

# Reduced Enzyme Activity and Starch Level in an Induced Mutant of Chloroplast Phosphoglucose Isomerase<sup>1</sup>

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## ABSTRACT

Ethyl methane sulfonate treatment was used to induce a mutation in the nuclear gene encoding the chloroplast isozyme of phosphoglucose isomerase in *Clarkia xantiana*. The mutation, which proved allelic to wild type activity, was backcrossed to wild type for five generations so that the two could be compared in a near isogenic background. An immunological analysis showed that the mutant, when homozygous, reduced the activity of the isozyme by about 50%. In contrast to wild type, the mutant showed little change in leaf starch level over a diurnal period or following a 72-hour continuous light treatment. By the end of the diurnal light period, the mutant accumulated only about 60% as much starch as wild type. However, mutant leaves had an increased sucrose level presumably because photosynthate was directly exported from the chloroplasts. The mutant also exhibited reduced leaf weight. These changes in metabolism and growth suggest that the wild type level of plastid phosphoglucose isomerase activity is necessary to achieve wild type carbohydrate status.

Phosphoglucose isomerase (D-glucose-6-P ketol isomerase, EC 5.3.1.9) catalyzes the reversible isomerization of fructose-6-P and glucose-6-P. Plant cells have two isozymes of PGI,<sup>2</sup> one located in the plastids and the other in the cytosol (5, 10, 11, 13), with both isozymes encoded by independent nuclear genes (5, 12). The plastid PGI governs an essential step in the synthesis and degradation of starch and the cytosolic isozyme carries out a reaction leading to the production (or degradation) of soluble carbohydrates, most generally sucrose. Plastid PGI activity constitutes about 30% of total cellular PGI (plastid plus cytosolic) in photosynthesizing leaves (7, 9, 10). Since a wide variety of plant species exhibits the same proportion of plastid to total PGI activity (7), the PGI activity ratio may be one of a number of specific consequences of a general mechanism that controls normal carbohydrate metabolism. However, PGI activity itself has not been implicated in the direct regulation of carbohydrate levels.

In this paper we describe the effects of a mutation of the gene encoding the plastid PGI on the levels of chloroplast starch, sucrose, and other soluble sugars, and on certain growth parameters. The mutation was induced by EMS treatment of seeds in *Clarkia xantiana* (Onagraceae) and has been sequentially back-

crossed to wild type for five generations so that it can be compared to wild type in a near isogenic background (98.4% wild type). The mutation reduces the activity of the plastid PGI isozyme by about 50% but has no effect on the activity of the cytosolic PGI.

## MATERIALS AND METHODS

**Plants, Growth Conditions and PGI Electrophoretic Pattern.** White-flowering plants of *Clarkia xantiana* Gray derived from seeds originally collected from a self-pollinating population (7436) in the Kern River Canyon, Tulare County, CA (4, 6) were studied. The plants have been maintained by self-pollination and are very highly homozygous (6). They were grown in a mixture of three parts sand to one part peat and supplied with Hoagland nutrient at weekly intervals. Controlled environment chambers providing a radiant flux density at plant level of 250  $\mu\text{E m}^{-2} \text{s}^{-1}$  (400–700 nm) and day/night temperatures of 19/14°C were used throughout the study. Day length was 11 h except for one set of experiments in which continuous illumination was provided for 72 h. All experiments were performed on 5 to 6 week old plants (5–7 cm).

The electrophoretic pattern following starch gel electrophoresis (7) consists of four bands of activity: the plastid PGI-1 which is 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 (4, 7, 8).

