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Evidence from subunit molecular weight suggests hybridization was the source of the phosphoglucose isomerase gene duplication in *Clarkia*

L. D. Gottlieb and R. C. Higgins
Department of Genetics, University of California, Davis, CA 95616, USA

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Summary. The apparent molecular weight of cytosolic phosphoglucose isomerase (PGI) subunits was evaluated in 18 species of *Clarkia* which have or do not have duplicated genes specifying this glycolytic enzyme. Species that lack the duplication had subunits of 59,000 or 60,400 whereas species with the duplication generally possessed two types of PGI subunits with these or closely similar molecular weights. The additive pattern in the species with the duplication suggests that the molecular weight divergence preceded the origin of the duplication, and that the duplication arose following hybridization between taxa that represented different lineages within *Clarkia*.

Key words: Gene duplication – Phosphoglucose isomerase isozymes – Subunit molecular weight – *Clarkia*

Introduction

The duplication of the 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 (Gottlieb 1977). The duplication is present in 12 diploid species which are morphologically and karyotypically diverse, but absent in a dozen other diploid species (Gottlieb and Weeden 1979). Linkage tests in four species revealed that the duplicated loci assort independently (Gottlieb 1977; Gottlieb and Weeden 1979; Weeden and Gottlieb 1979). This finding plus the substantial chromosomal rearrangements which distinguish species of *Clarkia*, and their self-compatibility, led to the hypothesis that the duplicated PGI loci mark duplicated segments on nonhomologous chromosomes that originated from a cross between partially overlapping reciprocal translocations or insertional translocations (Gottlieb 1977). In this paper we report evidence obtained by evaluating the apparent molecular weight of the PGI subunits which suggests that the ancestral PGI locus had diverged in different *Clarkia* lineages prior to the hybridization which brought them together into a common nucleus.

Materials and methods

Plants

The cytosolic isozymes of PGI were examined in extracts from plants of twelve species of *Clarkia* that possess duplicated PGI loci and from six species of *Clarkia* that lack the duplication. The species are listed in Table 1. Plants were grown from seeds collected in nature. The seeds were germinated in moistened vermiculite and the seedlings grown in growth chambers as described (Gottlieb 1977). They were harvested at about four–five weeks of age when they were about 3 to 4 cm tall.

PGI extraction

At harvest, leaves and stems were cut into 2 cm pieces and ground in a cold mortar or in a Waring blender. For each gram fresh weight, 6 ml cold extraction buffer and 0.4 g insoluble PVPP were used. The extraction buffer contained 0.05 M HEPES-KOH, 0.002 M EDTA, 100 μg/ml PMSF (previously dissolved in ethanol), 0.007 M or 0.042 M 2-mercaptoethanol, and 0.3% to 0.6% (v/v) Triton X-100, adjusted to pH 7.5. Extracts that were to be exposed to antiserum received the lower level of 2-mercaptoethanol. All operations were carried out at 0–3 °C, unless otherwise noted. After the grinding procedure, the extracts were centrifuged at 6,000 × g for 10 min and the supernatants recovered.

PGI subunit isolation

Subunits of the cytosolic PGI isozymes were isolated by an immunological procedure from the supernatants. The procedure made use of antibodies that had previously been generated in rabbits against the native cytosolic PGI from spinach (*Spinacia oleracea*) (Weeden et al. 1982). These anti-

bodies cross-reacted strongly with the native *Clarkia* cytosolic PGIs but did not react with the plastid PGI isozyme in the same extracts.

