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Genetic control of mitochondrial malate dehydrogenases: Evidence for duplicated chromosome segments

(gene duplication/gene evolution/mitochondrial genetics/gene-enzyme systems)

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ABSTRACT **The genetic control of the major mitochondrial isozymes of malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase; EC 1.1.1.37) has been investigated in *Zea mays*. The mitochondrial isozymes are coded at four nuclear gene loci. Two of the loci (*mdh1* and *mdh2*) are diallelic and tightly linked. The other two loci (*mdh3* and *mdh4*) appear to have arisen by duplication of the chromosome segment carrying *mdh1* and *mdh2*, but are not linked to them. The segregation of such a duplicate segment can explain anomalous backcross and F_2 segregation ratios.**

The occurrence of multiple molecular forms of enzymes (isozymes) is a common characteristic in most organisms (1, 2). Malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase; EC 1.1.1.37; MDH), in various animal and plant tissues, exists commonly in isozymic forms. Because malate dehydrogenase carries out several physiological roles within the cell (3–5) and occurs both in soluble cytoplasm and in mitochondria (3), it is important to know how the expression of MDH isozymes is controlled genetically.

Genetic variants of MDH isozymes have been observed in several animal (6–10) and plant tissues (11). The mitochondrial MDH (m-MDH) isozymes in maize (11) and in mouse (6) are controlled by nuclear genes; however, detailed genetic analysis of the m-MDH isozymes was not reported in either study. In vertebrates, it was reported that the structural genes coding for soluble MDHs may vary. In reptiles, birds, and mammals, soluble MDH (s-MDH) typically exists as a single major anodal form (12–14), which suggests single gene control. In fishes and amphibians (9, 15), the s-MDHs appear to be controlled by two unlinked loci.

The present study describes genetic analysis of the major m-MDH isozymes in maize.

MATERIALS AND METHODS

Maize Strains. The strains of maize used in these investigations were inbred for at least 15 generations. Appropriate genetic crosses involving these inbred lines were made. Ears were harvested between 16 and 20 days after pollination. MDH isozyme patterns and activity in the fresh liquid endosperm from individual kernels (16- to 20-days old) were examined. The maize ears were then stored at −50° within 24 hr after harvest. No changes in zymogram patterns or in levels of activity were observed after the immature maize kernels were frozen and thawed.

Electrophoresis and MDH Staining. MD isozymes were separated by electrophoresis on 12% starch gels as described (16), using the Tris-citrate buffer system (pH 7.0) of Meizel and Markert (17).

Liquid endosperm from individual kernels was squeezed onto a 4 × 6 mm piece of Whatman 3mm filter paper and inserted into a vertical slot in the gel. Horizontal starch gel electrophoresis and specific staining for MDH were conducted as described by Scandalios (16). Staining was completed in approximately 15 min to 1 hr at 37°. The gels were then washed several times with cold tap water, photographed, and preserved for future reference in a solution of 50% glycerol.

Identification of s-MDH and m-MDH Isozymes. The subcellular distribution of the MDH isozymes was determined after isolation of the various organelles by sucrose gradient centrifugation (18). The compartmentation patterns observed were consistent with those described in maize (11).

Scoring and Symbols for Phenotypes and Genotypes of the MDH Isozymes. Mitochondrial MDH isozymes will be designated as m-MDH^1, m-MDH^2, etc. The genes coding for these isozymes will be identified with a locus number followed by an allele designation. For example, *mdh1-m1* and *mdh1-m2* are alleles at the *mdh1* locus. The allele designations correlate with the isozyme coded by that allele (e.g., *mdh1*-m1 codes for isozyme m-MDH^1 and *mdh1*-m2 codes for isozyme m-MDH^2).

