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Linkage Relationships between Regulatory and Structural Gene Loci Involved in Zein Synthesis in Maize

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Summary. The synthesis of at least 15 zein polypeptides is under the control of regulatory gene loci. One of these, *Opaque-2* (chromosome 7, position 16) strongly reduces the zein accumulation without modifying the zein molecular components. The linkage relationship between the regulatory gene *02* and the 5 structural loci (*Zp1, Zp2, Zp3, Zp6, Zp12*) segregating with sample Mendelian ratios have been studied. *Zp1, Zp2, Zp3* are closely linked to each other; moreover this gene cluster is located on chromosome 7 at 5.5 cM from the *Opaque-2* locus. The structural loci *Zp6* and *Zp12* are not linked with each other, with the *02* locus or with *Zp1, Zp2, Zp3*. From our data it follows that the zein structural genes are located in at least three positions on the maize genome. The scattering in the genome of the genes controlled by the *Opaque-2* locus suggests a transacting role for this regulatory element.

Key words: Zein synthesis – Gene regulation – *Opaque-2* linkage relationships

Introduction

Our knowledge of the regulation of genes in prokaryotic organisms achieved substantial advancement with the operon theory of Monod and Jacob (1961). Similar genetic regulation may operate in higher eukaryotes (Paigen, 1971; Lewin 1975). However, a full understanding of gene regulation in higher eukaryotes requires the identification of genetic elements endowed with regulatory functions and controlling the expression of genes known to carry structural information. The zein-synthesizing system in maize endosperm offers the opportunity to investigate the linkage relationship between a transacting regulatory element and several structural genes under its control.

Zein is the major storage protein of maize endosperm. It is synthesized by membrane-bound polysomes and accumulated in protein bodies (Burr and Burr 1976; Larkins et al. 1976). Zein consists of a mixture of at least 15 polypeptide chains with MW of 23,000 or 21,000 daltons and exhibiting a large heterogeneity in charge as revealed by electrophoresis or isoelectric focusing (IEF) in polyacrylamide gels (Gianazza et al. 1976; Mossé 1966; Alexandrescu et al. 1976; Soave et al. 1976). This charge heterogeneity is not caused by artifacts such as deamidation or denaturation during extraction of the protein, Ampholine interaction or presence of non protein residues bound to the protein (Righetti et al. 1977b). Indeed, differences have been found in amino acid composition among the various IEF zein components (Gianazza et al. 1977) and the in vitro translation products of the polysomes bound to protein-bodies show the same heterogeneity as native zein (Viotti et al. submitted to Plant Science Letter). This last point strongly suggests the in vivo absence of a post-translational modification of this class of polypeptides. Furthermore, in reciprocal crosses between inbred lines with marked differences in zein IEF patterns, the amount of each component correlates with the gene dose present in the cross (Righetti et al. 1977a). The accumulated evidence strongly suggests that the IEF zein bands correspond to the products of a system of structural genes.

Several gene loci control the level of zein accumulation during endosperm development (Mertz et al. 1964; Nelson et al. 1965; McWhirter 1971; Ma and Nelson 1975). Among them, the *Opaque-2* (*02*) locus plays an important regulatory role (Nelson 1969). The recessive allele at this locus strongly reduces the zein accumulation (Mossé et al. 1966; Murphy and Dalby 1971; Soave et al. 1975) without modifying qualitatively the zein molecular components (Burr and Burr 1976; Gianazza et al. 1976; Soave et al. 1976; Lee et al. 1976; Misra and Mertz 1976; Di Fonzo et al. 1977). The inhibition is exerted on all the zein bands and is stronger on the more alkaline components (Soave et al. 1976).

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Recently it has been found that the zein IEF pattern is genotype dependent: A large variability among the patterns from several strains of maize has been observed (Gentinetta et al. 1975). This situation allowed us to investigate the linkage relationship between the regulatory gene *02* and the structural genes coding for some zein bands.

