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ALLOZYME SEGREGATION RATIOS IN THE INTERSPECIFIC CROSS *CUCURBITA MAXIMA* × *C. ECUADORENSIS* SUGGEST THAT HYBRID BREAKDOWN IS NOT CAUSED BY MINOR ALTERATIONS IN CHROMOSOME STRUCTURE

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ABSTRACT

The parentals of the interspecific cross *Cucurbita maxima* × *C. ecuadorensis* had different isozyme phenotypes for 12 enzyme systems. Characterization of the systems demonstrated that the expression and intracellular distribution of the isozymes were similar to those in other plant taxa; however, a considerable number of duplicate loci were identified, indicative of a polyploid ancestry for Cucurbita. Genetic analysis provided evidence for 20 loci segregating in F_2 and backcross populations. Five linkage groups were identified, consisting of the loci *Aat-mb* - - *Mdh-m2*; *Gal-1* - - *Gal-2*; *Aat-p2* - - *Gpi-c2*; *Acp-1* - - *Pgm-c2* - - *Pgm-p*; and *Est-1* - - *Tpi-c2*. Significant deviations from Mendelian segregation ratios were observed in 14% of the data sets for individual loci. However, these instances were scattered among the loci, no single locus consistently displaying skewed ratios. Recombination frequencies between linked loci were similar to those observed in intraspecific crosses, and the ratio of heterozygous to homozygous genotypes in backcross populations was very close to one. These results suggest that small differences in chromosome structure were not the major cause of the loss of fertility observed in F_2 and backcross populations.

The interspecific hybrid of *Cucurbita maxima* × *C. ecuadorensis* is relatively fertile, but both the F_2 and backcross populations display a severe reduction in fertility (CUTLER and WHITAKER 1969). Such loss of fertility has been described in other interspecific crosses, and several investigators have attributed this hybrid breakdown (dysgenesis) to the presence of many small differences in the structure of the parental chromosomes (SAX 1933; STEBBINS 1945; STEPHENS 1949; GRANT 1966). STEPHENS (1949, 1950) presented four lines of evidence which indicated that, in cotton, the loss of fertility in F_2's and backcrosses was due to minor alterations in chromosome structure. First, certain morphological characters exhibited simple Mendelian inheritance in intraspecific crosses but showed continuous variation in interspecific F_2 and backcross populations. These traits rapidly reverted to simple segregation pat-

terns upon repeated backcrossing. Second, there was a rapid polarization to the parental types in F_3 and more advanced generations. Third, in interspecific backcross populations, segregation ratios were skewed toward the recurrent parent. Finally, compared to results for intraspecific crosses, there was a reduction of crossover values between linked loci in interspecific crosses. Cytogenetic investigations (GERSTEL 1953; GERSTEL and SARVELLA 1956) also provided evidence for small chromosomal rearrangements among the interfertile species of Gossypium.

The phylogenetic relationship between *C. maxima* and *C. ecuadorensis* is unclear. It was concluded from the numerical taxonomy studies of RHOADES *et al.* (1968) and BEMIS *et al.* (1970) that *C. ecuadorensis* is unique, not belonging to any of the other nine groupings of Cucurbita species, and is most closely allied to *C. lundelliana* and *C. okeechobeensis*. However, the fertility of the hybrid between *C. maxima* and *C. ecuadorensis* as well as the similarity of pollinators have led others to propose a closer relationship between these two species (CUTLER and WHITAKER 1969; HURD, LINSLEY and WHITAKER 1971).

WALL and WHITAKER (1971) investigated the genetic basis of hybrid breakdown in the cross *C. ecuadorensis* × *C. maxima* by examining the inheritance of loci coding for α-naphthyl esterase and leucine aminopeptidase in F_2 and backcross generations. They demonstrated that the variation in the esterase and aminopeptidase phenotypes was produced by codominant alleles at the unlinked loci, *Est-1* and *Lap-1*, respectively. Distorted transmission ratios were observed at *Est-1* in backcrosses to *C. ecuadorensis*. The authors argued that these results were compatible with STEPHENS' structural hybridity hypothesis for Gossypium and suggested that a similar mechanism was operating in Cucurbita. WALL (1961) had previously reached the same conclusion when studying the fertility relationships of F_1, F_2 and backcross generations of the cross *Cucurbita pepo* × *C. moschata*.

A considerable number of other polymorphic isozyme loci have been identified in Cucurbita since the investigation by WALL and WHITAKER (DANE 1983; IGNART and WEEDEN 1984; KIRKPATRICK, DECKER and WILSON 1985; DECKER 1985). We felt a reinvestigation of the interspecific cross using an expanded set of genetic markers would provide a more critical test of the structural hybridity hypothesis. Additional information on the inheritance and expression of specific loci should also be useful to breeders attempting to transfer genes, such as those for resistance to zucchini yellow mosaic virus (PROVVIDENTI, GONSALVES and HUMAYDAN 1984), from *C. ecuadorensis* to *C. maxima*.

MATERIALS AND METHODS

Plant material: The *C. maxima* cv. Buttercup and *C. ecuadorensis* plants were obtained from seed maintained at the Department of Horticultural Sciences, New York State Agricultural Experiment Station, Geneva, New York. Hybrid plants were generated from a single cross in which *C. maxima* was the female parent. F_2 populations (referred to as 1 and 2) were generated by self-pollination of two F_1 plants. Backcross populations 3 and 5 were produced by fertilizing two hybrid plants with "Buttercup" pollen. Back-

cross population 4 was the reciprocal cross. Populations 1 and 5 were grown in greenhouse facilities, whereas populations 2, 3 and 4 were grown in the field.

