Loss of Cytosolic Phosphoglucomutase Compromises Gametophyte Development in Arabidopsis[1][W][OA]

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Cytosolic phosphoglucomutase (cPGM) interconverts glucose-6-phosphate and glucose-1-phosphate and is a key enzyme of central metabolism. In this study, we show that Arabidopsis (*Arabidopsis thaliana*) has two *cPGM* genes (*PGM2* and *PGM3*) encoding proteins with high sequence similarity and redundant functions. Whereas *pgm2* and *pgm3* single mutants were undistinguishable from the wild type, loss of both PGM2 and PGM3 severely impaired male and female gametophyte function. Double mutant pollen completed development but failed to germinate. Double mutant ovules also developed normally, but approximately half remained unfertilized 2 d after pollination. We attribute these phenotypes to an inability to effectively distribute carbohydrate from imported or stored substrates (e.g. sucrose) into the major biosynthetic (e.g. cell wall biosynthesis) and respiratory pathways (e.g. glycolysis and the oxidative pentose phosphate pathway). Disturbing these pathways is expected to have dramatic consequences for germinating pollen grains, which have high metabolic and biosynthetic activities. We propose that residual cPGM mRNA or protein derived from the diploid mother plant is sufficient to enable double mutant female gametophytes to attain maturity and for some to be fertilized. Mature plants possessing a single *cPGM* allele had a major reduction in cPGM activity. However, photosynthetic metabolism and growth were normal, suggesting that under standard laboratory conditions cPGM activity provided from one wild-type allele is sufficient to mediate the photosynthetic and respiratory fluxes in leaves.

Flowering plants have specialized tissues to support the development of the haploid male and female gametophytes (the pollen and ovules, respectively) during sexual reproduction and subsequently the developing seeds. Understanding the route of import, metabolism, and utilization of the delivered carbohydrates is fundamental for understanding plant reproduction and may inform strategies for improving yield and nutritional value in our seed crops. The gametophytes are essentially heterotrophic, dependent on the carbohydrate supply from the parent plant. However, current knowledge about the pathways by which carbohydrates are imported and used to support gametophyte and subsequent seed development is rather fragmented.

Suc is the predominant carbohydrate transported in the phloem of most higher plants (including Arabidopsis [*Arabidopsis thaliana*]) and is the primary source of energy for developing gametophytes and seeds. In general, Suc can enter heterotrophic cells (Fig. 1) either via symplastic connection to neighboring cells or via plasma membrane-localized Suc transporters (Sauer, 2007). As male and female gametophytes are symplastically isolated, the former route is excluded. Inside the cell, Suc can be catabolized either by Suc synthases (Susy; which converts Suc and UDP to Fru and UDP-Glc) or by invertases (cytosolic or vacuolar, which hydrolyze Suc to Fru and Glc). Alternatively, Suc can be hydrolyzed in the apoplast by cell wall invertases and the hexoses imported via monosaccharide transporters (Williams et al., 2000). Once in the cytosol, both Glc and Fru can be phosphorylated by hexokinase generating Glc-6-P and Fru-6-P, respectively (Fru can also be phosphorylated by fructokinase; Fig. 1). These metabolites, together with Glc-1-P, constitute the hexose-phosphate pool and represent an important node in primary metabolism. The major respiratory pathways use Glc-6-P (e.g. oxidative pentose phosphate pathway) and Fru-6-P (e.g. glycolysis), while major biosynthetic pathways use Glc-1-P (e.g. cell wall polysaccharide synthesis). Two enzymes catalyze the reversible interconversion of the hexose phosphates; phosphoglucose isomerase interconverts Glc-6-P and Fru-6-P, while phosphoglucomutase (PGM) interconverts Glc-6-P and Glc-1-P. These enzymes are thought to maintain the hexose phosphates in equilibrium with one another as the fluxes through the major metabolic pathways change.

In plants, the family of PGMs can be divided into plastid-localized (pPGM) and cytosol-localized (cPGM) isoforms (Herbert et al., 1979; Gottlieb, 1982). pPGM produces Glc-1-P from Glc-6-P, which is either

[1] This work was supported by the Swiss National Science Foundation (Sinergia grant no. CRSI33–127506) and ETH Zurich.

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.165027

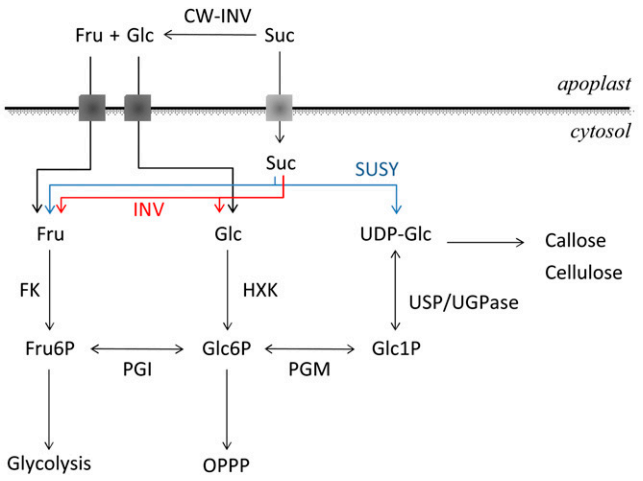

Figure 1. The pathways of Suc catabolism and the interconversion of components of the cytosolic hexose-phosphate pool. INV, Cytosolic invertase; CW-INV, cell wall invertase; HXK, hexokinase; FK, fructokinase; USP, UDP-sugar pyrophosphorylase; UGPase, UDP-Glc pyrophosphorylase; PGI, phosphoglucose isomerase; OPPP, oxidative pentose phosphate pathway.

imported from the cytosol (in heterotrophic tissues) or produced via photosynthesis (in autotrophic tissues). Glc-1-P serves as a substrate for ADP-Glc pyrophosphorylase, the first committed step in the starch biosynthesis pathway. If Glc-1-P is released from starch during degradation (i.e. by α-glucan phosphorylase; Steup, 1981), the net flux through pPGM would be in the direction of Glc-6-P production. Plants lacking pPGM have been widely studied. They have greatly reduced starch synthesis in leaves (Caspar et al., 1985; Hanson and McHale, 1988; Harrison et al., 1998, 2000; Fernie et al., 2001; Streb et al., 2009) and storage organs (Harrison et al., 1998; Tauberger et al., 2000). In contrast, the cPGMs have received relatively little attention, despite being located at a critical metabolic intersection. Cytosolic Glu-1-P is needed for sugar nucleotide (e.g. UDP-Glc) production, which serves as a precursor for the synthesis of Suc and cell wall constituents, including cellulose and callose (Koch, 2004). The only investigations of cPGMs were done with transgenic potato (*Solanum tuberosum*) lines in which cPGM was repressed using antisense constructs. In leaves, this led to a decreased rate of photosynthesis and many changes in metabolite levels, including slight decreases in Suc and starch. No changes in cell wall composition were observed (Fernie et al., 2002; Lytovchenko et al., 2002). However, no mutant entirely lacking cPGM activity has been reported for any plant species. It can be reasoned that abolishing cPGM activity would be lethal. If so, the effect may be seen first at the haploid gametophytic stage. Here, we show that the Arabidopsis genome encodes two highly redundant cPGMs and demonstrate the importance of this enzyme during the gametophyte development.

