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Genetic analysis of methionine-rich storage protein accumulation in maize

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Summary. Experiments were conducted to determine the chromosomal location of the gene conditioning overproduction of a methionine-rich, 10-K zein in maize kernels of line BSSS53. In addition, the chromosomal location of the structural gene encoding the overproduced protein was determined. Whereas the structural gene, designated *Zps10/(22)*, was found to be located on the long arm of chromosome 9 near the centromere, the locus regulating overproduction of the zein protein was mapped to the short arm of chromosome 4. This regulatory gene has been designated *Zpr10/(22)*. Regulation of 10-K zein production by *Zpr10/(22)* is, therefore, via a *trans*-acting mechanism.

Key words: *Zea mays* L. – Zein – Protein isoelectric focusing (IEF) – Restriction fragment length polymorphism (RFLP) mapping – *Trans*-acting factors

Introduction

Seed storage proteins serve as the major food reserve for germinating plant embryos. The composition of this reserve is responsible for determining the nutritional quality of the grain with respect to both human and livestock consumption. Understanding the genetic organization and regulation of genes involved in storage protein accumulation may result in novel approaches to increase nutritional value of cereal grain.

Cloning of major prolamine genes of maize has revealed a highly complex multigene family (Rubenstein 1982). In addition to describing structural gene organization, recent research also has focused on the elucidation of regulatory gene sequences (Schmidt et al. 1987). Relatively little, however, is understood of the organization and regulation of minor, low molecular weight zein proteins. Total zein extracts contain a mixture of proteins with apparent molecular weights of 27-, 22-, 19-, 16-, 15-, and 10-K. The 19- and 22-K proteins are found primarily in the alcohol-soluble (zein-1) fraction; the 27-, 16-, 15-, and 10-K proteins are extracted efficiently with the aid of a reducing agent (zein-2 fraction). While the compositions of the two fractions are similar, the 15- and 10-K proteins are characterized by a high level of S-containing amino acids, primarily methionine (Gianazza et al. 1977).

Zeins are heterogeneous for charge in addition to size (Righetti et al. 1977). At least 15 components are usually resolved by isoelectric focusing (IEF), differing both qualitatively and quantitatively among maize lines. Individual IEF bands correspond to the products of distinct structural genes (Soave et al. 1978). Variations in occurrence and intensity of protein bands make it possible to detect segregation of genes responsible for zein accumulation. In this manner, genes encoding 19- and 22-K proteins have been placed on chromosomes 4, 7, and 10 (Soave and Salamini 1984). A gene for a 15-K protein has been mapped to chromosome 7 (Soave and Salamini 1983). A 10-K protein gene has been mapped to chromosome 7 (Hastings et al. 1984; Wilson et al. 1989). Southern hybridization using probes for 19- and 22-K zein genes reveals approximately 100 zein sequences per maize genome (Hagen and Rubenstein 1981). Hybridizations with 27-, 15-, and 10-K protein gene clones each result in only one or few hybridizing sequences (Das and

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Messing 1987; Wilson and Larkins 1984; Kirihara et al. 1988).

The *opaque-2* (*o2*) endosperm mutant was the first variant recognized to modify the protein composition of maize kernels (Mertz et al. 1964). Additional endosperm mutants containing reduced accumulation of zeins have since been characterized. These genes have been termed 'regulatory', although the exact mechanisms underlying the protein changes have not been identified. Whereas some regulatory loci, such as *floury-2* (*fl2*), affect all subfamilies of zein, other loci, such as *o2,* are specific to a particular subfamily. Highly specific regulatory loci, affecting accumulation of only one or several polypeptides, have been proposed (Binelli et al. 1984).

Relatively little is known of the regulation of expression of genes encoding 27-, 16-, 15-, and 10-K storage proteins. Phillips and McClure (1985) have identified a maize inbred containing a two-fold elevation in the level of methionine-rich, 10-K protein. Differential accumulation of a 10-K protein mRNA appears to be responsible, at least in part, for the subsequent increases at the protein level (Kirihara et al. 1988). Objectives of this study were: first, to determine the number and chromosomal location of the gene(s) responsible for overproduction of 10-K zein, and second, to determine the location of the structural gene encoding the methionine-rich polypeptide. In addition to providing information regarding strategies for incorporation of methionine overproduction into elite maize lines, linkage relationships will indicate a *cis-* or *trans*-acting role for the overproduction factor(s). This knowledge is essential in the elucidation of the overproduction mechanism.