**Mutagenesis and Mutant Establishment.** Five hundred seeds were presoaked for 12 h in 0.1 M phosphate buffer (pH 7.0), and then for 3 h in the same buffer containing 0.1 M EMS. The seeds were washed, dried, and germinated according to normal procedures (4). Germination rates were within 5% of untreated controls. Seedlings were potted into 2 inch pots; 312 plants matured but, on the average, they set fewer than 10 seeds each. These seeds, all obtained by self-pollination, were sown, but only 25% germinated in contrast to 95% germination of wild type seeds. A total of 160 families (5 seedlings each) were eventually examined by starch gel electrophoresis (7). Several lines showed a marked decrease in the staining intensity of the plastid PGI band relative to that of the wild type, but only one line was recovered. It was crossed to wild type (always as the female parent), and the F<sub>1</sub> was then backcrossed to wild type. Heterozygous plants in the backcross progeny were backcrossed again to wild type for a total of five generations. The heterozygous plants in each backcross generation were identified in the following way. Each generation, 10 to 12 backcross seedlings were grown up, crossed to wild type, and also self-pollinated. The latter progenies were examined by electrophoresis. A 3:1 segregation (band of activity present *versus* band very weak to absent) in them identified the parent as a heterozygote. Therefore, the progenies between these backcross

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<sup>2</sup> Abbreviations: PGI, phosphoglucose isomerase; EMS, ethyl methane sulfonate.

plants and wild type plants constituted the next backcross generation. The progenies from backcross plants which did not segregate were thrown out. In this paper we describe results from homozygous mutant derivatives, obtained by self-pollination of the BC 5 generation. In principle, these plants are expected to be 98.4% wild type.

**Enzyme Extraction.** Leaves (60–100 mg) were homogenized in 0.6 to 1.0 ml of extraction buffer consisting of 0.1 M Hepes adjusted to pH 7.5 with KOH, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 50  $\mu$ g per ml of phenyl methyl sulfonyl fluoride (previously dissolved in ethanol). Extracts were centrifuged in a microcentrifuge at 8000g for 5 min and the supernatants used for analysis. All operations were carried out at 2 to 4°C.

**Spectrophotometric Assay.** PGI activity was assessed at 20°C by following the enzyme-linked reduction of NADP<sup>+</sup> at 340 nm in a Zeiss PM6 spectrophotometer. The reaction mixture (0.5 ml) consisted of 0.1 M Tris HCl (pH 8.3), 5 mM fructose-6-P, 1 mM NADP<sup>+</sup>, and 1 IU/ml of glucose-6-P dehydrogenase (ex *Torula* yeast, Sigma type XII). PGI activity was expressed as International units (IU) per g fresh weight, 1 IU converting 1.0  $\mu$ mol of fructose-6-P to glucose-6-P per min at 20°C and pH 8.3.

**Immunological Determination of Plastid PGI Activity.** Total cellular PGI activity is the sum of the activities of the plastid PGI and the cytosolic PGI isozymes. The analysis made use of an antiserum generated against native cytosolic PGI that had been fully purified from spinach (14). This antiserum inhibits 86% of the *Clarkia* cytosolic PGI activity and has no effect on the activity of the plastid PGI (7, 9). Thus, we estimated the plastid PGI activity by subtracting cytosolic PGI activity from total PGI. The standard test was to incubate 200  $\mu$ l of crude extract with 4  $\mu$ l of antiserum, to give a 1/50 dilution, for 90 to 120 min at 0°C and assay the change in PGI activity by spectrophotometry. The proportion of plastid PGI activity was calculated as follows:

% plastid PGI =

$$100 \times \left( 1 - \frac{\text{Activity in extract before incubation} - \left\{ \begin{array}{l} \text{Activity in} \\ \text{extract after} \\ \text{incubation} \end{array} \right\} - \text{Endogenous activity in antiserum}}{0.86} \right) \div \text{Activity in extract before incubation}$$

The level of plastid PGI activity can also be determined by an independent approach which takes advantage of the differential temperature stability of the plastid and cytosolic PGI isozymes (9, 13). Heating extracts to 50°C for 10 min completely inactivates the plastid PGI but has no effect on the cytosolic PGI activity (9). Thus, the plastid PGI activity can be determined as the difference between total PGI activity and the activity remaining after heat treatment. The heat treatment and immunological procedure gave nearly identical results (9).