RESULTS

Genetic Variants of Maize MDH Isozymes. In maize, there are two major classes of MDH isozymes (Fig. 1). The two soluble forms (s-MDHs) appear in all the inbred strains tested and are designated s-MDH^1 and s-MDH^2. Additional s-MDH isozyme variants have been observed occasionally in the inbred lines 59, 37, and T21; these variants are more anodal in their migration than is s-MDH^1. Seven distinct zymogram patterns of the mitochondrial MDH isozymes were observed in the 20 inbred lines examined (Fig. 1). The number of m-MDH isozymes in these patterns varied from 2 to 6. The isozymes are designated m-MDH^1, m-MDH^2, . . . m-MDH^7 in order of decreasing anodal mobility. The m-MDH isozyme pattern is fixed within each of the inbred lines examined.

Genetic Analysis of m-MDH Isozymes. Back crosses and F_2 crosses between inbred strains Oh51A and 59 were constructed to determine the genetic relationships of the various m-MDH isozymes. Oh51A has isozymes m-MDH^1 and m-MDH^3, while 59 carries m-MDH^2 and m-MDH^5 (Fig. 1). Four distinguishable m-MDH phenotypes were recovered from the back crosses (Fig. 2), the two parental types (A and B) and two hybrid patterns (C and D). The two hybrid patterns result from the fact that the triploid (3*n*) liquid endosperm was used in these studies. Since this tissue receives twice the genetic contribution from the female parent as from the male parent, dosage patterns will vary depending on the direction in which

Abbreviations: MDH, malate dehydrogenase; s-MDH, soluble MDH, m-MDH, mitochondrial MDH.

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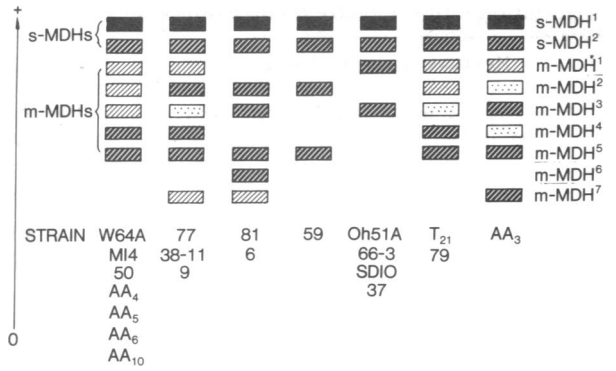

FIG. 1. Phenotypes of MDH isozymes observed in various inbred strains of maize.

the cross is made. F_1 progeny between Oh51A and 59 all showed pattern C when Oh51A was the female parent and pattern D when 59 was the female parent. The m-MDH[4] isozyme is a hybrid molecule containing one m-MDH[3] subunit and one m-MDH[5] subunit.

The results of the crosses are shown in Table 1. When Oh51A served as the female parent and the F_1 (59 × Oh51A or Oh51A × 59) as the male parent in the back crosses, parental and hybrid phenotypes (B and C, Fig. 2) were observed in the expected 1:1 ratio in the progeny. In all instances, m-MDH[1] and m-MDH[3] were inherited as a unit.

When the F_1 (59 × Oh51A or Oh51A × 59) was used as the female parent, three m-MDH phenotypes were observed in the progeny (Table 1, Fig. 2a): the Oh51A parental type and the two hybrid types. Segregation fits the 2:1:1 ratio predicted, assuming single locus segregation. The dosage patterns suggest that, like m-MDH[1] and m-MDH[3], m-MDH[2] and m-MDH[5] are not genetically independent.

When inbred 59 is crossed to the F_1 from either direction, the observed segregation patterns are not consistent with a single locus model (Table 1, crosses 5 and 6). The 1:3 ratios observed in these crosses suggest that a minimum of two genetically independent units are segregating in the system. The F_2 segregation ratios (Table 1, cross 7) are similarly inconsistent with single locus segregation because they deviate markedly from expected ratios of one Oh51A:one 59:two hybrid types in the progeny.

