Running title: Impact of cPGI on plant viability and fertility

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Research category: Biochemistry and Metabolism
Loss of Cytosolic Phosphoglucose Isomerase (cPGI) affects carbohydrate metabolism in leaves and is essential for fertility of Arabidopsis thaliana


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One-sentence summary:
An enzyme of cytosolic hexose phosphate metabolism is essential for plant reproduction and important for photosynthesis.
Funding:
This research was supported by the International Max Planck Research School Cologne (to S.Z.-N.), the DFG (Schwerpunktprogramm 1108) to U.-I.F. and R.E.H., the Human Frontiers Science Program, Alexander von Humboldt Foundation, and DAAD Rise program (to H.-H.K.) and in part by the National Science Foundation (MCB0918220) and fusion protein localization was supported by a grant from the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (DE-FG02-03ER15449) (to J.I.S.).

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Abstract

Carbohydrate metabolism in plants is tightly linked to photosynthesis and is essential for energy and carbon skeleton supply of the entire organism. Thus, the hexose phosphate pools of the cytosol and the chloroplast represent important metabolic resources that are maintained through action of phosphoglucone isomerase (PGI) and phosphoglucone mutase (PGM) interconverting glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate. Here, we investigated the impact of disrupted cytosolic \textit{PGI} (\textit{cPGI}) function on plant viability and metabolism. Over-expressing an artificial micro RNA targeted against \textit{cPGI} (amiR-\textit{cpgi}) resulted in adult plants with vegetative tissue essentially free of cPGI activity. These plants displayed diminished growth compared to wild type, accumulated excess starch in chloroplasts but maintained low sucrose content in leaves at the end of the night. Moreover, amiR-\textit{cpgi} plants exhibited increased non-photochemical chlorophyll \textit{a} quenching during photosynthesis. In contrast to amiR-\textit{cpgi} plants, viable T-DNA insertion mutants disrupted in \textit{cPGI} function could only be identified as heterozygous individuals. However, homozygous T-DNA insertion mutants could be isolated among plants ectopically expressing \textit{cPGI}. Intriguingly, these plants were only fertile when expression was driven by the ubiquitin 10 promoter but sterile when the seed-specific USP promoter or the cauliflower mosaic virus 35S promoter were employed. These data show that metabolism is apparently able to compensate for missing cPGI activity in adult amiR-\textit{cpgi} plants and indicate an essential function for cPGI in plant reproduction. Moreover, our data suggest a feedback regulation in amiR-\textit{cpgi} plants that fine tunes cytosolic sucrose metabolism with plastidic starch turnover.
Introduction

Starch and sucrose turnover are major pathways of primary metabolism in all higher plants. As such they are essential for carbohydrate storage, the energy supply of sink tissues and as building blocks for amino acid, fatty acid or cell wall biosynthesis (Stitt and Zeeman, 2012).

A core reaction in both starch and sucrose biosynthesis is the reversible interconversion of the hexose phosphate pool metabolites fructose 6-phosphate (Fru6P) and glucose 6-phosphate (Glc6P), which is mediated by phosphoglucone isomerase (PGI). *Arabidopsis thaliana* contains two isoforms of PGI, one in the plastids and one in the cytosol (Caspar et al., 1985).

During the light period, the plastid isoform of PGI (PGI1) is involved in starch biosynthesis by generating Glc6P from the primary photosynthetic product Fru6P. Glc6P is further converted to glucose 1-phosphate (Glc1P) and ADPglucose via action of phosphoglucomutase (PGM) and ADPglucose pyrophosphorylase (AGPase), respectively (Stitt and Zeeman, 2012). Finally, transfer of the glucosyl moiety of ADPglucose to the growing carbohydrate chain of starch is mediated by starch synthases. Any of the enzymatic reactions of this linear pathway is essential for starch synthesis as illustrated by the virtual absence of transitory starch in chloroplasts of mutant plant lines with impaired function of PGI1 (Yu et al., 2000; Kunz et al., 2010), PGM (Caspar et al., 1985; Kofler et al., 2000) or AGPase (Lin et al., 1988). Interestingly, in a few specific cell types, e.g. leaf guard cells and root columella cells, loss of PGI1 activity can be bypassed by the presence of the plastid Glc6P/phosphate translocator GPT1 (Niewiadomski et al., 2005; Kunz et al., 2010).

The cytosolic isoform of PGI (cPGI) is involved in anabolism and catabolism of sucrose, the major transport form of carbohydrates in plants. Glc6P and Fru6P interconversion is necessary for both sucrose synthesis during the day and during the night. During the day, sucrose synthesis in source leaves is fueled mainly by triose phosphates exported from chloroplast that are eventually converted to Fru6P in the cytosol. However, Fru6P is only one substrate for the sucrose-generating enzyme sucrose phosphate synthase (SPS). The second substrate, UDPglucose, is synthesized from Fru6P via Glc6P and Glc1P by the cytosolic isoenzymes of PGI1 and PGM as well as UDPglucose pyrophosphorylase.

