PHOSPHOGLUCOSE ISOMERASE EXPRESSION IN SPECIES OF CLARKIA WITH AND WITHOUT A DUPLICATION OF THE CODING GENE

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Manuscript received October 22, 1983
Revised copy accepted January 21, 1984

ABSTRACT

The duplication of the nuclear gene specifying the cytosolic isozyme of phosphoglucose isomerase (PGI; EC 5.3.1.9) arose within Clarkia, a genus of annual plants native to California, and now characterizes about half of the diploid species of this genus. Evidence obtained by immunological inhibition and titration of crude leaf extracts demonstrated that species with and without the duplication have the same levels of cytosolic to total PGI (the sum of the cytosolic and plastid PGI activities). The immunological studies were carried out with a specific anticytosolic PGI antiserum and were fully supported by a densitometric analysis of the electrophoretically separated isozymes. Densitometric examination of electrophoretically separated PGIs in 11 vegetable species revealed only two levels of cytosolic to total PGI activities, one of which was the same as in Clarkia. This suggests that only certain levels of the cytosolic isozyme are compatible with proper operation of the cytosolic PGI reaction and make it likely that some form of genic or metabolic regulation has evolved that compensates for the PGI duplication.

THE duplication of the nuclear gene specifying the cytosolic isozyme of phosphoglucose isomerase (PGI) (D-glucose-6-phosphate ketol-isomerase; EC 5.3.1.9) originated within Clarkia, a genus of annual plants native to California, and is now present in 12 of the diploid species of this genus but not in related genera (GOTTLIEB 1977; GOTTLIEB and WEEDEN 1979). Duplication of the PGI gene provides an unusual opportunity to examine early stages in gene evolution, since its effects can be assessed by comparison to closely related species that do not possess it. Diploid plants generally have a single gene for cytosolic PGI, but Clarkia species with the PGI duplication have two loci coding cytosolic PGI subunits. A different nuclear gene codes the plastid isozyme of PGI (WEEDEN and GOTTLIEB 1980). The duplicated gene products in homozygous individuals associate *in vivo* in all possible combinations to yield PGI-2 and PGI-3 homodimers and the intergenic PGI-2/3 heterodimer.

Previous studies have documented the mode of inheritance and independent assortment of the duplicated genes (GOTTLIEB 1977; GOTTLIEB and WEEDEN 1979; WEEDEN and GOTTLIEB 1979) and the electrophoretic variability of the isozymes in species with and without the duplication (GOTTLIEB and WEEDEN 1979). The duplicated isozymes in *Clarkia xantiana* have similar catalytic prop-

erties (GOTTLIEB and GREVE 1981; R. C. HIGGINS and L. D. GOTTLIEB, unpublished results), but can be distinguished immunologically by a modified enzyme-linked immunosorbent assay (ELISA). They also differ in the efficiency of reassociation into functional dimeric enzymes following *in vitro* dissociation (R. C. HIGGINS and L. D. GOTTLIEB, unpublished results).

In this paper we show by immunological procedures that Clarkia species with the duplication have the same levels of cytosolic PGI activity and protein as species without the duplication, suggesting that regulatory changes that restore the ancestral nonduplicate state have evolved in the former species. We also document that the observed PGI level in Clarkia is closely similar to that of numerous vegetable species suggesting that it is metabolically important.

MATERIALS AND METHODS

Species examined: Ten diploid species of Clarkia, representing the seven taxonomic sections of the genus that contain diploid species, were examined. The species with one gene specifying cytosolic PGI were *C. amoena*, *C. mildrediae*, *C. rostrata* and *C. williamsonii*. The species with duplicated loci coding the enzyme included *C. biloba*, *C. bottae* (formerly *C. deflexa*), *C. concinna*, *C. dudleyana*, *C. lewisii* (formerly *C. bottae*) and *C. xantiana*. Seeds of each of the species were germinated in cups of moistened vermiculite. The seedlings were grown in growth chambers under 12 hr of light at 20° and 12 hr of darkness at 15° until they were about 6 wk old (6–8 cm high), at which time they were harvested.

