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Genetics

Duplicated cytosolic malate dehydrogenase genes in *Zea mays*

(gene duplication/gene evolution/gene–enzyme system)

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ABSTRACT **Six inbred lines of *Zea mays* expressing different soluble (cytosolic) malate dehydrogenase (sMDH) zymogram phenotypes were analyzed genetically. sMDH was found to be coded for by unlinked duplicated loci in four of these inbred lines. The remaining two lines were found not to possess these duplicated loci. Furthermore, the duplicated loci, *sMdh1* and *sMdh2*, have been found to be located on different chromosomes: *sMdh1* on chromosome 1L linked to *Amp1*, and *sMdh2* on chromosome 5S linked to *Cat1* and *Amp3*. The importance of finding sMDH encoded by duplicated loci is discussed in relation to the role of chromosomal rearrangements, the relationship between the cytoplasmic and mitochondrial enzymes, and the evolution of *Z. mays*.**

Multiple molecular forms of malate dehydrogenase (MDH) occur in the mitochondria, glyoxysomes, and cytoplasm of *Zea mays* (1, 2). Mitochondrial MDH (mMDH) participates in the mitochondrial half of the malate shuttle and is an essential enzyme of the tricarboxylic acid cycle (3). Extensive biochemical analysis of mMDH in *Z. mays* has been described (1). Genetic analysis has shown that mMDH in maize is coded for by nuclear genes (4) on unlinked, duplicated chromosome segments (5). Soluble MDH (sMDH) is involved in transporting NADH equivalents, in the form of malate, from the cytoplasm across the mitochondrial membrane. Therefore, sMDH and mMDH are intimately related by means of their individual roles in the malate shuttle. This close relationship and the fact that mMDH was found to be coded for by duplicated loci led to the question of whether or not selection might favor duplication of the sMDH loci as well. The present study found extensive genetic evidence that sMDH is coded for by unlinked duplicated loci in some inbred lines of maize; in other inbred lines of maize, the duplication does not exist. The significance of this finding is discussed in relation to mMDH duplications, gene evolution, and the evolution of *Z. mays*.

MATERIALS AND METHODS

Genetic Stocks. The inbred lines used in this study are listed first by our own laboratory designation followed by the breeder's designation in parentheses. Inbred lines A188 (Yu Bc 8) and A187 (Yu Bc 14) were obtained from D. Palaversic (Institute for Breeding and Production of Field Crops, Zagreb, Yugoslavia). Inbred lines A119 (Gr9) and A123 (Gr14524) were obtained from J. Karayiannis (Cereal Institute, Thessaloniki, Greece). Inbred line A215 (4 Co 82) was obtained from W. A. Russell (Iowa State University).

Electrophoresis and Staining Procedures. After seeds were imbibed for 24 hr, scutella were carefully removed from the endosperm and pericarp and homogenized in 0.025 M glycylglycine buffer (pH 7.4) in a mortar and pestle chilled on ice. The extracts were applied to 5 × 7 mm Whatman 3MM filter paper sections which were inserted into vertical slots cut into 12% starch gels. Horizontal starch gel electrophoresis and specific staining for MDH were conducted as described (6).

Mitochondrial Isolation. Six-day-old dark grown maize scutella (10 g) was minced (razor blade) in a petri dish with 8 ml of cold grinding medium. The grinding medium was modified from that used by Briedenbach and Beevers (7). Instead of 0.4 M sucrose in Tris buffer, 25% sucrose in 0.05 M Hepes buffer (pH 7.5) was used. The homogenate was passed through four layers of Miracloth and the filtrate was centrifuged at 1500 × *g* for 15 min. The supernatant was layered into a 25–60% continuous sucrose gradient and centrifuged at 113,000 × *g* in an SW 27 rotor for 4 hr. After centrifugation, the bottom of the tube was punctured with a dissecting needle and 0.5-ml fractions were collected.

