ENZYME ACTIVITY IN EMS-INDUCED NULL MUTATIONS OF DUPLICATED GENES ENCODING PHOSPHOGLUCOSE ISOMERASES IN CLARKIA

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ABSTRACT

The duplication of the nuclear gene encoding the cytosolic isozyme of phosphoglucose isomerase (PGI; EC 5.3.1.9) originated within Clarkia, a genus of annual plants native to California. Previous immunological studies showed that species with and without the duplication have the same levels of cytosolic PGI activity (relative to that of the plastid PGI isozyme), as well as similar levels of cytosolic PGI protein. In the present study, we characterized seven EMS-induced null activity mutations in both duplicate PGI genes. The mutations reduced PGI activity levels in direct proportion to the normal contribution of each gene. Homozygous mutants at *Pgi-3* had 64% of wild-type activity, whereas those at *Pgi-2* had only 36%. The effects of the mutations at the two loci were additive, as shown by further reductions in activity in certain progeny classes in F_2 progenies between them. The homozygous double null mutant class was not recovered and is presumably lethal. All of the mutants appear to be CRM+. The results account for the previously observed differences in *in vivo* accumulation of the duplicate isozymes in numerous Clarkia species. The results further show that PGI activity is not directly regulated by metabolic factors and suggest that the reduced PGI levels in Clarkias with the duplication probably evolved by regulatory changes in transcription or translation. The study also demonstrates a novel method to evaluate whether a particular enzyme activity is essential.

WHEN gene dosage is increased in plants by chromosomal manipulations, a proportional increase is generally observed in the level of enzyme product (FOBES 1980; BIRCHLER 1981). This is not the case for the gene duplication that encodes the cytosolic isozyme of phosphoglucose isomerase (PGI; EC 5.3.1.9) in diploid species of the wildflower genus Clarkia, native to California. PGI catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. Immunological analyses (GOTTLIEB and HIGGINS 1984a) revealed that Clarkia species with and without the PGI duplication have the same levels of cytosolic PGI activity (relative to that of the plastid PGI isozyme) and similar levels of cytosolic PGI protein. The PGI activity characteristic of Clarkia was also found in a number of diploid vegetable spe-

cies, indicating that it is generally maintained within narrow limits (GOTTLIEB and HIGGINS 1984a). The result suggested that a genic or metabolic regulatory mechanism had evolved that reduced the accumulated cytosolic PGI protein per coding gene in Clarkia species with the duplication, effectively restoring a conserved enzyme level.

The present study examined a number of null activity mutations induced by ethyl methanesulfonate (EMS) treatment in both cytosolic PGI genes of *C. xantiana* to test the hypothesis that PGI activity is regulated by metabolic factors. The hypothesis is rejected if lesions induced in either duplicated gene affect PGI levels.

Previous studies of PGI in Clarkia have documented the mode of inheritance and independent assortment of the duplicated genes (GOTTLIEB 1977; GOTTLIEB and WEEDEN 1979; WEEDEN and GOTTLIEB 1979), electrophoretic variability in natural populations of the genus (GOTTLIEB and WEEDEN 1979) and close similarities between the duplicated isozymes in kinetic and immunological properties (HIGGINS and GOTTLIEB 1984). The previous studies also described differences in the molecular weight of the subunits coded by the duplicated genes (GOTTLIEB and HIGGINS 1984b) and in the efficiency of subunit reassociation following *in vitro* dissociation of the isozymes (HIGGINS and GOTTLIEB 1984). Proposals regarding the mode of origin and phylogenetic implications of the PGI duplication were presented in GOTTLIEB and WEEDEN (1979) and GOTTLIEB and HIGGINS (1984b).

MATERIALS AND METHODS

Plants: White- and pink-flowering plants of *Clarkia xantiana* Gray were studied. These plants derive from seeds originally collected from the self-pollinating populations 7436 (white) and 7437 (pink) in the Kern River Canyon, Tulare County, California (GOTTLIEB 1977), and have been maintained by self-pollination. Electrophoretic analysis of isozyme variation coded by 40 loci in the two populations revealed that both were completely monomorphic and had the same genes at 32 loci and different ones at eight loci (GOTTLIEB 1984). White- and pink-flowering plants have identical electrophoretic mobilities for both plastid and cytosolic PGI isozymes. The pattern consists of four bands: the plastid PGI-1 (the most anodal band) and the duplicated cytosolic isozymes PGI-2, PGI-3, and their heterodimer PGI-2/3, which migrates to an intermediate position between them. The genotype at the loci coding the cytosolic isozymes in these plants is designated *Pgi-2*^{b}*2*^{b}, *Pgi-3*^{a}*3*^{a} and is referred to as the wild type. In the studies described below, the same single genotype, derived from 7436, was always used as the wild type control.