**Starch Determination.** All the true leaves from 5 to 6 week old plants were harvested, weighed, and plunged immediately into 10 volumes of boiling 80% ethanol. They were extracted a further three times with boiling ethanol, dried at 50°C, and homogenized in a total volume of 2 ml of 0.2 N KOH for each 100 mg leaf fresh weight. Samples were heated in boiling water for 30 min, cooled, and 200  $\mu$ l of 1 N acetic acid added for each 1 ml. An equal volume of dialyzed amyloglucosidase (35  $\mu$ l/ml in 0.05 M acetate [pH 4.5]) was added and samples incubated for 30 min at 55°C followed by 5 min in boiling water. Cooled samples were centrifuged for 20 min at 12,000g and the glucose levels in the supernatants assessed by the hexokinase/fructose-6-P/dehydrogenase enzymic method. The reaction mixture, in 0.1 M Tris HCl (pH 8.0), was 1 mM ATP, 1 mM NADP, 2 mM MgCl<sub>2</sub> with 1 unit/ml of hexokinase and 1 unit/ml of glucose-6-P dehydro-

genase. Levels were calculated from glucose standards and converted to starch by multiplying by 0.9.

**Sugar Determinations.** The sugars present were initially identified by TLC on 20 × 20 cm cellulose sheets using a solvent system consisting of tertiary butanol:methyl ethyl ketone:HCOOH:water (40:30:15:15). Two g of leaf tissue was extracted three times with 30 ml of boiling 80% ethanol and the combined extracts reduced to 2 ml on a rotary film evaporator at 28°C. Samples were spot loaded onto the thin-layer sheets together with a range of known carbohydrates. Separation was achieved by two ascending runs and carbohydrates visualized using a mixture of equal volumes of 0.2% naphthoresorcinol in ethanol and 24% H<sub>2</sub>SO<sub>4</sub> followed by heating at 100°C for 5 to 10 min. Both wild type and mutants had three major spots which co-chromatographed with and had color reactions corresponding to sucrose, fructose, and glucose, respectively. Quantitative determinations of these sugars were subsequently carried out as follows: leaves were harvested, rapidly weighed, and plunged into liquid N<sub>2</sub> in a mortar, homogenized with 1.25 ml of cold 5% HClO<sub>4</sub> per 1 g leaf fresh weight, and centrifuged at 15,000g for 15 min at 4°C. The supernatant was neutralized with 8 N KOH and the precipitated perchlorate removed by centrifugation, reacidified (pH 5.0–5.5) with 0.1 N HCl, cleared with activated charcoal, and the pH adjusted to 6.0 with 0.2 N NaOH. Following another 15,000g/15 min centrifugation, the supernatant was used as the sample. Glucose and fructose as well as glucose-6-P and fructose-6-P were determined in the same sample using the reaction mixture employed in the starch determination with the addition of 0.5 units per ml of PGI, essentially as described in Cooper *et al.* (2). Sucrose levels were assessed by measuring the increase in fructose and glucose following inversion with equal volumes in invertase (1000 units/ml in 0.1 M acetate [pH 4.5]) for 3 h at 37°C. Levels were determined by reference to glucose standards and expressed as mg glucose per g fresh weight.

## RESULTS

**PGI Activity in the Mutant and Its Genetic Basis.** The immunological analysis of wild type plants revealed that about 29% of total cellular PGI activity was contributed by the plastid PGI, and 71% by the cytosolic PGI (Table I). A study in spinach, based on activities recovered following DEAE-cellulose chromatography, partitioned the activities as 35% plastid and 65% cytosolic (10).

The relative proportion of the two PGI activities differed in homozygous mutant plants. In them, the activity contributed by the plastid PGI was reduced by about half, but that contributed by the cytosolic PGI was unchanged (Table I). The result indicated the plastid PGI activity in the mutant accounted for 16% of total PGI.