Because sucrose is the major long-distance carbon transport form, its synthesis has to continue throughout the night to supply energy and carbohydrates to all tissues. The nocturnal synthesis of
sucrose is dependent on breakdown and mobilization of transitory starch from chloroplasts (Zeeman et al., 2007) via export of maltose and glucose (Weber et al., 2000; Niittylä et al., 2004; Weise et al., 2004; Cho et al., 2011). Exported maltose is temporarily integrated into cytosolic heteroglycans (Fettke et al., 2005) mediated by disproportionating enzyme 2 (DPE2) (Chia et al., 2004; Lu and Sharkey, 2004) yielding glucose and a heteroglycan molecule elongated by an α1-4-bound glucosyl residue. Cytosolic glucose can directly be phosphorylated to Glc6P by the action of hexokinase while temporarily stored glucose in heteroglycans is released as Glc1P mediated by cytosolic glucan phosphorylase 2 (PHS2) (Fettke et al., 2004; Lu et al., 2006). Both Glc6P and Glc1P can then be converted to UDPglucose as during the day.

Generation of Fru6P, the second substrate for sucrose synthesis, can proceed only to a limited extent from triose phosphates during the night. This limitation is caused mainly by the nocturnal inactivation of fructose 1,6-bisphosphatase (Cseke et al., 1982; Stitt, 1990), a key enzyme in sucrose biosynthesis during the day. Hence in contrast to the situation in the light, cPGI activity is now crucial for providing Fru6P from Glc6P.

On the catabolic side, degradation of sucrose into its monosaccharides in sink tissues yields both, Glc6P and Fru6P of which only Fru6P can be utilized in glycolytic degradation. Therefore cPGI is also required for Glc6P conversion to Fru6P in glycolysis, which, in combination with respiration, is the major path of energy production in heterotrophic tissues.

Impairment or loss of function of enzymes contributing to the cytosolic hexose phosphate pool has recently been investigated for the Glc1P-forming enzyme PGM (Egli et al., 2010). The A. thaliana genome encodes three PGM isoforms with PGM1 localized to plastids and PGM2 and PGM3 localized to the cytosol (Caspar et al., 1985; Egli et al., 2010). Analyses of T-DNA mutants showed that homozygous pgm2/3 double mutants were non-viable because of impaired gametophyte development. However, pgm2 and pgm3 single mutants grew like Col-0 wild-type plants indicating overlapping functions of PGM2 and PGM3 (Egli et al., 2010).

In contrast, cPGI is encoded only by a single locus in A. thaliana (Kawabe et al., 2000). Higher plant mutants reduced in cPGI activity have so far been characterized only in EMS-mutagenized Clarkia xantiana (Jones et al., 1986a; Kruckeberg et al., 1989; Neuhaus et al., 1989). The C. xantiana genome encodes for two isoenzymes of cPGI and homozygous point mutations in each individual cPGI led to significant decrease in cPGI enzyme activity, which was further reduced to a residual activity of 18% in cpgi2/3 double mutants where the cPGI3 locus was heterozygous
for the mutation (Jones et al., 1986a; Kruckeberg et al., 1989). Detailed physiological analyses of these mutants indicated a negative impact on sucrose biosynthesis and elevated starch levels when cPGI activity was decreased at least 3-5 fold (Kruckeberg et al., 1989).

The physiological impact of decreased or even absent cPGI activity has not been characterized in the genetic model organism A. thaliana. Here we show that homozygous T-DNA insertion mutants in the cPGI locus are non-viable and present data from analyses of mature Arabidopsis plants constitutively expressing artificial micro RNAs (amiRNAs) targeted against cPGI. These mutants reveal altered photosynthesis, a strong impact on nocturnal leaf starch degradation and impaired sucrose metabolism.

**Results**

*Tissue-specific expression and subcellular localization of PGI isoenzymes*

According to publicly available expression data (eFP-Browser; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi, Winter et al. (2007)), both PGI homologues are expressed at a similarly high level throughout somatic leaf tissue. Intriguingly, while cPGI (At5g42740) transcript is also highly abundant in developing embryos and seeds, plastidic PGI1 (At4g24620) expression is much lower in these tissues. In roots, highest transcript accumulation for the plastidic isoform is found in vascular tissues and columella cells which build starch statoliths to perceive gravitropism. Expression of the cytosolic isoform in roots is strongest in the meristematic, elongation and maturation zone.