Materials and Methods

Three crosses were studied to estimate linkage relationships between *Opaque-2* and the *Zp1, Zp2, Zp3, Zp6, Zp12* loci. In the first cross, the inbred N28 normal was pollinated with pollen from Oh45 *Opaque-2*. The F1 was self-fertilized or crossed to the inbred A69Y *opaque-2* in order to obtain the F2 and the BC generation respectively. F2 plants were also grown and self-fertilized to produce F3 seeds. In the second cross, the inbred OH43 normal was pollinated with pollen from A69Y *opaque-2*. In the third cross, the inbred N28 *opaque-2* was pollinated with A69Y *opaque-2* pollen. The genotypes of the mentioned inbreds were: N28 normal = *02, zp1, zp2, zp3, zp6, zp12*; Oh45 *opaque-2* = *o2, Zp1, Zp2, Zp3, Zp6, Zp12*; A69Y *o2* = *o2, zp1, zp2, zp3*; Oh43 normal = *02, Zp1, Zp2, Zp3*; N28 *opaque-2* = *o2, zp1, zp2, zp3*.

The endosperm of the kernels from the segregating ears was classified with respect to the normal or *opaque-2* phenotype. The endosperm of individual kernels was powdered with a mortar and the meal (about 200 mg) was extracted twice with 3 ml of 0.5M NaCl by shaking for 2 hours at 4°C. The suspension was centrifuged at 5000 x g for 5 min and the pellet washed twice with water. Zein was extracted from the residue with 3 ml of 70% (v/v) ethanol containing 1% (v/v) of 2-mercaptoethanol by shaking for 2 h. The extraction was repeated twice. The alcohol extraction dissolves only the zein protein. The extract was evaporated in vacuo and the zein dissolved at a concentration of about 7 mg/ml with 0.01 M Trisglycine, pH 8.2, 6 M Urea and 1% 2-mercaptoethanol.

Thin-layer polyacrylamide gel isoelectric focusing of zein was performed as described by Righetti et al. (1977b). The gel slab contained 5% acrylamide (Bis being 1:26 of acrylamide), 2% carrier ampholytes prepared with equal volume of pH 6-8 and pH 7-9 range Ampholine (LKB Products) and 6 M Urea. Protein samples (about 150 µg) were applied in small pockets precast in the gel and the run was for four hours at 20°C with a constant wattage of 13 W. Staining and destaining were as described by Righetti et al. (1974).