Following electrophoresis of crude extracts, the homozygous mutant exhibited weak to no visible plastid PGI activity upon histochemical staining (Fig. 1). A similar faint band of activity also resulted when crude wild type extract was diluted in half by the addition of buffer. The result showed that failure to detect

Table I. Total PGI Activity and Subcellular Distribution in Wild Type and Homozygous Mutant

		Values represent the mean $\pm$ SE.		
	N <sup>a</sup>	Total Activity	Activity in Plastid Isozyme	Activity in Cytosolic Isozyme
IU/g fresh wt				
Wildtype	3	8.4	2.4 $\pm$ 0.2	6.0 $\pm$ 0.8
Mutant	5	7.3	1.2 $\pm$ 0.1	6.1 $\pm$ 0.8

<sup>a</sup> Number of replicates. Each replicate is the mean of three separate determinations.

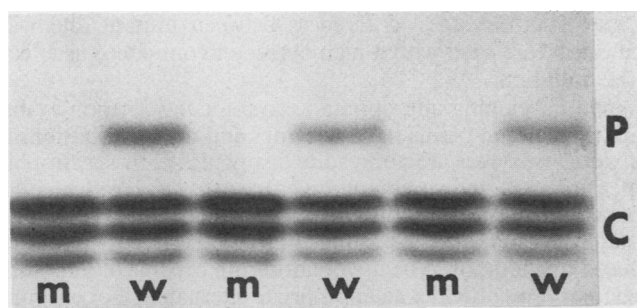


FIG. 1. PGI isozymes in wild type and mutant *C. xantiana*. Whole leaf extracts from the wild type (W) and mutant (M) were subjected to electrophoresis in a starch gel which was then sliced and stained for activity. The chloroplast isozyme (P) is the most anodal band at the top. The cytosolic PGI isozymes (C) in this species are encoded by duplicated genes and include two homodimers and a heterodimer with intermediate mobility.

Table II. Progeny Segregation from Self-Pollination of Backcross 5 Plants Heterozygous for Mutant Allele at Gene Encoding Chloroplast PGI

	Number Expected (out of 491 plants)	No. Observed	$\chi^2$
Wild type	123	120	0.07
Heterozygous for the mutation	245	250	0.10
Homozygous for the mutation	123	121	0.03

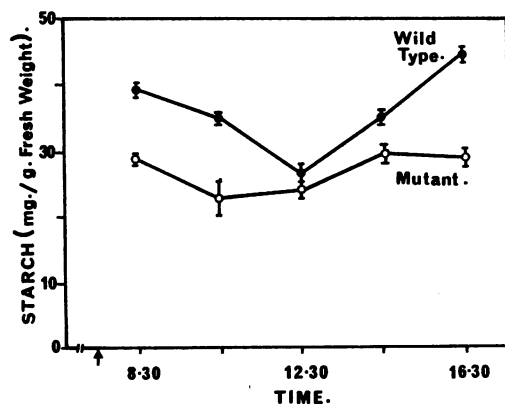


FIG. 2. Diurnal changes in leaf starch levels in wild type and mutant *C. xantiana*. The plants were grown in an 11-h photoperiod. The arrow indicates the start of the light period. The symbols represent the means of three replicates  $\pm$  1 SE.

enzyme activity in crude extracts by histochemical staining after electrophoresis need not indicate absence of enzyme. It may also be that the plastid PGI isozyme, which is more heat-labile than the cytosolic PGI (13), is more subject to degradation during the electrophoresis. The plastid PGI activity was readily visible on gels containing extracts of heterozygous plants.

Table II presents the segregation of the mutant in the progeny of self-pollinated heterozygous plants obtained in backcross 5. As expected, 1/4 were homozygous mutant, 1/2 were heterozygous, and 1/4 were homozygous wildtype ( $\chi^2=0.21$ ;  $p>0.75$ ).

**Chloroplast Starch Levels.** Since the plastid PGI isozyme catalyzes an essential reaction in the synthesis and degradation of starch, we assessed effects of the mutation on chloroplast starch levels. Initial studies compared the mutant and wild type at five time points during an 11 h light period (following 13 h of darkness). In the wild type, leaf starch level declined gradually after the onset of the light period, reached a minimum at midday, and rose steadily to a point above the morning level (Fig. 2). In

contrast, the mutant plants showed little fluctuation in starch level during the light period and exhibited lower values than wild type at every measurement. By the late afternoon, the mutants had accumulated only about 60% as much starch as wild type (Fig. 2).