To study the subcellular localization of both PGI isoenzymes in vivo, the Venus reporter protein was fused to the C-terminus of either PGI protein. According to activity gel assays, PGI1 localizes to the chloroplast (Caspar et al., 1985; Yu et al., 2000). Indeed yellow fluorescence signal for the PGI1::Venus fusion protein was detected specifically in chloroplasts of transiently expressing Nicotiana benthamiana leaves as demonstrated by the co-localization with chlorophyll autofluorescence (Fig. 1A). For the cPGI::Venus fusion protein on the other hand, fluorescence could be detected in the cytosol, clearly not co-localizing with the red autofluorescence from chlorophyll in the chloroplasts (Fig. 1B). Cytosolic localization of cPGI is in agreement with in silico predictions (Schwacke et al., 2003) and proteome studies of the Arabidopsis cytosol (Ito et al., 2011). In contrast, free Venus protein was detected in the cytosol and also strongly in the nucleus (Fig. 1C).
Isolation of T-DNA insertion lines and generation of cPGI amiRNA plants

Two independent T-DNA insertion lines from the Salk collection (Alonso et al., 2003) were obtained from the Arabidopsis stock center. T-DNA insertion sites were located in the ninth exon (cpgi-1) and the seventh intron (cpgi-2), respectively (Fig. 2A). Heterozygous cpgi plants could be identified from the segregating progeny of self-pollinated heterozygous parent plants at a ratio of 148/156 and 159/146 (heterozygotes/wild type) for cpgi-1 and cpgi-2, respectively. However, no homozygous individuals could be recovered from the same segregating seed population (Fig. 2B, Supplemental Table SI), suggesting that a homozygous mutation in cPGI is lethal. To exclude that homozygous individuals are missed due to the inability of homozygous seeds to germinate we compared the germination rates of seeds from heterozygous cpgi mutants and Col-0 wild type plants. Results show that germination rates of segregating cpgi seeds were not different from the wild type (Supplemental Table SII) indicating that no homozygous seeds are among the segregating cpgi seed population.

To overcome lethality in homozygous T-DNA insertion mutants, two independent artificial micro RNAs were designed targeting different regions of the cPGI coding region (Fig. 2C). In contrast to loss-of-function T-DNA mutants, amiRNA mutants allow analysis of only partial or regulated functional loss of the gene of interest when full “knock-out” mutants are lethal (Schwab et al., 2010). Plants expressing these amiRNAs under the control of the cauliflower mosaic virus (CaMV) 35S promoter were isolated on selective medium and the progeny of individual plants analyzed. In total five independent lines of the two amiRNA target sites (two lines of amiRNA1 and three lines of amiRNA2) were analyzed for core metabolic data. Two independent lines carrying the amiRNA1 transgene, designated as amiR-cpgi 6 and amiR-cpgi 10 were studied in detail further.

Mature amiR-cpgi plants display reduced growth and increased non-photochemical Chl a quenching in the virtual absence of cPGI activity.

At first glance no growth abnormalities were found in amiR-cpgi mutants (Fig. 3A). However, when measuring rosette leaf area or rosette fresh weight, amiR-cpgi mutants displayed slight but significant growth retardation compared to wild-type plants that was comparable to that of starch-free pgi1-1 plants (Fig. 3B, Yu et al., 2000). In addition, growth was even further diminished under short-day compared to long-day conditions (Supplemental Fig. S1B and C).
This is characteristic for mutants impaired in carbohydrate metabolism like pgi1-1 or pgm (Gibon et al., 2009; Izumi et al., 2013).

Analyses of total PGI activity (i.e. activity of the plastidic and cytosolic isoform) in leaf extracts of wild-type, amiRNA-cpgi and pgi1-1 plants revealed that total PGI activity is strongly decreased in amiR-cpgi 6 and amiR-cpgi 10 lines to less than 40 % of wild-type activity (Fig. 4A, Supplemental Fig. S2A). These measurements also showed that total PGI activity in pgi1-1 plants is decreased to approximately 80 % of the wild-type level. The plastidic PGI can be heat-inactivated by incubating the protein extract for 10 min at 50°C prior to the enzyme assay (Jones et al., 1986b; Yu et al., 2000). Leaf extracts pretreated accordingly showed no detectable residual PGI activity in amiR-cpgi plants (Supplemental Fig. S2B) suggesting that leaves were essentially free of cPGI activity. The residual total PGI activity found in those plants (Fig. 4A) may be attributed to plastidic PGI activity.