In addition to the Clarkia species, we also examined PGI activities in seedlings of 11 diploid vegetable species: *Brassica oleracea* cv. Copenhagen market (cabbage); *Capsicum annuum* cv. Yolo wonder (bell pepper); *Citrullus vulgaris* cv. Jubilee (watermelon); *Cucumis sativus* cv. Long green black spine (cucumber); *Lactuca sativa* cv. Great Lakes 118 (lettuce); *Lycopersicon esculentum* cv. Ace (tomato); *Pastinaca sativa* cv. Improved hollow crown (parsnip); *Pisum sativum* cv. Little marvel (pea); *Raphanus sativus* cv. Early scarlet globe (radish); *Spinacia oleracea* cv. Bloomsdale or savoy leaved (spinach); *Zea mays* cv. Jubilee hybrid (maize).

Preparation of extracts for analysis of PGI: Extracts were prepared by grinding 80–100 mg of leaves in a chilled mortar with pestle in 0.8–1.0 ml of cold extraction buffer containing 32–40 mg of polyvinylpolypyrrolidone. The buffer was 0.1 M HEPES, adjusted to pH 7.5 with KOH, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.45% (v/v) Triton X-100 and 50 μl/ml of phenylmethylsulfonylfluoride. The extracts were centrifuged in a microcentrifuge at 8000 × *g* for 5 min at 4°, and the supernatants were removed for analysis. The samples were maintained on ice during all subsequent treatments.

Spectrophotometric assay: PGI activity was determined by measurement at 340 nm in the enzyme-coupled assay containing 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).

Starch gel electrophoresis: Horizontal starch gel electrophoresis was used to separate the PGI isozymes for densitometry and to demonstrate the specificity of the anticytosolic PGI antiserum. Gel preparation, PGI assay and the electrophoretic procedure were similar to those described by GOTTLIEB (1977) with the following changes: (1) The gel buffer pH was lowered from pH 8.3 to between pH 7.5 and 7.8 by the addition of citric acid, which increased the electrophoretic separation between adjacent isozyme bands. The ionic strength of the buffer was held constant by adding water to the buffer to compensate for the added citric acid. (2) In the gel assay, MTT was increased to 16 mg/100 ml and was dissolved in a minimum volume of dimethylformamide before being added to the assay; fructose-6-phosphate was increased to 50 mg/100 ml, and NADP was increased to 10 mg/100 ml; magnesium sulfate was omitted, since its inclusion appeared not to affect the assay.

Densitometry: Densitometry was carried out on electrophoretically separated PGI isozymes with a Helena Laboratories QuickScan equipped with a 595-nm transmission filter. For electrophoresis, 0.02 ml of extract, diluted with extraction buffer to 0.05 IU/ml, was loaded onto 4- × 8-mm

Beckman paper wicks by pipette. The wicks were placed in a vertical slot cut across the gel 4 cm from its cathodal end and left there for the initial 50 min of the electrophoresis to ensure that all PGI entered the gel (initial electrical conditions were 35 mA and 250 V). Electrophoresis was continued for approximately 6 hr or until the bromphenol blue front had migrated 10 cm from the position of wick insertion. The gels were agitated gently in PGI assay solution for 50 min at 30°, rinsed in three changes of cold water and stored in the refrigerator until densitometry was carried out (usually within 2 hr). The amount of PGI loaded was within the linear range of the densitometry. The area under the peaks was determined by weighing cut-out Xerox copies of the gel tracings.

Immunological determination of levels of cytosolic PGI activity: Antiserum against the native form of purified spinach cytosolic PGI was obtained as previously described (WEEDEN, HIGGINS and GOTTLIEB 1982). The ability of the antiserum to inhibit Clarkia cytosolic PGI was evaluated by incubating purified PGI isozymes from *C. xantiana* with a range of antiserum dilutions for 90–120 min at 0° and measuring the level of PGI activity remaining by spectrophotometric assay. The maximum inhibition of the cytosolic PGI activity was 86% and was obtained with levels of antiserum dilution as low as 1/150 (Figure 1). Figure 2 shows that cytosolic PGI was not present in starch gels following electrophoresis of crude extracts incubated with antiserum. Figure 3 shows that the level of plastid PGI activity was unchanged following incubation with the antiserum.

