–	3.3	–	27.5	–	25.8	–	–	–	–
A3/14[a]	7	–	–	0–0.8	0–1.8	–	0–1.8	26.2	24.6	21.2	25.0	26.2	27.1	28.1	21.9
B9/35	7	–	0 (2)	–	0	–	0.2	25.4	23–28	20.4	20.8	26–27	26.2	31.0	18.8
B6/36	6 (2)	3	1 (3)	0 (2)	–	3.3	0–0.4	–	23–28	–	22–28	26–27	–	31.0	18.8
A1/37	6	–	–	–	3	–	3.3	–	27.5	–	25.8	–	–	–	–
B8/32[b]	6 (2)	3	1 (2)	0	0 (5)	3	–	–	22–28	–	21–27	26.2	–	30.6	18.2
B8/32y[b]	–	–	26	25	–	–	–	–	–	4.5–7.5	9.6	–	7–15	–	–
A3/33.5	26 (3)	28	25 (2)	25 (2)	25 (3)	28	24 (3)	–	–	–	5–8	4.2	–	3–10	–
A3/33	–	–	21	20	–	–	–	6 (2)	–	–	5.4	6.2	10–12	–	–
A2/28	26 (2)	26	25 (2)	21	26 (3)	26	25 (3)	10	6 (2)	5	–	14.0	13	14.0	14.0
B9/49[c]	32 (2)	–	26 (2)	27 (2)	27 (2)	–	26	–	4	6	14 (2)	–	6.2	14.0	14.0
B8/54.5	–	–	27	26	–	–	–	11 (2)	–	11 (2)	13	6	–	–	–
su	–	–	28	31	31	–	31	–	6 (2)	–	14	14	–	–	0
A1/30.5[d]	22	–	22	19	19	–	18	–	–	–	14	14	–	0 (2)	–

[a] Zein band 14 was always accompanied by band A3/19
[b] Zein band 32 gave two widely different sets of values, as if two genes were involved (32y is found in the *y8* stock)
[c] Zein band 49 gave widely variable values in several crosses (see text)
[d] Zein band 30.5 occurred only in seeds homozygous for *su*. However, this band was scored for the occurrence of 1 or 3 doses of protein

The *y8* marker for chromosome 7 (at position 18 on the short arm) (Sheridan 1982) produces lemon yellow seeds when homozygous. Only homozygous seed was assayed for BC × P2 of Oh43 × *y8*, where *y8* is the MGSC line in an unknown inbred background. Band 10 from Oh43 occurred in only 4 of 97 seeds (seed 6 in Fig. 2b), suggesting linkage to chromosome 7 near the *y8* locus. All other scorable bands (Table 1) showed segregation for expression of two doses from the maternal F1 parent. In the cross B57 × *y8* (Fig. 2c), band 22, from *y8*, had a high dosage level in 105 of 120 mutant seeds and in 10 of 120 normal seeds of BC × P2. Low levels of band 38 (from B57) occurred in the same seeds, except for one crossover (among 240 seeds total).

Figure 2d shows zein patterns of lines used in the experiment with the chromosome 7–9 *wx* translocation, near the centromere of chromosome 7. The translocation lines were crossed to produce vigorous plants for the final crossing experiment. Although M14 and W23 differed in two prominent zeins, they were on chromosome 4 and were not linked to the translocation. Thus the hybrid was satisfactory even though it showed some segregation of zeins. The seed produced by pollinating inbreds WF9, Oh43, and R802 with the translocation hybrid were selfed, and the resulting seeds were scored for zeins. When WF9 was the normal parent, bands 10 and 22 occurred together in 4 of 61 waxy seeds and in 62 of 63 normal phenotype seeds. In contrast, band 60 was present, in 1, 2, or 3 doses, in about equal numbers in both waxy and normal seeds. Band 60 occurred in 94 of 124 seeds, close to the theoretical 3:1 ratio expected if it were not linked to the 7–9 translocation. Segregation for bands 10 and 22 from OH43 was similar to that from WF9. Zein D/55 from Oh43 was also linked to the 7–9 translocation. Zein D/55 from the agarose gel was analyzed by SDS-PAGE to confirm that the bands which were scored were properly identified (zein B5/55 was not present in these seeds, see below). When R802 was the normal parent, only band 55 was linked to the translocation and it was confirmed as D/55 (not shown).