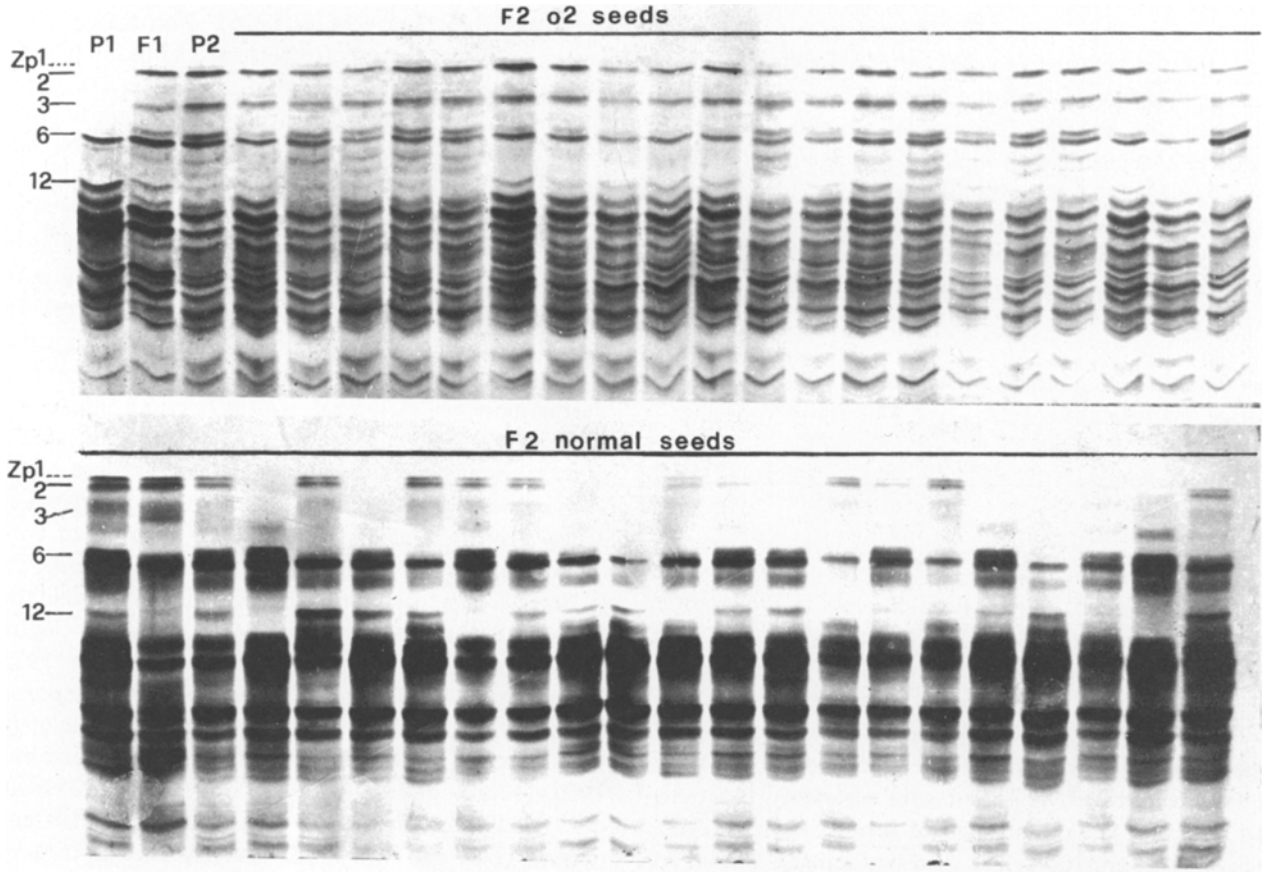

Fig. 1. Zein IEF phenotypes of parents, F1 and F2 generations of the cross N28 normal (P1) X OH45 *opaque-2* (P2). The band Zp3 is relatively faint in the photograph but its presence or absence was easily detectable in the gels. When necessary, overloading resolved doubtful cases

Results and Discussion

As observed during the work with various genotypes of maize, the bands coded Zp1, Zp2, Zp3 (Zp is for zein polypeptide), in Fig. 1 of this paper, show a coordinated absence or presence suggesting that the genes coding for them are closely linked to each other. These bands are characterized by a M.W. of 21 Kds and focus at the most alkaline pH values. Their linkage relationship with the locus *02* was studied in three crosses. In cross number 1, the parental inbred OH45 (P2 in Fig. 1) was homozygous for the *o2* allele and characterized by the presence of the bands Zp1, Zp2, Zp3 (genotype *o2*, Zp1, Zp2, Zp3); the second parent was N28 (P1), homozygous for the normal *O2* allele and lacking the above mentioned bands (genotype *O2, zp1, zp2, zp3*). Fig. 1 shows the zein IEF patterns of parents, F1 and F2 seeds. Several differences can be observed between the two parents, while the F1 pattern is the combination of the patterns of the two parental genotypes. The presence of the bands Zp1, Zp2 and Zp3 is dominant over the absence. In the *opaque-2* seeds (*o2*, upper panel, Fig. 1) of the F2 generation, the bands Zp1, Zp2 and Zp3 are always present while in normal seeds they are not always present (*O2*, bottom panel, Fig. 1). The quantitative data of cross 1 are reported in Table 1. The linkage between the *O2* locus and the three structural loci *Zp1, Zp2, Zp3* is suggested by the discrepancy between expected and observed number of F2 and backcross (BC) phenotypes. This linkage, however, is not absolute since some recombinant phenotypes have been observed (5 out 58 in the BC generation). When calculated from F2 and BC data, the estimates of the crossover values were 5.1 and 6.5 centiMorgans (cM) respectively. The F3 data of cross 1 were in accord with the data of the F2 or BC.