The mitochondrial fraction was identified by using cytochrome oxidase as a marker enzyme (8). Because, in maize, both the glutamate-oxaloacetic transaminase (GOT) and superoxide dismutase (SOD) systems contain isozymes specifically localized within the mitochondria [i.e., mGOT and SOD-3 (9, 10)] these were also used as markers of mitochondrial purity.

RESULTS

A number of inbred lines from around the world were screened for sMDH. The most common sMDH phenotype among strains of *Z. mays* is phenotype A (Fig. 1 *Upper*). Organelle isolation has confirmed that isozymes sMDH1 and sMDH2 are localized in the soluble fraction of cellular extracts (1, 2). Inbred lines A215 and A187 exhibited phenotype A. Inbred line A188 exhibited two additional isozymes (sMDH3 and sMDH4) which migrated more anodally than sMDH1 and sMDH2 (phenotype B); inbred lines A123 and A119 exhibited two isozymes (sMDH5 and sMDH6) which migrated more cathodally than sMDH1 and sMDH2 (phenotypes C and D).

The isozymes (sMDH5, sMDH6) that migrated cathodally to sMDH1 and sMDH2 in lines A123 and A119 overlapped with some of the mMDH isozymes. To confirm that these two isozymes were indeed cytosolic variants, A123 extracts were subjected to sucrose gradient centrifugation to separate cytosol and mitochondria. The two isozymes were recovered in the cytosolic fraction but never in the mitochondrial fraction. Therefore, it was concluded that sMDH5 and sMDH6 are indeed cytosolic variants (Fig. 2 *Top*). In addition to cytochrome *c* oxidase (data not shown), maize SOD-3 and mGOT were used as markers to determine mitochondrial purity.

Crosses were made between different sMDH variants to determine the genetic control of sMDH. The relatively weak activity of mMDH isozymes on zymograms made it possible to score the sMDH phenotypes resulting from genetic crosses without difficulty.

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Abbreviations: MDH, malate dehydrogenase; mMDH, mitochondrial MDH; sMDH, soluble MDH; GOT, glutamate-oxaloacetic transaminase; SOD, superoxide dismutase.

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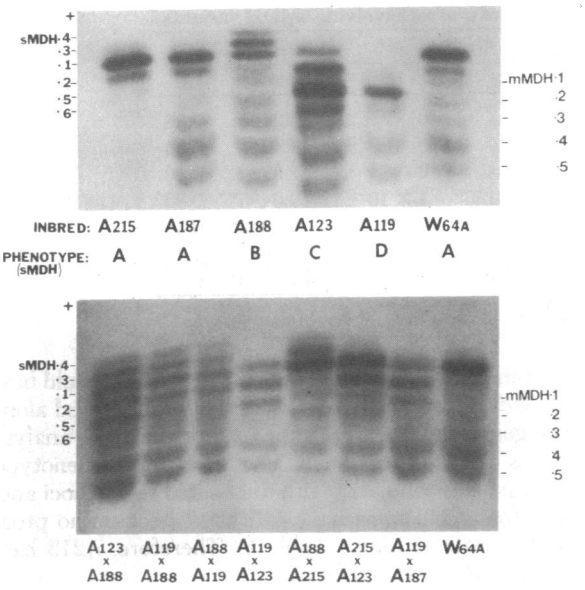

FIG. 1. Zymograms showing variant sMDH phenotypes used in analyzing the genetic control of sMDH isozymes (*Upper*) and the resulting F_1 sMDH phenotypes from the crosses (*Lower*). sMDH, cytosolic forms of MDH; mMDH, mitochondrial forms of MDH. Migration is anodal.

Both inbred lines A123 and A188 expressed sMDH1 and sMDH2 as well as two additional sMDH isozymes. A188 exhibited two sMDH isozymes anodal to sMDH1 and sMDH2 (Fig. 1 *Upper*; phenotype B). These isozymes have been labeled sMDH3 and sMDH4. A123 expressed two sMDH isozymes (sMDH5 and sMDH6) which are cathodal to sMDH1 and sMDH2 (phenotype C). F_1 progeny of the cross A123 × A188 expressed six sMDH isozymes (Fig. 1 *Lower*). The F_2 progeny expressed five distinct sMDH phenotypes (Table 1; Fig. 3 *Upper*). The phenotypes in Fig. 3 and Table 1 are based solely on the presence or absence of sMDH isozymes and not on the dosage differences expected in the F_2 progeny (Table 2).