Zein gene linkages from additional crosses

Fig. 3 shows examples of IEF gels for three crosses involving B57. B57 was chosen because it has band 28, a zein well separated from other bands by IEF. B57 also lacks several zeins common among many inbred lines. Results similar to those reported for W64A × A619 were obtained from B57 × WF9 (Fig. 3a) for bands 32 and 33.5 (in coupling phase in WF9 and W64A) on chromosome 4. Bands 10, 22 (in WF9 and A619), and 38 (intense band in repulsion on B57 and W64A) on chromosome 7 showed no crossing over in either B57 × WF9 or W64A × A619. The close linkage among these three zeins is illustrated by the presence of bands 10 and 22 in seeds 2, 4, and 7 along with relatively low levels of band 38. Inbred W64A was developed from WF9, which accounts for the similar linkages of 32 and 33.5 on chromosome 4. WF9 and A619 (derived from Oh43) are not related, but share similar chromosome 7 zeins.

Figure 3b shows IEF patterns for F2 seeds from B57 × Pa91; backcross seeds which were scored for

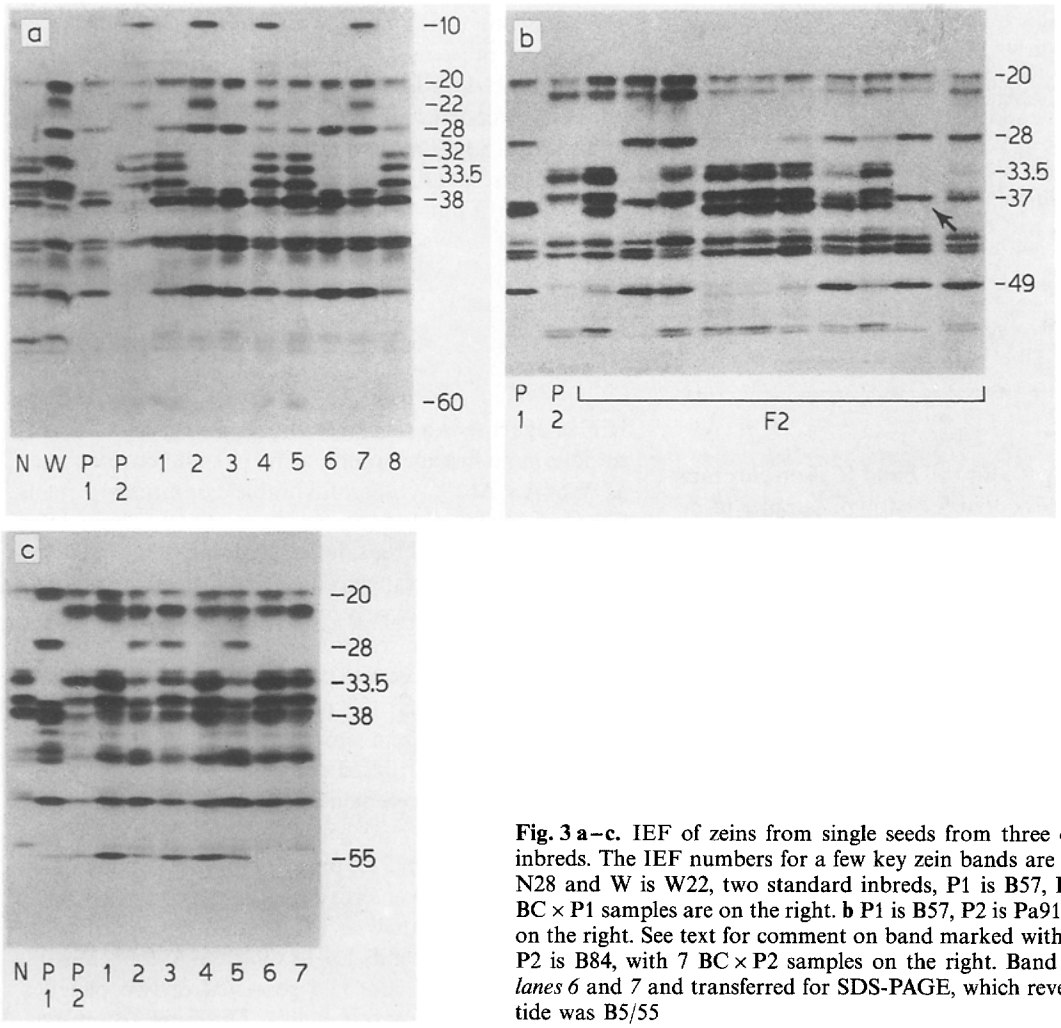

Fig. 3 a–c. IEF of zeins from single seeds from three crosses among normal inbreds. The IEF numbers for a few key zein bands are given (cf Fig. 1). **a** N is N28 and W is W22, two standard inbreds, P1 is B57, P2 is WF9, while eight BC × P1 samples are on the right. **b** P1 is B57, P2 is Pa91 and 10 F2 samples are on the right. See text for comment on band marked with an *arrow*. **c** P1 is B57, P2 is B84, with 7 BC × P2 samples on the right. Band 55 was removed from *lanes 6* and *7* and transferred for SDS-PAGE, which revealed that the polypeptide was B5/55