Electrophoretic analysis: Young leaf tissue was crushed in ice-cold extraction buffer, then the extracts were loaded on horizontal starch gels and subjected to electrophoresis at 5° as described previously (WEEDEN 1984a) with several minor modifications. Two extraction buffers were used to optimize allozyme resolution and enzyme stability. Buffer A consisted of 0.07 M Tris-maleate, pH 8.0, containing 10% glycerol (v/v), 10% soluble polyvinylpyrrolidone (PVP-40), 0.5% Triton X-100 and 14 mM 2-mercaptoethanol. Buffer B was 0.08 M potassium phosphate, pH 7.0, containing the same additional components as buffer A. Three gel buffer systems were used. System I was a discontinuous Tris citrate/lithium borate mixture described by SELANDER *et al.* (1971); system II was the histidine gel, pH 6.5, used by CARDY *et al.* (1980); and system III was a 0.04 M citric acid/*N*-(3-aminopropyl) morpholine buffer, pH 6.1 (CLAYTON and TRETIAK 1972). Extraction buffer A was used for samples loaded on system I gels, buffer B for system II and III gels.

Enzyme assays: Slices from the Tris/borate gel were assayed for glucosephosphate isomerase (GPI, EC 5.3.1.9), aspartate aminotransferase (AAT, EC 2.6.1.1), triosephosphate isomerase (TPI, EC 5.3.1.1) and superoxide dismutase (SOD, EC 1.15.1.1); those from the histidine gel were assayed for esterase (EST, EC 3.1.1.–), leucine aminopeptidase (LAP, EC 3.4.11.–), malate dehydrogenase (MDH, EC 1.1.1.37), phosphoglucomutase (PGM, EC 2.7.5.1) and shikimate dehydrogenase (SKDH, EC 1.1.1.25); and those from the citrate/*N*-aminopropyl morpholine gel were assayed for acid phosphatase (ACP, EC 3.1.3.2), β-galactosidase (GAL, EC 3.2.1.23) and peroxidase (PER, EC 1.11.1.7). The ACP, LAP, and AAT assays were taken from SHAW and PRASAD (1970). The GPI, MDH, PGM, PER, SKDH and TPI assays have been described previously (WEEDEN and GOTTLIEB 1980a; WEEDEN 1984a; IGNART and WEEDEN 1984). SOD bands could be observed as colorless regions in a violet background on gels stained for TPI activity. Esterase was assayed using the fluorogenic substrate 4-methylumbelliferyl acetate (BENDER, NAGEL and GUNTHER 1982). The β-galactosidase isozymes were also visualized using a fluorogenic substrate 4-methylumbelliferyl β-D-galactoside (1.5 mM) in a 0.1 M sodium citrate buffer, pH 4.5. The assay solution was poured over the surface of the gel and allowed to stand for 8 min before observing the gel under 302 nm ultraviolet light.

Subcellular localization: Certain cytosol-specific isozymes (GPI, PGM, TPI) could be identified by their appearance in pollen leachates (WEEDEN and GOTTLIEB 1980a). Other cytosolic isozymes (AAT, MDH, EST) were identified by their absence in any of the particulate fractions isolated. Chloroplast, mitochondrial and microbody pellets were obtained by differential centrifugation. The chloroplast pellet was obtained as described for pea chloroplasts (WEEDEN and GOTTLIEB 1980b), except that the initial pellet was resuspended in 20 ml of ice-cold chloroplast extraction buffer and was centrifuged a second time for 90 sec at 1000 × *g*. This pellet was suspended in 200 μl of 0.05 M Tris/HCl, pH 8.0, containing 14 mM 2-mercaptoethanol and 0.3% Triton X-100. Mitochondria were obtained by homogenizing 25 g of 1-wk-old hypocotyl tissue in 250 ml of 50 mM Tricine, pH 7.6, containing 0.35 M sorbitol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 4 mM cysteine and 0.5% (w/v) bovine serum albumin. The hypocotyls were subjected to ten 1-sec bursts in a homogenizer and were filtered through four layers of cheesecloth. The filtrate was centrifuged for 2 min at 5000 × *g*. The resulting pellet was discarded, and the supernatant was centrifuged for 5 min at 20,000 × *g*. This second pellet was resuspended in 200 μl of 50 mM Tris/HCl, pH 8.0, containing 14 mM 2-mercaptoethanol and 0.3% Triton X-100. A microbody-enriched pellet was obtained from young root tissue. Approximately 25 g of root tissue was partially disrupted by homogenization (7 × 0.5 sec bursts) in 250 ml of ice-cold mitochondrial extraction buffer. The slurry was filtered through cheesecloth, and the filtrate was centrifuged at 7000 × *g* for 5 min. The small pellet was discarded, and the supernatant

was centrifuged for 40,000 × g for 30 min. This pellet was resuspended in the same solution described above for the mitochondrial pellet.

Catalase, a marker enzyme for microbodies, and fumarase, a marker enzyme for mitochondria, were assayed on a Gilford Model 250 spectrophotometer by the method of LÜCK (1965) and RACKER (1950), respectively. Initially, we used NAD-specific glyceraldehyde dehydrogenase as a cytosolic marker. The spectrophotometric assay for this enzyme measured the change in absorbance at 340 nm when 100 μl of a mixture of 0.1 M Tris-HCl, pH 8.0, 7 mM fructose 1,6-diphosphate and 3 units/ml aldolase (preincubated for 10 min at 37°) was added to 2 ml of 0.1 M Tris-HCl, pH 8.0, containing 0.45 mM NAD, 6 mM sodium arsenate and 100 μl of sample. Blanks lacking substrate were run to allow correction for interfering reactions. For later extractions of chloroplasts and mitochondria we found it more practical to use the cytosolic isozymes of GPI as a cytosolic marker. These could be conveniently observed after electrophoresis of the sample using a Tris citrate/lithium borate buffer system.