The expression of sMDH1 and sMDH2 appeared to be coordinate. Therefore, it is possible that sMDH2, which stains less intensely on zymograms than does sMDH1, may be a modification of sMDH1. An alternative possibility is that sMDH1 and sMDH2 may be products of closely linked loci. sMDH6 is always expressed with sMDH5, but as a lighter staining isozyme. Because sMDH2 and sMDH6 do not form detectable hybrids with other sMDH isozymes, the assumption has been made that sMDH2 and sMDH6 may be products of secondary modification.

The phenotypes observed in the F_2 progeny of A123 × A188 illustrate that sMDH3 is a hybrid isozyme between sMDH1 and sMDH4, because sMDH3 is absent when sMDH1 and sMDH4 are not present simultaneously. sMDH2 is greatly enhanced in F_1 hybrids and in F_2 progeny which exhibit both sMDH1 and sMDH5, suggesting that the hybrid between sMDH1 and sMDH5 overlaps with sMDH2 (Figs. 1 and 3). Phenotype F (Fig. 3) suggests that the hybrid isozyme between sMDH5 and sMDH4 migrates close to the position of sMDH1. Because phenotype F was observed in the F_2 progeny of A123 × A188, isozyme sMDH4 cannot be allelic to sMDH5.

The appearance of five distinct phenotypes in the F_2 progeny of A123 × A188 cannot be explained by the assumption that one gene codes for sMDH. However, the hypothesis that two loci code for sMDH will explain the observed data (Table 1). Although A123 and A188 both possess sMDH1 and sMDH2, the appearance of sMDH1 and sMDH2 alone (phenotype A, Fig. 3) in the F_2 progeny is possible only if sMDH1 and sMDH2 in A123 are coded for by a different locus than are the counterparts in line A188 (Table 1). These data strongly suggest that there are two duplicated loci coding for sMDH in maize. Furthermore, the two duplicated loci must be unlinked to give the ratio observed.