Following purification of the antibodies from the whole serum by passage through DEAE-Affigel-Blue (BioRad), the antibodies were covalently coupled to cyanogen-bromide activated Sepharose 4B (Cuatrecasas 1970). The IgG fraction from preimmune serum was similarly attached to activated Sepharose 4B. The extracts were initially incubated for 30 min at 0 °C with the pre-immune-Sepharose 4B. The Sepharose resin was then removed by filtration through a Whatman glass fiber filter Type B, and the extract passed three times through a small column containing the anti-PGI-Sepharose (volume about 1/5th that of the sample). The column was washed with 10 bed volumes of extraction buffer, followed with two bed volumes of 0.05 M Tris-HCl, pH 6.8. The bound cytosolic PGI was then eluted from the column as subunits by a buffer consisting of 0.05 M Tris-HCl, pH 6.8, 2% SDS, 5% (v/v) 2-mercaptoethanol, and 10% glycerol, at 25 °C. The eluted subunits were thoroughly denatured by heating for 5–10 min at 80 °C.

Subunit molecular weight

The molecular weight of the duplicated PGI-2B and PGI-3A subunits of the white-flowered self-pollinating line of *C. xantiana* (Gottlieb 1977) was assessed by polyacrylamide gel electrophoresis in the presence of SDS (7.5% monomer) (Laemmli 1970). Prior to the electrophoresis, the two isozymes were highly purified by a series of steps that included DE52 and DE53 chromatography, heat denaturation, hydroxylapatite chromatography, and gel filtration on Sephacryl S-200 (Higgins and Gottlieb, in preparation). The gels were calibrated using four markers including phosphorylase B (94,000), BSA (68,000), yeast PGI (57,000 as determined by us), and ovalbumin (43,000). The gels were stained for protein with Coomassie Blue R. The molecular weight of the PGI subunits, extracted from the other *Clarkia* species by the immunological procedures, were determined by comparison of their mobilities to those of the two subunits of *C. xantiana* after they had been separated electrophoretically and blotted to nitrocellulose membranes (Schleicher and Schuell). The blotting buffer was 20% methanol, 0.025 M Tris, 0.195 M glycine: blotting was carried out at 350 mA for 100 min at 4 °C.

The membrane-bound PGI subunits were detected immunochemically (Renart et al. 1979) by incubating the blots with a mixture of the antisera that had been prepared in rabbits against each of the denatured cytosolic PGIs from *C. xantiana* as described elsewhere (Higgins and Gottlieb, in preparation). These antibodies were labeled with ^{125}I-protein A so that the position of the antigen-antibody complexes on the membrane could be determined by exposing X-ray film with intensifying screens.

Results

Molecular weight calibration

As shown in Fig. 1, the subunits of PGI-2B and PGI-3A from *Clarkia xantiana* differed in apparent molecular weight, measuring 60,400 and 59,000, respectively. The heterodimeric enzyme PGI-2B/3A showed both molecular weight bands in equal amounts (Fig. 2).

Previous estimates of the molecular weights of the native proteins by the less precise methods of gel

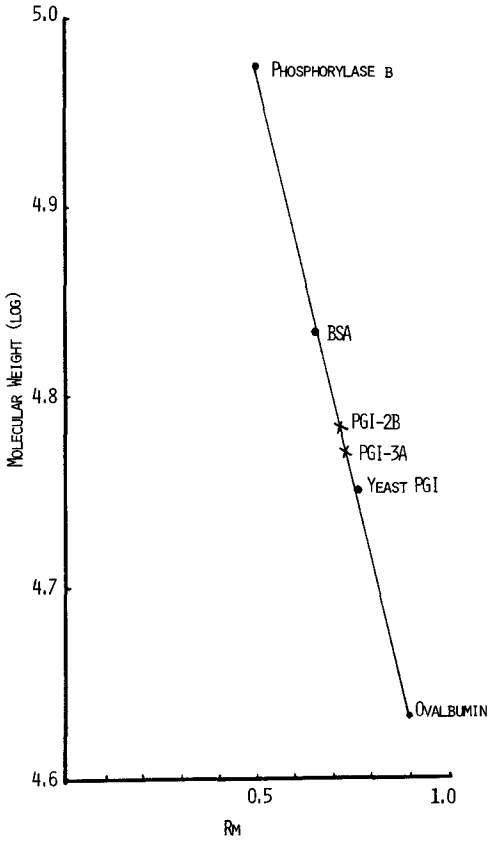

Fig. 1. Molecular weight determination of subunits of cytosolic PGI of *Clarkia xantiana*. Standards are phosphorylase B (94,000), bovine serum albumin (68,000), yeast PGI (57,000 as calibrated by us), and ovalbumin (43,000)