PGI stability during the incubation test was evaluated by determining activity after 120 min of incubation at 0° with and without preimmune serum. The extracts showed no change in activity in the two tests. Endogenous PGI activity in the antiserum was determined at each test and subtracted from the test results. Such activity was less than 3% of the PGI activity in Clarkia extracts examined. The standard test was to incubate 200 μl of extract with 4 μl of antiserum, to give a 1/50 dilution, for 90–120 min at 0°. Total initial PGI activity in the extracts usually was 50–75 spectrophotometric units/ml, equivalent to 0.8–1.2 IU/ml. After incubation, PGI activity was measured in three 10-μl aliquots, and the values were averaged. The test was carried out in four to 20 individuals of each of the ten Clarkia species.

The values were corrected to reflect the fact that the antiserum inhibits only 86% of the cytosolic PGI activity. The proportion of such activity in each crude extract was calculated as follows:

$$\% \text{ Cytosolic PGI} = \frac{100 \times \left(\begin{array}{l} \text{Activity in} \\ \text{extract before} \\ \text{incubation} \end{array} - \begin{array}{l} \text{Activity in} \\ \text{extract after} \\ \text{incubation} \end{array} - \begin{array}{l} \text{Endogenous} \\ \text{activity in} \\ \text{antiserum} \end{array} \right)}{0.86}}{\text{Activity in extract before incubation}}$$

Immunological determination of levels of cytosolic PGI protein: The amount of cytosolic PGI protein was determined in the closely related *C. lewisii* and *C. rostrata*, species with and without the duplication, respectively. The extracts were adjusted to equal total spectrophotometric PGI activity by dilution with buffer. Aliquots were then incubated over a wide range of antiserum dilutions (1/50 to 1/1500), and the level of PGI inhibition at each dilution was determined as described earlier. The results were plotted on a log scale to permit graphical determination of the antiserum dilution required to inhibit 50% of the cytosolic PGI activity.

RESULTS

Table 1 shows the mean proportion of cytosolic to total PGI activity in the leaf extracts of six species of Clarkia that have the duplicated loci and in four species that do not have the duplication. The mean values for the two groups of species were nearly identical: 67.8% for those with the duplication and 68.2% for those without it. Standard errors both within and among the species were uniformly low (Table 1). Thus, doubling the number of genes coding cytosolic PGI in these species has no affect on the level of PGI activity in the

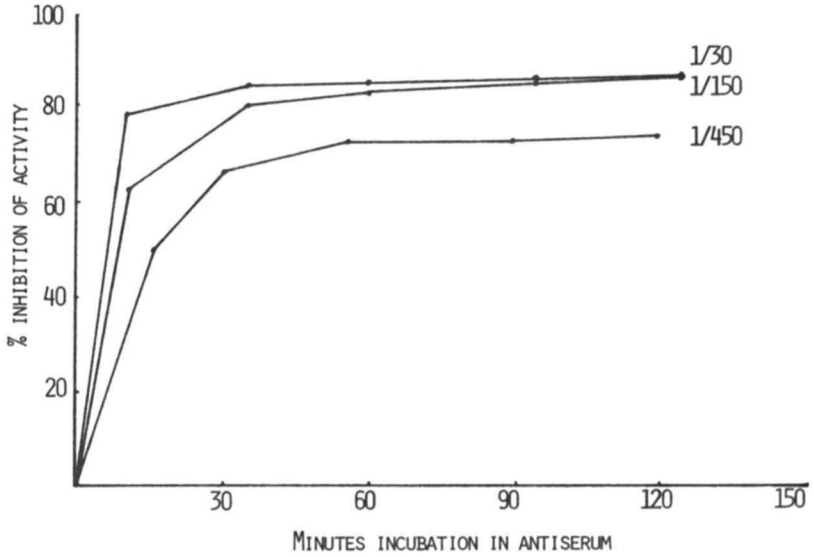

FIGURE 1.—Inhibition of activity of purified cytosolic PGI from *C. xantiana* at three antiserum dilutions over time.