We have devised a model by which the aberrant ratios may be explained. The salient features of the model are that maize mitochondrial MDH isozymes are coded at four distinct loci in two linkage groups. Two of the loci, which comprise the first linkage group, code for isozymes m-MDH[1] and m-MDH[3]. Allelic alternatives at these loci code for m-MDH[2] and m-MDH[5], respectively. The two additional loci are carried on a

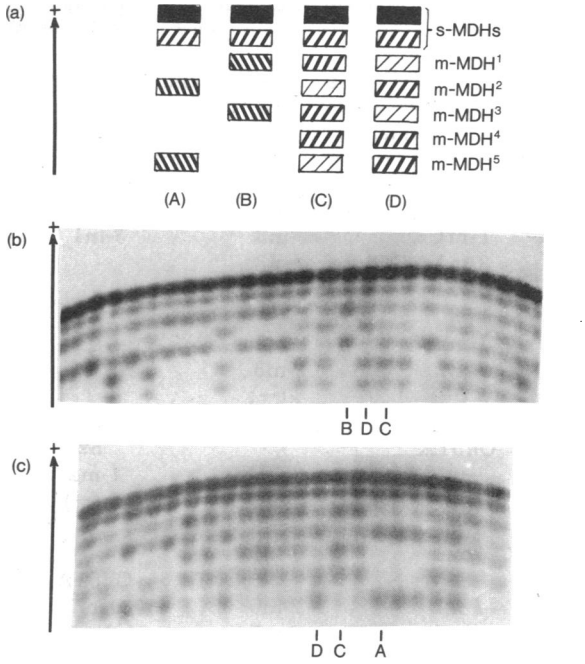

FIG. 2. MDH phenotypes of the back crosses involving strains 59 and OH51A (see Table 1). (a) Diagram of all four phenotypes observed in the back crosses described in Table 1. Type A and type B are the same as those found in inbred lines 59 and Oh51A, respectively. (b) MDH zymogram of the progeny from the back cross, (Oh51A × 59) × Oh51A. (c) MDH zymogram of the progeny from the back cross, (59 × Oh51A) × 59. Type B, C, and D were observed. In (b) and (c), lettered channels indicate representative examples of the phenotypes in (a).

genetically independent chromosome segment and are duplicates of the first two loci. These two loci carry only the alleles for m-MDH[1] and m-MDH[3].

The loci and their corresponding alleles have been designated as follows: *mdh1* and *mdh2* are the original linked loci. Alleles at these two loci are: *mdh1*-m1, which codes for isozyme m-MDH[1], *mdh1*-m2, which codes for isozyme m-MDH[2], *mdh2*-m3, which codes for m-MDH[3], and *mdh2*-m5, which codes for m-MDH[5]. The duplicated loci are designated *mdh3* and *mdh4* and correspond to *mdh1* and *mdh2*, respectively. In the inbred lines examined to date only one allele has been recovered for each of the duplicate loci. Allele *mdh3*-m1 codes for isozyme m-MDH[1], and *mdh4*-m3 codes for isozyme m-MDH[3].

According to the above model, any given inbred line should either show only m-MDH[1] and m-MDH[3] (genotype *mdh1*-m1, *mdh2*-m3, *mdh3*-m1, *mdh4*-m3) or should show m-MDH[1],

Table 1. Summary of back crosses and F_2 generation invoking an independently segregating duplicated chromosome segment

Cross	Parents			Phenotypes of progeny*				Total	Expected ratio of phenotypes†	χ^2	P
	Female		Male	(A)	(B)	(C)	(D)				
1	Oh51A	×	(59 × Oh51A)		118	103		221	1B:1C	1.02	>0.30
2	Oh51A	×	(Oh51A × 59)		73	74		147	1B:1C	0.007	>0.95
3	(59 × Oh51A)	×	Oh51A		85	41	38	164	2B:1C:1D	0.329	>0.85
4	(Oh51A × 59)	×	Oh51A		118	60	57	235	2B:1C:1D	0.09	>0.95
5	(59 × Oh51A)	×	59	56		126	59	241	1A:2C:1D	0.58	>0.75
6	59	×	(59 × Oh51A)	25			81	106	1A:3D	0.113	>0.74
7	(59 × Oh51A)	×	(59 × Oh51A)	69	236	669		974	1A:4B:11C+D	1.32	>0.52

* See Fig. 2 for schematic representation of the phenotypes.
† Expected ratios determined as in Table 2.