Statistical analysis of linkage was done by the maximum likelihood method, using the computer program LINKAGE-1 (SUITER, WENDEL and CASE 1983). In order to avoid the generation of inaccurate recombination values, single-locus segregation data that gave highly significant ($P < 0.01$) distortions from the expected ratios were not used in the calculation of linkage.

RESULTS

Comparisons of the isozyme phenotypes obtained from *Cucurbita maxima* and *C. ecuadorensis* extracts revealed differences in 12 enzyme systems (Figure 1). In the hybrid the additive expression of these phenotypes was observed, including the formation of novel, heterodimeric bands in systems containing dimeric enzymes (Figure 1). Many of the systems exhibited multiple activity bands that, in several cases, overlapped each other. The analysis of such systems was often made possible only through subcellular localization of specific isozymes and observation of the segregation patterns in F_2 progeny.

The chloroplast pellet was judged to be free of cytosolic and mitochondrial contaminants by the absence of NAD-specific glyceraldehyde 3-phosphate dehydrogenase and fumarase activity. Further verification of the purity of the chloroplast pellet was obtained when, after electrophoresis, only a single GPI band was observed with a mobility of 0.60. The mobility of the chloroplast GPI has been shown to be highly conserved in all angiosperms examined (GOTTLIEB and WEEDEN 1981), and thus the isozyme serves as an excellent marker for chloroplast preparations. Both the mitochondrial and microbody preparations appeared to be free of cytosolic and chloroplast enzymes, and the mitochondrial pellet displayed very low levels of catalase activity, thereby being judged relatively free of microbody contamination. The microbody extract was contaminated with mitochondrial enzymes; however, markers specific for the microbody fraction (*e.g.*, catalase) showed increased activity in the extract from the pellet relative to a total cellular extract, whereas the mitochondrial enzymes displayed reduced activity.

Acid phosphatase: In spite of the separation of six bands of acid phosphatase activity by electrophoresis, only two genetic differences were observed between the parental lines (Figure 1). The most anodal acid phosphatase stained faintly in leaf extracts and was difficult to score in F_2 populations. In backcross populations, the homozygous phenotype gave a more intense stain than the het-

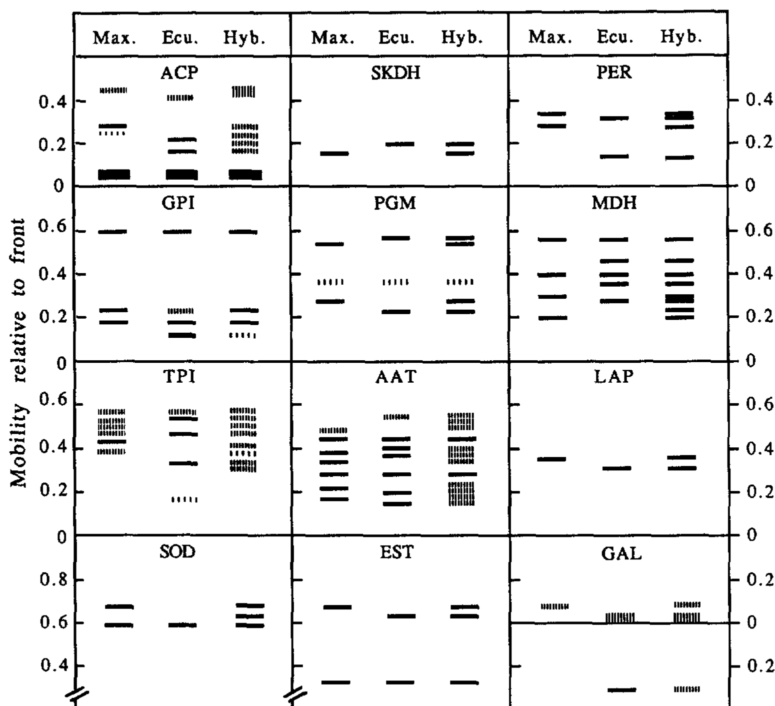

FIGURE 1.—Schematic of isozyme phenotypes for each of the 12 enzyme systems investigated. For each enzyme system, the phenotype characteristics of *C. maxima* (Max.) is shown to the left; that for *C. ecuadorensis* (Ecu.) is in the middle; and that for the F_1 hybrid (Hyb.) to the right. Position of origin is marked by 0 on the vertical axis. Anode is toward top of figure.

erozygous, and this difference in intensity was more useful for distinguishing phenotypes than was band position. Thus, segregation data are presented only for backcross populations (Table 1). The locus responsible for this isozyme was designated *Acp-1*.

The second zone of variability contained several activity bands. *C. ecuadorensis* extracts showed an intense doublet with a mobility of about 0.2, whereas *C. maxima* extracts produced a more anodal doublet. The hybrid exhibited all parental bands. None of the ACP bands were observed in organellar extracts.

Aspartate aminotransferase: Seven major bands of AAT activity were observed in each parent (Figure 1). The fainter, most anodal band cosedimented with the 40,000 × *g* pellet, indicative of a microbody localization. The activity of this isozyme was significantly greater in root extracts. The microbody AAT possessed different mobilities in the two parents, and the hybrid phenotype was a wide blur. Segregation in the F_2 or backcross populations could be easily observed, and the locus coding subunits of this isozyme was designated *Aat-mb*.