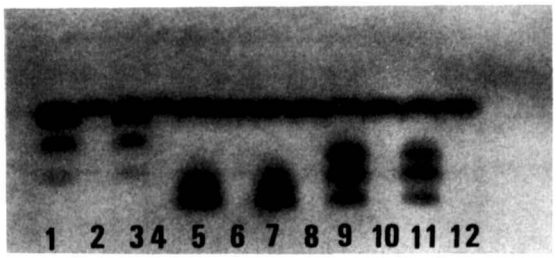

FIGURE 2.—Starch gel electrophoresis of Clarkia leaf extracts after incubation with anticytosolic PGI antiserum. The photo shows three different extracts, each repeated twice. For each extract, the enzymes in the odd-numbered columns were incubated with preimmune serum, and the enzymes in the even-numbered columns were incubated with antiserum. The direction of mobility is toward the top (anode) of the photo; the anodal band, present in all extracts and unaffected by incubation with either the preimmune or immune sera, is the plastid PGI isozyme.

leaves. This also appears to be the case for stem extracts, although only a few species were tested (Table 1).

Densitometric analysis of the same ratio also showed near identical values for the species with and without the duplication, although the values were about 9% higher than obtained by the immunological study (Table 2). The higher values may reflect slight losses of plastid PGI activity because of its greater sensitivity to heat and pH fluctuations that occur during the electrophoresis, but the matter was not studied further. The ratios in the stem extracts were higher than in the leaf extracts probably as a result of the reduced number of chloroplasts in stem cells, which are mostly nongreen tissue, and consequently have a reduced level of plastid PGI.

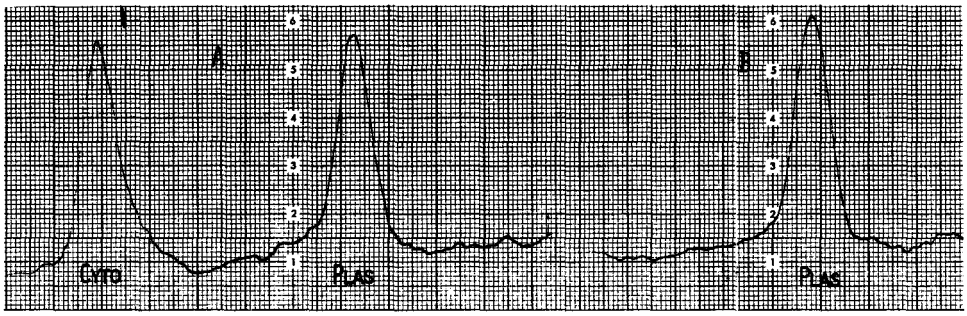

FIGURE 3.—Densitometric tracings of electrophoretically separated PGI isozymes in a Clarkia leaf extract incubated with preimmune serum (A) and anticytosolic PGI antiserum (B). Equal amounts of total PGI activity were loaded on the wicks for each separation. Note that the level of plastid PGI was not affected by having been incubated with the anticytosolic antiserum.

TABLE 1

Proportion of cytosolic to total PGI activity in young leaves (and stems) of Clarkia species evaluated by immunological inhibition

Species	No. of individuals	Leaves	No. of individuals	Stems
Without PGI duplication				
C. amoena	8	64.9 ± 1.31		
C. mildrediae	6	69.7 ± 0.49		
C. rostrata	10	67.2 ± 0.94	6	70.2 ± 1.99
C. williamsonii	4	71.1 ± 0.62		
		$\bar{X}$ = 68.2 ± 1.37		
With PGI duplication				
C. biloba	4	68.4 ± 1.20		
C. bottae	8	70.1 ± 0.73		
C. concinna	9	70.2 ± 2.08	4	74.4 ± 1.06
C. dudleyana	14	62.8 ± 0.99	3	71.6 ± 2.60
C. lewisii	4	70.9 ± 1.11		
C. xantiana	20	64.2 ± 0.92	9	69.7 ± 1.03
		$\bar{X}$ = 67.8 ± 1.40		

Values for leaves and stems are means ± SE.