Mutagenesis and mutant establishment: Five hundred seeds of the white- and pink-flowering plants were presoaked for 12 hr in 0.1 M phosphate buffer, pH 7.0, and then for 3 hr in the same buffer containing 0.1 M EMS. The seeds were washed, dried and germinated according to normal procedures (GOTTLIEB 1977). Germination rates were within 5% of untreated controls. Seedlings were potted into 2-inch pots. Three hundred twelve white- and 324 pink-flowering plants matured, but on the average, they set fewer than ten seeds each. These seeds, all obtained by self-pollination, were sown; but only 5% of those from 7437 and 25% of those from 7436 germinated, in contrast to 95% germination of wild-type seeds.

A total of 160 families of the white-flowered mutants and 188 families from the pink-flowered mutants (five seedlings each) were eventually examined by starch gel electrophoresis (procedure described in GOTTLIEB and HIGGINS 1984a). A number of

plants mutant at PGI were identified in these families, and each of them was crossed to wild type (always as the female parent). The F_1's were then backcrossed to wild type. Heterozygous plants (identified by segregation analysis) in the backcross progenies were backcrossed again to wild type, for a total of five generations. In principle, the resulting progeny is expected to be 98.4% wild type.

The results reported in this paper were obtained from selfed progenies of BC 4 and BC 5 lines, as well as from two F_2 progenies. The first F_2 was obtained from F_1's between homozygous mutants at *Pgi-2* (line 90-5) and *Pgi-3* (line 141-6) from selfed BC 2 progenies, and the second F_2 from a similar cross starting with homozygous plants obtained from selfed BC 4 progenies (lines 9-13 and 90-5). Mutants in a number of early generations were also examined. Throughout the studies, the seedlings for the analysis were grown in controlled environment chambers providing 12-hr light at 20° and 12-hr dark at 15°.

Preparation of extracts: Young leaves (60–100 mg) were harvested from 5- to 6-week-old plants (5–7 cm in height) and then homogenized in 0.6–1.0 ml of extraction buffer at 2–4°. The buffer was 0.1 M HEPES adjusted to pH 7.5 with KOH, 1 mM EDTA, 5 mM 2-mercaptoethanol and 50 μg/ml of phenylmethylsulfonylfluoride (previously dissolved in ethanol). Extracts were centrifuged in a microcentrifuge at 8,000 × *g* for 5 min at 4°, and the supernatants were used for the analyses.

Electrophoresis and densitometry: Starch gel electrophoresis was performed and separated isozymes visualized according to the procedures described by GOTTLIEB and HIGGINS (1984a). For the densitometric studies, 0.03 ml of extract, diluted with extraction buffer to 0.075 IU/ml, was loaded onto 6- × 8-mm Beckman paper wicks. The rest of the procedure was as described in GOTTLIEB and HIGGINS (1984a).

Spectrophotometric assay: PGI activity was assessed at 20° in a Zeiss PM6 spectrophotometer by measuring the enzyme-coupled reduction of NADP at 340 nm. The assay mixture (0.5 ml) consisted of 0.1 M Tris HCl, pH 8.3, 5 mM fructose-6-phosphate, 1 mM NADP and 1 IU/ml of glucose-6-phosphate dehydrogenase (Torula yeast, type XII; Sigma). Activity was expressed as international units per gram of fresh weight, with 1 IU converting 1.0 μM fructose-6-phosphate to glucose-6-phosphate per minute under the above conditions.

Immunology: Immunological methods were used to determine the proportions of cytosolic PGI to the total PGI activity in the extract and to measure the amount of cytosolic PGI protein. The system employed was that of GOTTLIEB and HIGGINS (1984a), but the formula used to calculate the percentage of cytosolic PGI was the corrected version published in a corrigendum (1984, Genetics **108:** 522).

Heat treatment of samples: JONES (1985) showed that plastid and cytosolic PGI isozymes from Lolium (ryegrass) was differentially inactivated at temperatures above 40°, and he suggested that this property might be utilized as a simple method to assess the relative contribution of the two isozymes to the total PGI activity in the crude Clarkia homogenates. The usefulness of this system for Clarkia PGI was investigated as follows: Samples of 100 μl of enzyme extract were heated to 50° for 10 min, and the effects were assessed by densitometry. The plastid PGI was completely inactivated, but the recovery of each of the cytosolic PGI isozymes was near 100% (data not shown). Further incubation of the heated sample at 4° with or without substrate did not reactivate any portion of the plastid isozyme. Similar tests on extracts of each of the null activity mutants also showed that the heat treatments had no effect on cytosolic PGI activity, but completely eliminated the plastid PGI activity. In view of these results, the heat treatment was also included as an additional measure of the relative proportions of plastid and cytosolic isozymes in the various extracts tested. The immunological procedure and the heat treatment represented two independent approaches to assess the accumulated levels of the cytosolic PGI activity.