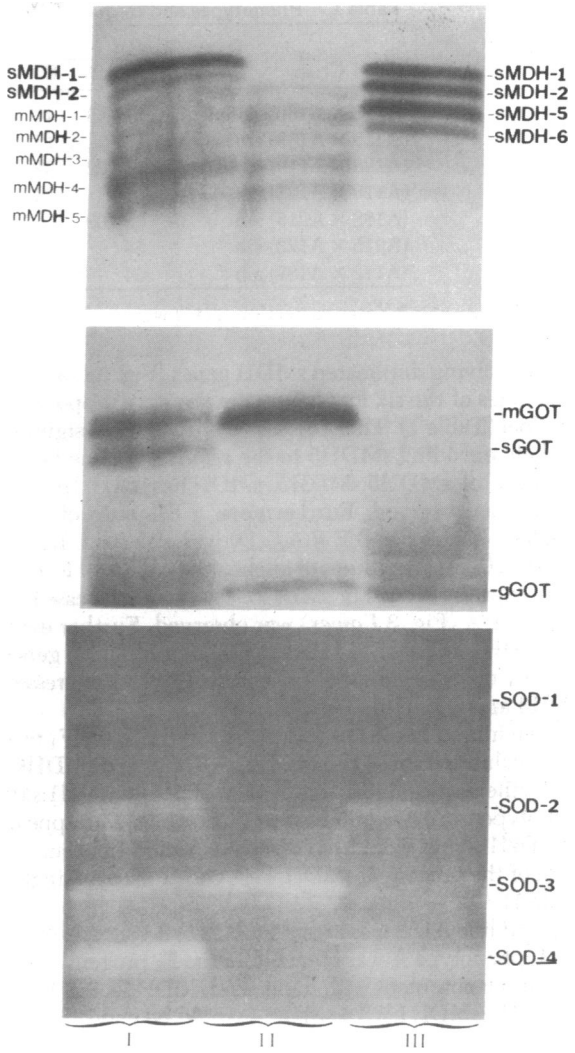

FIG. 2. Zymograms of mitochondrial and cytosolic fractions following cell fractionation. (*Top*) MDH; (*Middle*) GOT; (*Bottom*) SOD. I, crude extract of W64A as marker; II, mitochondrial fraction from line A123; III, cytosolic fraction from line A123. Migration is anodal.

The isozyme and gene notation used throughout this paper is as follows: isozyme sMDH1 is coded for by the genes *sMdh1-s1* and *sMdh2-s1*; isozyme sMDH5, gene *sMdh1-s5*; isozyme sMDH4, gene *sMdh2-s4*; isozyme sMDH8, gene *sMdh2-s8*.

Inbred line A119 expressed only sMDH5 and, therefore, would have the genotype *sMdh1-s5, sMdh2-s0* (phenotype D, Fig. 1). Inbred line A188 expressed phenotype B and had the genotype *sMdh1-s1, sMdh2-s4*. For the other inbred lines: A123, *sMdh1-s5, sMdh2-s1*; A187, *sMdh1-s1*, sMdh2-s0; A215, *sMdh1-s1, sMdh2-s1*; A205, *sMdh1-s1, sMdh2-s8* [A205 has a new variant that is anodal to sMDH-4 and is allelic to sMDH-4 (data not shown)]. Reciprocal F_1 hybrids between A119 and A188 showed phenotype E. Crossing the F_1 plants resulted in six F_2 phenotypes. This result can be explained only with a

Table 1. Phenotypes and frequencies observed for F_2 progeny of crosses involving sMDH variants

Cross	Phenotype							Single gene		Duplicated loci	
	A	B	C	D	E	F	Total	χ^2	P	χ^2	P
(A123 × A188) selfed	14	28	41		76	12	171	—	0	5.2495	0.26
(A119 × A188) sib	13	21	15	7	42	28	126	—	0	4.421*	0.54
(A188 × A119) sib	14	52	32	15	85	53	251	—	0	2.375	0.80
(A119 × A123) sib			84	26			110	41.3136*	1×10^{-9}	0.0485*	0.83
(A188 × A215) sib	32	80					112	37.2533*	8×10^{-9}	0.5833*	0.45
(A215 × A123) sib	54		140				194	68.6418*	0	0.6873	0.41
(A119 × A187) sib	64		140	72			276	0.5217	0.77	187.1178	0

* Yates correction factor was used with one degree of freedom or with sample sizes <140.

model involving duplicated sMDH genes (Fig. 3 *Lower*). The frequencies of the six F_2 phenotypes strongly supports duplicated loci (Table 1). The cross also confirms the assignment of the allele encoding sMDH5 to the *sMdh1-s5* gene because a 1:2:1 ratio of sMDH5:sMDH5, sMDH1:sMDH1 (phenotype D:C:A) was observed. Furthermore, a 3:1 ratio of sMDH4:sMDH0 (phenotypes B,E,F:A,C,D) was observed. If sMDH4 and sMDH5 were products of allelic genes, then all F_2 progeny should express sMDH4 or sMDH5. This is not the case because phenotype A (Fig. 3 *Lower*) was observed. Further evidence that sMDH4 and sMDH5 are not products of allelic genes was shown by the appearance of phenotype F, which expresses both sMDH4 and sMDH5.

When inbred line A119 was crossed to A123, the F_1 progeny always exhibited sMDH1, sMDH2, sMDH5, and sMDH6 (Fig. 1). Furthermore, a 3:1 ratio of sMDH5, sMDH1:sMDH5 (phenotype C:D) was obtained in F_2 progeny. The appearance of sMDH1 alone would have been expected to occur in one-fourth of the progeny if sMDH were coded for by a single locus (Table 1).