To assess the relationship between PGI activity and protein level, quantitative immunological titrations were carried out on *C. lewisii* and *C. rostrata*. These species were studied because they are more closely related (DAVIS 1970) than any other species pair with and without the duplication (GOTTLIEB and WEEDEN 1979). Figure 4 shows that 50% inhibition of cytosolic PGI in *C. lewisii* occurred at a mean dilution of 1/692 and in *C. rostrata* at 1/860. If *C. lewisii* contained two times more cytosolic PGI protein per unit activity than *C. rostrata*, then an antiserum dilution of approximately 1/430 would be required to obtain 50% inhibition; but this was not observed. The extract from *C. lewisii* required only a 25% antiserum dilution relative to that from *C. rostrata*.

Cytosolic PGI protein levels were also evaluated by a mixing experiment in which equal spectrophotometric activities of extracts of the two species were

TABLE 2

Proportion of cytosolic to total PGI activity in young leaves and stems of Clarkia species evaluated by densitometry of electrophoretically separated isozymes

Species	No. of individuals	Ratio of enzyme activities ± SE	
		Leaves	Stems
Without PGI duplication			
C. amoena	6	76.2 ± 2.2	81.3 ± 2.0
C. mildrediae	3	82.3 ± 2.9	76.0 ± 3.6
C. rostrata	6	74.7 ± 1.2	79.1 ± 0.9
C. williamsonii	3	73.9 ± 0.3	80.0 ± 1.2
		$\bar{X}$ = 77.0 ± 1.9	79.1 ± 1.1
With PGI duplication			
C. bottae	3	82.3 ± 2.3	81.6 ± 1.9
C. breweri	3	76.9 ± 2.7	78.9 ± 2.7
C. lewisii	6	75.4 ± 1.3	82.3 ± 1.0
C. xantiana	18	74.4 ± 1.6	83.0 ± 1.3
		$\bar{X}$ = 77.2 ± 1.6	81.4 ± 0.9

mixed and incubated over the same range of antiserum dilutions (Figure 4). The 50% inhibition values of the mixed extracts were similar to the values for the two species. Since species with and without the duplicated PGIs were equivalently titrated by the antiserum, the levels of PGI activity and protein in the extracts appear proportional.

The proportion of cytosolic to total PGI activity was also obtained in young leaves of seedlings of 11 vegetable species by densitometry of the electrophoretically separated isozymes (Table 3). The electrophoretic PGI pattern in each of the species was similar and contained only two bands, the anodal plastid isozyme and the less anodal cytosolic isozyme. The immunological procedure was not used with the vegetables because the antiserum, which had been generated against the cytosolic PGI of spinach, cross-reacted differently against the various cytosolic PGIs. This was not surprising in view of the diversity of the phylogenetic lineages represented.

The results divided the vegetables into two groups. Seven species had a mean value of 66.3% cytosolic PGI, and four species had a mean of 76.5% (Table 3), the latter value closely similar to that obtained by densitometry for Clarkia species. A physiological rationale to account for the presence of a species in one group or the other is not now available. The proportion of cytosolic PGI to total PGI from spinach leaves has previously been reported as 65% (based on activities recovered following DEAE-cellulose chromatography of a crude extract) (SCHNARRENBERGER and OESER 1974), almost identical with the value we obtained by densitometry (Table 3).

DISCUSSION

The evidence from immunological inhibition and titration studies demonstrates that the duplication of the gene specifying the cytosolic isozyme of PGI