RESULTS

The mutants: Two classes of cytosolic PGI mutants were obtained. The first included mutants which, following electrophoresis of crude extracts, showed

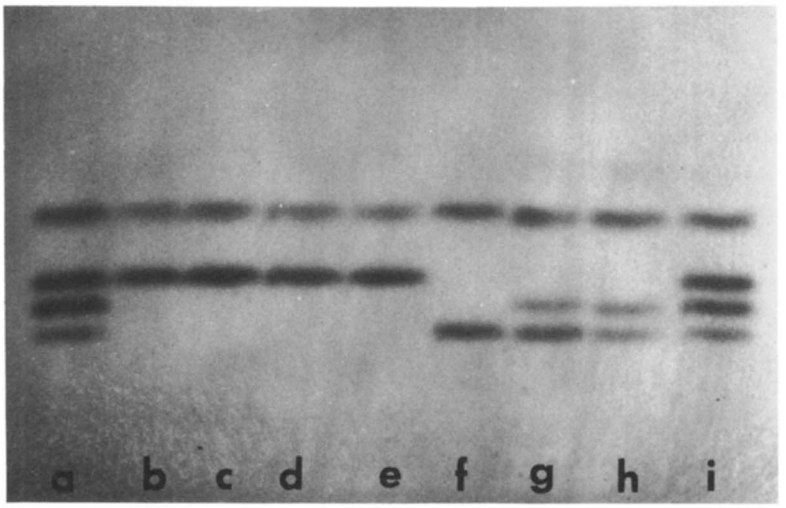

FIGURE 1.—Starch gel electrophoresis and activity staining of seven PGI mutants and wild type. a and i, Wild type. b–e, *Pgi-3* null genotypes: b, line 8-29; c, line 9-13; d, line 141-6; e, line 109-5. f, *Pgi-2* null line 90-5. g and h, "Two-banded" *Pgi-2* null genotypes: g, line 141-9; h, line 148-7. The most anodal band in each separation is the plastid PGI isozyme.

no cytosolic PGI activity in regions of the gel corresponding to PGI-2 and PGI-2/3 or PGI-3 and PGI-2/3, or to PGI-2 alone (*i.e.*, only this single band absent). The former two mutant types are referred to as "one-banded" and the latter as "two-banded." The second class included a number of mobility variants of both PGI-2 and PGI-3. (In addition to these mutations affecting cytosolic PGI, several mutants with reduced plastid PGI activity were observed. One of these survived and will be described in a separate publication.) In addition, we recovered a number of morphological variants. The high frequency of mutations and the sharp reduction in the number of seeds set on the plants grown from treated seeds, and in the germinability of these seeds (values given in MATERIALS AND METHODS), suggest that EMS was an extremely effective mutagen. The null activity mutants form the subject of this report. The other mutants were not further examined.

Seven independently obtained null mutants were carried through to the BC 5 generation (Figure 1). Four of them were obtained from white-flowering lines (population 7436); these included one mutant at *Pgi-2* (line 90-5) and two mutants at *Pgi-3* (lines 8–29 and 9–13). Each of these one-banded mutants exhibited only a single cytosolic PGI isozyme after electrophoresis; this corresponded to PGI-3 for the former mutant and to PGI-2 for the latter two mutants. A fourth two-banded mutant at *Pgi-2* derived from 7436 (line 141-9) had no activity corresponding to PGI-2, but did possess a catalytically active PGI-2/3 heterodimer with the same mobility as the wild-type heterodimer (in addition to the PGI-3).

Three mutants were obtained from population 7437. Two were one-banded mutants at *Pgi-3* (lines 109-5 and 141-6), and each exhibited only a single PGI-2 band following electrophoresis. The third was a two-banded mutant at *Pgi-*

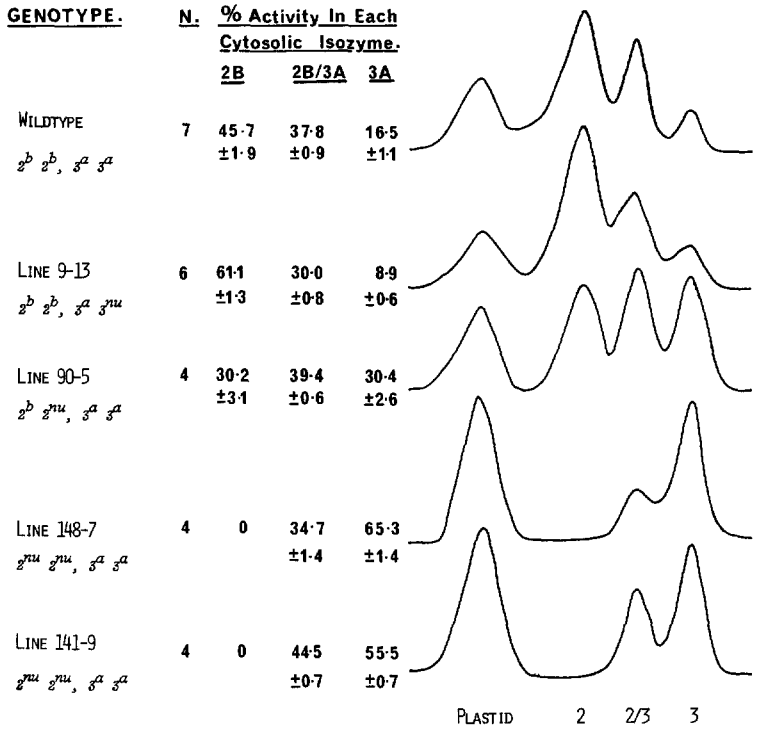

FIGURE 2.—Densitometry of PGI activity in wild-type and mutant plants. For each genotype, four or more independent electrophoretic separations and two scans of each separation were carried out.