RESULTS

Identification of Genes Coding for cPGMs

Based on activity gel assays and cell fractionation studies, it has been reported that some plant species contain two cPGM isoforms (including tobacco and Arabidopsis; Hanson and McHale, 1988; Caspar et al., 1985), whereas others (e.g. spinach [*Spinacia oleracea*] and potato; Muhlbach and Schnarrenberger, 1978; Fernie et al., 2002) have only one. This is consistent with more recently available genome sequence information, indicating that some plant genomes contain duplicated PGM isoforms (Fig. 2). In Arabidopsis, it has not been shown whether the two cytosolic activities correspond to proteins encoded by different genes. We searched the DNA databases and the literature and found six genes predicted to encode PGMs in Arabidopsis (At1g23190, At1g70730, At1g70820, At4g11570, At5g17530, and At5g51820; Sergeeva et al., 2004; Keurentjes et al., 2008; Mentzen et al., 2008). The plastidial isoform, PGM1, is encoded by At5g51820 (Kofler et al., 2000; Periappuram et al., 2000). At1g23190 (*PGM3*) and At1g70730 (*PGM2*) have similar exon/intron structures (Fig. 3A) and reside on segmental duplications within the Arabidopsis genome (Arabidopsis Genome Initiative, 2000). They encode putative cytosolic isoforms with 91% sequence identity to each other and high similarity to plastidial isoforms and to other eukaryotic PGMs. The remaining three genes are similar to each other but share less sequence similarity with the other Arabidopsis PGMs (Fig. 2). Their closest homologs outside the chloroplastida are prokaryotic phosphohexose mutases.

We isolated two T-DNA insertion mutant lines for *PGM2* and one for *PGM3* (Fig. 3A) and confirmed that in each case, the transcript levels of the affected gene was absent (Supplemental Fig. S1). To determine which enzymatic activities these genes encode, proteins in crude extracts of leaves were separated by native PAGE and the gels stained for PGM activity. Three distinct bands can be separated in extracts of the wild type (Caspar et al., 1985; Fettke et al., 2008). Consistent with previous observations, the fastest migrating activity is attributable to chloroplastic PGM1, as it is missing in the *pgm1* mutant (Fig. 3B; Caspar et al., 1985). In *pgm2*-1 (and *pgm2*-2), the middle band was missing, whereas in *pgm3*-1 plants, the slowest migrating activity band vanished (Fig. 3B). We prepared leaf mesophyll protoplasts and subfractionated them into chloroplast and cytosol fractions. Native PAGE analyses revealed that PGM2 and PGM3 are intracellular but not chloroplastic (Fig. 3C). These data show that each of the three major bands of PGM activity can be assigned to a specific gene product. The other predicted phosphoglucomutase genes either encode minor isoforms, which cannot be detected in our analyses, or enzymes with different substrate specificities.

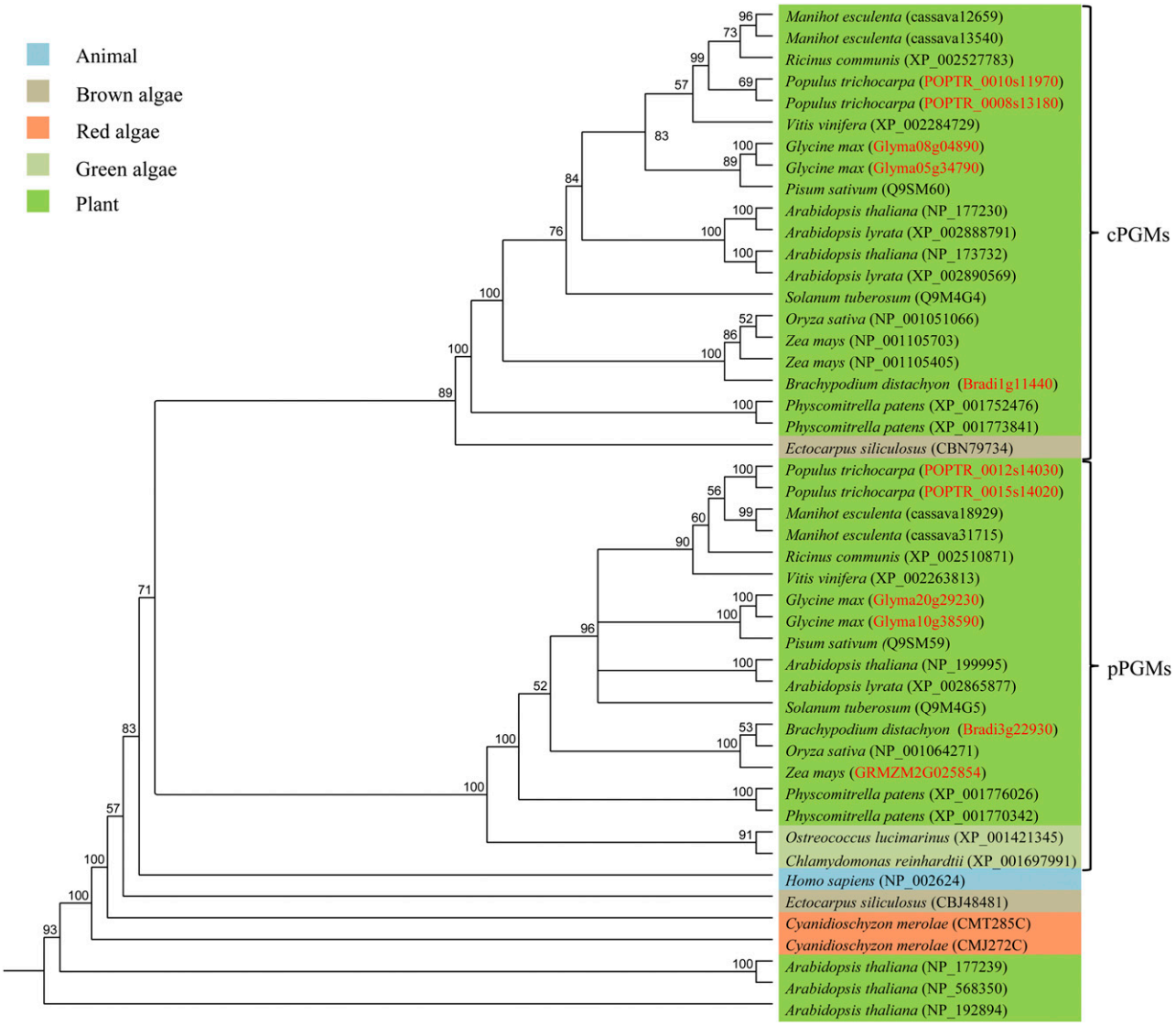

Figure 2. Unrooted phylogenetic tree of proteins annotated as PGMs from higher plants and algal species. Amino acid sequences were obtained from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov; black accession nos.) or from the Phytozome database (www.phytozome.net; red accession nos.). Full-length sequences were aligned and used to construct a phylogenetic tree based on the genetic Jukes-Cantor distance-based model. PGMs fall into two distinct clusters within the plant kingdom. One cluster contains Arabidopsis *PGM1* (NP_199995), known to be plastidial. The second cluster contains *PGM2* (NP_177230) and *PGM3* (NP_173732). All sequenced genomes analyzed contain at least one plastidial and one cytosolic PGM. Some species contain two closely related isoforms, indicating recent gene duplication events. The three Arabidopsis genes At1g70820 (NP_177239), At5g17530 (NP_568350), and At4g11570 (NP_192894) do not fall into either of these clusters, indicating that they may not code for PGMs. Numbers next to the branch points indicate bootstrap support for the robustness of the tree.