Table 2. Method of determining expected phenotypes and segregation ratios in maize liquid endosperm (3*n*)

	Potential gametic combinations*				Relative isozyme dosage					
	Female		Male		m-MDH[1]	m-MDH[2]	m-MDH[3]	m-MDH[5]	Phenotype†	Ratio of phenotypes
1.	Oh51A	×	Oh51A							
	mdh1-m1	*mdh2*-m3	*1*-m1	*2*-m3						
	1-m1	*2*-m3	*3*-m1	*4*-m3	6	0	6	0	B	—
	3-m1	*4*-m3								
	3-m1	*4*-m3								
2.	59	×	59							
	1-m2	*2*-m5	*1*-m2	*2*-m5						
	1-m2	*2*-m5	*3* (—)	*4* (—)	0	3	0	3	A	—
	3 (—)	*4* (—)								
	3 (—)	*4* (—)								
3.	Oh51A	×	59							
	1-m1	*2*-m3	*1*-m2	*2*-m5						
	1-m1	*2*-m3	*3* (—)	*4* (—)	4	1	4	1	C	—
	3-m1	*4*-m3								
	3-m1	*4*-m3								
4.	59	×	Oh51A							
	1-m2	*2*-m5	*1*-m1	*2*-m3						
	1-m2	*2*-m5	*3*-m1	*4*-m3	2	2	2	2	D‡	—
	3 (—)	*4* (—)								
	3 (—)	*4* (—)								
5.	Oh51A	× (Oh51A × 59) or (59 × Oh51A)								
	1-m1	*2*-m3	*1*-m2	*2*-m5						
	1-m1	*2*-m3	*3* (—)	*4* (—)	4	1	4	1	C	
	3-m1	*4*-m3								
	3-m1	*4*-m3								
	1-m1	*2*-m3	*1*-m2	*2*-m5						
	1-m1	*2*-m3	*3*-m1	*4*-m3	5	1	5	1	C	
	3-m1	*4*-m3								
	3-m1	*4*-m3								1B:1C
	1-m1	*2*-m3	*1*-m1	*2*-m3						
	1-m1	*2*-m3	*3* (—)	*4* (—)	5	0	5	0	B	
	3-m1	*4*-m3								
	3-m1	*4*-m3								
	1-m1	*2*-m3	*1*-m1	*2*-m3	6	0	6	0	B	
	1-m1	*2*-m3	*3*-m1	*4*-m3						
	3-m1	*4*-m3								
	3-m1	*4*-m3								
6.	(Oh51A × 59) or (59 × Oh51A) ×		59							
	1-m1	*2*-m3	*1*-m2	*2*-m5	4	1	4	1	C	
	1-m1	*2*-m3	*3* (—)	*4* (—)						
	3-m1	*4*-m3								
	3-m1	*4*-m3								
	1-m1	*2*-m3	*1*-m2	*2*-m5	2	1	2	1	C	
	1-m1	*2*-m3	*3* (—)	*4* (—)						
	3 (—)	*4* (—)								
	3 (—)	*4* (—)								1A:2C:1D
	1-m2	*2*-m5	*1*-m2	*2*-m5	2	3	2	3	D	
	1-m2	*2*-m5	*3* (—)	*4* (—)						
	3-m1	*4*-m3								
	3-m1	*4*-m3								
	1-m2	*2*-m5	*1*-m2	*2*-m5	0	3	0	3	A	
	1-m2	*2*-m5	*3* (—)	*4* (—)						
	3 (—)	*4* (—)								
	3 (—)	*4* (—)								

* Genotypic configurations of loci and alleles in possible gametes from various crosses. Italicized number preceding hyphen represents the locus number, and that after the hyphen represents the allele. No allele is listed for *mdh3* and *mdh4* in line 59 since no isozyme activity is observed for these loci. Since the isozyme patterns are scored in the triploid endosperm, each chromosome from the female gamete is represented twice. *mdh1* and *mdh2* are tightly linked, as are *mdh3* and *mdh4*.