2 (line 148-7); it lacked PGI-2, but had a PGI-3 and an active PGI-2/3 heterodimer with wild-type mobility similar to that of mutant 141-9 derived from 7436 (Figure 1).

To test the initial possibility that the mutants were not true nulls, but merely that the mutant isozymes overlapped active isozymes. they were evaluated under a number of electrophoretic conditions that varied in buffer components, molarity and pH. In addition, following electrophoresis, they were examined under several different assay conditions (temperature ranging from 15 to 35°, and pH from 7.0 to 9.0). In no case was any PGI activity detected, other than in the band coded by the alternate duplicated wild-type gene.

Seedling plants of each mutant line were also grown under cool conditions (11-hr light at 12° and 13-hr dark at 10°) for 14–20 days to test the possibility that the mutants were sensitive to temperature. Extracts from leaves formed during this time period were then subjected to electrophoresis. No additional isozyme bands were found in any of the lines.

Thus each mutation, in homozygous condition, could be reliably scored and identified by electrophoresis. The heterozygous condition for each mutant could also be distinguished from wild type by densitometry. In each case, the activities of the additional bands in the heterozygote showed a clear diminution of activity relative to the corresponding wild-type bands (Figure 2).

TABLE 1

Progeny segregations from self-pollination of plants obtained in BC 4 and BC 5 which were heterozygous for null alleles at *Pgi-2* or *Pgi-3*

Mutant line	Genotype	Generation	*N*	No. observed		χ^2
				Mutant	Wild type	
One-banded						
90-5 (white)	$2^b\ 2^{nu},\ 3^a\ 3^a$	BC 4	144	36	108	0
		BC 5	123	25	98	1.44
8-29 (white)	$2^b\ 2^b,\ 3^a\ 3^{nu}$	BC 4	45	7	38	2.14
		BC 5	24	5	19	0.23
9-13 (white)	$2^b\ 2^b,\ 3^a\ 3^{nu}$	BC 4	108	20	88	2.42
		BC 5	122	31	91	0.01
141-6 (pink)	$2^b\ 2^b,\ 3^a\ 3^{nu}$	BC 4	36	15	21	5.33
		BC 5	111	26	85	0.15
109-5 (pink)	$2^b\ 2^b,\ 3^a\ 3^{nu}$	BC 4	71	19	52	0.12
		BC 5	117	34	83	1.03
Two-banded						
141-9 (white)	$2^b\ 2^{nu},\ 3^a\ 3^a$	BC 4	36	10	26	0.15
		BC 5	36	10	26	0.15
148-7 (pink)	$2^b\ 2^{nu},\ 3^a\ 3^a$	BC 4	36	8	28	0.15
		BC 5	36	10	26	0.15

Genetics of the mutant lines: The inheritance of each of the seven mutants was examined in progenies from self-pollinated heterozygous plants obtained in the BC 4 and BC 5 generations. Crude extracts were obtained from the plants and were then subjected to electrophoresis. For each mutant line, ¼ of the progeny was expected to lack PGI activity in one or two band positions, depending on the line examined, and ¾ of the progeny were expected to display all three isozyme bands. These expectations, of course, depended on the model that the loss of PGI activity in one or the other homodimer band position was allelic to the presence of that activity. The segregation in progenies of all seven lines in both BC 4 and BC 5 was extremely close to the 3:1 ratios expected (Table 1) and confirm that each of the null mutants is allelic to wild-type activity at a single locus, either *Pgi-2* or *Pgi-3*.

Two different F_2 progenies were constructed by self-pollinating F_1 plants obtained by intercrossing homozygous *Pgi-2* null and *Pgi-3* null individuals from selfed BC 2 progenies and, again, from selfed BC 4 progenies. Since the background genomes of BC 2 plants are expected to be 87.5% wild type, 25% of the genomes of their F_1 and F_2 derivatives could possess mutations. This is the maximum estimate and is based on the assumption that a different 87.5% of the genome in each mutant parent had been made wild type by the backcrossing. The maximum mutant proportion of the background genome constructed from BC 4 plants was substantially less. Thus, the genome of BC 4 is expected to be 96.8% wild type, so that the F_1 and F_2 descendants should have no more than a 6.26% mutant background.

TABLE 2

Observed and expected numbers of plants having 2 null, 3 null, and double null phenotypes in F_2 progenies constructed by crossing homozygous *Pgi-2*^{null} × homozygous *Pgi-3*^{null} individuals obtained from BC 2 and from BC 4

Cross	*N*	Progeny phenotype			
		2 null	3 null	Double null	All others
141-6 × 90-5	599				
(BC 2 parents)	(ob)	30	104	0	465
	(ex)	112.3	112.3	37.4	337
9-13 × 90-5	327				
(BC 4 parents)	(ob)	41	50	0	236
	(ex)	61.3	61.3	20.4	184

ob = observed numbers; ex = expected numbers.