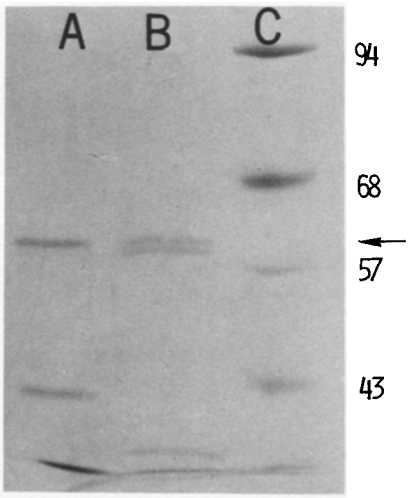

Fig. 2. SDS-polyacrylamide electrophoresis of purified subunits of cytosolic PGI from *Clarkia xantiana*. (*A*) PGI-2B; (*B*) PGI-2B/3A; (*C*) Molecular weight markers

electrophoresis and gel filtration on Sephacryl S-200 yielded values of approximately 130,000 (Gottlieb and Greve 1981). In spinach, the native cytosolic PGI has an apparent molecular weight of 120,000 (Schnarrenberger and Oeser 1974) and its subunit 61,000 (Herbert and Schnarrenberger 1982).

PGI subunit molecular weight in Clarkia species

Table 1 summarizes the results of molecular weight comparisons of the cytosolic PGI subunits in 18 species of the seven sections of *Clarkia* that contain diploid species. The subunits in ten of the 12 species with duplicated PGI loci had two molecular weights, usually 59,000 and 60,400 (or values close to these). The PGI subunits in each of the six species without the duplication had only a single molecular weight, either 59,000 or 60,400. Figure 3 shows blots of the subunits of several of the species.

Small differences were observed in the values of the molecular weight of both subunits among the species with the duplication. Thus among the ten species with two subunits, the smaller subunit had a value of 59,000 in eight species and 59,400 or 60,000 in one species each. The larger subunit had a value of 60,400 in five species, 59,400 in three species, and 60,000 or 61,000 in one species each. Only a single subunit class was detected in *C. biloba* and *C. unguiculata.* Since both of these species have two loci specifying cytosolic PGI (Gottlieb and Weeden 1979), it is likely that the two gene products have the same or extremely similar molecular weight. The situation in *C. biloba* was unexpected because its derivative, *C. lingulata* (Lewis and Roberts 1956), had two distinguishable subunits.

Table 1. Molecular weight of cytosolic PGI subunits extracted from species of *Clarkia* with and without a duplication of the coding gene. The collection number for each species is given before the number of plants sampled (in parentheses)

Without PGI duplication	Subunit molecular weight(s)
Godetia:	
C. speciosa 120 (2)	59,000
Rhodanthos:	
C. amoena 147 (1)	59,000
C. arcuata 157 (1)	59,000
Myxocarpa:	
C. mildrediae 50 (2)	60,400
C. virgata 67 (1)	60,400
Peripetasma:	
C. rostrata 97A (5)	60,400
With PGI duplication	
Peripetasma:	
C. biloba 739 (1)	59,400
C. cylindrica 104B (1)	59,000, 60,400
C. dudleyana 7311 (2)	59,000, 59,400
C. epilobioides 40 (1)	59,400, 60,400
C. lewisii 796 (1)	59,000, 60,400
C. lingulata 737 (1)	59,000, 59,400
C. modesta 127E (1)	60,000, 60,400
Phaeostoma:	
C. unguiculata 36, 76, 90A (3)	59,400
C. xantiana 7436, 7437 (numerous)	59,000, 60,400
Fibula:	
C. bottae 34X, 35 (2)	59,000, 60,000
Eucharidium:	
C. breweri 103 (2)	59,000, 59,400
C. concinna 146 (1)	59,000, 61,000