The *pgm2* and *pgm3* Single Mutant Phenotypes

We compared the wild type and the two *cpgm* mutants for several phenotypic parameters. No changes in shoot growth or flowering time of soil-grown *pgm2*-1 (and *pgm2*-2) or *pgm3*-1 mutant plants were observed compared to the wild type (Supplemental Fig. S2; data not shown). Root growth, visualized by growing plants on vertical agar plates, was also unchanged (data not shown). The starch content was slightly elevated in *pgm2*-1 and in *pgm3*-1 (Supplemental Fig. S3A). However, these differences were not substantiated in subsequent experiments (see below, Fig. 7, and Supplemental Fig. S5). The Suc content was not altered (Supplemental Fig. S3B). These data suggest that the two cPGMs are redundant such that, under our growth conditions, the loss of one does not affect plant fitness. Furthermore, publicly available transcript data suggest that both genes are expressed

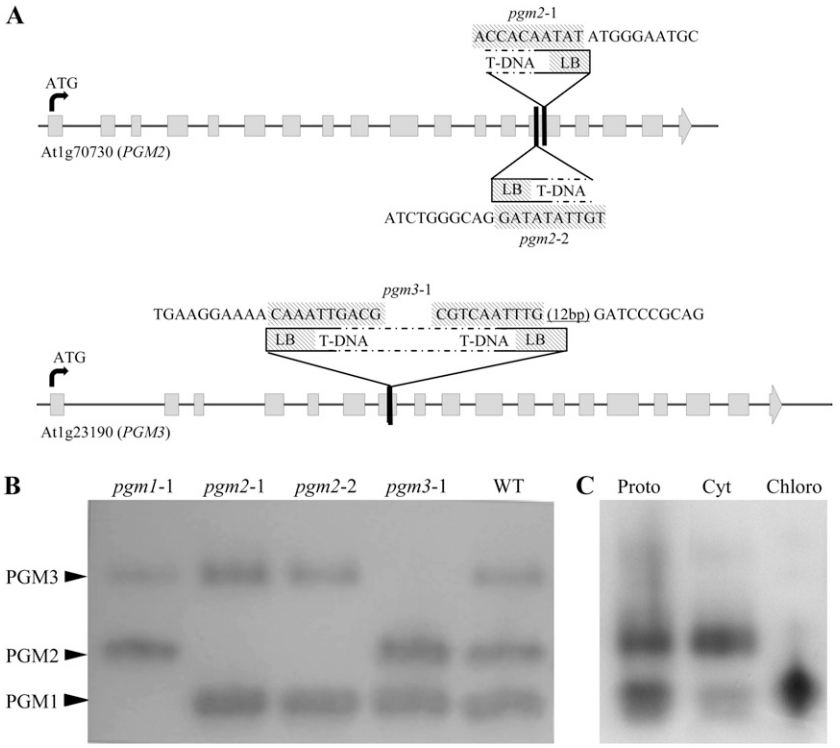

Figure 3. Gene structure and assignment of PGM activities to *PGM2* and *PGM3*. A, Gene structure of the cytosolic PGMs showing similar exon/intron structure and the same number of exons. The exons are shown as boxes, and both genes are at the same scale. Triangles illustrate T-DNA insertion sites in exons of *PGM2* and *PGM3* in the mutant lines. T-DNA-specific sequences at the left border (LB) are given above (hatched boxes). The following sequence is gene specific. In the case of *pgm3*-1, the T-DNA has two left borders, suggesting a tandem inverted repeat. B, PGM isoform separation by native PAGE, followed by enzyme activity staining. Three distinct activities are visible. In each of the single mutants, one specific enzyme activity is completely missing. The band with the lowest mobility is assigned as PGM3, that with the highest mobility as PGM1, and the activity in between as PGM2. WT, Wild type. C, Protein localization of the PGMs in wild-type leaf extract. All three enzymes are contained in isolated protoplast (Proto). Protoplast fractionation into cytosol-enriched (Cyt) and chloroplast-enriched (Chloro) fractions shows PGM1 to be plastidial, whereas PGM2 and PGM3 are cytosolic.

ubiquitously, though not always at the same level (Supplemental Fig. S4A). We performed native PAGE on extracts of leaf, root, and developing seeds. In all cases, the three PGM activities were present (Supplemental Fig. S4B).

To test the hypothesis that cPGMs catalyze an essential enzymatic step in primary carbohydrate metabolism, we attempted to generate double mutants lacking PGM2 and PGM3 by crossing *pgm2*-2 and *pgm3*-1 single mutants (hereafter named *pgm2* and *pgm3* for simplicity). Although both genes are present on chromosome one, the large genetic distance (approximately 70 centimorgans) between them should ensure a relatively high rate of recombination between the two genes. However, we were not successful in identifying double mutant plants in the F2 generation, and only rarely were plants homozygous for one mutation and heterozygous for the other obtained (either *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3*). We genotyped the offspring of self-pollinated *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* plants. Again, no double mutants were found and segregation was strongly distorted in favor for the single mutants (Table I). Furthermore, we tested the seed germination rate but found it comparable to that of the wild type (*pgm2pgm2/PGM3pgm3*, 98%, $n = 384$; *PGM2pgm2/pgm3pgm3*, 98%, $n = 385$; wild type, 99%, $n = 403$). The isolation of the *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* genotypes revealed that the double mutant alleles can be passed through at least one gametophytic stage. However, the reduced transmission of the mutant alleles (Table I) suggests a gametophytic or a seed defect. We considered it possible that male or female double mutant gametophyte may be nonviable and that the double mutant combination causes embryo lethality.

Reduced Transmission of *pgm2* and *pgm3* Double Mutant Combinations through Male and Female Gametes

In siliques of self-pollinated *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3* mutants, we detected 35% and 30% undeveloped seeds, respectively (the mean of three experiments; Table II; Fig. 4A). To determine the cause of this phenotype, we performed reciprocal crosses of *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* mutants to wild-type plants. The number of successfully developing seeds per silique when using the mutant pollen did not differ from the control crosses (Table III), and all seeds were able to germinate. However, when genotyping the progeny, we failed to detect plants containing both mutant alleles, revealing that transmission of the double mutant *pgm2/pgm3* through pollen did not occur (Table III). By contrast, when pollinating *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3* mutants with wild-type pollen, about one-third of the ovules remained undeveloped, indicating that the double mutant combination impairs female gametophyte development (Table III). However, transmission analysis revealed that about 22% of the female gametophytes lacking both *PGM2* and *PGM3* were able to complete development and gave rise to viable seeds after fertilization (Table III). This suggests that the requirement for functional *PGM2* and *PGM3* genes in female gametophytes can be bypassed.

Table I. *Distorted segregation in the offspring of self-pollinated PGM2pgm2/pgm3pgm3 and pgm2pgm2/PGM3pgm3 plants*

Genotype frequencies among the progeny of self-fertilized *PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3* plants. *n*, Number of plants genotyped by PCR as described in "Materials and Methods."

Genotype Parent Plant	Observed/Expected Genotype of Progeny in %		
PGM2pgm2/pgm3pgm3	*PGM2pgm2/pgm3pgm3* 18/50 (*n* = 297)	*pgm2pgm2/pgm3pgm3* 0/25 (*n* = 297)	*PGM2PGM2/pgm3pgm3* 82/25 (*n* = 297)
pgm2pgm2/PGM3pgm3	*pgm2pgm2/PGM3pgm3* 21/50 (*n* = 277)	*pgm2pgm2/pgm3pgm3* 0/25 (*n* = 277)	*pgm2pgm2/PGM3PGM3* 79/25 (*n* = 277)

Impaired Fertilization of *pgm2pgm3* Female Gametophytes

We did not detect morphological differences between ovules of *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3* mutants, even though half of them are expected to be double mutant for *pgm2* and *pgm3* (Fig. 4, B and C). This implied normal development up to the mature gametophyte stage, irrespective of the number of functional *cPGM* alleles present. Nevertheless, the loss of cPGM may render the double mutant ovules less viable, despite appearing morphologically intact. Alternatively, if all the double mutant ovules are fertilized, a fraction of the embryos derived from the double mutant ovules may abort thereafter. To distinguish between these possibilities, we investigated a series of self-pollinated siliques of the wild type and of *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* mutants 2 d after pollination. Wild-type embryos were at the two- to four-cell stage, whereas seeds in siliques of the mutants could be classified into three fractions: wild-type-like seeds containing a visible embryo and syncytial endosperm, unfertilized ovules, and collapsed ovules that likely aborted without being fertilized (Fig. 4, D–F). The frequency of wild-type-like embryos and unfertilized ovules at this early stage (Fig. 4G) resembled the distribution of developed and undeveloped seeds seen previously in the mature siliques (Table II). Therefore, we conclude that double mutant ovules, despite appearing normal before pollination, are less viable and/or less likely to be fertilized than those containing a functional *cPGM* gene. Those double mutant ovules that are fertilized and develop into seeds are presumably rescued by the wild-type c*PGM* allele transmitted by the pollen.