crossing-over are not shown. The arrow indicates zein A1/37, a common zein usually found in small amounts between B6/36 and B8/38 in many inbreds (Wilson 1985a). Band 37 occurs in 33 of about 50 inbreds which have been mapped by IEF, but always with band 36, 38 or both (data not shown). This F2 seed and a few others had recombination patterns in which band 37 appeared by itself. F3 progeny from one of these seeds proved to be homozygous for a pattern showing band 37 without either 36 or 38. Thus, it is possible to select for combinations of zeins which may not otherwise occur. Zein 33.5 was found in more than 60% of seeds from backcrosses of B57 × Pa91. Hartings et al. (1984) and Soave and Salamini (1984) noted that single bands may represent two or more polypeptides which are products of different genes. Band 33.5 from Pa91 was found to contain both A1 and A3 zeins upon serial analysis by SDS-PAGE (Wilson 1986). Several F2 seeds were planted and self-pollinated to produce F3 seed. One F3 ear showed various intensities for band 33.5 in different kernels. Of five kernels assayed by IEF and then SDS-PAGE, one had only A1/33.5, two had only A3/33.5, and two had both. These two zeins moved to the same position by IEF, but had different mobilities by SDS-PAGE. Because only a few seeds were tested by SDS-PAGE, it was not possible to determine if the two genes were linked on chromosome 4. The inbred L289 contains only A1/33.5 (Wilson 1986) and could be used in a cross to determine linkage for this version of IEF band 33.5. Band 21.5 also gave aberrant ratios in backcross seed of B57 × Pa91, being found in 115 of 188 backcross seeds (61.2%). Zein 21.5 from Pa91 contained A1/21.5 and B9/21.5 when tested by SDS-PAGE.

Figure 3c shows the cross B57 × B84. B84 also has the double band A1/33.5 and A3/33.5. This gel shows a band at IEF position 55, which in most inbreds is a D-zein (high methionine, with apparent mass near 10 kDa, Wilson 1987). Band 55 was intense in samples 1, 4, 6, and 7 (and others on this gel) in which band 28 was absent, and band 55 was weak in samples in which band 28 occurred.

Table 5. Zein gene linkages on chromosome 7. Crossover percentages were determined for up to five crosses. The average values are given in the lower left, with the number of crosses (if not one) in parentheses. Ranges are given in the upper right

Zeins	*wx*	55	22	10	38	*y8*
	Recombination %					
7–9 tr, *wx*	–	0–3.2	6.5	4–8	–	–
D/55	2 (2)	–	5.6	7.3	–	–
B9/22	6	6	–	0–1.2	0–0.8	10.4
B9/10	6	7	0 (5)	–	0–0.8	4.1
B8/38	–	–	0 (4)	0	–	10.8
y8, *y8*	–	–	10	4	11	–

This suggests that the gene for this band 55 is on chromosome 4. It could not be scored for enough samples to be certain of this determination. This contrasted with assignment of the gene for D/55 to chromosome 7 using the waxy translocation. Band 55 samples taken from lanes 6 and 7 (Fig. 3c) yielded a B5 zein and a weak D-zein when analyzed by SDS-PAGE (not shown). The samples assayed included some of those scored as more intense. When similar SDS-PAGE assays were run on band 55 zeins from the waxy translocation experiment, B5 zein was too weak to interfere with D-zein scoring.