The mitochondrial AAT isozymes migrated as a set of bands immediately cathodal to the microbody AAT and were designated AAT-2 and AAT-3 (Figure 2A). A triplet of bands was observed in each parent. Genetic analysis

TABLE 1

Segregation analysis for allozyme polymorphisms

Isozyme (locus)	Population[b]	Phenotype[a] Ecu.	Hyb.	Max.	χ^{2}[c]
AAT-1	1	39	46	6	23.9**
(*Aat-mb*)	2	27	48	18	1.8
	3		17	28	2.7
	4		16	19	0.3
AAT-3	1	22	32	39	15.2**
(*Aat-m2*)	2	20	55	26	1.5
	3		11	34	11.8**
	4		47	32	2.8
	5		17	13	0.5
AAT-5	1	24	48	20	0.5
(Aat-p2)	2	23	47	34	3.3
	3		20	25	0.6
	4		39	41	0.05
	5		21	14	1.4
ACP-1	3		16	24	1.6
(*Acp-1*)	4		21	12	2.5
	5		16	21	0.7
ACP-2	1	18	21	12	3.0
(*Acp-2*)	2	27	39	20	1.9
	3		23	22	0.2
	4		9	9	0.0
	5		4	32	21.8**
EST-1	1	8	19	7	0.5
(*Est-1*)	2	24	51	22	0.3
	3		33	12	9.8**
	4		21	15	1.0
	5		17	20	0.2
GAL-1	1	33	37	14	9.8**
(*Gal-1*)	2	15	31	13	0.3
	5		11	11	0.0
GAL-2	1	58		32	5.3*
(*Gal-2*)	2	68		28	0.9
	3	19		20	0.03
	4	18		21	0.2
	5	18		19	0.03
GPI-3	1	20	55	15	5.0
(*Gpi-c2*)	2	16	25	21	3.1
	3		6	9	0.6
	4		17	22	0.6
	5		21	15	1.0
LAP	1	17	52	23	2.3
(*Lap-1*)	2	23	48	31	1.6
	3		22	18	0.6
	4		42	32	1.4
	5		18	22	0.4
MDH-2	2	69		18	0.9
(*Mdh-c2*)	3	28		17	3.9*
	4	22		18	0.5
MDH-4	2	21	50	16	2.5
(*Mdh-m2*)	3		16	29	3.8
	4		13	23	2.8
PGM-1	1	21	38	16	0.7
(*Pgm-p*)	2	21	32	23	2.0
	3		19	24	0.6
	4		21	18	0.2
	5		15	18	0.3
PGM-3	1	17	51	20	2.4
(*Pgm-c2*)	2	30	35	30	6.6*
	3		21	24	0.2
	4		20	18	0.1
PER-1	3		24	20	0.4
(*Per-1*)	4		10	12	0.2
	5		10	21	0.1
PER-3	1	63		26	0.8
(*Per-3*)	2	24		11	0.8
	3	21		24	0.2
SKDH	1	12	29	14	0.3
(*Skdh*)	2	4	20	2	7.8*
	3		15	30	5.0
	4		17	17	0.0
SOD-1	2	9	48	25	8.6*
(*Sod-1*)	3		9	8	0.03
	4		38	41	0.1
	5		25	14	3.1
TPI-2	1	6		13	0.4
(*Tpi-c2*)	2	22		69	0.03
TPI-4	2	20	56	27	1.7
(*Tpi-p2*)	3		12	13	0.04
	4		44	36	0.8
	5		13	26	4.3

[a] Ecu. = *C. ecuadorensis* phenotype; Hyb. = hybrid phenotype; Max. = *C. maxima* phenotype.
[b] Populations 1 and 2 = F_2; 3 and 5 = $F_1 \times$ *C. maxima*; 4 = *C. maxima* $\times F_1$.
[c] Expected ratios were 1:2:1 in the F_2 populations (1 and 2) and 1:1 in the backcross populations (3, 4 and 5), except for Gal-2, Mdh-c2, Per-3 and TPI-c2, for which the expected ratio in the F_2 populations was 3:1.
* $P < 0.05$; ** $P < 0.01$.

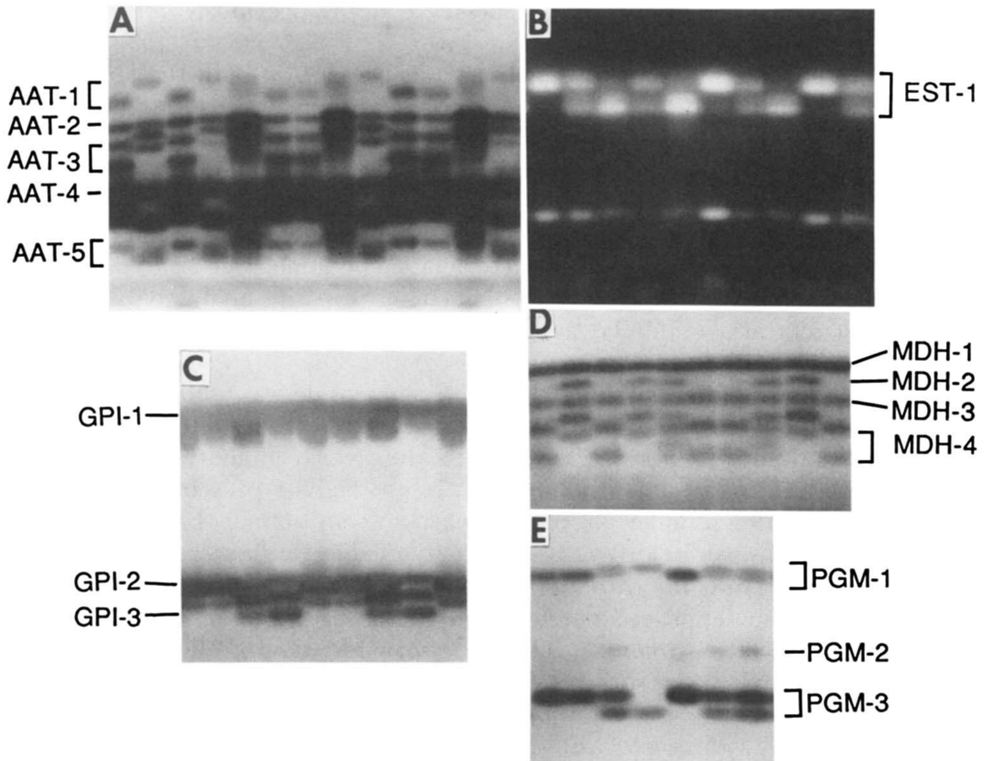

FIGURE 2.—Isozyme designations and segregation patterns observed in F_2 populations for aspartate aminotransferase (A), esterase (B), glucosephosphate isomerase (C), malate dehydrogenase (D), and phosphoglucomutase (E). Anode is toward top of figure.

indicated that these triplets were produced by dimerization of subunits specified by two loci, *Aat-m1* and *Aat-m2*. The mobility of the AAT-3 homodimers differed between the two species, as did the respective AAT-2/AAT-3 heterodimers (Figure 2A).