Pollen Germination Requires cPGM Activity

Transmission failure of the double mutant combination through pollen might result from dysfunctional pollen development, dysfunctional pollen tube germination or growth, or an inability to fertilize the ovule (Johnson et al., 2004). We used light microscopy to examine the morphology of mature pollen grains. Both mutants (*pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3*) produced mature pollen indistinguishable from that of the wild type (and the single mutant) in respect of shape and size. The 4′,6-diamino-phenylindole (DAPI) staining showed a normal tricellular stage, with two sperm nuclei and one vegetative nucleus (Fig. 5A). Furthermore, testing pollen viability by Alexander staining (Alexander, 1969) revealed that all mature pollen had intact protoplasm (Fig. 5B), suggesting that pollen development was not impaired by lack of PGM2 and PGM3 function. We tested the germination ability of pollen using in vitro germination experiments and observed that 81% of wild-type pollen (*n* = 370) but only 37% of the *pgm2pgm2/PGM3pgm3* (*n* = 902) and 38% of *PGM2pgm2/pgm3pgm3* (*n* = 847) pollen produced pollen tubes. Similar results were obtained in three separate experiments. These data, together with the backcrossing experiments described above, suggest that only the pollen carrying at least one functional *cPGM* gene produced pollen tubes, explaining why no *pgm2pgm2/pgm3pgm3* double mutant plants were identified.

cPGM Activity Is Not Limiting for Carbon Metabolism

The interconversion of Glc-6-P and Glc-1-P is probably an essential step in sporophytic as well as game-

Table II. *Undeveloped seeds in siliques of the wild type and pgm mutants*

Mutants have two or one functional *cPGM* (*PGM2* or *PGM3*) alleles remaining. The percentages of undeveloped seeds are given for each genotype from three different experiments. *n*, Number of seeds counted; n.d., not determined.

Genotype of Parent Plant	% of Undeveloped Seeds (Seeds Examined)			
	Experiment 1	Experiment 2	Experiment 3	Mean
PGM2pgm/2pgm3pgm3	27 (*n* = 463)	32 (*n* = 170)	31 (*n* = 691)	30 (*n* = 1,324)
pgm2pgm2/PGM3pgm3	30 (*n* = 453)	34 (*n* = 169)	38 (*n* = 627)	35 (*n* = 1,249)
pgm2pgm2/PGMPGM3	6 (*n* = 448)	n.d.	n.d.	6 (*n* = 448)
PGM2PGM2/pgm3pgm3	4 (*n* = 353)	n.d.	n.d.	4 (*n* = 353)
Wild type	2 (*n* = 247)	1 (*n* = 188)	3 (*n* = 692)	2 (*n* = 1,127)

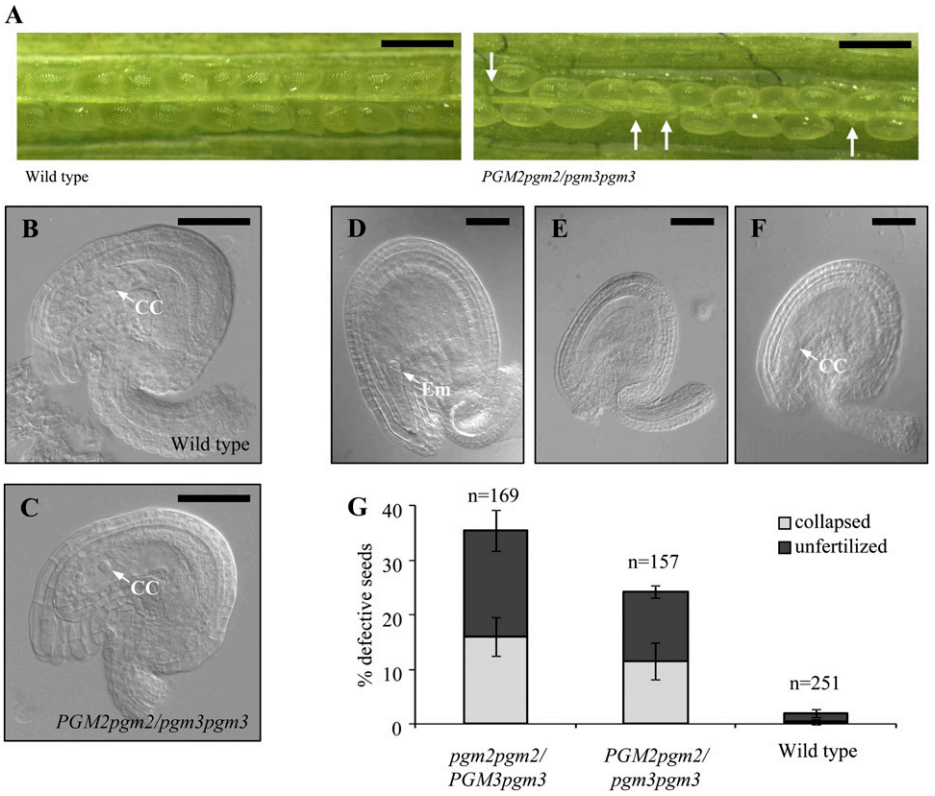

Figure 4. Seed and ovule development in the wild type compared to *PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3* mutants. Results for *PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3* mutants were the same. Only *PGM2pgm2/pgm3pgm3* is shown. A, Siliques of the wild type and the *PGM2pgm2/pgm3pgm3* mutant. White arrows indicate aborted seeds. Bars = 1 mm. B, Representative light microscopy picture of unfertilized ovules of the wild type. CC, Central cell. C, Representative light microscopy picture of unfertilized ovules of the *PGM2pgm2/pgm3pgm3* mutant. Ovules from *pgm2pgm2/PGM3pgm3* mutants were indistinguishable from the other two genotypes. D to F, Seeds of self-pollinated *PGM2pgm2/pgm3pgm3* mutants 3 d after pollination were categorized into normal wild-type-like (D), collapsed (E), and unfertilized (F) seeds. Em, Embryo. *pgm2pgm2/PGM3pgm3* mutants showed essentially a comparable distribution into the same categories. In B to F, bars = 50 μm. G, Quantification of the percentage of wild-type-like and defective seeds (as shown in D–F) in the three genotypes. n, Number of seeds counted.

tophytic metabolism. To test whether cPGM levels exert any control over the carbon flux from Calvin cycle-derived sugars to Suc, we performed cPGM gene dosage experiments, analyzing plants with only one functional copy of either *PGM2* or *PGM3*. First, we tested if the mutants lacking either *PGM2* or *PGM3* show a compensatory transcriptional response (i.e. up-regulation of the remaining *PGM* gene transcript or increased PGM activity). Quantitative reverse transcription-PCR did not reveal substantial transcriptional changes (Supplemental Fig. S1). We also examined the activities of the different PGM isoforms in the single mutants, in *pgm2pgm2/PGM3pgm3*, and in *PGM2pgm2/pgm3pgm3* using native PAGE. This was possible as staining intensity changed linearly in relation to protein amount and incubation time (data not

Table III. *Results of reciprocal crosses of PGM2pgm2/pgm3pgm3 and pgm2pgm2/PGM3pgm3 with wild-type plants*

The percentages of undeveloped seeds given as a percentage of the total seeds, corrected by subtracting the observed percentage of undeveloped seeds in manually pollinated wild type. For comparisons with self-pollinated plants, see Table II. Simultaneous transmission of both mutant alleles (*pgm2* and *pgm3*) to the offspring, detected by genotyping the offspring, is given as a percentage of the plants analyzed. *n*, Number of seeds and ovules counted or plants genotyped; n.d., none detected.