We report here that while overproduction of 10-K protein is determined by a single gene mapping to chromosome 4, the structural gene encoding the overproduced protein is located on chromosome 9. A *trans*-acting mechanism for the overproduction of 10-K protein in line BSSS53 is, therefore, implied.

Materials and methods

Inheritance of 10-K protein overproduction

Inbred maize lines BSSS53, W23, and B73 along with resulting F_1, F_2, and backcross progenies were used to determine the inheritance of 10-K protein overproduction. Zein-2 proteins were extracted and separated as described below.

Protein extraction and isoelectric focusing

Individual kernels were crushed in a mill similar to that described by Paulis and Wall (1979). From 25 to 100 mg meal was placed in microcentrifuge tubes for extraction. The zein-1 fraction was extracted twice with 70% ethanol at 25 °C for 30 min (1 ml per 100 mg). The zein-2 fraction was then extracted twice with 70% ethanol, 1% 2-mercaptoethanol at 25 °C for 30 min using the same solvent : meal ratio. In preparation for isoelectric focusing, 50–100 µl zein-2 extract was dried in vacuo and resuspended in 10 µl 70% ethanol, 1% 2-mercaptoethanol. Proteins were loaded onto gels consisting of: 5% acrylamide, 0.15% bisacrylamide, 6.4 *M* urea, 2% carrier ampholytes (Servalyte 5–8), and 0.3 ml 10% ammonium persulfate per 60-ml gel to initiate polymerization (Kirihara et al. 1988). Isoelectric focusing was performed for 3 h at 10 °C with 25-W constant power on an LKB Multiphor apparatus. Proteins were visualized by precipitation with 10% trichloroacetic acid.

Mapping of the 10-K protein overproduction locus

For gene mapping to a chromosome arm, a series of *waxy* (*wx*)-linked reciprocal translocation stocks (in inbred background W23) were crossed with line BSSS53. F_1 progeny were selfed or backcrossed to *wx*. Subsequent analyses utilized crosses between BSSS53 and stocks homozygous for *fl2* and *Gametophytic factor 1* (*Ga1*). F_1 progeny were selfed or backcrossed to *Fl2* or *Ga1* stocks. Individual kernels or portions thereof were screened for segregation of 10-K protein overproduction and specific marker phenotypes. Extraction of zein-2 proteins and subsequent separation were as previously described. Chi-square analyses were performed to test whether allelic segregations fit theoretical distributions, corrected for continuity when degrees of freedom equaled one (Zar 1974). Chi-square contingency tables were used to test for independent assortment of alleles, corrected for continuity when 2×2. Recombination frequencies were determined by the method of maximum likelihood (Allard 1956). A combined *p*-value and associated variance were calculated by weighting (Kramer and Burnham 1947). Recombination frequencies were converted into map distances using Kosambi's mapping function:

$$x = 1/4 \ln[(1+2y)/(1-2y)]$$

where x represents map distance in centiMorgans (cM) and y represents recombination frequency (Bailey 1961).

Restriction fragment length polymorphism mapping of the 10-K structural gene

Kernels previously scored for 10-K overproduction or endosperm mutations were germinated in a growth chamber. DNA was extracted from 1–2 gm leaf tissue (Shure et al. 1983). Approximately 5 µg DNA was digested with EcoRI (Promega Biotec). Restriction reactions were loaded onto 0.8% agarose gels and fractionated with 20–40 V in TBE buffer. Gels were soaked in 0.25 *M* HCl prior to DNA transfer to Zetabind nylon membranes (Cuno Laboratory Products) with 0.4 *M* NaOH. Membranes were prehybridized in 6× SSC, 10× Denhardt's solution, 1% SDS, 500 µg/ml salmon sperm DNA at 65 °C. Hybridizations were performed in 6× SSC, 1% SDS, 50 µg/ml salmon sperm DNA with 50–250 ng DNA probe at 65 °C. Probe DNA was labeled via the random primer method, as described by Feinberg and Vogelstein (1983). Following hybridization, membranes were washed twice at 65 °C for 15 min with 2× SCP, 1% SDS, and once at 65 °C for 15 min with 0.2× SCP, 1% SDS. Hybridized membranes were exposed to Kodak XAR-5 X-ray film.