Table 1. Estimates of the crossover values between the regulatory gene *Opaque-2* (chromosome 7, position 16) and the loci *Zp1,Zp2,Zp3* coding for the IEF zein subunits Zp1, Zp2 and Zp3

Generation		Number of seeds in the segregating generations Phenotypes (a)				Crossover Value (cM ± s.e.)
		02 Zp1,Zp2,Zp3	*02 zp1,zp2,zp3*	*o2 Zp1,Zp2,Zp3*	*o2 zp1,zp2,zp3*	
Cross § 1 (N28 Normal X Oh45 *opaque-2*)						
F2 (121 seeds)	expected (b)	68.1	22.7	22.7	7.5	
	observed	56	35	30	0 (d)	5.1 ± 6.1 (c)
BC (58 seeds)	expected	14.5	14.5	14.5	14.5	
	observed	3	26	27	2	6.5 ± 2.1 (c)
F3 7 Normal ears		All the seeds of each ear had the phenotype *02,zp1,zp2,zp3*				
2 Ears segregating 3 : 1, *02:o2*		The frequencies of the 4 phenotypes were similar to those reported for the F2.				
9 Ears homozygous *o2*		8 ears had seeds all of the phenotype *o2,Zp1,Zp2,Zp3*				
		1 ear had seeds of the phenotype *o2,Zp1,Zp2,Zp3* (77%) and *o2,zp1,zp2,zp3* (23%).				5.5 (e)
Cross § 2 (Oh43 Normal X A69Y *opaque-2*)						
F2 (188 seeds)	expected	105.7	35.2	35.2	11.8	
	observed	140	1	6	41	3.0 ± 0.8 (c)
BC (59 seeds)	expected	14.7	14.7	14.7	14.7	
	observed	29	2	3	26	7.5 ± 2.3 (c)
Cross § 3 (N28 *opaque-2* X A69Y *opaque-2*)						
F1 (10 seeds)		All the seeds had the phenotype *o2,zp1, zp2,zp3*				
F2 (20 seeds)		All the seeds had the phenotype *o2,zp1,zp2,zp3*				
Mean Crossover Value (cM)						5.5

a) Since in our crosses the linkages among Zp1,Zp2 and Zp3 were absolute, they were considered altogether in studying their relationship with the *02* locus
b) In case of independence
c) Calculated according to the product method (Immer 1930)
d) This value was considered equal to 0.1 for the sake of calculation
e) The number of gametes sampled (9 *opaque-2* F3 ears) was 18. The crossover values was then estimated as 1/18 (= 0.055)

Table 2. Estimates of the crossover values and their standard errors among *Opaque-2*, the gene cluster *Zp1, Zp2, Zp3* and the genes *Zp6* and *Zp12* coding for the IEF zein subunits *Zp6* and *Zp12*

Segregating genes	cM ± s.e.
02 – Zp6	47 ± 4.4
02 – Zp12	52 ± 4.5
Zp1, Zp2, Zp3 – Zp6	43 ± 4.2
Zp1, Zp2, Zp3 – Zp12	51 ± 4.5
Zp6 – Zp12	42 ± 4.9

Data from 121 F2 seeds of the cross 1

Specifically, out of 9 F3 *opaque-2* ears, one ear segregated Zp1, Zp2, Zp3: zp1, zp2, zp3. This permitted a third estimate of the crossover values corresponding to 5.5 cM.

In the second cross, the parents were OH43 normal (genotype *O2, Zp1, Zp2, Zp3*) and A69Y *opaque-2* (genotype *o2, zp1, zp2, zp3*). In this cross, the recessive *o2* allele was coupled with the null alleles *zp1, zp2, zp3*. The crossover values between the *O2* locus and the region associated with the three bands were 3.0 cM. and 7.5 cM, respectively for the generations F2 and BC (see Table 1). These data are in close agreement with those from cross 1. Cross 3 was performed to demonstrate the allelic nature of the absence of the bands Zp1, Zp2, Zp3 in the genotypes N28 and A69Y, two inbreds involved in crosses 1 and 2 respectively. The F1 showed no Zp1, Zp2 and Zp3 bands and no transgressive phenotypes were present in the F2 generation.

The linkage relationships between the cluster *Zp1, Zp2* and *Zp3* and other structural loci have been followed utilizing the F2 generation of cross 1. In this cross, two other bands, Zp6 and Zp12, clearly segregated as separate monogenic traits. Table 2 shows that the genetic elements *Zp6* and *Zp12* coding for Zp6 and Zp12 are not linked with each other or with *O2, Zp1, Zp2* or *Zp3* loci (the crossover values are all in the 50 cM range). These results indicate that the structural genes coding for zein proteins are identified with more than one linkage group (from the available data, it follows that the minimum number of groups is three; of these, two are not linked with the regulatory gene *O2*).