† See Fig. 2 for schematic representation of the phenotypes.

‡ Isozymes m-MDH[2] and m-MDH[5] stain somewhat more heavily on gels and at equal dosage with m-MDH[1] and m-MDH[3] would be scored as a D phenotype.

m-MDH[2], m-MDH[3], and m-MDH[5] (genotype *mdh1*-m2, *mdh2*-m5, *mdh3*-m1, *mdh4*-m3). As seen in Fig. 1, 17 of the 20 inbred lines examined can be placed into one of these two classifications. Of the remaining three, two lines (81 and 6) are missing isozyme m-MDH[1] but have m-MDH[3]. These lines therefore appear to have the duplicated segment (at least that portion carrying *mdh4*), and the absence of isozyme m-MDH[1] may be due to a null allele at the *mdh3* locus, partial deletion of the duplicated segment, or to perturbation of regulation of a normal *mdh3* gene. In certain types of crosses at least one of the two lines (81) shows m-MDH[1] expression in a low percentage of the progeny (N. S. Yang and J. G. Scandalios, unpublished observation), suggesting that the latter explanation may be the more likely. It is also of interest to note that the hybrid isozyme m-MDH[4] is not observed in these lines even though m-MDH[3] and m-MDH[5] are present. This suggests that the m-MDH[3] isozyme in these lines may differ from normal m-MDH[3].

The only line that appears to be inconsistent with the model is line 59. The apparent absence of the duplicated segment in inbred strain 59 might be due to the occurrence of "null" alleles at the *mdh3* and *mdh4* loci, a regulatory or transcriptional block for the duplicate segment, or to the physical absence of the duplicate loci. The exact nature of the lesion is not critical to the present analysis.

As seen in Table 1, the backcross and F_2 segregation ratios are in excellent agreement with values predicted by the model values. The method for predicting expected ratios from the various crosses is shown in Table 2.

DISCUSSION

Current evidence suggests that mitochondrial and nuclear DNAs share in supplying gene products for structure, function, and control of mitochondria (19, 20). The mitochondrial gene products that have been identified are ribosomal RNA, transfer RNA (21), and probably some ribosomal proteins (22), although the coding capacity of mitochondrial DNA would be large enough to code for at least an additional 20 proteins. Maternal (cytoplasmic) inheritance would prevail in any case where genes were encoded in mitochondrial DNA. Some examples of cytoplasmic inheritance have been described in microorganisms, insects, and plants (23, 24). However, there is still no clear evidence that enzymes localized within mitochondria are coded by the mitochondrial genome. On the other hand, there is overwhelming evidence that most proteins in mitochondria are coded by nuclear DNA. Mitochondrial leucyl-tRNA synthetase from *Neurospora* (25) and two mitochondrial peptide chain elongation factors from yeast (26) appear to be coded by nuclear DNA. Mitochondrial malate dehydrogenase (m-MDH) in maize (11) and mouse (6) are coded by nuclear genes.

Results from this investigation indicate that multiple structural genes are involved in the expression of maize m-MDH isozymes. They are inherited according to Mendelian rules, thus providing further support that maize m-MDHs are controlled by nuclear genes (11). In addition, our studies of the turnover of the various m-MDH isozymes (27) and the intracellular site of their synthesis (28) suggest strongly that the maize mitochondrial MDH isozymes are synthesized in the cytoplasm and then become incorporated into mitochondria. This result is consistent with the fact that the m-MDHs are controlled by nuclear genes (11).

Segregation ratios observed in the Oh51A and 59 back crosses and F_2 generation can be readily explained by invoking the presence of an independently segregating chromosomal segment carrying duplicates of the *mdh1* and *mdh2* loci. Duplication of a specific chromosome segment in maize has been reported in at least 15 cases (29), although this is the first example to our knowledge in which biochemical markers have been used in such investigations.