The 10-K zein cDNA clone, 10kZ-1, has been previously described (Kirihara et al. 1988). Southern hybridizations were performed with a deletion subclone designated 10kZ-1Δ43 as a probe. Mapped genomic clones were kindly provided by D. Hoisington, Department of Agronomy, University of Missouri. Bacteria were grown in LB broth containing 50 µg/ml ampicillin. Plasmid DNA was purified as described in Promega Biotec Technical Bulletin No. 009. Insert DNA was separated from

vector sequence by gel electrophoresis following restriction and prior to labeling.

Statistical analyses of linkage relations were as previously described.

Results

Inheritance of 10-K protein overproduction

F_2 segregation of 10-K protein overproduction as determined by visualization of the major 10-K protein on IEF gels did not significantly differ from a 3:1, high:low ratio (Table 1). Different levels of overproduction, however, were observed, suggesting a dosage response due to the triploid nature of the endosperm. Likewise, backcross segregation did not significantly differ from a 1:1 ratio. Levels of 10-K protein from reciprocal F_1 kernels from the cross BSSS53 × W23 appeared to be correlated with the gene dose present in the endosperm, although a linear dosage response cannot be concluded due to lack of quantitation (Fig. 1). W23, the low 10-K protein parent, contained trace amounts of the major 10-K protein; thus, segregation in the F_2 population was not for presence versus absence, but for high versus low levels of accumulation. Additional inbred maize lines examined also contained low levels of the 10-K protein (B37, W64A, Oh43). The gene responsible for overproduction of the 10-K protein was designated *Zpr10/(22)*, to represent a regulator of the production of zein polypeptide with a molecular weight of 10-K and tentative isoelectric focusing position 22.

Linkage analysis of Zpr10/(22)

Segregation of *Zpr10/(22)* was compared to segregation of *wx* among backcross progeny from crosses between BSSS53 and *wx*-linked translocations. Linkage was detected with *wx*T4-9g (4S.27:9L.27) (Table 2). A recombination frequency of 0.36 was calculated with data from 124 kernels (S.E. = 0.04). Independent segregation of *Zpr10/(22)* with *wx*T4-9(5657) (4L.33:9S.25) and *wx*T4-9b (4L.90:9L.20) was observed among 35 and 48 kernels, respectively. F_2 kernels from the cross of BSSS53 with B37*fl2* were screened for segregation of *Zpr10/(22)* and *fl2*. Due to the semidominant nature of *fl2*, kernels were placed into one of three phenotypic classes: floury, semi-floury, or non-floury, corresponding to the genotypes *fl2/fl2*, *fl2/+*, and +/+, respectively. Similarly, kernels were classified as containing high, medium, or low 10-K protein levels, corresponding to genotypes *Zpr10/(22)/Zpr10/(22)*, *Zpr10/(22)/+*, and +/+, respectively. Thus, nine phenotypic classes were observed. A significant deviation from independent segregation was detected among 209 kernels (Table 3). A recombination frequency of 0.20 was calculated (S.E. = 0.02). Recombination among 192 backcross progeny was calculated as 0.25 (S.E. = 0.03). A combined *p*-value from F_2 and backcross data was calculated as 0.22 (S.E. = 0.02). F_1 individuals from the cross of BSSS53 with *Ga1-s* were backcrossed as male to a homozygous *Ga1-s* stock. Since only *Ga1-s*-containing pollen will successfully compete on *Ga1-s*-containing silks, F_2 progeny were all homozygous at the *Ga1* locus. Among 55 F_2 kernels, only 4 contained *Zpr10/(22)*, indicating a recombination frequency of 0.07 (S.E. = 0.04) between *Ga1* and *Zpr10/(22)*.

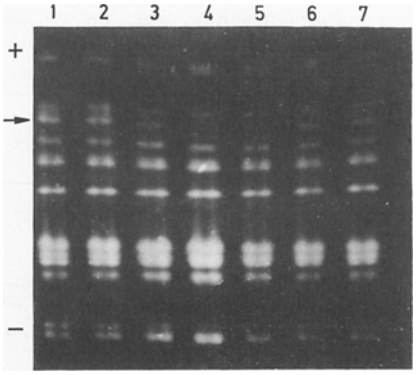

Fig. 1. IEF of zein-2 protein from: *lane 1*, BSSS53; *lane 2*, BSSS53 × W23; *lane 3*, W23 × BSSS53; *lane 4*, W23; and *lanes 5–7*, three representative F_2 individuals (*arrow* indicates isoelectric focusing point of 10-K protein)