The slowest migrating set of bands included both cytosolic and plastid isozymes. In leaf tissue the activity of the plastid isozymes was much greater than that of the cytosolic forms; thus, by using leaf extracts we were able to obtain clearly interpretable plastid AAT phenotypes. As was the case for the mitochondrial AAT isozymes, the plastid isozymes formed triplets with the most anodal band (AAT-4) exhibiting the same mobility in both parents. The mobility differences observed between parents for the other bands was due to polymorphism at a single locus, *Aat-p2*, again paralleling the mitochondrial system. The cytosolic AAT isozymes were not investigated because of the interference by the plastid forms.

Esterase: Two zones of methylumbelliferyl esterase activity were observed in leaf extracts (Figure 2B). The mobility of the EST-1 isozyme differed between the two parents and gave a 1:2:1 phenotypic ratio in the F_2 generation (Table 1). EST-1 isozymes were also active when α-naphthyl acetate was used as the substrate, indicating that this esterase is identical to that described by

WALL and WHITAKER (1971) and is specified by the previously defined locus, *Est-1*.

β-Galactosidase: β-galactosidase activity was present on both the anodal and cathodal sections of the pH 6.1 gel. Anodally, a single wide zone of activity (GAL-1) was observed in each parent, that in *C. maxima* possessing the greater mobility. Although the diffuse nature of the bands often made scoring the phenotypes difficult, repetitive testing of each plant permitted us to determine the segregation ratios in several populations (Table 1). Segregation in the GAL-1 phenotype appeared to be controlled by a single gene, *Gal-1*.

The cathodal activity (GAL-2) was much more easily analyzed because *C. ecuadorensis* extracts exhibited a single intense band, whereas *C. maxima* extracts contained no detectable cathodal isozyme. The *C. ecuadorensis* isozyme was also active when 4-methylumbelliferyl β-D-glucoside was used as a substrate, again giving an intense band. The hybrid exhibited a less intense band, but we did not attempt to distinguish between the hybrid phenotype and that of the *C. ecuadorensis* parent, grouping both in an "activity present" class. In the F_2 populations the number of plants displaying an activity band was about threefold the number lacking GAL-2 activity (Table 1). Backcross populations segregated 1:1. Thus, the cathodal activity is apparently controlled by a single locus, *Gal-2*, which appeared not to be expressed in the *C. maxima* parent.

Glucosephosphate isomerase: The GPI isozymes could be divided into two sets. The more anodal plastid-specific isozyme gave a broad zone of activity at a position approximately midway between the origin and the front. The other set, consisting of a doublet in *C. maxima* and a triplet in *C. ecuadorensis*, was cytosolic in origin (Figure 2C). Genetic analysis demonstrated that the difference in the cytosolic GPI phenotype between the two parental lines was due to a different allele being present at one of the two loci specifying cytosolic GPI subunits. This locus, *Gpi-c2*, codes for subunits which, in *C. ecuadorensis*, dimerize to form the most slowly migrating band, GPI-3 (Figure 2C). The GPI-2 isozyme was the homodimeric combination of subunits from the invariant locus, *Gpi-c1*. Evidence that the *C. maxima Gpi-c2* locus produces a polypeptide is derived from the presence of an active GPI with the same mobility as the GPI 2/3 heterodimer observed in *C. ecuadorensis*. This GPI heterodimer stained less intensely than the GPI-2 homodimer, again contrasting with the *C. ecuadorensis* phenotype.

Leucine aminopeptidase: A single band of LAP activity was observed in leaf extracts, the position of the band being different in each parent. Segregation analysis revealed that these bands behaved as allozymic forms, and they were presumed to be coded by the same locus, *Lap-1*, identified by WALL and WHITAKER (1971). The enzyme was not associated with any of the particulate fractions examined.

Malate dehydrogenase: *C. maxima* exhibited four major zones of MDH activity. The intensely staining, anodal band (MDH-1) was cytosolic. The remaining three bands all coprecipitated with the mitochondrial pellet and were not present in the chloroplast fraction. Minor MDH bands could occasionally be seen in leaf extracts, but the inconsistency of their appearance precluded

further analysis. The MDH phenotype of *C. ecuadorensis* differed from that of *C. maxima* in two respects: a second "cytosolic" isozyme (MDH-2) was present, and the mobilities of two mitochondrion-specific bands were faster (Figure 2D).

Genetic studies demonstrated that two loci were responsible for the mitochondrial MDH phenotype (Table 1). In each species, the most anodal mitochondrial isozyme was designated MDH-3, and the homodimer with the slower mobility, MDH-4 (Figure 2D). The genes specifying the subunits were labeled *Mdh-m1* and *Mdh-m2*, respectively. MDH-2, the "cytosolic" form unique to *C. ecuadorensis*, clearly segregated as a distinct isozyme, the ratio of the number of progeny displaying this form to the number lacking the isozyme being close to 3:1 in F_2 populations and 1:1 in the backcross populations (Table 1). The isozyme was assigned to the cytosol because it was not associated with any of the particulate fractions; however, the formation of the expected intergenic hybrid dimer between the two cytosolic MDHs was not observed. It is possible that MDH-2 is the hybrid dimer, the slower homodimer in *C. ecuadorensis* being too faint to be seen. The locus responsible for the polymorphism in MDH-2 was designated *Mdh-c2*.

