Loss of Cytosolic Phosphoglucomutase Compromises Gametophyte Development in Arabidopsis

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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 symbiotically 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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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; PGI, phosphoglucose isomerase; OPP, oxidative pentose phosphate pathway.

**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 cysotolic 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 (Koffler 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 cysotolic 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 plastilidae 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 chloroplast 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 1.** The pathways of Suc catabolism and the interconversion of components of the cytosolic hexose-phosphate pool. INV, Cytosolic invertase; CW-INN, cell wall invertase; HXK, hexokinase; FK, fructokinase; USP, UDP-sugar pyrophosphorylase; UGPase, UDP-Glc pyrophosphorylase; PGI, phosphoglucose isomerase; OPP, oxidative pentose phosphate pathway.
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.
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 or PGM2pgm2/pgm3pgm3 mutants, 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 of 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.
**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 cPGM 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-

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**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.”

<table>
<thead>
<tr>
<th>Genotype of Parent Plant</th>
<th>Observed/Expected Genotype of Progeny in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGM2pgm2/pgm3pgm3</td>
<td>pgm2pgm2/pgm3pgm3 0/25 (n = 297)</td>
</tr>
<tr>
<td>pgm2pgm2/PGM3pgm3</td>
<td>pgm2pgm2/PGM3pgm3 0/25 (n = 277)</td>
</tr>
<tr>
<td></td>
<td>PGM2PGM2/pgm3pgm3 82/25 (n = 297)</td>
</tr>
</tbody>
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**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.

<table>
<thead>
<tr>
<th>Genotype of Parent Plant</th>
<th>% of Undeveloped Seeds (Seeds Examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>PGM2pgm2/pgm3pgm3</td>
<td>27 (n = 463)</td>
</tr>
<tr>
<td>pgm2pgm2/PGM3pgm3</td>
<td>30 (n = 453)</td>
</tr>
<tr>
<td>pgm2pgm2/PGMPG3</td>
<td>6 (n = 448)</td>
</tr>
<tr>
<td>PGM2PGM2/pgm3pgm3</td>
<td>4 (n = 353)</td>
</tr>
<tr>
<td>Wild type</td>
<td>2 (n = 247)</td>
</tr>
</tbody>
</table>
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 shown).

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

<table>
<thead>
<tr>
<th>Parental Genotypes (Female × Male)</th>
<th>% Undeveloped Seeds (Seeds Examined)</th>
<th>% Transmission Observed/Expected (Plants Genotyped)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGM2PGM2/PGM3PGM3 × PGM2pgm2/pgm3pgm3</td>
<td>0 (n = 189)</td>
<td>0/50 (n = 152)</td>
</tr>
<tr>
<td>PGM2PGM2/PGM3PGM3 × pgm2pgm2/PGM3pgm3</td>
<td>0 (n = 206)</td>
<td>0/50 (n = 104)</td>
</tr>
<tr>
<td>PGM2pgm2/pgm3pgm3 × PGM2PGM2/PGM3PGM3</td>
<td>28 (n = 329)</td>
<td>22/50 (n = 228)</td>
</tr>
<tr>
<td>pgm2pgm2/PGM3pgm3 × PGM2PGM2/PGM3PGM3</td>
<td>38 (n = 249)</td>
<td>23/50 (n = 119)</td>
</tr>
</tbody>
</table>
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 development.
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 [Oryza 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 isozyme 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 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.
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 photosimilates) 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; Lytvchenko 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; Wilhelm 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 remineralized 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 effect 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, Töllner 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 pathway 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 light microscopes (DM2500 fluorescence microscope fitted with a DFC300 FX digital color camera). DM2500 fluorescence microscope fitted with a DFC300 FX digital color camera.

**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 MgCl2, 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-bis-acrylamide) and 37% (v/v) 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 MgCl2, for 1 min and then incubated in staining solution containing 50 mM Tris-HCl, pH 7.0, 5 mM MgCl2, 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 N2 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 a-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 cellulose, as an internal standard and applied to a 1.5-M column 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 (column reequilibration). Peaks were identified by coelution with known Glc, Fru, Suc, and cellubiose standards. Peak areas were determined using Chromleon software. All peaks were normalized to the internal cellubiose 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 Buivid and McCormick (2007). Filter-sterilized liquid pollen germination medium containing 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1.6 mM H3BO3, 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 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 µM 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 microscope 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 vacuum infiltrated for 10 min with protoplast isolation medium containing 10 mM MES-NaOH, pH 6.0, 1 mM CaCl₂, 0.3% (w/v) pectinase (Rhizopus sp.; Serva), 1% (w/v) cellulase (Trichoderma viride; Serva), and 0.5 mM 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 (100g, 5 min, 22°C). Protoplasts were resuspended in 5 mL of protoplast storage medium containing 10 mM MES-NaOH, pH 6.0, 0.5 mM sorbitol, and 1 mM CaCl₂ and overlaid on a Percoll cushion (50% [v/v] Percoll, 10 mM MES-NaOH, pH 6.0, 0.5 mM sorbitol, and 1 mM CaCl₂). After centrifugation (100g, 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 mM sorbitol, 10 mM EDTA, 10 mM NaHCO₃, 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 (300g, 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 AtG3320 (FP24A3). Primers used are given in Supplemental Table S2.

Radioactive Labeling

Labeling of the products of photosynthesis was achieved by incorporation of 14CO₂. 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 14CO₂ circulated from a closed reservoir. 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 (2,400g, 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%, 5% [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 14C 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 Phytozone). 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 14C 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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