Inbred line A188 was crossed to A215. A 3:1 ratio of phenotype B:phenotype A was obtained in the F_2 progeny (Table 1). This ratio is consistent with duplicated sMDH loci. A 1:2:1 ratio of sMDH1:sMDH1,2,3,4:sMDH4 would be expected under a single gene model. The data indicate that A215 would have to have *sMdh-s1* because sMDH4 was never recovered alone.

The genotype of A215 can be established by F_2 analysis of the cross (A215 × A123) sib. The 3:1 ratio of phenotype C:phenotype A is consistent with duplicated sMDH loci and indicates that A215 must have *sMdh2-s1* because no progeny exhibited sMDH5 alone (Table 1). Therefore, A215 has the genotype *sMdh1-s1, sMdh2-s1*.

Inbred line A119 was crossed to A187. The F_1 progeny expressed a phenotype expected from a single gene locus (Fig. 1 *Lower*). The F_2 progeny gave a 1:2:1 segregation ratio consistent with a single sMDH locus segregating between lines A119 and A187 (Table 1). The genotype of A187 is therefore *sMdh1-s1, sMdh2-s0*, which indicates that both A119 and A187 lack detectable duplicated *sMdh* loci.

The chromosomal locations of *sMdh1* and *sMdh2* have been established. *sMdh1* has been found to be linked to an aminopeptidase gene, *Amp1*, by 7.59 ± 2.1 map units (Table 3). *Amp1* had previously been shown to be on chromosome 1L, 17 map units from the marker *an1* (anther ear) and 15 map units from another aminopeptidase gene, *Amp2* (11). *Amp2* is located close to the centromere. *sMdh2* is 4.46 ± 1.9 map units from a catalase gene, *Cat1*. *Cat1* has been located on chromosome 5S, 9.1 map units from the marker *bt* (brittle endosperm) (unpublished data).

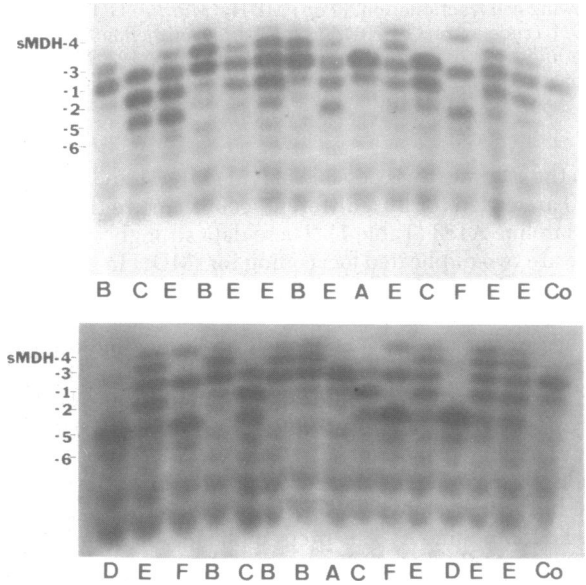

FIG. 3. Zymograms showing the five sMDH phenotypes (indicated by letters on horizontal axis) observed in F_2 progeny of A123 × A188 (*Upper*) and the six sMDH phenotypes observed in F_2 progeny of the cross A119 × A188 (*Lower*). Phenotypes are based on the presence or absence of sMDH isozymes and not on dosage intensities (see Table 2). Co, control (W64A). Migration is anodal.