To investigate these differences further, photosynthetic input was increased by subjecting the plants to a pretreatment of 72 h continuous illumination prior to measuring leaf starch. After the second measurement, wild type plants displayed a strong surge of starch accumulation, resulting in levels about 25% higher than those detected in plants growing under light-dark cycles (Fig. 3). The starch level in the mutant, however, was unaffected and remained similar to that previously recorded (approximately 30 mg/g fresh weight). Leaf sucrose levels were also measured in additional plants from the same experimental batch. Figure 4 shows that the mutant always accumulated more sucrose than wild type. Levels of intermediates (glucose, fructose, glucose-6-P, and fructose-6-P) were similar in both types (Fig. 4).

**Whole Plant Growth.** The effect of the mutation on several measures of whole-plant growth is summarized in Figure 5 which compares leaf and root weights. At all stages of development, the leaf/root ratio was higher in the wild type than in the mutant. The difference was almost entirely attributable to a marked reduction in mutant leaf weight to about 56% of that of wild

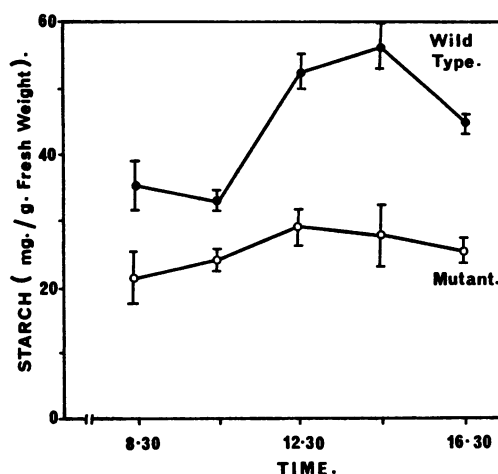


FIG. 3. Changes in leaf starch levels in wild type and mutant *C. xantiana* following a 72-h continuous light treatment. The symbols represent the means of three replicates  $\pm$  1 SE.

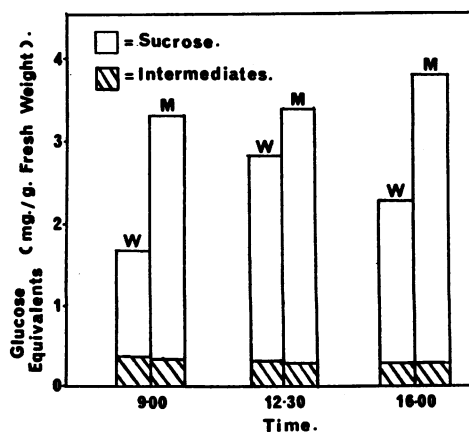


FIG. 4. Levels of sucrose and intermediates (glucose, fructose, glucose-6-P, fructose-6-P) in wild type and mutant *C. xantiana*. The histograms show the means of two replicates, each consisting of leaves from five plants.

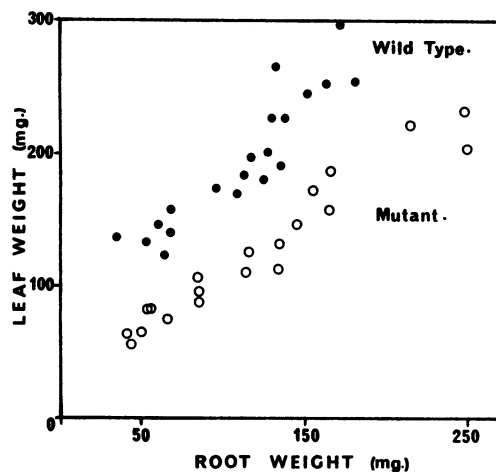


FIG. 5. Plot of leaf weight and root weight in wild type and mutant *C. xantiana*.

type. In contrast, root weight in the mutant was only slightly reduced ( $\leq 10\%$ ) relative to wild type.