Zein gene linkages, combined data

Data on recombination fractions for all pairs of zeins scorable in the 11 crosses described above are summarized in Tables 4 and 5. Eight crosses gave useful data for chromosome 4 linkages while nine gave data for chromosome 7. The lower left halves of the tables give the average values (rounded off) for each pair of zeins, with the number of crosses yielding useful data in parentheses. The ranges are given when a pair was determined in more than one cross. Not all combinations of zeins represented in Table 1 are reported in Table 4. Table 3, data from W64A × A619, was quite straightforward, with clear-cut differences between the scorable zeins, and with segregation occurring according to expectations for Mendelian genes. Also, each band appeared to represent a single gene product. With one exception, the cross B57 × WF9 produced data which agreed. There was 32.9% recombination between bands 60 and 49 in W64A × A619 (and 30.7% in M14-*su* × WF9), but only 11.7% in B57 × WF9. Additional discrepancies occurred among some zein pairs in other crosses. The recombination percentages for bands 35 and 32 were 0 and 0.4 for W64A × A619 and Oh43*su* × N28, respectively, but was 25.4 for B57 × *y8*. The calculated crossovers between bands 32 and 49 and other zeins were also different in the same crosses. These bands were checked for relative mobility by SDS-PAGE, but no differences could be detected. The most simple explanation is that two genes code proteins which migrate to position 32 and two or more code proteins at position 49. It was possible to combine the data for band 32 into two sets of results, which are reported in Table 4. The zein in *y8* stock is identified as 32y. The data for band 49 was quite variable among crosses, and so only results which agreed with the W64A × A619 data are presented at this time. All data for band 49 showed linkages to other zeins on chromosome 4, but with variations of up to 20% among crosses.

Discussion

IEF in agarose is a relatively simple and efficient means to determine linkage among zeins in some crosses, such as W64A × A619. Under favorable conditions 9 zeins were separated in W64A and 12 in A619. Three zeins occurred in both inbreds, leaving 18 zeins for which segregation would be expected in F2 and backcross seeds. Eleven zeins were clearly distinguishable in one backcross, and 10 in the reciprocal backcross. No problems occurred when linkage was calculated, assuming that each IEF band represented the product of a single gene, and that the amount of zein was proportional to the number of genes present in triploid endosperm.

Zeins are produced by a large multi-gene family, with possibly more than 100 genes (Heidecker and Messing 1986; Rubenstein and Geraghty 1986). As many as 70 zeins were identified among different inbred lines by IEF/SDS-PAGE serial analysis (Wilson 1986). Aberrant ratios for some zein bands led to the finding that, in some inbreds, bands 21.5 and 33.5 consisted of two peptides, presumably with separate genes. Differing recombination fractions for bands 32 and 49 in different crosses suggests that each of these zeins may be specified by two genes.

Extensive studies on zein linkages were conducted previously (Soave et al. 1981; Soave et al. 1982; Hartings et al. 1984). Unfortunately, the nomenclature system in these studies cannot be correlated with the present results, with two exceptions. Three tightly linked genes, Zp 20/1, Zp 20/2, and Zp 20/3 (Hartings et al. 1984), may be the same as the gene for the zein B9/10 reported here, because all are linked to chromosome 7, and the corresponding zeins are quite basic and are in the same size class. Agarose IEF showed multiple bands for IEF band 10 until DTT was incorporated into the gel. Thus, polyacrylamide IEF bands 1, 2, and 3 may be artifacts of separation rather than separate zeins (Wilson 1985a). Band D/55 studied here is probably the same as the zeins from Zp 10/16–20 (Hartings et al. 1984), though only one gene product was noted here.

Ottoboni and Steffensen (1987) reported that several zein genes were located on chromosome 4 and 10. They used the same agarose IEF system as in the present pa-

per, but used reciprocal translocations to try to reduce the number of assays needed. Some results depended upon determination of dosage levels in duplicate deficient progeny. Our experience with hybrids suggests that band intensities in hybrids may be relatively greater than in the corresponding inbreds. Thus duplicate-deficient progeny cannot be reliably distinguished from normal progeny on the basis of band intensity. Zeins segregating with chromosome 7 were confused with zeins segregating with the chromosome 4 translocation, causing incorrect assignments of chromosome location. Additional confusion resulted from the use of three parental inbreds in each experiment, which greatly reduced the potential number of segregating zeins which could be scored and increased the number of bands for which intermediate levels of zein were found. The data in figures and tables of Ottoboni and Steffensen (1987) can be reconciled with our chromosome assignments, even where the conclusions are different. The present paper also uses more assays and straightforward genetic calculations (Immer 1930; Bailey 1961). Only one zein gene (Zp 22/2) is definitely located on chromosome 10 (Valentini et al. 1979; Binelli et al. 1984). It has been found only in a trisomic chromosome 10 stock from MGSC and in diploids derived from this source and is closely linked to the Rst locus. A zein designated A1/7 in the agarose IEF system, the most basic zein yet detected, was found in the same source material, but the chromosomal location is not yet confirmed (Wilson, unpublished results).