Pgi-2 and *Pgi-3* were already known to assort independently (GOTTLIEB 1977), and a number of different allelic combinations at the two loci in simple Mendelian proportions could be expected in their F_2. Thus, it became possible to determine whether the homozygous null genotype at both loci was viable; such a plant would have no cytosolic PGI activity. The possibility of interactions between the loci and/or their products could also be assessed in the same segregating genetic background.

Following electrophoresis of the F_2 progenies, three different isozyme patterns were scored: a single band of PGI-2, a single band of PGI-3 and the three-banded wild-type pattern (Table 2). The former two patterns could each result from two genotypes and the latter from four genotypes. Since densitometric analyses would have been necessary to distinguish the several genotypes responsible for each pattern, and since we wished to score very large numbers of individual plants, it was decided to score only the three patterns and not to distinguish individual genotypes within them.

A plant with a single fast PGI-2 band could be either *Pgi-2*^{b} *2*^{b}, *Pgi-3*^{null} *3*^{null} or *Pgi-2*^{b} *2*^{null}, *Pgi-3*^{null} *3*^{null}. The former genotype was expected to constitute 1/16 of the entire F_2, and the latter genotype 2/16. Similarly, a total of 3/16 of the F_2 was expected to show a single slow PGI-3 band, 9/16 would have three bands and 1/16 would have no cytosolic PGI bands (the double homozygous null genotype).

Results from the two F_2 progenies were fully concordant and revealed very large deviations from expected in several of the progeny classes (Table 2). The double null was never recovered, suggesting that it was lethal. A severe deficit characterized the *Pgi-2* null category (a single PGI-3 band). We show below that *Pgi-3* specifies sharply less PGI activity than does *Pgi-2*; presumably this difference reduces the viability of *Pgi-2* nulls and contributes to their poor recovery in these progenies. In contrast, the observed number of plants in the *Pgi-3* null category (a single PGI-2 band) was close to expected.

PGI activity levels in the one-banded mutants: Table 3 presents the level

TABLE 3

PGI activities (international units per gram of fresh weight) in crude extracts of wild-type and mutant plants determined by immunological procedures and by heat treatment (see text)

Line	Genotype	*N*	Total activity (IU/g fresh weight)	Activity in plastid isozyme	Activity in each cytosolic isozyme			Contribution of subunit	
					2B	2B/3A	3A	2B	3A
Part A									
Wild type	$2^b\ 2^b,\ 3^a\ 3^a$	7	8.2 ± 0.4	2.3 (2.3)[a]	2.7 ± 0.23	2.2 ± 0.12	1.0 ± 0.07	3.8	2.1
9-13 (BC 4)	$2^b\ 2^b,\ 3^a\ 3^{nu}$	6	7.2 ± 0.4	2.1 (2.1)[a]	3.1 ± 0.20	1.6 ± 0.15	0.4 ± 0.05	3.9	1.2
	$2^b\ 2^b,\ 3^{nu}\ 3^{nu}$	4	6.0 ± 0.2	2.2 (2.2)[a]	3.8 ± 0.11	0	0	3.8	0
90-5 (BC 4)	$2^b\ 2^{nu},\ 3^a\ 3^a$	4	6.4 ± 0.1	2.1 (2.1)[a]	1.3 ± 0.10	1.7 ± 0.13	1.3 ± 0.14	2.15	2.15
	$2^{nu}\ 2^{nu},\ 3^a\ 3^a$	5	4.6 ± 0.2	2.5 (2.3)[a]	0	0	2.1 ± 0.12	0	2.1
Part B									
Wild type	$2^b\ 2^b,\ 3^a\ 3^a$	4	8.5 ± 0.8	2.4 ± 0.2	2.8 ± 0.3	2.3 ± 0.3	1.0 ± 0.1	3.95	2.15
148-7 (BC 5)	$2^{nu}\ 2^{nu},\ 3^a\ 3^a$	4	3.3 ± 0.3	2.1 ± 0.2	0	0.4 ± 0.02	0.8 ± 0.08	0.20	1.00
141-9 (BC 5)	$2^{nu}\ 2^{nu},\ 3^a\ 3^a$	4	3.2 ± 0.2	1.9 ± 0.1	0	0.6 ± 0.04	0.7 ± 0.04	0.30	1.00

The activities of the individual cytosolic isozymes were determined by densitometry. Since lines 148-7 and 141-9 were studied at a different time from the other two lines, activities were determined a second time in newly grown wild-type plants (part B).