DISCUSSION

The genetics of the sMDH system of *Z. mays* have been investigated and the results suggest that, during the evolution of maize, one sMDH gene (*sMdh-s1*) had been duplicated and translocated to another chromosome to give rise to an unlinked set of duplicate genes. A similar phenomenon has already been shown to have occurred for the mMDH system in maize (5). Subcellular fractionation experiments on sucrose gradients have confirmed the cytosolic location of all of the sMDH isozymes. This confirmation was necessitated by the fact that some sMDH and mMDH isozymes comigrate to the same position on zymograms. An interesting fact revealed by these experiments was that a mitochondrial enzyme with MDH activity migrates to the same electrophoretic position as sMDH1. It may be another

Table 2. sMDH phenotypes

Phenotype	sMDH isozymes expressed					
	sMDH-1	sMDH-2	sMDH-3	sMDH-4	sMDH-5	sMDH-6
A	+	+				
B	+	+	+	+		
C	+	+			+	+
D					+	+
E	+	+	+	+	+	+
F*				+	+	+

* Phenotype F has an additional isozyme which migrates near sMDH-1.

Table 3. Linkage of *sMdh1* with *Amp1* and of *sMdh2* with *Cat1*

	sMdh1 and *Amp1*	*sMdh2* and *Cat1*
Cross	A188 × (A188 × A119)	$\frac{\text{A205 × D10}}{\text{A119}}$ selfed
Genotype	$\frac{sMdh1\text{-}s1,\ Amp1F}{sMdh1\text{-}s1,\ Amp1F} \times \frac{sMdh1\text{-}s1,\ Amp1F}{sMdh1\text{-}s5,\ Amp1V}$	$\frac{sMdh2\text{-}s8,\ Cat1\text{-}F}{sMdh2\text{-}s\text{-}0,\ Cat1\text{-}V}$
Phenotypes observed*	sMDH1, AMP1F (68) sMDH1, AMP1F/V (6) sMDH1/5, AMP1F (6) sMDH1/5, AMP1F/V (78)	sMDH8, CAT1F (31) sMDH8, CAT1F/V (68) sMDH8, CAT1V (4) sMDH0, CAT1F (0) sMDH0, CAT1F/V (2) sMDH0, CAT1V (30)
Chi square test	1:1 segregation of sMDH1:sMDH5, $P = 0.47$ 1:1 segregation of AMP1F:AMP1-FV, $P = 0.47$ Independence of *sMdh1* and *Amp1*, $P < 0.001$	3:1 segregation of sMDH8:sMDH0, $P = 0.73$ 1:2:1 segregation of CAT1, $P = 0.86$ Independence of *sMdh2* and *Cat1*, $P < 0.001$
Conclusion	*sMdh1* is linked with *Amp1*	*sMdh2* is linked with *Cat1*
Maximum likelihood estimation of linkage	Between *sMdh1* and *Amp1*, 7.59 ± 2.1 map units	Between *sMdh2* and *Cat1*, 4.46 ± 1.9 map units

* Numbers in parentheses are numbers of progeny.

dehydrogenase with some MDH activity or it may be another mMDH.

It previously was assumed that sMDH2 and sMDH6 are modified forms of sMDH1 and sMDH5, respectively. This assumption is not completely unwarranted because it has been shown that the sMDH isozymes of *Ilyanassa* can be modified with the use of 2-mercaptoethanol (12).

The finding that duplicated loci code for sMDH in maize is important. Duplications have been reported in other organisms. For instance, gene duplication has been shown to be the underlying mechanism for the evolution of myoglobin, hemoglobin, and the proteolytic enzymes (13, 14). Duplications have also been reported for indophenol oxidase in *Sceloporus undulates* (15), α-glycerophosphate dehydrogenase in *Sceloporus grammicus* (16), isocitrate dehydrogenase in *Scaphiopus* (17), alcohol dehydrogenase in *Clarkia franciscana* (18), and esterase in Z. *mays* (19). However, the sMDH system of maize is unique in several ways. First, duplicated sMDH loci may not exist in all inbred lines of maize; this is shown in the F_2 progeny of the cross A119 × A187. This fact may prove to be important in future studies concerning the evolution of present day Z. *mays*. Second, the duplicated sMDH system is intimately related to the duplicated mMDH system of maize via the malate shuttle. This provides an excellent opportunity for the study of cytoplasm–organelle interactions. Could duplication of the mMDH locus have led to selection favoring duplication of the sMDH locus? Or could the reverse have occurred?