Phosphoglucomutase: Two major zones of PGM activity were observed (Figure 2E). PGM-1 was specific to the plastid compartment, whereas PGM-3 was cytosolic. Genetic analysis demonstrated that each zone segregated in a Mendelian fashion, indicating that two loci, *Pgm-p* and *Pgm-c2*, were involved in the determination of the phenotype. The locus coding the segregating cytosolic isozyme was given the "c-2" suffix because a second, more anodal, cytosolic form gave faint activity in leaf extracts and could be easily observed in extracts from crushed pollen grains.

Peroxidase: Although several peroxidase isozymes could be observed in leaf extracts, only two gave consistent patterns that were easily interpreted (Figure 1). The most anodal peroxidase, PER-1, displayed a simple segregation pattern in F_2 and backcross populations, the parental phenotypes being single banded and the hybrid exhibiting both parental bands. The locus responsible for this polymorphism was designated *Per-1*. The other confirmed peroxidase locus, *Per-3*, produced a strong band in *C. ecuadorensis* extracts with a mobility (R_f) of 0.15, but appeared not to be expressed in *C. maxima* (Figure 1). The ratio of phenotypes obtained in the two F_2 populations did not differ significantly from the 3:1 ratio expected, and the backcross 3 gave a 1:1 ratio (Table 1).

Shikimate dehydrogenase: A single SKDH band, occasionally with an anodal shadow band, was observed in leaf extracts (Figure 1). Segregation in the F_2 and backcross generations indicated that a single locus, *Skdh*, was responsible for the difference between parental phenotypes. The isozyme was found to be specific to the plastid compartment.

Superoxide dismutase: On gels assayed for triosephosphate isomerase a conspicuous zone of SOD activity could be seen immediately anodal to the TPI bands (Figure 1). Extracts from *C. maxima* displayed two widely spaced SOD bands. In contrast, *C. ecuadorensis* extracts obtained only the slower migrating band. The triple-banded hybrid phenotype and the segregation pattern in the F_2 and backcross populations indicated that two SOD isozymes were present.

One was monomorphic, forming the slower band of the doublet in *C. maxima*. The other isozyme overlapped the first in *C. ecuadorensis* and produced the faster SOD band in *C. maxima*. The difference in mobility of this second SOD isozyme was caused by polymorphism at the locus *Sod-1*. The intracellular location of the SOD isozymes was not determined.

Triosephosphate isomerase: Two differences in TPI phenotype were found between the two parents, one in the upper cluster of cytosolic forms and the second in the mobility of the more slowly migrating of the two plastid specific isozymes (Figure 1). The former polymorphism (TPI-2) displayed three phenotypes in F_2 populations, but the hybrid phenotype was often difficult to distinguish from that of *C. maxima*. These two phenotypes were grouped together for genetic analysis. The ratio of the segregating phenotypes was close to 3:1 in F_2 and 1:1 in backcross populations (Table 1), suggesting that a single locus was involved in producing the observed polymorphism. This locus was tentatively labeled *Tpi-c2*, pending further analysis of the other activity bands that did not segregate.

The phenotypes for the plastid-specific isozymes were more easily interpreted. Both species exhibited three-banded patterns, with the most anodal (TPI-3) band possessing the same mobility (Figure 1). The genetic analysis suggested that the difference in mobility of the two most slowly migrating bands was due to variation at a single locus, *Tpi-p2*.

Linkage relationships among loci: Pairwise comparisons of the segregation pattern at the loci investigated revealed consistent deviations from random assortment in seven sets involving 11 loci (Table 2). These loci could be placed into five linkage groups: *Aat-mb – – Mdh-m2*, *Gal-1 – – Gal-2*, *Aat-p2 – – Gpi-c2*, *Acp-1 – – Pgm-c2 – – Pgm-p*, and *Est-1 – – Tpi-c2*. The recombination frequency between linked loci varied among the different populations (Table 2). However, the linkage groups were observed in every population for which data had been collected on the appropriate loci.

Examination for inconsistent linkages and skewing in backcross data: Deviations from random assortment were observed between other locus pairs, but only in isolated populations (data not shown). *Mdh-m2* and *Acp-2* exhibited linkage in the backcross population 3 (25 ± 10% recombinants). In this same population the *Gal-1 – – Gal-2* group appeared to be linked to *Per-1* (17 ± 8%), whereas in population 1 with both galactosidase loci showed linkage (25 ± 7%) with *Skdh*. Such linkages were not observed in any of the other populations and were attributed to peculiarities in the segregation patterns of nonhomologous chromosomes.

The backcross segregation data were further tested for skewness, specifically toward the *C. maxima* genotypes. For each plant produced by a backcross, the number of loci homozygous for the allele derived from the recurrent parent was divided by the total number of scored loci. The quotient was multiplied by 100 to give an overall percentage of *C. maxima* phenotypes for each plant. The distribution of these percentages is presented in Table 3. The distribution of the 124 plants was nearly symmetric about the mean, and the mean (50.8)

TABLE 2

Joint segregation data for pairs of loci exhibiting significant deviations from random assortment