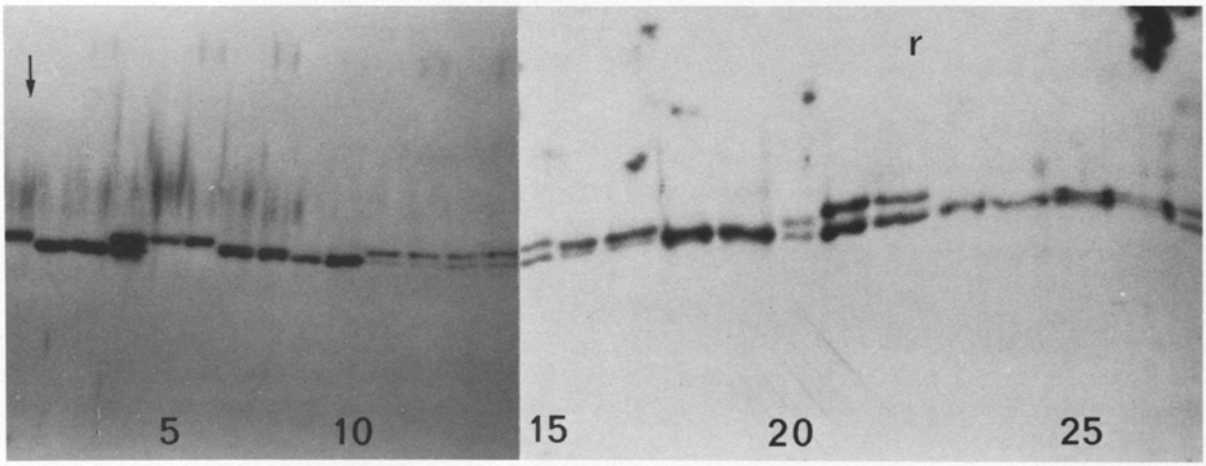

Fig. 3. Blots of electrophoretically separated PGI subunits visualized with ^{125}-I-protein A labeled antibodies. (1) *C. rostrata;* (2, 3) *C. amoena;* (4) *C. xantiana* 2B/3A; (5, 6) *C. mildrediae;* (7, 8) *C. dudleyana;* (9, 10) *C. amoena;* (11, 12) *C. lewisii;* (13, 14) *C. xantiana;* (15) *C. xantiana* 2B/3A; (16, 17) *C. bottae;* (18, 19) *C. breweri;* (20) *C. xantiana* 2B/ 3A; (21, 22) *C. concinna;* (23, 24) *C. dudleyana;* (25, 26) *C. modesta;* (27) *C. xantiana* 2B/3A

The apparent molecular weight variability among the duplicate subunits indicates that this attribute is subject to change presumably by mutation. It is possible that the variability reflects mutational change to certain amino acids which are particularly subject to proteolysis, or that a stop mutation causes slightly premature termination of translation. However, the present data do not exclude the possibility that the observed differences result from SDS binding properties rather than amino acid number. A final determination cannot be made until protein sequences are available. Therefore the values given must be regarded as apparent molecular weights.

Examination of purified subunits from *C. xantiana* and *C. lewisii* revealed that the catalytically active dimer of the subunits with the higher molecular weight had a more anodal electrophoretic mobility than that of the dimer of the lower molecular weight subunit. It seems likely that this relationship is general among the species with the duplication.

Isozymes from two species were examined to determine if the molecular weight differences reflected differences in isoelectric point of the native proteins, the main determinant of their mobilities during electrophoresis. PGI-2A, -2B, and -2C from *C. xantiana* which differ in isoelectric point from 5.25 to 5.70 (Gottlieb and Greve 1981), showed the same molecular weight of 60,400. PGI-3A, -3B, and -3C, also from *C. xantiana,* with isoelectric points from 5.95 to 6.40 (Gottlieb and Greve 1981) had a single molecular weight of 59,000. The subunits of two cytosolic PGIs from *C. rostrata,* a species without the duplication, with electrophoretic mobilities of 58 and 82 relative to that of the plastid PGI (Gottlieb and Weeden 1979), also had a single weight class (Table 1). Thus the difference in subunit mobility is not correlated with isoelectric point of the native proteins.