Markert *et al.* (20) proposed that the evolution of a gene involves two steps. After gene duplication, mutations accumulate which change the structure and function of the gene product. Further evolution causes the spatial and temporal regulation of the gene to change. There may be maize lines, with respect to the sMDH system, which represent each step of this proposed gene evolution scheme (20). For example, our genetic data confirm that line A215 possesses duplicated *sMdh* genes. However, their enzymatic products (i.e., MDH isozymes) comigrate on zymograms and cannot be differentiated visually. A parallel situation has been reported for the duplicated *B* genes for sMDH in salmon (21). Furthermore, whereas lines A188 and A123 represent duplicated genes in which structural changes have occurred, lines A187 and A119, which reveal only one sMDH gene, may actually contain two loci. It is possible that the second locus has diverged to a point where its product is no longer recognizable as MDH, or it could have evolved in such a way that its temporal and spatial specificity has been altered with respect to the other sMDH locus. Of course, it is possible that A187 and A119 could have originated from maize lines in which the duplication event did not occur.

The possibility exists that the duplicated sMDH loci could be a result of a B chromosome/A chromosome translocation. If this were the case, then the lines that had duplicated sMDH loci would also have B chromosomes present. Cytological analysis of root tips collected from all the lines used in this study has confirmed that the lines that have duplicated loci (A215, A123, A188, A205) do not have B chromosomes. Furthermore, when the lines with one sMDH locus were examined, A187 was found to have B chromosomes but A119 did not. Therefore, the duplication of sMDH loci is not the result of a B/A translocation.

The chromosome location for most of the duplicated loci has been determined. By the nomenclature of Yang *et al.* (5) the gene set *mMdh1, mMdh2* has been located on the very distal end of chromosome 6L (22). *sMdh1* is located on chromosome 1L and *sMdh2* is located on chromosome 5S. The other mMDH gene set has not been mapped, but the genetic data indicate that it is not linked to *mMdh1 mMdh2, sMdh1*, or *sMdh2*. *sMdh1* is located approximately 7.59 ± 2.1 map units from *Amp1* on chromosome 1L; *sMdh2* is 4.46 ± 1.9 map units from *Cat1* on chromosome 5S. This is interesting because *Cat1* is 3 map units from *Amp3* (11), suggesting that the sMDH duplication may have resulted from a duplication of a portion of chromosome 1L or chromosome 5S. Duplications involving portions of a chromosome arm are an important mechanism for increasing the number of genes or blocks of genes (23). This seems to be the case with mammals in which it has been observed that the rapid rate of anatomical evolution parallels the rate of gene rearrangements (24). The MDH system in Z. *mays* will provide an opportunity to test the effect of chromosome segment duplication in plants.

The evolution of Z. *mays* has not been conclusively established. Its closest relative is presumed to be teosinte (Z. *mexicana*) (25) with which it can readily cross to form 10 bivalents (26). Because sMDH and mMDH have been found to be duplicated and the map locations of most of the loci are known, the MDH system can be used as a diagnostic tool to determine whether the teosinte races closest to Z. *mays* have the same duplications. It is possible that the more maize-like teosinte may express duplicated MDH loci whereas the ancient teosinte races

would not. The MDH gene–enzyme system may serve as an important tool in elucidating the evolution of Z. *mays*; however, because the most common duplicated *sMdh* loci code for variants that migrate to the same position on zymograms, formal genetic analysis must be conducted. A homozygous line with phenotype F (*sMdh1-s5, sMdh2-s4*) would be an excellent diagnostic probe for genetic studies of sMDH because these lines have variants at both *sMdh1* and *sMdh2*.

The duplicated *sMdh* and *mMdh* genes in maize represent a unique system for further studies of the role gene duplication has played in plant evolution, the role of chromosome rearrangements in evolution, the relationship between the cytoplasm and mitochondria, and the evolution of maize MDH.

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