Parental Genotypes (Female × Male)	% Undeveloped Seeds (Seeds Examined)	% Transmission Observed/Expected (Plants Genotyped)
PGM2PGM2/PGM3PGM3 × *PGM2pgm2/pgm3pgm3*	0 (*n* = 189)	0/50 (*n* = 152)
PGM2PGM2/PGM3PGM3 × *pgm2pgm2/PGM3pgm3*	0 (*n* = 206)	0/50 (*n* = 104)
PGM2pgm2/pgm3pgm3 × *PGM2PGM2/PGM3PGM3*	28 (*n* = 329)	22/50 (*n* = 228)
pgm2pgm2/PGM3pgm3 × *PGM2PGM2/PGM3PGM3*	38 (*n* = 249)	23/50 (*n* = 119)

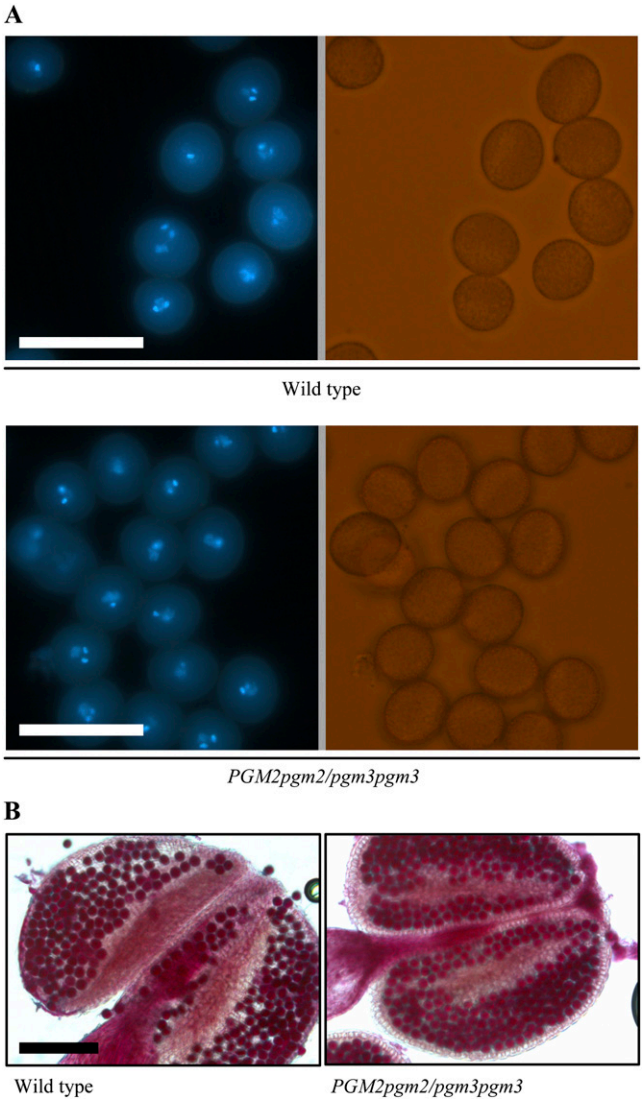

Figure 5. Normal pollen phenotype of *PGM2pgm2/pgm3pgm3* plants. Results for *PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3* mutants were the same. Only *PGM2pgm2/pgm3pgm3* is shown. A, Fluorescence of mature pollen nuclei stained with DAPI (left) and differential interference contrast picture (right) of the same pollen. All pollen has one vegetative nucleus and two sperm nuclei, irrespective of the genotype. Bars = 50 μm. B, Alexander staining for viability of mature pollen. Viable pollen stains purple, whereas dead pollen would stain green. All pollen stained purple, irrespective of the genotype. Bar = 200 μm.

shown). The activities correlated with the number of wild-type alleles present (Fig. 6). Compared with the wild type, the calculated residual cPGM activities were 53% in *pgm2*, 35% in *pgm2pgm2/PGM3pgm3*, 56% in *pgm3*, and 23% in *PGM2pgm2/pgm3pgm3*. Again, there was no obvious compensatory effect for the loss of one cPGM on the activity of the remaining cPGM.

We took advantage of the strongly reduced cPGM activity in the *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* mutants to elucidate the possible effects on primary carbohydrate metabolism in mature plants. Previous studies showed that down-regulating or abolishing enzymes or transporters involved in converting Calvin cycle products to Suc decreases Suc synthesis and increases partitioning of photoassimilates into starch. Examples include the mutation of the plastidial triose phosphate/Pi translocator (Schneider et al., 2002; Walters et al., 2004) and repression of either cytosolic Fru-1,6-bisphosphatase or Suc phosphate synthase (Strand et al., 2000). As cPGM resides in the same pathway, we measured Suc, Glc, and Fru in the middle of the light period. Interestingly, no significant changes were observed between the wild type and the different mutants (Fig. 7A). Next, we measured starch content in all mutants at three stages during plant

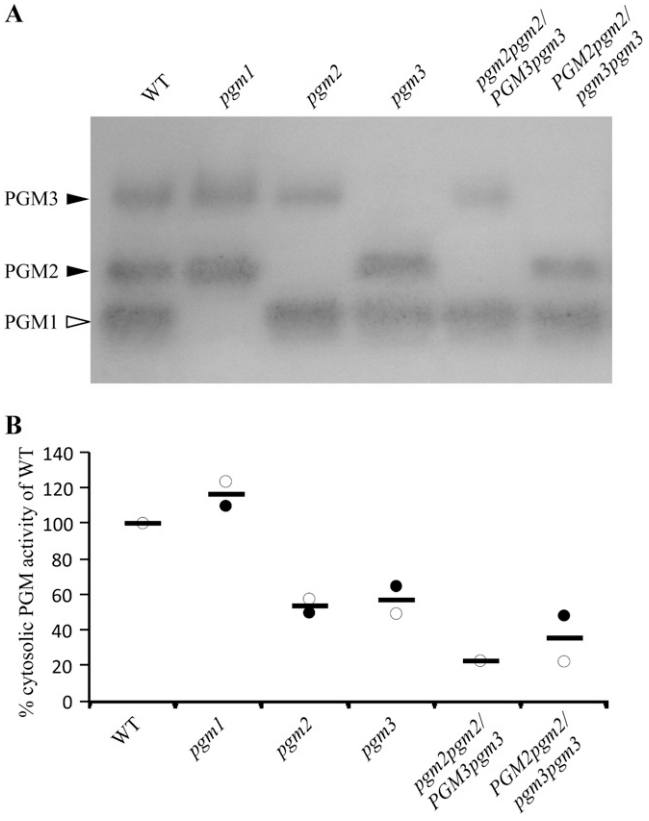

Figure 6. Total cPGM activity correlates to the number of wild-type (WT) alleles. A, Proteins were separated by native PAGE and stained for PGM activity. Homozygous single mutants (*pgm1*, *pgm2*, and *pgm3*) lack the corresponding enzyme activity completely, whereas plants with one functional allele (*PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3*) have around half the activity of the corresponding single mutants in which two functional allele remains. B, Total cPGM activity (PGM3 plus PGM2) was quantified based on mean pixel density (see "Materials and Methods") and expressed as percentage of the wild type. Two independent experiments (first, white circles; second, black circles) gave similar results. The mean of both experiments is shown as a horizontal black bar. Homozygous single mutants have about half the wild-type cPGM activity. Plants with only one functional *cPGM* allele have further reduced activity.