Discussion

The discovery that the apparent molecular weight of cytosolic PGI subunits in species of different sections of *Clarkia* without the PGI duplication is either 59,000 or 60,400, and that species with the duplication generally synthesize two types of PGI subunits with these or similar values has two important implications: (1) The divergence in molecular weight preceded the origin of the duplication; and (2) The duplication arose following hybridization between taxa that either gave rise to or already represented the Rhodanthos/Godetia lineage and the Myxocarpa lineage.

In all, five different apparent molecular weights were observed. In the species without the duplication, only two molecular weight forms were found and, significantly, these were conserved within sections. Variation within sections occurs only in species with the duplication. But, even here, such species each contained at least one of the molecular weight forms that characterized species lacking the duplication.

The presence of two molecular weight forms in species with the duplication is consistent with the previous proposal (Gottlieb 1977) that the duplication arose following cross(es) between individuals which carried the locus coding cytosolic PGI on nonhomologous chromosomes, reflecting prior translocation events. Chromosomal rearrangements are not infrequent in *Clarkia* (Lewis 1973). Self-pollination or intercrossing of chromosomal heterozygotes from such hybridization presumably led to the segregation of a progeny which was duplicated for the segments carrying both PGI loci (this model is further described in Gottlieb 1977; Gottlieb and Weeden 1979).

The subunit molecular weight in *C. rostrata* is particularly interesting. *Clarkia rostrata* is the only species in section Peripetasma without duplicated PGI isozymes (Gottlieb and Weeden 1979). Since it is closely related to *C. lewisii* and *C. cylindrica* (Davis 1970) that have the duplication, the suggestion was made that the duplication originated in one of these species or their common ancestor with *C. rostrata* (Gottlieb and Weeden 1979). The alternative hypothesis was that one of the duplicated loci in *C. rostrata* was silenced by mutation.

The present evidence shows that the PGI subunit of *C. rostrata* is the same as one of the subunits characteristic of *C. lewisii* and *C. cylindrica,* but its molecular weight resembles that of subunits found in Myxocarpa rather than in Rhodanthos. Since Rhodanthos is thought to be ancestral to Peripetasma (Lewis and Lewis 1955), the structural evidence suggests that *C. rostrata* may have lost the duplicate locus coding the 59,000 subunit.

The suggestion that hybridization between different lineages was involved in the origin of the duplication rather than crosses from within a single lineage was not previously considered because little information was initially available regarding structural properties of the isozymes. But since an additive pattern like that found for the subunit molecular weights is unlikely to have evolved independently, the hybridization model now seems appropriate. The two PGI loci presumably became associated in a single genome following hybridization between the translocations as outlined above. Thus the model of the origin of the PGI duplication is *not* the classic one in which a duplicated gamete is initially produced by unequal crossing-over (as in Bar Eye in *Drosophila melanogaster*). The evolutionary divergence between the duplicated PGI genes appears to have taken place before the loci were brought together into a common genome. That divergence may precede duplication is certainly the case in allopolyploids, and has been clearly documented for

alcohol dehydrogenase in a comparison of the tetraploid *Tragopogon miscellus* and its diploid progenitors (Roose and Gottlieb 1980). Since a number of other duplicated genes which have nonduplicated homologues in related species have been identified in diploid plants (Gottlieb 1982; Pichersky and Gottlieb 1983), it is not unlikely that additional examples of divergence preceding duplication will be forthcoming. In the present example of *Clarkia* PGI, it may eventually be possible to identify other genes (or characters) in species with the duplication that are combinations of divergent traits in the Rhodanthos/Godetia lineages and the Myxocarpa lineage.

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