Previous experimental evidence demonstrated the existence of several zein proteins possibly coded for by different genetic loci (Gianazza et al. 1976; Soave et al. 1976; Righetti et al. 1977a, 1977b). The present data show a clear Mendelian segregation for at least 5 IEF zein bands, suggesting a close relation between single IEF components and zein structural loci. Moreover some of these genes are clustered as *Zp1, Zp2* and *Zp3*. The two other structural genes identified in this analysis, *Zp6* and *Zp12*, do not show linkage between each other or with the cluster *Zp1, Zp2, Zp3* even if we can not exclude that other zein genes are clustered with *Zp6* and *Zp12*. Our data, however, show that *Zp6, Zp12* and the cluster *Zp1, Zp2, Zp3* are located in three different positions on the genome. This situation allows a reconsideration of the relationship between the regulatory locus *O2* and the structural genes under its control. The dominance of the *O2* wild type allele, together with the scattering in the genome of the structural genes under its control, strongly suggest a trans-acting role for this regulatory gene. From this point of view, the *O2* locus is different from other eukaryotic regulatory elements which are cis-acting and are located close to the structural genes they control (Swank et al. 1973; Arst and Scazzocchio 1975; Chovnik 1976). In contrast, the *O2* type of regulation fits well the models of gene regulation described in maize (McClintock 1956) or in lower eukaryotes (Douglas and Hawthorne 1972; Littlewood 1975; Arst 1976).

A second interesting point emerges from our results and this concerns the linkage relationship between *O2* and the cluster *Zp1, Zp2, Zp3*. Since there is some recombination between them, it appears that the regulatory function is located beyond the boundaries of the cluster of structural genes. Nevertheless we could suppose that the two elements evolved independently from a common ancestor endowed with both regulatory and structural functions; the first element retaining and improving the regulatory function, the second stregthening its structural role by redundancy.

Literature

Alexandrescu, V.; Chihaia, C.; Cosmin, O.: Zein specificity in inbred lines of maize. Rev. Roum. Biochim. **13**, 169-172 (1976)

Arst, H.N.; Scazzocchio, C.: Initiator constitutive mutation with an 'up-promoter' effect in *Aspergillus nidulans*. Nature **254**, 31-34 (1975)

Arst, H.N.; Scazzocchio, C.: Integrator gene in *Aspergillus nidulans*. Nature **262**, 231-237 (1976)

Burr, B.; Burr, F.A.: Zein synthesis in maize endosperm by polyribosomes attached to protein bodies. Proc. Natl. Acad. Sci. (USA) **73**, 515-519 (1976)

Chovnick, A.; Gelbart, W.; McCarron, M.; Osmond, B.; Candido, E.P.M.; Baillie, D.L.: Organization of the *rosy* locus in *Drosophyla melanogaster*: evidence for a control element adjacent to the xanthyne deydrogenase structural element. Genetics **84**, 233-255 (1976)

Di Fonzo, N.; Fornasari, E.; Salamini, F.; Soave, C.: SDS-protein subunits in normal, opaque-2 and floury-2 maize endosperms. Maydica **22**, 77-88 (1977)

Douglas, H.C.; Hawtorne, D.C.: Uninducible mutants in the *gal i* locus of *Saccharomyces cerevisiae*. J. Bacteriol. **109**, 1139-1143 (1972)

Gentinetta, E.; Maggiore, T.; Salamini, F.; Lorenzoni, C.; Pioli, F.; Soave, C.: Protein studies in 46 'opaque-2' strains with modified endosperm texture. Maydica **20**, 145-165 (1975)

Gianazza, E.; Righetti, P.G.; Pioli, F.; Galante, E.; Soave, C.: Size and charge heterogeneity of zein in normal and opaque-2 maize endosperms. Maydica **21**, 1-17 (1976)

Gianazza, E.; Viglienghi, V.; Righetti, P.G.; Salamini, F.; Soave, C.: Amino acid composition of zein molecular components. Phytochemistry **16**, 315-317 (1977)

Immer, F.: Formulae and tables for calculating linkage intensities. Genetics **15**, 81-98 (1930)