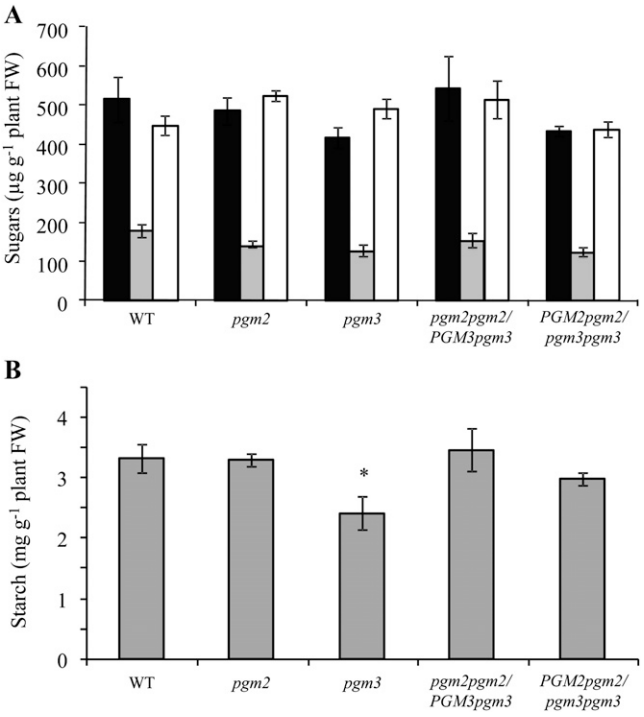

Figure 7. Effect of reduced cPGM activity on sugar and starch content in the leaf. Five-week-old plants were harvested 4 h into their 12-h photoperiod, and starch and sugar contents were determined. Homozygous single mutants (*pgm2* and *pgm3*) and plants with one functional allele (*PGM2pgm2/pgm3pgm3* and *pgm2pgm2/PGM3pgm3*) were analyzed. Values are the means of four biological replicates ± SE. FW, Fresh weight. Asterisks indicate values that differ significantly from the wild type (WT; Student's *t* test; $P \leq 0.05$). A, Glc (black bars), Fru (gray bars), and Suc (white bars) content in different mutants lacking PGM2 and PGM3. B, Starch content from the same plants as used for sugar measurements in A. The starch content of *pgm3* was slightly reduced. However, when considered together with other experiments (see Supplemental Figs. S3 and S5), no consistent changes were observed between mutant and wild-type plants.

growth and at different time points during the diurnal cycle. No consistent changes were detected in any mutant compared to the wild type (Fig. 7B; Supplemental Figs. S3 and S5).

It is possible that carbon fluxes in the mutants may differ from the wild type, even if the absolute amounts of sugars and starch remain unchanged. For example, in the triose phosphate/Pi translocator mutant, photoassimilates that cannot be exported to the cytosol are redirected into the starch pool and then released from the starch through simultaneous starch degradation (Schneider et al., 2002). This allows export of carbon from the chloroplast during the day in the form of Glc or maltose (Weber et al., 2000; Niittylä et al., 2004) circumventing the block in triose phosphate export. Therefore, we determined the carbon flux into neutral compounds (including the sugars Suc, Glc, and Fru) and starch using short-term $^{14}CO_2$ radiolabeling experiments. However, labeling single leaves of intact, mature plants revealed no differences in partitioning into neutral sugars and starch between mutants and the wild type (Supplemental Fig. S6). Overall, these data suggest that cPGM activity exceeds by far the minimum level required under laboratory growth conditions. It is likely that further reduction would lead to dramatic changes in plant fitness or even prevent plant survival, as the failure to interconvert Glc-1-P and Glc-6-P is predicted to have serious negative implications for metabolism.

DISCUSSION

Identification of Genes Encoding Active PGMs

Here, we showed that the Arabidopsis genome encodes three active PGMs: one plastidial (*PGM1*, At5g51820) and two cytosolic isoforms (*PGM2*, At1g70730; *PGM3*, At1g23190). In databases and the literature, three other genes (At1g70820, At4g11570, and At5g17530) have been annotated as encoding PGMs (Sergeeva et al., 2004; Keurentjes et al., 2008; Mentzen et al., 2008), but our experiments do not support this, as all three PGM activities detected using native PAGE can clearly be associated to *PGM1*, *PGM2*, and *PGM3*. Furthermore, At1g70820 and At5g17530 are predicted to be located in the plastid (Zybailov et al., 2008). If they were functional PGMs and could catalyze the conversion of Glc-6-P to Glc-1-P, mutants lacking PGM1 should not be as strongly impaired in starch synthesis as they are; *pgm1* mutants contain very little residual starch (0.3% of the wild type; Streb et al., 2009). Therefore, we conclude that PGM1, PGM2, and PGM3 are the only enzymes in Arabidopsis able to catalyze the conversion of Glc-6-P to Glc-1-P at physiologically relevant rates.

The presence of two genes encoding cPGMs is not ubiquitous within the plant kingdom. Some sequenced plant genomes encode only one cPGM (e.g. rice [*Orzya sativa*] and grapevine [*Vitis vinifera*]; see Fig. 2). Our analysis of knockout mutants indicate that the two isoforms in Arabidopsis are functionally redundant, as single mutants were indistinguishable from the wild type, and the mutant combinations *pgm2pgm2/PGM3pgm3* and *PGM2pgm2/pgm3pgm3* showed similar defects in male and female gametophyte function. Both genes seem ubiquitously expressed, and both enzyme activities were detected in the tissues we examined. However, we cannot exclude that each isoform may have a specific role under some growth conditions. In cases where gene duplication does not result in an advantage, mutations resulting in dysfunction of one the genes will not affect plant performance. Indeed, mining publicly available microarray data revealed that the Cape Verde Island Arabidopsis accession (Cvi) expresses only one of its two *cPGM* genes (*PGM3*; Lempe et al., 2005), resulting in only one detectable cPGM activity (Supplemental Fig. S7). Thus, either there was no advantage to retain both gene copies specifically in Cvi or the gene duplication

event in Arabidopsis was so recent that the sporadic mutations have affected the cPGM genes in some accessions but not others. We favor the latter hypothesis as many plants appear to have just one *cPGM* gene.

Function of cPGM in Primary Carbon Metabolism

In photosynthetic tissues, the main role of cPGMs is probably to convert Glc-6-P (derived from photoassimilates) to Glc-1-P, which will mainly be used to generate nucleotide sugars, such as UDP-Glc. This can be catalyzed either by UDP-Glc pyrophosphorylase or UDP-sugar pyrophosphorylase, both of which will also catalyze the reverse reaction (Sheu and Frey, 1978; Kotake et al., 2004). Nucleotide sugars are the substrates for several essential reactions, such as Suc, cell wall, and callose synthesis (Koch, 2004). The extent of the flux through cPGM will depend on the fate of the assimilated carbon, which in turn will be affected by the growth stage of the tissue. By contrast, heterotrophic tissues rely on imported Suc, which can be converted into hexose phosphates by different enzymes (Fig. 1). Here, the flux through cPGM will depend on the pathway of Suc catabolism. If Suc is catabolized by invertases, the entire flux through cPGM will be in the direction of Glc-1-P. By contrast, if Suc is catabolized by Susy, up to half of the imported carbon will reach the hexose-phosphate pool via Glc-1-P (some UDP-Glc formed by Susy may be used directly in biosynthesis). In either case, cPGM will maintain Glc-6-P and Glc-1-P in equilibrium to ensure that the different hexose phosphate-using pathways are all supplied, regardless of changes in their respective fluxes. The effect of the *cpgm* mutations on gametophyte development demonstrates that cPGM plays an important or essential role in heterotrophic cells. However, mutants with one functional *cPGM* allele had normal gametophytic growth, consistent with the idea that cPGM activity is not limiting.