Loci	Population	N	No. observed with designated phenotype[a]									χ^{2b}	Recombinant fraction
			E/E	E/H	E/M	H/E	H/H	H/M	M/E	M/H	M/M		
Aat-mb:Mdh-m2	2	76	9	14	2	6	32	1	1	1	10	49	21 ± 4
	3	45					14	3		2	26	26	11 ± 5
Aat-p2:Gpi-c2	1	89	13	10	0	7	39	1	0	6	13	67	15 ± 3
	2	48	13	0	0	3	22	5	0	2	16	75	9 ± 3
	4	39					17	2		0	20	32	5 ± 4
	5	33					17	3		2	11	16	15 ± 6
Acp-1:Pgm-c2	3	38					13	1		3	21	23	11 ± 5
	4	32					15	2		5	10	10.2	22 ± 7
	5	23					3	2		3	15	3.8[c]	22 ± 8
Acp-1:Pgm-p	3	36					11	3		4	18	13	19 ± 7
	4	33					14	3		7	9	5.3	30 ± 8
	5	32					8	7		4	13	3.0[c]	34 ± 8
Est-1:Tpi-p2	1	17	2	2	0	0	11	0	0	1	1	15	10 ± 5
	2	95	15	7	0	4	34	13	0	11	11	48	21 ± 3
	4	36					12	0		3	21	25	8 ± 5
	5	36					17	7		2	10	9.4	25 ± 7
Gal-1:Gal-2	2	59	12	0	1	24	0	7	4	0	11	17	21 ± 6
	5	21	0	0	0	0	9	2	0	2	8	8.0	19 ± 9
Pgm-c2:Pgm-p	1	75	7	4	2	11	27	6	3	7	8	13	32 ± 5
	2	70	13	5	0	6	16	6	0	8	16	39	19 ± 4
	3	43					15	5		4	19	14	21 ± 6
	4	37					14	5		6	12	6.1	39 ± 8
	5	23					5	4		1	13	6.7	22 ± 9

[a] Phenotypes designations: E = *C. ecuadorensis* parent; M = *C. maxima* parent; H = hybrid phenotype.

[b] Expected ratios for independent assortment were 1:2:1:2:4:2:1:2:1 for the F_2 populations (1 and 2) and 1:1:1:1 for the backcross populations (3, 4 and 5), except for the *Gal-1:Gal-2* results in the F_2 populations, for which the expected ratio was 3:1:6:2:3:1.

[c] Not significant at $P \leq 0.05$.

TABLE 3

Frequency distribution of *C. maxima* phenotypes in plants from backcross populations

	Percentage of *C. maxima* allozyme phenotypes per plant										Total plants
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100	
No. of plants	3	5	6	17	29	30	18	10	4	2	124

was very close to and not significantly different from 50.0, the expected mean in the absence of skewing. The results from the populations produced using the hybrid plant as the female parent were similar to those from the population derived from the reciprocal cross, and only the combined data from all backcross populations are presented in Table 3.

DISCUSSION

Analysis of isozyme phenotypes in 12 enzyme systems in the interspecific cross *C. maxima* cv. Buttercup × *C. ecuadorensis* permitted the identification of 20 segregating loci which could be assembled into 14 independently assorting groups. In all except two cases, the interpretation of the isozyme patterns was straightforward and conformed to patterns seen in many intraspecific crosses in other plant species. Codominance of the allelic forms and interaction of subunits to produce the respective heteromeric enzymes were generally observed. The major exceptions were the cytosolic forms of MDH and TPI. In these systems the products of two loci appeared to be expressed, but hybrid dimers were not observed. Further biochemical or genetic analysis, perhaps using other interspecific or intraspecific crosses, may be required for a complete understanding of the MDH and TPI phenotypes.

The subcellular localization studies only distinguished four compartments (*e.g.*, plastid, mitochondrial, microbody and cytosol) and thus did not differentiate enzymes of vacuolar origin (which were presumably grouped with the cytosolic fraction) or those which were bound to membranes (which again were grouped in the cytosolic fraction or eliminated during the centrifugation or electrophoretic procedures). Distribution of isozymes in the two species studied corresponded closely to results in many other plant species. For instance, distinct plastid and cytosolic isozymes existed for the three enzymes (GPI, PGM and TPI) catalyzing reactions in the Embden-Meyerhof pathway. In many plant species it appears that the entire pathway is present in both of these compartments (SIMCOX and DENNIS 1978; WEEDEN 1983). Isozymes of AAT exist in four subcellular locations in Cucurbita, comparable to the results in spinach (HUANG, LIU and YOULE 1976) and pea (WEEDEN and MARX 1984). At least two sets of MDH isozymes are present in Cucurbita, one localized in the mitochondria and one in the cytosol. In watermelon cotyledons, distinct MDH isozymes were found in the cytosol, mitochondria and microbodies (glyoxysomes) (WALK, MICHAELI and HOCK 1977); however, we could not distinguish a microbody-specific form in Cucurbita using our procedures. The SKDH

isozyme was plastid-specific, in agreement with the subcellular location of similar enzymes in pea (FEIERABEND and BRASSEL 1977; WEEDEN and GOTTLIEB 1980b) and spinach (FIEDLER and SCHULTZ 1985).

Although the intracellular locations of the isozyme systems studied were generally as expected, the number of isozymes was greater than commonly encountered in diploid plants. GOTTLIEB (1982) indicated that usually only one structural gene codes for a particular enzyme localized in one subcellular compartment. In Cucurbita, however, numerous cases of apparent duplications have been observed (WEEDEN 1984b; KIRKPATRICK, DECKER and WILSON 1985). In the cross described in this paper, we identified at least six clear examples (mitochondrial AATs, plastid AATs, cytosolic GPIs, mitochondrial MDHs, cytosolic PGMs and plastid TPIs) of two loci specifying isozymes sharing the same intracellular location. According to GOTTLIEB (1982), such a multitude of "duplicate" loci would support the contention of WEILING (1959) and SINGH (1979) that Cucurbita ($2n = 40$) is an ancient polyploid that has undergone extensive diploidization.