[a] Numbers in parentheses indicate value from heat treatment.

of PGI activity of each cytosolic isozyme in wild-type plants and in homozygous and heterozygous mutants at *Pgi-2* and *Pgi-3*. The results for wild type show that the plastid PGI isozyme accounted for 28% of the total extractable PGI activity in seedling leaves and that, among the cytosolic PGIs, the 2B homodimer contributed the most activity, the heterodimer somewhat less and the 3A homodimer the least (only 12% of total PGI activity and 17% of the cytosolic PGI activity). Previous analyses of the relative activities of the three cytosolic PGI isozymes gave very similar results (HIGGINS and GOTTLIEB 1984).

The presence of a mutant null allele at either *Pgi-2* or *Pgi-3* always reduced the cytosolic PGI activity levels. Comparison of the activity levels in heterozygous *vs.* homozygous *Pgi-3* mutants showed that presence of one mutant allele reduced the PGI-3 level slightly less than one-half (43%), and when both alleles were mutant, no PGI-3 or PGI-2/3 activity could be detected.

When *Pgi-3* was omozygous mutant, PGI-2 levels increased (Table 3). Presumably, this reflected an increased number of dimerizations between 2B subunits that became possible either because 2B did not associate with 3A (evidence described below suggests 3A was present) or because such hetrodimers, if formed, were unstable, so that all 2B subunits eventually formed homodimer associations

The con bution of the 2B subunit to the total cytosolic PGI activity did not change n the *Pgi-3* mutant relative to its contribution in wild-type and regardless of allelic state at *Pgi-3*. This observation is critical because it argues persuasively against the possibility that PGI level is affected by metabolic factors.

Similar results were recorded for mutants at *Pgi-2* (Table 3). The presence of one null allele reduced PGI-2 activity by nearly one-half, and when both alleles were null there were no PGI-2 or PGI-2/3 activities. The contribution of 3A subunits to the total cytosolic activity did not change when *Pgi-2* was either homozygous or heterozygous. Also, similar to the *Pgi-3* mutant just described, 3A-3A dimerization increased the level of PGI-3A compared to its level in wild type.

The mutations at *Pgi-2* led to greater reductions in cytosolic PGI activity than did the mutations at *Pgi-3*. Homozygosity for the null at *Pgi-2* resulted in a 64% loss (3.8 of 5.9), whereas homozygosity for the null at *Pgi-3* gave a 36% deficit (2.1 of 5.9) (Table 3). That the mutations affected only the cytosolic PGI levels was demonstrated by the lack of change in the plastid PGI levels in the mutants and wild type (Table 3). The increased level of the normal homodimer in each mutant is also consistent with the mutant effect acting only at the structural locus.

The same reduction in PGI level was obtained for *Pgi-3* null mutants grown from the BC 1 and BC 2 generations (data not shown); stocks of early generations of $Pgi\text{-}2^{null}$ were not available and therefore could not be tested.

The effects of the mutations were also examined in extracts from root tissue. Cytosolic PGI constituted a higher proportion of total PGI activity in the root extracts than in leaves of the same plants (79% *vs.* 73%), but homozygosity

TABLE 4

Proportion of total PGI activity in cytosolic isozymes in leaves and roots of wild-type and mutant plants

Line	Genotype	*N*	Proportion of total PGI activity in cytosolic isozymes	
			Leaves	Roots
Wild type	$2^b\ 2^b,\ 3^a\ 3^a$	2	73.4 ± 1.2	78.9 ± 0.9
9-13 (BC 5)	$2^b\ 2^b,\ 3^{nu}\ 3^{nu}$	2	69.7 ± 2.4	72.8 ± 1.5
109-5 (BC 5)	$2^b\ 2^b,\ 3^{nu}\ 3^{nu}$	2	64.4 ± 2.2	71.8 ± 1.4
90-5 (BC 5)	$2^{nu}\ 2^{nu},\ 3^a\ 3^a$	2	43.4 ± 2.3	47.6 ± 2.5

for null alleles at either locus caused comparable reductions in cytosolic PGI levels in the two organs (Table 4).

PGI activity in the two-banded mutants: The cytosolic PGI activity levels were also examined in the two-banded mutants (lines 148-7 and 141-9) that lacked only PGI-2 homodimer (Table 3, part B). Since these analyses were carried out at a different time from the previous ones, extracts from newly grown wild-type plants were made; the distribution of activities between the plastid and cytosolic isozymes was closely similar to that previously obtained. PGI levels were the same in both two-banded *Pgi-2* mutants (Table 3, part B); however, in contrast to the one-banded homozygous *Pgi-2* mutant (line 90-5), the PGI-3 level in them was less than that in wild type. Presumably this was because many 3A subunits formed heterodimers with 2B subunits and were not available for 3A-3A associations. Since less activity was present in the heterodimer band than in the 3A homodimer band (Table 3, part B), it is likely that the mutant heterodimer had reduced specific activity relative to wild-type heterodimer (*i.e.*, only the PGI-3 active site was functional), or fewer 2B subunits were available or competent to form heterodimers. The matter was not studied further.

The mutant heterodimer proved to be unstable at elevated temperatures that did not affect wild-type heterodimer. Thus, raising the temperature of extracts of two-banded mutants to 50° completely inactivated the heterodimer (Figure 3), suggesting that the mutant subunit formed less stable associations with 3A than those formed by normal 2B subunits.