Interestingly, greenhouse-grown transgenic potato lines with reductions in cytosolic PGM activity of between 61% and 85% exhibited reduced plant growth, reduced tuber yield, and altered metabolite levels (Fernie et al., 2002; Lytovchenko et al., 2002). Although we observed a comparable reduction in activity (*PGM2pgm2/pgm3pgm3* has a 77% reduction in activity), there was no effect on vegetative growth. It seems likely that if activity were reduced further in Arabidopsis (e.g. via gene silencing approaches), metabolism would be affected and plant growth would be impaired. It is also possible that under growth conditions different to the ones used in this study, a single functional *cPGM* allele could also limit growth.

cPGM in Male Gametophyte Development and Pollen Germination

We showed that *pgm2/pgm3* double mutant pollen does not fertilize ovules, most likely due to an inability to germinate and establish pollen tubes. During this process, the rate of cell growth is enormous (5.3 μm min^{-1}; Wilhelmi and Preuss, 1996). The plasma membrane and cell wall have to be constantly synthesized, requiring a large amount of energy, reducing power, and precursors for biosynthesis. The resources for respiration and growth are either remobilized inside the pollen or imported. In Arabidopsis pollen, there is evidence that Suc import is necessary. Mutating the SUC1 Suc transporter, which is expressed at a late stage in mature pollen and in the pollen tube, reduces pollen germination (Sivitz et al., 2008). It is plausible that abolishing cPGM prevents the generation of enough Glc-1-P, restricting UDP-Glc production and, therefore, cell wall biosynthesis. This would be especially true if invertase rather than Susy is responsible for the metabolism of the imported Suc. Given that mutants lacking the four major soluble isoforms of Susy do not have a reported defect in fertility (Barratt et al., 2009), invertases are probably responsible. Therefore, the products of Suc catabolism would enter the hexose-phosphate pool as Glc-6-P or Fru-6-P, but not Glc-1-P. The impact on Arabidopsis pollen function of blocking subsequent steps in metabolism or cell wall biosynthesis itself has been demonstrated. First, mutants lacking both UDP-Glc pyrophosphorylases (1 and 2) are male sterile (Park et al., 2010). Second, mutants lacking components of the cellulose synthase complex are impaired in pollen development and are also male sterile (Persson et al., 2007). Third, mutations in several callose synthase (GSL) genes result in male sterility (e.g. GSL2, Dong et al., 2005; GSL1 and GSL5, Enns et al., 2005; GSL8 and GSL10, Töller et al., 2008), affecting different stages of pollen development.

Despite the effect on pollen germination, the *pgm2/pgm3* double mutant pollen develops normally to maturity. During these steps, it is also likely that invertase rather than Susy is responsible for Suc metabolism. Repression of cell wall invertase isoforms has been shown to disrupt pollen development (Goetz et al., 2001; Hirsche et al., 2009). Thus, Suc would be cleaved outside the cell and the resulting monosaccharides imported, again raising the question as to how Glc-1-P is synthesized. Theoretically, there are indirect pathways for Glc-1-P production. For example, Glc-6-P could be imported into plastids and support starch synthesis via the pPGM (Niewiadomski et al., 2005; Tsai et al., 2009; Kunz et al., 2010). Maltose derived from subsequent starch breakdown and exported from the plastid can be partially converted to heteroglycans by the cytosolic glucosyltransferase DPE2 (Chia et al., 2004). Cytosolic phosphorylase can then release Glc-1-P from the heteroglycan (Fettke et al., 2008). Although some starch does accumulate transiently during Arabidopsis pollen development (Kuang and Musgrave, 1996), there is no evidence that the partway outlined above operates, and we doubt that this would efficiently supply Glc-1-P. A simpler explanation is that during pollen development, *cPGM* mRNAs and proteins produced in the

diploid, heterozygous pollen mother cell are passed on to the pollen during meiosis. Thus, although the pollen does not carry a functional cPGM gene, the inherited protein (or mRNA) may be sufficient for Glc-1-P production during development, resulting in viable pollen. This hypothesis seems plausible as our experiments revealed that cPGM activity is present in excess. However, the inherited mRNAs or proteins are unlikely to be sufficient for the increased metabolic demands associated with pollen germination and pollen tube growth.

cPGM in Female Gametophyte and Seed Development

In contrast with the male gametophyte, *pgm2/pgm3* double mutant alleles can be partially transmitted through the female gametophyte. Ovules of *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3* plants developed normally, and around half of the expected number of double mutant ovules were fertilized and developed to mature seeds. The transmitted wild-type cPGM allele from the pollen is presumably sufficient for all further metabolic processes. Although the route of carbohydrate import and metabolism in Arabidopsis female gametophytes and seeds are poorly understood, the same arguments posed above for developing pollen can be applied to the developing female gametophyte. It is conceivable that the metabolic demands for female gametophyte development is small compared with pollen during tube growth and that inherited cPGM is sufficient to interconvert Glc-6-P and Glc-1-P, at least initially. However, we propose that cPGM activity is close to a threshold level, below which the gametophytes become nonviable. This threshold could be influenced by environmental and developmental factors, which could explain why the fraction of gametophytes of *pgm2pgm2/PGM3pgm3* or *PGM2pgm2/pgm3pgm3* that failed to give rise to seeds varied slightly between experiments (Table II).

To conclude, our data revealed an essential function of cPGM activity for male and female gametophyte development. We suggest that complete loss of cPGM activity during vegetative development would also have serious negative implications for metabolism and dramatically reduce plant fitness or even prevent plant survival.

MATERIALS AND METHODS

Growth Conditions and Plant Material

Arabidopsis (*Arabidopsis thaliana*) plants, ecotypes Columbia, Landsberg *erecta*, and Cvi, were grown in a nutrient rich, medium-grade, peat-based compost at 20°C, 70% relative humidity, with a 12-h photoperiod in a Percival AR95 growth chamber (CLF Plant Climatics). Light intensity was uniform at 150 μmol quanta m^{-2} s^{-1}. Seed were sown out by hand. After seedling establishment, individual plants were transferred into 200-mL pots. The mutations in At1g70730 (*PGM2*) and At1g23190 (*PGM3*) result from T-DNA insertions in exons (Fig. 3A). The mutation in At5g51820 (*PGM1*) is a single base substitution in exon 7 leading to a premature stop codon (Periappuram et al., 2000). Primers used for identification of *pgm2*-1 (Salk_146133), *pgm2*-2 (Salk_053655), *pgm3*-1 (Salk_034720), and *pgm1*-1 (TC75) are given in Supplemental Table S1. All mutants used in this study were in Columbia background.

Protein Extraction and Analysis of PGM Activity by Native PAGE

Plant material was collected and immediately homogenized in ice-cold extraction medium containing 100 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 100 mM KCl, 42 mM β-mercaptoethanol, and 15% (v/v) glycerol (250 mg plant mL^{-1} extraction medium). Proteins were separated in nondenaturing polyacrylamide gels containing 6% (w/v) acrylamide (30:0.8 acrylamide:bis-acrylamide) and 375 mM Tris-HCl, pH 8.8. The stacking gel contained 3.75% (w/v) acrylamide and 125 mM Tris-HCl, pH 6.8. After separation, the gels were washed in 50 mM Tris-HCl, pH 7.0, and 5 mM $MgCl_2$ for 1 min and then incubated in staining solution containing 50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 5.3 mM Glc-1-P, 0.25 mM NADP, 0.25 mM NAD, 0.1 mM phenazine methosulfate, 0.25 mM nitroblue tetrazolium, and 40 units Glc-6-P dehydrogenase at 37°C until bands appeared. Band density was quantified using Adobe Photoshop CS4 and corrected for the background.

Starch and Sugar Measurements

Samples comprising all the leaves of individual rosettes were frozen in liquid N_2 and extracted in ice-cold 1.12 M perchloric acid as described by Delatte et al. (2005). The insoluble material was washed once with water and three times with 80% (v/v) ethanol to remove residual soluble glucans and pigments. Starch in the insoluble fraction was digested with α-amylase (pig pancreas; Roche) and amyloglucosidase (*Aspergillus niger*; Roche) and the released Glc measured spectrophotometrically, as described previously (Smith and Zeeman, 2006). Sugars (Glc, Fru, and Suc) in the soluble fraction were determined using high performance anion-exchange chromatography with pulsed amperometric detection. Volumes of the soluble fraction containing the equivalent of 5 mg plant fresh weight were spiked with 5 nmol cellobiose as an internal standard and applied to sequential 1.5-mL columns of Dowex50W and Dowex1 (Sigma-Aldrich). Neutral sugars were eluted with 4 mL of water, lyophilized, and redissolved in 100 μL of water. Sugars were separated on a CarboPac PA-20 column from Dionex with the following conditions: eluent A, 100 mM NaOH; eluent B, 150 mM NaOH and 500 mM sodium acetate. The flow rate was 0.5 mL min^{-1}. The gradient was 0 to 15 min, 100% A (monosaccharide elution); 15 to 26.5 min, linear gradient to 20% A and 80% B (disaccharide elution); 26.5 to 32.5 min, 20% A, 80% B (column wash step); and 32.5 to 40 min step to 100% A (column reequilibration). Peaks were identified by coelution with known Glc, Fru, Suc, and cellobiose standards. Peak areas were determined using Chromeleon software. All peaks were normalized to the internal cellobiose standard, and the amount of each was calculated based on standard curves of the pure standards run in parallel.