Larkins, B.A.; Bracker, C.E.; Tsai, C.Y.: Storage protein synthesis in maize. Plant Physiol. **57**, 740-745 (1976)

Lee, K.H.; Johns, R.A.; Dalby, A.; Tsai, C.Y.: Genetic regulation of storage protein content in maize endosperm. Biochem. Genet. **14**, 641-650 (1976)

Lewin, B.: Units of transcription and translation: the relationship between heterogeneous unclear RNA and messenger RNA. Cell **4**, 11-20 (1975)

Littlewood, B.S.; Chia, W.; Metzenberg, R.L.: Genetic control of phosphate-metabolizing enzymes in *Neurospora crassa*: relationships among regulatory mutations. Genetics **79**, 419-434 (1975)

Ma, Y.; Nelson, O.E.: Amino acid composition and storage proteins in two new high-lysine mutants in maize. Cereal Chem. **52**, 412-419 (1975)

McClintock, B.: Controlling elements and the gene. Cold Spring Harbor Symp. Quant. Biol. **21**, 197-216 (1956)

McWhirter, K.S.: A floury endosperm, high lysine locus on chromosome 10. Maize Genet. Coop. News Letter **45**, 184 (1971)

Mertz, E.T.; Bates, L.S.; Nelson, O.E.: Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science **145**, 279-280 (1964)

Misra, P.S.; Mertz, E.T.: Studies on corn proteins. X Polypeptide molecular-weight distribution in Landry – Moreaux fractions of normal and mutant endosperms. Cereal Chem. **53**, 705-711 (1976)

Monod, J.; Jacob, F.: General conclusions: teleonomic mechanisms in cellular metabolism, growth and differentiation. Cold Spring Harbor Symp. Quant. Biol. **26**, 389-401 (1961)

Mossé, J.: Alcohol-soluble proteins of cereal grains. Fed. Proc. **25**, 1663 (1966)

Mossè, J.; Baudet, J.; Landry, J.; Moreaux, T.: Etude comparée sur les proportions et les compositions en acides aminés des fractions protéiques de grains de maïs normaux et mutants. C. Red. Acad. Sci. **263**, 788-791 (1966)

Murphy, J.J.; Dalby, A.: Changes in the protein fractions of developing normal and 'opaque-2' maize endosperm. Cereal Chem. **48**, 336-349 (1971)

Nelson, O.E.; Mertz, E.T.; Bates, L.S.: Second mutant gene affecting the amino acid pattern of maize endosperm proteins. Science **150**, 1469-1470 (1965)

Nelson, O.E.: Genetic modification of protein quality in plants. Adv. Agron. **21**, 171-194 (1969)

Paigen, K.: The genetics of enzyme realization. In: Enzyme Synthesis and Degradation in Mammalian Systems. (Ed. Rechcigl, M.) pp 1-46. Basel: S. Karger 1971

Righetti, P.G.; Drysdale, J.W.: Isoelectric focusing in polyacrylamide gels. Biochim. Biophys. Acta **236**, 17-28 (1974)

Righetti, P.G.; Gianazza, E.; Salamini, F.; Galante, E.; Viotti, A.; Soave, C.: Zein: macromolecular properties, biosynthesis and genetic regulation. In: Electrofocusing and Isotachophoresis. pp. 199-211. Berlin-New York: Gruyter & Co. 1977a

Righetti, P.G.; Gianazza, E.; Viotti, A.; Soave, C.: Heterogeneity of Storage Proteins in Maize. Planta **136**, 115-123 (1977b)

Soave, C.; Pioli, F.; Viotti, A.; Salamini, F.; Righetti, P.G.: Synthesis and heterogeneity of endosperm proteins in normal and 'opaque-2' maize. Maydica **20**, 83-94 (1975)

Soave, C.; Righetti, P.G.; Lorenzoni, C.; Gentinetta, E.; Salamini, F.: Expressivity of the opaque-2 gene at the level of zein molecular components. Maydica **21**, 61-75 (1976)

Swank, R.T.; Paigen, K.; Gansschow, R.E.: Genetic control of glucuronidase induction in mice. J. Mol. Biol. **81**, 225-243 (1973)

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