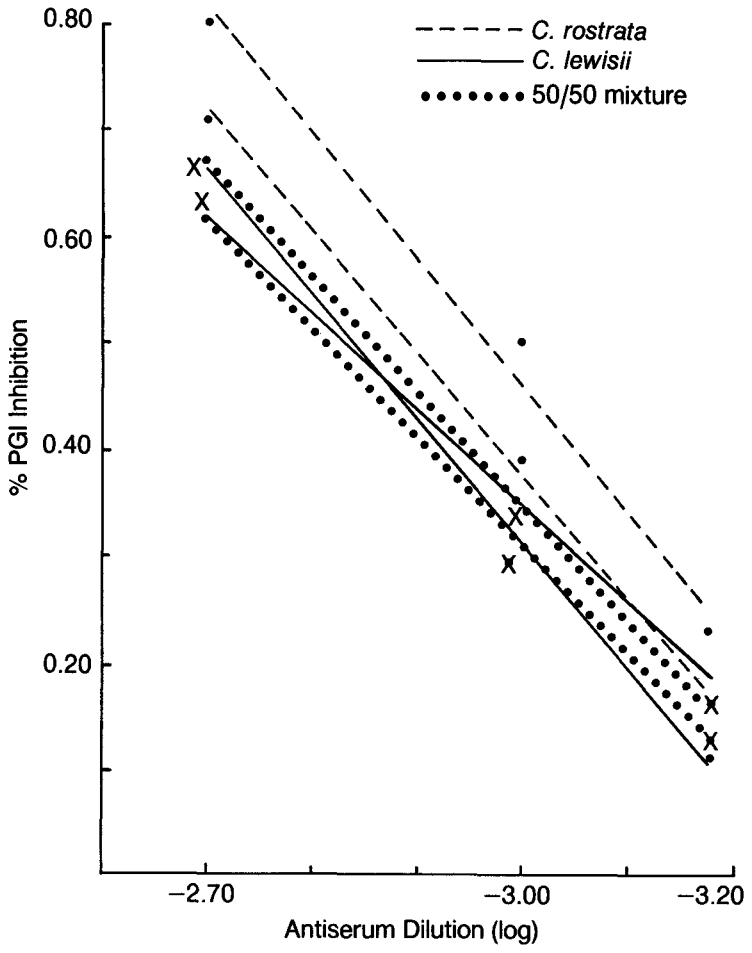

FIGURE 4.—Quantitative immunotitration of cytosolic PGI activity in leaf extracts of *C. lewisii*, *C. rostrata* (species with and without the PGI duplication, respectively), and two 50:50 mixes of extracts of the two species. The 1/50 antiserum dilution, which resulted in maximum inhibition, was not plotted.

in Clarkia does not increase either the activity or protein levels of the enzyme relative to that of the plastid PGI isozyme. The immunological results were fully supported by densitometric analysis of the electrophoretically separated isozymes. Since a large number of allelic and nonallelic PGIs in ten species of several sections of the genus were examined, the result is not likely to be overthrown by additional sampling.

The proportionality between PGI activity and protein levels is consistent with previous results with the duplicated PGI-2 and PGI-3 from *C. xantiana*, which showed that equal spectrophotometric activities of the purified enzymes, subjected to polyacrylamide electrophoresis in the presence of SDS, yielded peak areas for the subunits with equal amounts of protein staining (R. C. HIGGINS and L. D. GOTTLIEB, unpublished results).

TABLE 3

Proportion of cytosolic to total PGI activity in young leaves of 11 vegetable species evaluated by densitometry of electrophoretically separated isozymes

Plant	Ratio of enzyme activities ± SE
Group I	
Cabbage	63.3 ± 1.3
Cucumber	67.0 ± 2.1
Pepper	66.5 ± 1.5
Radish	67.0 ± 0.6
Spinach	64.3 ± 1.8
Tomato	66.7 ± 0.9
Watermelon	69.3 ± 0.3
	$\bar{X}$ = 66.3 ± 0.74
Group II	
Lettuce	79.3 ± 0.9
Maize	75.3 ± 2.4
Parsnip	75.3 ± 0.3
Pea	76.0 ± 1.0
	$\bar{X}$ = 76.5 ± 1.0

Densitometric analysis was also carried out in 11 vegetable species in order to determine whether the cytosolic PGI level in Clarkia was typical of other plants. Only two levels were found, one of which was the same as in Clarkia. This suggests that only certain levels of PGI activity are compatible with the proper operation of the cytosolic PGI reaction during gluconeogenesis, the most likely direction of flux in the photosynthesizing leaf. Thus, it appears that some form of genic or metabolic regulation has evolved that compensates for the PGI duplication.