### DISCUSSION

The mutation of the gene encoding the chloroplast PGI isozyme reduces its accumulated activity by 50% and appears to be responsible for reducing the level of chloroplast starch and increasing the level of sucrose in the leaf. Segregation analysis showed that the mutant is allelic to wild type. The lesion is probably in the structural gene although biochemical characterization remains incomplete. The changed levels of starch and sucrose are thought to be direct consequences of the mutation because they occur in a backcross line that is nearly isogenic to wild type. A further consequence of the mutation is a reduction in leaf weight.

Starch accumulation in the mutant was always less than that in wild type. Increasing photosynthetic input by exposing the mutant to continuous light did not increase its starch level. Rather, the presumed additional photosynthate was apparently exported into the cytosol where it was converted into sucrose, giving the mutant a higher sucrose level than found in wild type. The abundant sucrose permitted a near normal rate of root growth in the mutant which, otherwise, might have been adversely affected (3). These changes in metabolism and growth suggest that the wild type level of plastid PGI activity is necessary to achieve the carbohydrate status of wild type plants. The result is surprising since PGI has not been considered rate-limiting.

Although the activities of the cytosolic PGI isozymes are not affected by the mutation, our analysis does not address the question whether the metabolic changes reflect the reduction in plastid PGI level itself or the consequent change in the ratio of plastid to total PGI activity. It is particularly interesting that carbohydrate levels are not compensated by adjustments in the flux of other reaction steps.

The utilization of induced mutations to study the effects of particular reaction steps in carbohydrate and other metabolism is clearly worthwhile though few previous studies in higher plants have been carried out. Analyses of this type require that care be taken in isolating the effects of particular mutations and in assaying those effects. Attaining the former objective requires that a mutation of interest be backcrossed to wild type. This must be done for a number of generations to eliminate other mutations likely to have also been induced during the mutagenesis which could confound metabolic or physiological measurements. After five backcross generations, the mutation will be on a 98.4% wild type background. Under such nearly

isogenic circumstances, differences between mutant and wild type can be assigned with a high degree of confidence as effects of the mutation.

When developing appropriate assays, a consideration is that many enzymes in plants have isozymes and the contributions of individual isozymes are not readily distinguished by spectrophotometric activity measurements on crude extracts. Histochemical localization following resolution of an isozyme by electrophoresis in starch gels may also be insufficient when the activity is sharply reduced by mutation. The use of antiserum capable of inhibiting single isozymes provides an appropriate alternative. For enzymes of glycolysis, each of which is present as at least two isozymes in plants, one located in the plastids and the other in the cytosol (5), an analysis can be performed with antiserum against either isozyme (by direct inhibition or by subtraction of one activity from the total activity as in this paper).

Neglect of these issues can confound otherwise interesting results. For example, a recent claim that an EMS-induced loss of the chloroplast phosphoglucomutase isozyme was responsible for a near absence of starch (1) is questionable because additional mutations induced by the mutagenesis may have been fixed in the tested lines since they were advanced by self-fertilization and not carried through a backcross program. In principle, following self-fertilization, 1/4 of any heterozygous mutations induced by mutagenesis will be made homozygous each generation and, at the limit, each derived line is expected to be homozygous for 1/2 of the mutations originally present. Thus, mutagenized lines advanced by self-fertilization are not isogenic to wild type and, consequently, it is difficult to assign a particular mutation as the sole cause of an unusual or novel phenotype. Indeed, the starchless mutant described in Casper *et al.* (1) also exhibited large changes in the activities of three other enzymes, effects that may also have been induced by the EMS treatment. Regardless of the exact cause of the lack of starch in the phosphoglucomutase mutant (1), it displayed a marked increase in sucrose and hexose sugars similar to the plastid PGI mutant in *Clarkia*. Clearly, mutagenesis can induce sharp reductions and/or loss of particular enzyme activities and loss or reduced levels of major metabolic products. We believe that combined genetic and physiological studies can contribute substantial information to our understanding of plant metabolism.

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