PGI protein levels in the one-banded mutants: The cytosolic PGI protein levels were evaluated by quantitative immunological titration procedures, as previously described (GOTTLIEB and HIGGINS 1984a), in extracts from homozygous one-banded *Pgi-2* and *Pgi-3* mutants. The antibody dilution required for 50% inhibition of cytosolic PGI in the wild type was 1.38×10^{-3}, nearly the same value as that previously reported (GOTTLIEB and HIGGINS 1984a). The comparable value for the *Pgi-2* null was 1.16×10^{-3} and for the *Pgi-3* null was 1.17×10^{-3}. The standard errors ranged between 2 and 5% of these mean values. The results suggested that the mutants have about 85% of the cytosolic PGI protein present in the wild type, although they exhibit only 36 and 64%, respectively, of the PGI activity (Table 3). The implication is that

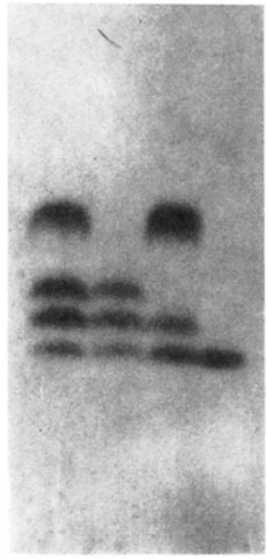

FIGURE 3.—Demonstration of temperature sensitivity of PGI-2/3 heterodimer in *Pgi-2* null line 148-7. The two columns on the left show activity stain after electrophoresis of wild type before and after heat treatment (which denatures the anodal plastid PGI isozyme). The two columns on the right show the mutant before and after heat treatment and reveals that the PGI-2/3 heterodimer is inactivated.

the mutants are CRM+, although they do not have active dimers. Direct tests to determine if the mutants synthesize PGI polypeptides were not carried out. The two-banded *Pgi-2* mutants certainly synthesize subunits, because they accumulate the PGI-2/3 heterodimer.

PGI activity in segregating F_2 progenies: The results for the analyses of the single mutants were fully upheld by the findings in the F_2 progeny between them (from BC 2 plants). Four progeny classes were studied: *Pgi-*2^b2^b, *Pgi-*$3^{null}3^{null}$; *Pgi-*2^b2^{null}, *Pgi-*$3^{null}3^{null}$; *Pgi-*$2^{null}2^{null}$, *Pgi-*3^a3^a; and *Pgi-*$2^{null}2^{null}$, *Pgi-*3^a3^{null}. Extracts from each of these types exhibit a single band of cytosolic PGI after electrophoresis, a fast band for the former two classes and a slow one for the latter two classes.

The *Pgi-3* homozygous null plants with two active *Pgi-2* alleles showed a similar PGI level as the same genotype from BC 4 previously described: 67.7% of wild-type cytosolic PGI (Table 5) *vs.* 64% for the latter genotype (Table 3). The *Pgi-3* homozygous null with only a single active *Pgi-2* allele had 28.8% of wild-type activity (Table 5).

Much larger reductions in PGI activity were found in the F_2 classes in which *Pgi-2* was homozygous null. With two active *Pgi-3* alleles, the cytosolic PGI activity was reduced to 35.6% of wild type (also comparable to the same genotype in BC 4), and with a single active *Pgi-3* allele, it was reduced to only 13.6% of the wild-type activity (Table 5). A similar reduction was also observed for the *Pgi-*$2^{null}2^{null}$, 3^a3^{null} genotype in the F_2 constructed from BC 4 plants. Thus, the F_2 data were fully concordant with the results of the analyses of

TABLE 5

PGI activities (international units per gram of fresh weight) in certain mutant genotypes from F_2 progeny (constructed from BC 2 parents; see text)

Genotype	N	Total activity (IU/g fresh weight)	Activity in plastid isozyme	Activity in cytosolic isozyme	Wild-type cytosolic activity (%)
$2^b\ 2^b,\ 3^a\ 3^a$	3	8.3 ± 0.2	2.4	5.9	100.
$2^b\ 2^b,\ 3^{nu}\ 3^{nu}$	5	6.9 ± 1.1	2.9	4.0	67.8
$2^b\ 2^{nu},\ 3^{nu}\ 3^{nu}$	3	4.9 ± 0.3	3.2	1.7	28.8
$2^{nu}\ 2^{nu},\ 3^a\ 3^a$	5	5.0 ± 0.9	2.9	2.1	35.6
$2^{nu}\ 2^{nu},\ 3^a\ 3^{nu}$	3	3.5 ± 0.2	2.7	0.8	13.6

single mutants obtained from the backcrosses and clearly demonstrate the independence of the duplicated *Pgi* loci.