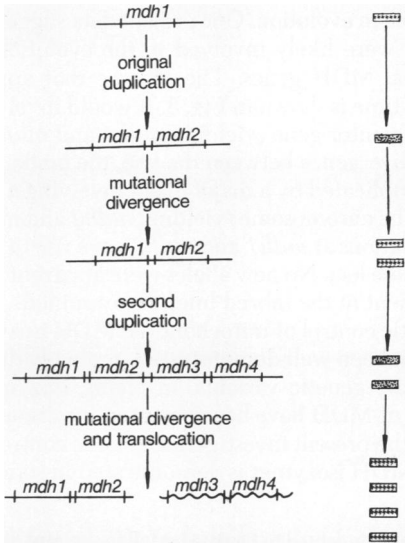

FIG. 3. Proposed evolutionary progression of multiple genes coding for mitochondrial MDH isozymes in maize. The presumed isozyme patterns for each of the ancestral stages is indicated at the right of the figure. The intensity of shading of each band reflects the number of gene copies coding for that isozyme. Each isozyme is presumed to be a dimer composed of subunits from one of the structural genes.

We have observed three recombinants between *mdh1* and *mdh2* in the 977 F_2 progeny examined in this study (genotype: *mdh1*-m1/m1, *mdh2*-m3/m5, *mdh3*-m1/-, *mdh4*-m3/-). Linkage values cannot be calculated from these data since 1/2 of the potential recombinant phenotypes would be indistinguishable from non-recombinant hybrid individuals, and since some of the detectable recombinants would be difficult to recognize in the maternally dosed liquid endosperm. Linkage analysis would be further confounded by any recombination between *mdh3* and *mdh4*. Even assuming that a significant amount of recombination went undetected, it is obvious that the two loci are distinct and very tightly linked.

Further evidence that *mdh1* and *mdh2* are discrete loci comes from the fact that the two appear to be under different regulatory control (N. S. Yang and J. G. Scandalios, unpublished observation) and from extensive biochemical characterization of the gene products (5). The putative alleles at the *mdh1* locus (isozymes m-MDH[1] and m-MDH[2]) have very similar thermostability, kinetics of NAD inhibition, K_m values for substrate and coenzyme, and K_m dependency on pH. The allelic products of the *mdh2* locus (isozymes m-MDH[3] and m-MDH[5]) also are very similar to each other in these parameters, but differ substantially from the *mdh1* allelic products (5). Studies on the developmental expression of isozymes m-MDH[2] and m-MDH[5] (coded by *mdh1* and *mdh2*, respectively) showed that the two isozymes are turning over at dramatically different rates (27), suggesting that rates of transcription and/or translation may differ for the two. Finally, exhaustive attempts to convert one isozymic form to another by various treatments proved unsuccessful (5). This further argues that the isozymes m-MDH[1,2,3,5] are separate genetic entities rather than conformers or artifacts.

Ohno (30) has proposed that gene duplication may play an

important role in evolution. Our present data suggest that such mechanisms were likely involved in the evolution of maize mitochondrial MDH genes. The scheme that appears most likely at this time is shown in Fig. 3. It would involve duplication of a progenitor gene (yielding *mdh1* and *mdh2*). After a mutational divergence between the two, the tandem pair may have been duplicated by a major event involving a substantial segment of the chromosome (yielding *mdh3* and *mdh4*). Subsequent mutations at *mdh1* and *mdh2* gave rise to new alleles at each of these loci. No new alleles were apparent in the duplicated segment in the inbred lines we examined.

The genetic control of mitochondrial MDH isozymes in animals has not been well demonstrated, probably due to a lack of appropriate genetic variants. In plants, isozymes of both s-MDH and m-MDH have been observed in various organisms (31), but in the present investigation genetic control of the polymorphic MDH isozymes is demonstrated in some detail.

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