Pollen Germination

The protocol was adapted from Boavida and McCormick (2007). Filter-sterilized liquid pollen germination medium containing 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1.6 mM H_3BO_3, and 292 mM Suc was adjusted to pH 7.5 with NaOH and mixed with 1.5% (w/v) low-melting agarose. The mixture was heated to dissolve the agarose, applied to a microscope slide while still warm, spread evenly, and allowed to solidify. Eight mature flowers (carpels perpendicular to pistil) per genotype were collected and incubated in a moisture box (a plastic box containing moistened filter paper) for at least 30 min at 22°C. Pollen was brushed evenly onto the slides and the flowers placed in a circle around the pollen. Slides were in incubated in the moisture box for 35 min at 30°C and then 14 h at 22°C. The flowers were removed. A drop of liquid pollen germination media was added and a coverslip applied. Pollen germination was analyzed with a bright-field microscope. Pollen possessing a tube longer than the diameter of the pollen grain itself was considered germinated.

Light Microscopy

Prior to differential interference contrast microscopy using a Leica DM2500 fitted with a DFC300 FX digital color camera, ovules and seeds were cleared as previously described (Liu and Meinke, 1998). To visualize nuclei of mature pollen, open flowers were collected into a solution containing 0.1 M sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.4 μg mL^{-1} DAPI, and mixed by vortexing. The pollen released was pelleted by centrifugation and mounted onto a microscopy slide. DAPI fluorescence was observed with a Leica DM2500 fluorescence microscope fitted with a DFC300 FX digital color camera.

To examine pollen viability, Alexander's staining was performed (Alexander, 1969). Viable pollen stains purple, whereas dead pollen stain green. Inflorescences were collected in 10% (v/v) ethanol and the anthers dissected on the microscopy slide. Alexander solution containing 9.5% (v/v) ethanol, 25% (v/v) glycerol, 2% (v/v) glacial acetic acid, 5% (v/v) phenol, 0.01% (w/v) malachite green, 0.05% (w/v) fuchsin acid, and 0.005% (w/v) orange G in 0.3 M chloral hydrate was applied, and the anthers and pollen were stained for 15 min at 22°C. Pollen was analyzed by differential interference contrast microscopy.

Protoplast and Chloroplast Isolation

Protoplasts were isolated from Arabidopsis leaves using a protocol adapted from Kunst (1998). Ten grams of freshly harvested leaves were cut into thin strips and vaccum infiltrated for 10 min with protoplast isolation medium containing 10 mM MES-NaOH, pH 6.0, 1 mM $CaCl_2$, 0.3% (w/v) pectinase (*Rhizopus* sp.; Serva), 1% (w/v) cellulase (*Trichoderma viride*; Serva), and 0.5 M sorbitol. The strips were incubated for 2 h at 22°C with occasional agitation. Protoplasts were isolated by filtration through cheesecloth and subsequently collected via centrifugation (100*g*, 5 min, 22°C). Protoplasts were resuspended in 5 mL of protoplast storage medium containing 10 mM MES-NaOH, pH 6.0, 0.5 M sorbitol, and 1 mM $CaCl_2$ and overlaid on a Percoll cushion (50% [v/v] Percoll, 10 mM MES-NaOH, pH 6.0, 0.5 M sorbitol, and 1 mM $CaCl_2$). After centrifugation (100*g*, 10 min, 22°C), intact protoplasts formed a band at the interface between the storage medium and the Percoll. Protoplasts were collected and resuspended in protoplast lysis buffer containing 20 mM Tricine-KOH, pH 8.4, 0.3 M sorbitol, 10 mM EDTA, 10 mM $NaHCO_3$, and 0.1% (w/v) BSA and washed twice. To fractionate chloroplast and cytosolic compartments, the protoplasts were passed gently through a 15-μm nylon mesh. The filtrate was subject to centrifugation (300*g*, 2 min, 22°C) whereby the chloroplasts were pelleted and the cytosolic fraction was obtained from the supernatant.

Quantitative Reverse Transcription-PCR

For each genotype, three plants were pooled and RNA was extracted with TRIzol. cDNA synthesis was performed with the SuperScript III first strand kit (Invitrogen) using an oligo(dT) primer. Quantitative reverse transcription-PCR was carried out with Fast SYBR Green Master Mix on a 7500 Fast Real-Time PCR instrument (Applied Biosystems) according to the supplier's instructions. The mean values of three technical replicates were normalized to the transcript of At1g13320 (*PP2AA3*). Primers used are given in Supplemental Table S2.

Radioactive Labeling

Labeling of the products of photosynthesis was achieved by incorporation of $^{14}CO_2$. Plants were labeled 6 h into the photoperiod. Single attached leaves (leaf no. 8) were clamped into a custom-built, sealable cuvette and exposed to a constant stream of $^{14}CO_2$ circulated from a closed reservoir ($^{14}CO_2$ with a specific activity of 2.18 GBq mmol^{-1}was liberated within the reservoir by the acidification of $NaH^{14}CO_3$). After 5 min of exposure, the leaves were removed from the chamber and allowed to photosynthesize for a further 5 min in air. Labeled leaves were detached and immediately transferred to 5 mL 80% (v/v) ethanol at 80°C and incubated for 20 min. The samples were homogenized using an all-glass homogenizer. Centrifugation (2,400*g*, 12 min, 22°C) separated the insoluble starch-containing fraction from the soluble fraction. The insoluble fraction was resuspended and washed in a declining ethanol series (5 mL 50% [v/v] ethanol, 5 mL 20% [v/v] ethanol, and 5 mL water) and each time collected by centrifugation. All soluble fractions were pooled and dried under an airstream. The residue was dissolved in 2 mL of water and then further separated into basic, acidic, and neutral fractions by ion-exchange chromatography (using Dowex50 and Dowex1 resins) as described by Quick et al. (1989). The insoluble fraction was dissolved in 1 mL of tissue solubilizer (NCS; GE Healthcare) for 24 h at 22°C. The ^{14}C present in all fractions was determined by liquid scintillation counting.

Phylogenetic Tree Construction

Full-length protein sequences from different species were obtained from public databases (National Center for Biotechnology Information and Phytozome). Phylogenetic analysis was performed using Geneious 5.0.3 Pro software (Drummond et al., 2009). Sequences were aligned using MUSCLE with default settings and iterations set to 100. The consensus tree was created with Geneious Tree Builder using the genetic Jukes-Cantor distance-based model and UPGMA tree-building method. The robustness of the tree was assessed using 100 bootstrap replicates.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of the *PGM* genes in the wild type and *pgm* mutants.

Supplemental Figure S2. Growth phenotype of the wild type and *pgm* mutants.

Supplemental Figure S3. Starch and Suc content in wild-type and homozygous single mutant (*pgm2* and *pgm3*) plants.

Supplemental Figure S4. Expression and activity of PGMs in different tissues.

Supplemental Figure S5. Effect of reduced cPGM activity on starch content in the leaf at different time points throughout the light period and in plants of different ages.

Supplemental Figure S6. Photosynthetic partitioning of ^{14}C into major metabolite pools in different cPGM mutants.

Supplemental Figure S7. Loss of function of the *PGM2* gene in the Arabidopsis Cvi ecotype.

Supplemental Table S1. Primers for mutant genotyping.

Supplemental Table S2. Primers used for quantitative reverse transcription-PCR.

ACKNOWLEDGMENTS

We thank Lynette Brownfield, Aurélien Boisson-Dernier, and Elisabeth Hehenberger for advice with the analysis of Arabidopsis embryos and pollen germination and Stefan Herwig for advice in the design of the radiolabeling apparatus.

Received August 31, 2010; accepted October 14, 2010; published October 19, 2010.

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