DISCUSSION

Each of the seven EMS-induced mutants at the duplicated *Pgi-2* and *Pgi-3*, in homozygous state, completely lacked the homodimer activity normally specified by the affected locus, and five of the mutants also failed to accumulate active PGI-2/3 heterodimers. The mutations reduced PGI activity levels in direct proportion to the normal contribution of each gene. The homozygous mutants at *Pgi-3* reduced cytosolic PGI activity to 64% of wild type, and the mutant at *Pgi-2* reduced it to 36%. The effects of mutations at the two loci were additive. Thus, $Pgi\text{-}2^{null}2^{null}$, $Pgi\text{-}3^a3^{null}$ plants synthesized in several F_2 progenies from experimental hybrids between them exhibited only 14% of wild-type cytosolic PGI activity. Since the PGI-2 and PGI-3 isozymes have closely similar specific activities and catalytic properties (HIGGINS and GOTTLIEB 1984), the analysis demonstrates that wild-type plants normally accumulate substantially more PGI-2 than PGI-3 protein. This is now the best explanation for the previously observed difference (HIGGINS and GOTTLIEB 1984) in *in vivo* accumulation of the duplicate isozymes in numerous Clarkia species.

The present results demonstrate that PGI activity is not regulated by metabolic factors. Thus, the near identity of PGI levels in Clarkias with and without the PGI duplication (GOTTLIEB and HIGGINS 1984a) probably evolved by changes that regulate transcription or translation in the former species. Since *Pgi-3* contributes less than *Pgi-2* does to the total cytosolic PGI activity, the regulatory factors must operate to a greater extent on the former locus. The mechanism of this regulation remains to be determined. In this regard, an interesting experiment can be carried out in which the $Pgi\text{-}2^{null}2^{null},\ 3^a3^a$ genotype is subjected to mutagenesis to introduce new genetic variability and then is screened for regulatory mutations that increase the level of PGI-3 protein. If such mutations can be recovered, they might reveal how the output of *Pgi-3* was decreased during the evolution of the PGI duplication.

At the present time no information is available regarding the nature of the lesions in the one-banded mutants. It is likely that they synthesize PGI protein, because the immunotitration experiment revealed that they required 85% as

much antibody as did the wild type to inhibit their cytosolic PGI, even though their level of activity was substantially less than that of wild type. Since the increased activity of the normal homodimer in the mutants suggests that mutant subunits cannot form stable associations with normal subunits, it is likely that they also do not form stable dimers with each other and, thereby, are subjected to more rapid degradation.

The two-banded *Pgi-2* mutants are able to synthesize polypeptides, and these form heterodimers with PGI-3 subunits. Since the electrophoretic mobility of these heterodimers is the same as that of wild-type heterodimers, the mutant polypeptides are probably full length. However, since the heterodimers are heat-labile, the mutation may have affected intersubunit binding sites. Of interest is the fact that two mutations with such similar effects were recovered. The exact nature of the mutant lesions can be determined when the coding genes are cloned and sequenced.

The mutations confirm the previous genetic analyses (GOTTLIEB 1977; GOTTLIEB and WEEDEN 1979) that the cytosolic PGI isozymes in *C. xantiana* are coded by two structural genes, the products of which interact. Thus, a single mutation at *Pgi-2* removed the PGI-2 homodimer and the PGI-2/3 heterodimer, but had no effect on the PGI-3 isozyme. Likewise, a single mutation at the *Pgi-3* gene removed the PGI-3 homodimer and the heterodimer, but did not affect PGI-2.

The complete absence of the homozygous double mutant in the F_2 progenies grown from hybrids between the single mutants demonstrates that cytosolic PGI carries out an essential reaction that cannot be bypassed or compensated by other pathways. The PGI reaction in the cytosol is one of the steps preceding the synthesis of sucrose, the major transport carbohydrate in plants. The reaction is also required during the glycolytic degradation of sugar moieties. Since the $Pgi\text{-}2^{null}2^{null}$, $Pgi\text{-}3^{a}3^{null}$ genotype in the F_2 was viable, although it accumulated only 14% of the cytosolic PGI activity characteristic of wild type, physiological studies can now be undertaken to assess whether such a sharply reduced level of enzyme affects metabolite levels (sucrose and associated metabolites). The results will make it possible to evaluate the significance of the wild-type PGI level in these plants.

Our demonstration that cytosolic PGI activity is essential could not have been carried out in species which have only a single locus encoding the enzyme, the normal case for many isozymes in diploid plants (GOTTLIEB 1982). Failure to recover homozygous null individuals following mutagenesis of plants having only a single gene does not constitute proof that its loss was lethal; this is because null mutations at a gene of interest would necessarily be confounded with lethal mutations elsewhere in the genome. In contrast, the present results show that mutations can be independently induced in duplicate loci, removed from the mutant background by backcrossing to wild type and then intercrossed to determine if plants homozygous null at both duplicate loci are viable. Since duplicate genes coding enzymes are now being frequently discovered in diploid flowering plants (PICHERSKY and GOTTLIEB 1984; TANKSLEY 1984;

TANKSLEY and KUEHN 1985), our method may be applicable to a large number of genes.

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