Table 1. F_2 and BC segregation ratios of 10-K zein overproduction resulting from the crosses (BSSS53 × B73) × self and (BSSS53 × W23) × W23, respectively

	Zpr10/(22) / *Zpr10/(22)*	*Zpr10/(22)* / +	+/+	Total
F_2:*	55	102	52	209
BC:**	–	219	179	398

* Chi-square (1:2:1) = 0.21 ($0.75 > p > 0.50$)
** Chi-square (1:1) = 3.82 ($0.10 > p > 0.05$)

Table 2. BC segregation resulting from the cross of (*Zpr10/(22)* × *wx*T4-9g) × *wx*

	Zpr10/(22)/+	+/+	Total
+/*wx*	42	19	61
wx/*wx*	25	38	63
Total	67	57	124

Chi-square *Zpr10/(22)* (1:1) = 0.65 ($0.50 > p > 0.25$)
Chi-square *wx* (1:1) = 0.01 ($0.95 > p > 0.90$)
Chi-square independence = 9.48 ($p < 0.005$)

Table 3. F_2 and BC segregation resulting from the crosses (*Zpr10/(22)* × B73*fl2*) × self and (*Zpr10/(22)* × B73*fl2*) × wild-type

		Zpr10/(22) / *Zpr10/(22)*	*Zpr10/(22)* / +	+/+	Total
F_2*:	+/+	36	10	0	46
	fl2/+	17	70	23	110
	fl2/*fl2*	2	22	29	53
	Total	55	102	52	209
BC**:	+/+	–	78	19	97
	fl2/+	–	28	67	95
	Total	–	106	86	192

* Chi-square *Zpr10/(22)* (1:2:1)=0.21 ($0.75>p>0.50$)
Chi-square *fl2* (1:2:1)=1.05 ($0.5>p>0.25$)
Chi-square independence=105.88 ($p<0.001$)
** Chi-square *Zpr10/(22)* (1:1)=1.88 ($0.25>p>0.10$)
Chi-square *fl2* (1:1)=0.01 ($0.95>p>0.90$)
Chi-square independence=32.29 ($p<0.001$)

Table 4. BC segregation of *Zpr10/(22)* and *Zps10/(22)* resulting from the cross of (BSSS53 × W23) × W23

	Zps10/(22)		Total
	BSSS53/W23	W23/W23	
Zpr10/(22)/+	12	6	18
+/+	7	4	11
Total	19	10	29

Chi-square *Zps10/(22)* (1:1)=2.21 ($0.25>p>0.10$)
Chi-square *Zpr10/(22)* (1:1)=1.24 ($0.5>p>0.25$)
Chi-square independence=0.03 ($0.90>p>0.75$)

Table 5. F_2 segregation of BNL3.06 and *Zps10/(22)* resulting from the cross of BSSS53 and W23

		BNL3.06			Total
		BSSS53/BSSS53	BSSS53/W23	W23/W23	
Zps10/(2)	BSSS53/BSSS53	13	4	0	17
	BSSS53/W23	2	22	4	28
	W23/W23	2	2	10	14
	Total	17	28	14	59

Chi-square BNL3.06 (1:2:1)=0.46 ($0.90>p>0.75$)
Chi-square *Zps10/(22)* (1:2:1)=0.46 ($0.90>p>0.75$)
Chi-square independence=48.58 ($p<0.001$)

Table 6. F_2 segregation of UMC81 and *Zps10/(22)* resulting from the cross of BSSS53 and W23

		UMC81			Total
		BSSS53/BSSS53	BSSS53/W23	W23/W23	
Zps10/(22)	BSSS53/BSSS53	10	2	0	12
	BSSS53/W23	0	18	5	23
	W23/W23	0	2	9	11
	Total	10	22	14	46

Chi-square UMC81 (1:2:1)=0.78 ($0.75>p>0.50$)
Chi-square *Zps10/(22)* (1:2:1)=0.04 ($0.99>p>0.975$)
Chi-square independence=51.01 ($p<0.001$)

Linkage analysis of the 10-K structural gene

Southern hybridization of the cDNA subclone, 10kZ-1Δ43, to EcoRI digests of genomic DNA from BSSS53 and W23 revealed restriction fragment length polymorphisms as reported by Kirihara et al. (1988). Both inbreds contained one strong and one weak hybridizing fragment. Due to its greater homology, the strong hybridizing restriction fragment was assumed to represent the major 10-K structural gene, designated *Zps10/(22)*. It was not determined whether the weak hybridizing fragment represented a second 10-K protein gene, a pseudogene, or a partially homologous gene encoding a zein of higher molecular weight.