IEF reveals a wide divergence in intensities of individual zeins (Fig. 1–3). If the assumption is correct that each gene occurs three times in homozygous endosperms of inbreds, then some regulatory mechanism(s) must differentially control zein gene expression. Although some IEF bands show approximately the same relative intensities in the various inbreds in which they appear, others range from very weak to very intense (Wilson 1985a). We assumed that genes being mapped are structural genes, but in three cases the supposed mapping units might have been regulatory genes. Band A1/30.5 was present only in seeds homozygous for *su*, but, in addition, the relative amount of zein was linked to chromosome 4 (Table 4). Zein B8/38 occurred in high or low amounts in different inbreds, with no data to indicate that two different zeins were involved. Crosses between two such inbreds in some cases showed segregation for the amount of zein, and this was mapped to chromosome 7. The zein at IEF position 38 is a major zein of many inbreds (Wilson 1985a), so knowledge of the regulatory mechanisms for this zein is of interest. Zein D/55 also mapped to chromosome 7 on the basis of high and low amounts of zein. This small zein is of practical importance because it has a high methionine content which would increase the nutritive value of maize if present in large amounts (Phillips and McClure 1985). All inbred lines may contain zein D/55, but in some its amount may be too low to be noticed. Thus, it is possible that the segregation we noted in the present work might be for a regulatory rather than a structural gene. Analysis by SDS-PAGE is needed to be sure that the analyses for D/55 are not confused by B5/55, also detected in only some lines.

Three groups of zeins, which were linked on the same chromosome and which occurred together in the same inbred lines (Table 1), were compared with records of zein banding patterns of 50 inbred lines, as in Fig. 4 of Wilson (1985a). Zeins B8/32 and B6/36 (linked on chromosome 4 with 0.1% crossing over) occurred together in 36 inbreds. Each occurred alone in only 2 inbreds. One exception was B8/32y in the MGSC line for *y8*, the gene for which was some distance from the gene for B6/36 (Table 4). Zeins A3/14, A3/19, and B9/35 (linked, repulsion phase, on chromosome 4 to B8/32 and B6/36 with only 0.4% crossing-over) occurred together in 10 inbreds (five were Oh43 and related inbreds), while B9/35 occurred alone in only one inbred. Zein A3/18 is always found in inbreds with this group, but it could not be scored for linkage in the crosses used in this experiment. Bands B9/10 and B9/22 (linked on chromosome 7 with 0.2% crossing over) occurred together in 9 inbreds, while B9/22 occurred alone in 2 inbreds. Further, zein B8/38 showed relatively low intensity in all inbred lines in which bands 10 and 22 were found. B57 was the only inbred of the 50 which lacked all three zein groups mentioned above, though it does have a major band at position 38. We conclude that blocks of DNA coding for several zeins are the units which undergo segregation in most crosses. Cluster analysis of zein IEF banding patterns can suggest relationships among inbreds (Nucca et al. 1978), but differences and similarities will be exaggerated by linkages of zein genes. For example, B57 lacks six zeins found in Oh43, but the difference may be only the lack of two inheritable zein groups.

The *opaque-2* (*o2*) mutation strongly inhibits synthesis of certain zeins, including bands B7/17.5, A1/20, A3/33.5, and A2/60 in W64A and A1/20, A2/28, A1/43.5, and A2/44.5 in other inbreds. The *o2* mutation has little or no effect on zeins B8/32 and B6/36 (Wilson 1984, 1985b, 1986). Genes for these zeins are all located on chromosome 4, but are scattered on the chromosome. Control is exerted at several points on chromosome 4. Other factors which control the synthesis of a limited number of zeins are known, yet in a normally developing endosperm all zeins appear to be produced synchronously (Soave and Salamini 1984).

The zein multigene family consists of more than 100 members, but these can be grouped into four to eight subfamilies with high levels of sequence similarity (Heidecker and Messing 1986; Marks et al. 1985; Rubenstein and Geraghty 1986). Clustering of zein genes is suggested from hybridization patterns of cloned zein inserts to

genomic DNA (Wilson and Larkins 1984). Although zeins can be divided into two groups on the basis of size (relative mobility in SDS-PAGE), related genes may code for zeins which vary in both length and charge (Esen et al. 1987; Viotti et al. 1985). It would be interesting to determine if the linkage groups here determined are related to known zein subfamilies.

The zeins, with genes on chromosome 4 and 7, are good electrophoretic markers which may supplement isozymes for identification or fingerprinting of lines and hybrids, because few isozymes are known for these chromosomes (Stuber and Goodman 1983). Seeds may be assayed for zeins at any time, then the same seeds may be planted in the field or germinated for isozyme studies.

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