We assessed the amount of cytosolic PGI relative to total PGI (the sum of cytosolic and plastid PGI activities) rather than to total soluble protein because our studies were done in crude leaf or stem extracts in which it is notoriously difficult to quantitate protein amounts because of the presence of phenolics and other protein-complexing compounds (LOOMIS 1974). In contrast, studies of gene dosage in maize have generally been conducted on extracts of scutella, a nongreen embryonic organ in which protein levels are more readily assayed (for example, BIRCHLER 1979, 1981; BIRCHLER and NEWTON 1981; MCMILLIN and SCANDALIOS 1982).

We selected total PGI activity because it can be accurately determined by routine spectrophotometric means, and the assay is not complicated by positive or negative effectors in the extracts. Our design requires that the level of plastid PGI be conserved, an assumption that seems reasonable for metabolically active leaves in the congeneric Clarkia species. In addition, the plastid isozyme served as a convenient internal control in the densitometry, because its product is also visualized by the activity stain. Since the proportion of cytosolic to total PGI activity was the same in species with and without the duplication, the level of the plastid PGI in the former species, if not maintained, would have had to increase by a precise doubling. Such a requirement merely shifts the target of regulation and, although it cannot now be rejected, it seems unlikely.

Other studies of the amount of enzyme correlated with gene duplication in plants appear to have been done only on maize mitochondrial and cytosolic malate dehydrogenases (MDH) and maize catalase. Rocket immunoelectrophoresis revealed that lines with three "active" mitochondrial MDH genes had less mitochondrial MDH protein on the average than lines with four such genes, and lines with a single cytosolic MDH gene averaged about half the amount of cytosolic MDH protein as those with two coding genes (McMillin and Scandalios 1982). Similar gene dosage responses were also obtained by manipulating the dosage of each of the two catalase genes (Tsaftaris, Scandalios and McMillin 1981).

The data from these studies show a substantial amount of variability of protein level among lines having the same gene dosage (as high as fourfold for the cytosolic maize MDH), suggesting that the gene products were differentially stable in the various lines or that the lines had different modifying genes. The latter seems to explain the expression of alcohol dehydrogenase-1 (ADH-1) levels in maize. Thus, ADH-1 activity levels were initially thought to show compensation, since varying the number of doses of the long arm of chromosome *1*, which includes the *Adh1* locus, did not lead to changes in ADH protein (Birchler 1979). But when the chromosome arm was subdivided so that the *Adh1* locus was included in a smaller 18 cM segment, changes in the number of these segments yielded a strict dosage effect, whereas changes in the dosage of segments without the locus had a negative regulatory effect on ADH level (Birchler 1981). The dosage response of *Adh1* was later confirmed in anaerobic root tissue by measuring the incorporation of tritiated leucine in a euploid line and its segmental tetrasomic derivative (Johns, Alleman and Freeling 1983). In other studies, gene dosage effects in primary trisomics of tomato (Fobes 1980), *Datura stramonium* (Carlson 1972) and barley (Nielsen and Frydenberg 1971) have been sufficiently clear-cut to enable mapping of a number of genes. Thus, in general, increased gene dosage in these plants appears to be correlated with proportional increases in the level of enzyme product.

However, the studies in maize ADH and in the several trisomic series are not comparable to the PGI duplication in Clarkia because these cases involve chromosomally manipulated stocks, whereas the PGI duplication occurred anciently (it now characterizes 12 species in four sections) and has since been subject to natural selection. At present, the mode of regulation of the constancy of PGI levels is not known. Whether it is similar to dosage compensation in Drosophila (Lucchesi 1974, 1977) remains to be determined.

We are approaching the problem with a series of null activity mutants of *Pgi-2* and *Pgi-3* in *C. xantiana* that were generated by EMS treatment of seeds. The mutants are presently being transferred to wild-type backgrounds, after which they will be intercrossed to construct plants having zero to four doses of the gene coding cytosolic PGI. Evaluation of the consequences of this dosage series on sucrose synthesis may provide important information about the significance of PGI level in Clarkia.

This research was supported by National Science Foundation grant BSR 82-15363.

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