Independent segregation was observed between *Zps10/(22)* and *Zpr10/(22)* among 25 BC kernels; this indicated separate loci (Table 4). Linkage was initially detected between *Zps10/(22)* and *wx*T7-9a (7L.63: 9S.07). No recombination between *wx* and *Zps10/(22)* occurred among 16 backcross kernels. Close linkage of *Zps10/(22)* was also detected with *wx*T4-9g; no recombinants were found among 22 backcross progeny. Because of observed linkage with both *wx*T4-9g and *wx*T7-9a, restriction fragments hybridizing to random genomic clones from chromosome 9 were used as markers (Burr et al. 1988; D. Hoisington, unpublished data). An RFLP which showed linkage with *Zps10/(22)* was detected with clone BNL3.06 (Table 5). A recombination fre-

quency of 0.15 (S.E. = 0.04) was calculated with data from 59 F_2 progeny. Linkage with *Zps10/(22)* was also demonstrated with clone UMC81 (Table 6). A recombination frequency of 0.03 (S.E. = 0.02) was calculated from 52 F_2 progeny.

In addition, *Zps10/(22)* segregated independent of three chromosome 7 markers: *o2* and RFLPs detected with clones UMC110 and BNL15.4 (data not shown). No linkage was detected between the restriction fragment showing weak hybridization with 10kZ-1Δ43 and *Zps10/(22)* or any of the above mentioned markers.

Discussion

The genetic analysis of methionine-rich storage protein accumulation may provide information useful in the improvement of maize nutritional quality. Unsupplemented corn feed is limiting in lysine and tryptophan, whereas corn-soybean mixtures are deficient in methionine (Paulis et al. 1978). Maximum feed performance for poultry nutrition is usually attained by the addition of synthetic methionine. Cereal-legume mixtures developed for human consumption may also be limiting in methionine (Doggett 1975). The Latin American corn-bean diet, e.g., generally contains sufficient quantities of lysine. Research into the organization of genes involved in methionine-rich storage protein production will yield information relevant to the incorporation of increased nutritional quality into elite germplasm, along with providing data concerning gene action.

F_2 and backcross populations were utilized to demonstrate that a single gene was responsible for the increased accumulation of methionine-rich, 10-K protein. F_1 patterns were additive and exhibited dosage dependency, although a linear response could not be concluded. Since differences in zein synthesis may be the result of altered regulation at the structural locus or, alternatively, a distinct 'regulatory' gene, experiments were undertaken to map both the structural gene and the regulatory locus.

Linkage of *Zpr10/(22)* and *wx*T4-9g implied one of four map locations: (1) on the short arm of chromosome 4, distal to the breakpoint, (2) on the long arm of chromosome 4, (3) on the short arm of chromosome 9, or (4) on the long arm of chromosome 9. Tight linkage with *wx*T4-9(5657) would be expected if *Zpr10/(22)* was located on the long arm of chromosome 4; however, as mentioned previously, tight linkage was not detected with either *wx*T4-9(5657) or *wx*T4-9b. A chromosomal position for *Zpr10/(22)* on the short arm of chromosome 4 was indicated by linkage data from populations segregating for *fl2* and *Ga1-s*. Due to the relative location of chromosome 4 markers, placement of *Zpr10/(22)* between *fl2* and *Ga1* was dictated by map distances of

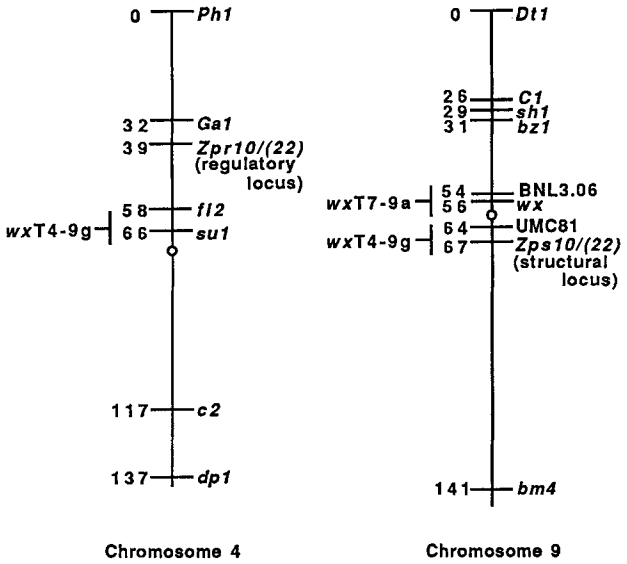

Fig. 2. Diagrammatic representation of maize chromosomes 4 and 9 (short arm oriented upwards) indicating placement of 10-K zein regulatory and structural genes in relation to additional morphologic, molecular and cytogenetic markers