Duplicate isozyme systems provide interesting evolutionary systems, for only one of the isozymes is presumably required to fulfill the metabolic requirements of the plant. The second locus is theoretically redundant and should, therefore, be free to diverge and develop a different function or become silenced (OHNO 1970). The *Gpi-c2* locus in *C. maxima* and *Tpi-p2* in *C. ecuadorensis* appear to have initiated this divergence process, displaying a dramatic drop in the catalytic activity of their product at normal assay conditions. The complementary locus (*e.g.*, *Gpi-c1* in *C. maxima* and *Tpi-p1* in *C. ecuadorensis*), as well as both loci in the alternate species, exhibited relatively normal levels of expression, suggesting that the biochemical changes occurred after the formation of the two species. It is not known whether these differences in activity are due to alterations in the kinetic properties of the enzymes or to changes in the level of gene expression. In either case these systems merit further study as models of gene evolution.

Divergence between the two species could be assessed both biochemically, by changes in isozyme expression, and genetically, by deviations from normal segregation patterns at the various loci. The divergence in the cytosolic GPI and plastid TPI isozymes has been discussed primarily at the intraspecific level. The loss of *Gpi-c2* expression appears to have occurred within *C. maxima*, for other accessions of this species exhibit a normal three-banded phenotype for the cytosolic isozymes (data not shown). The difference in the plastid TPI phenotype could represent interspecific variation; however, few *C. ecuadorensis* accessions have been examined. Additional examples of divergence, at least between the two accessions used in the crosses, were found in the peroxidase, β-galactosidase, malate dehydrogenase and triosephosphate isomerase isozyme systems. *C. maxima* did not express an isozyme comparable to PER-3, GAL-2, or MDH-2 isozymes in *C. ecuadorensis*, and the TPI-2 isozyme present in *C. maxima* did not have a counterpart in *C. ecuadorensis*.

Divergence between the genomes of the two species may also be indicated by the numerous cases of significant deviations from expected segregation

ratios in the F_2 and backcross populations (Table 1). More than half the loci displayed significant deviations from the expected 1:2:1, 3:1 or 1:1 ratio in at least one population. Conversely, normal segregation was displayed at every locus in at least one F_2 and/or one backcross population. A relatively high frequency of distorted segregation ratios also has been recently observed in *Citrus grandis* × *Poncirus trifoliata* progeny (TORRES *et al.* 1985), and interspecific crosses in both *Lycopersicon* (VALLEJOS, TANKSLEY and BERNATZKY 1986) and *Lens* (ZAMIR and LADIZINSKY 1984).

Considering only those cases from Table 1 in which significant deviations occurred, neither of the parental types appeared to be favored. In the F_2 populations significant deviations resulted from an excess of the *C. maxima* allele in two cases, an excess of the *C. ecuadorensis* allele in one case, a surplus of heterozygotes in one case and a dearth of heterozygotes in one case. Similarly, in the backcross populations there were two cases in which the *C. maxima* allele was favored and two in which the *C. ecuadorensis* was favored. *Est-1,* the locus WALL and WHITAKER (1971) concluded to be linked to a rearrangement, segregated normally in all populations except backcross 3 (Table 2). Normal segregation in F_2 populations was also reported by WALL and WHITAKER, and the abnormal ratios they observed were in a backcross to *C. ecuadorensis*, which we did not investigate. Yet, the results we obtained for *Est-1* were similar to those for all the other loci we screened. Consistent deviations from expected ratios were not observed at any of the loci.

Examination of backcross populations for possible skewing of the genotypes in favor of the recurrent parent did not produce results parallel to those obtained by STEPHENS (1949). Although backcrosses to only one of the parents were investigated, the skewing effect should be manifested in either backcross, and we examined over twice the number of loci reported by STEPHENS. Although the length of the linkage map in Cucurbita is unknown, if we take the approach used by BECKMAN and SOLLER (1983) and assume that Cucurbita possesses a medium to large genome relative to other vascular plants, a sample of 20 loci should cover (lie within 20 map units of) approximately one-fourth of the linkage map. With such coverage, we would expect at least one isozyme locus to be linked to a rearrangement, and thus display consistent skewing, if there were a significant number (*e.g.*, ten or more) of such rearrangements, as has been assumed in most models (STEBBINS 1945; STEPHENS 1950; GRANT 1966). Our results demonstrate that *C. maxima* homozygotes were not in excess in backcross populations. A similar finding was made by RICK (1969) in his studies on the cross *Lycopersicon esculentum* × *L. pennellii.* Indeed, his results showed a slight skewing of allele frequencies toward the nonrecurrent parent. Thus, the model used by STEPHENS (1949) to explain the Gossypium results does not appear to be generally applicable to all cases of hybrid breakdown.

Some of the linkages observed in this cross could conceivably be artifacts generated by chromosomal structural differences, such as inversions or reciprocal translocations. The demonstration that one or more of the linkage groups was not maintained in an intraspecific cross or the backcross to *C. ecuadorensis* would indicate that the linkage was due to structural rearrangements or genetic

factors other than physical linkage. The only relevant results involve the linkage group containing *Gpi-c2* and *Aat-p2*, which has also been identified in *C. palmata*, using trisomic alien addition lines (WEEDEN, GRAHAM and ROBINSON 1986), and in *C. pepo* in intraspecific crosses (WEEDEN, ROBINSON and SHAIL 1986). The percentage of recombinants observed between the two loci in *C. pepo* was 10–13%, very close to that observed in the interspecific cross and giving no indication that chromosomal repatterning between the two species has lead to a suppression of recombination in the *Aat-p2–Gpi-c2* region of the genome. We conclude that the basis for hybrid breakdown in interspecific Cucurbita hybrids is more likely to be a dispersed set of genetic factors, as originally postulated by HARLAND (1936), than to be the presence of many small structural differences in the chromosomes.

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