23 cM and 7 cM from the regulatory locus, respectively. Due to its relative proximity, linkage of *Zpr10/(22)* with *Ga1* is taken to best represent the actual location of the regulatory locus, at map position 39 on chromosome 4 (Fig. 2).

A map location for *Zps10/(22)* on chromosome 9 was implied by linkage with both *wx*T4-9g and *wx*T7-9a. Placement of the gene at position 67 on chromosome 9 was dictated by map distances of 15 cM from BNL3.06 (position 54) and 3 cM from UMC81 (position 64) (Fig. 2). A 10-K zein gene was previously mapped to chromosome 7 by Hastings et al. (1984) and recently Wilson et al. (1989). Both studies, however, utilized a translocation involving chromosomes 7 and 9. Linkage of *Zps10/(22)* to chromosome 7-specific markers via a 7–9 translocation may have led to the erroneous placement of the 10-K structural gene in these previous reports. The present study has confirmed placement on chromosome 9 by utilizing both chromosome 7- and chromosome 9-specific RFLPs.

The 10-K zein overproduction phenotype was not dependent upon the allelic constitution of the 10-K zein structural gene (chromosome 9), but rather was dependent upon a distinct 'regulatory' locus (chromosome 4). This putative regulatory gene, *Zpr10/(22)*, may represent a direct modifier of 10-K protein accumulation. Maier et al. (1987) have identified an interaction between the 5′ region of a zein gene and nuclear proteins, and suggest that zein genes are controlled through interactions between *cis* elements in flanking DNA and products from *trans*-acting genes. Specific regulation of 10-K

zein levels by *Zpr10/(22)* via a *cis* element would, however, require a sequence not shared by other zein structural genes. Alternatively, *Zpr10/(22)* may influence zein accumulation by indirect effects, perhaps by regulating availability of amino acids. In soybean, the rate of methionine synthesis limits aminoacylation of methionyl-transfer RNA in developing cotyledons (Thompson et al. 1981). Should an analogous rate limitation exist in maize, then *Zpr10/(22)* may represent a gene which regulates the supply of methionine to the developing endosperm. Levels of methionine-rich, 15-K zeins, however, appear unchanged in the *Zpr10/(22)*-containing line BSSS53 (Phillips and McClure 1985). Specific regulation of 10-K zein levels via alterations in methionine availability is, therefore, not likely.

Assuming a two-gene model (one structural and one regulatory gene), the inbred line BSSS53 contains the overproduction allele *Zpr10/(22)* and a *Zps10/(22)* allele capable of participating in the overproduction of protein. Differences between inbred lines, which result in segregation of the 10-K protein overproduction phenotype in appropriate crosses, may exist at the structural gene, the regulatory gene, or both. A 3:1 F_2 ratio would indicate a single-gene difference, whereas a two-gene difference would be characterized by a 15:1 ratio. The presence of a structural gene in kernels of line W23 was indicated by trace amounts of the major 10-K protein. A difference only at the *Zpr10/(22)* locus was indicated by single-gene segregation ratios observed in progeny resulting from crosses between BSSS53 and W23; the W23-derived *Zps10/(22)* allele is, therefore, capable of participating in overproduction when combined with the appropriate regulatory allele. In addition, crosses for the linkage analysis of *Zpr10/(22)* and *Zps10/(22)* generated kernels homozygous for the W23-derived *Zps10/(22)* allele which contained elevated levels of 10-K protein, providing further evidence that the W23-derived structural allele is capable of participating in protein overproduction. The presence of a 10-K zein structural locus in lines B37, W64A, and Oh43 is indicated by trace amounts of 10-K protein in mature kernels. Single-gene segregation of overproduction was observed in crosses of BSSS53 with A619, A632, B73, and Mo17. This accumulated evidence has led us to speculate that a *Zps10/(22)* allele capable of participation in overproduction of protein may be present in many inbred maize lines. Overproduction, however, has only been observed in line BSSS53; this suggests limited distribution of *Zpr10/(22)*.

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