To investigate whether reduced leaf area and rosette fresh weight might be the result of changes in photosynthetic performance, photosystem II properties were investigated by Pulse Amplitude Modulation fluorometry (PAM). Determination of the Fv/Fm ratio in dark-adapted leaves as a measure of the potential efficiency of photosystem II showed no difference between wild type and mutants (Fig. 4B). Thus the Fv/Fm ratio of approximately 0.8 indicated full functional integrity of photosystem II for all genotypes. Intriguingly, PAM measurements of photosynthetic induction curves revealed increased non-photochemical quenching (qN) for all amiRNA lines (Fig. 4C, Supplemental Fig. S3A). Concomitantly, the photochemical quench coefficient (qP) and the electron transport rate (ETR) were decreased in all amiR-cpgi lines compared to wild-type and pgi1-1 plants (Fig. 4C, Supplemental Fig. S3A). These data indicate that decreased cytosolic PGI activity exhibits an effect on the photosynthetic light reaction in the thylakoids of chloroplasts.

amiRNA-cpgi plants display a starch excess phenotype in mature leaves

In addition to the macroscopic growth retardation and photochemical phenotypes of amiR-cpgi plants, whole rosette starch staining revealed strongly increased starch levels at the end of the night in amiR-cpgi plants (Fig. 5A, Supplemental Fig. S3B). Usually transitory leaf starch is depleted at the end of the night period as it was found in wild-type plants (Fig. 5A, Supplemental Fig. S3B). Measurement of starch content in leaves of mutants and wild type over the course of a
day confirmed significant starch excess in amiR-cpgi lines of both amiRNA targets at the end of the night (Fig. 5B, Supplemental Fig. S3C). Moreover, starch levels were generally elevated in leaves of amiR-cpgi plants compared to those in the wild type even at the end of the day, when starch levels are usually highest in wild-type leaves (Fig. 5B, Supplemental Fig. S3C). In contrast pgi1-1 plants, used as an almost starch-free reference, displayed only very low starch levels in leaves (Fig. 5B, Supplemental Fig. S3C).

To investigate if leaf starch excess (Fig. 5A) and the increased non-photochemical quenching phenotype (Fig. 4C) are functionally connected, mutants with impaired activity of other cytosolic enzymes known to display high-starch phenotypes such as fum2 mutants (Pracharoenwattana et al., 2010), defective in cytosolic fumarase activity or dpe2 mutants, defective in cytosolic heteroglycan metabolism (Lu and Sharkey, 2004), were analyzed. Although all different plant lines were grown side-by-side under the same conditions, we did not detect elevated starch in rosettes of three independent fum2 mutant lines and hence focused on dpe2 mutants that showed a starch excess phenotype as reported before (Supplemental Fig. S1A). dpe2 mutants just as amiR-cpgi plants displayed reduced growth in long-day and short-day conditions compared to their respective wild type. However, contrary to amiR-cpgi plants, dpe2 mutants displayed no or only mildly increased non-photochemical quenching compared to wild type (Supplemental Fig. S4). In contrast to the starch-free and starch-excess mutants investigated, fum2 mutants appeared to produce more biomass and rosette leaf area compared to the wild type (Supplemental Fig. S1B and C).

*Leaf sucrose concentration and glucose content of cytosolic heteroglycans is altered in amiR-cpgi plants*

Because cPGI is an important enzyme in sucrose biosynthesis and the starch-excess phenotype of amiR-cpgi plants indicated impaired starch turnover, we analyzed leaf sucrose at different time points during the day/night cycle and the monosaccharide composition of cytosolic heteroglycans. At the end of the night, the sucrose concentration in leaves was significantly reduced compared to the wild type in all amiRNA lines and pgi1-1 (Fig. 6A, Supplemental Fig. S3D). No significant difference in sucrose content could be observed at any other time point during the light period (Fig. 6A).
Cytosolic heteroglycans are involved in turnover of the starch degradation product maltose and serve as a temporary storage for glucosyl-moieties. Analyses of the cytosolic fraction of low (<10 kDa, SHGs) and high (>10 kDa, SHGL) molecular weight soluble heteroglycans revealed that both amiR-cpgi 6 and amiR-cpgi 10 harbored significantly increased glucose concentrations in their SHGs at the end of the night (Fig. 6B). The content of the major heteroglycan monomer galactose was constant between ecotypes and time points and used for normalization. Sugar monomers other than glucose were not consistently changed but showed considerable variation. The observed elevated glucose content in SHGs may result from diminished cPGI activity leading to decreased carbon flux through sucrose synthesis at night and hence to maltose-derived glucose being accumulated in heteroglycans.

Simultaneous loss of transitory starch formation and cPGI activity severely impairs viability of amiR-cpgi plants on soil

Since loss of cPGI activity apparently resulted in starch over accumulation, we investigated whether the absence of transitory starch had an impact on the severity of the amiR-cpgi plant phenotype. Homozygous plants defective in either plastidic PGI1 (pgi1-1) or AGPase (adg1-1) were transformed with cPGI amiRNA1 and transformed individuals identified on selective half-strength MS medium (supplemented with 2% sucrose). In initial experiments plants transferred from selective media to soil hardly developed into adult plants. Rosette leaves remained very small and the plants eventually died before producing seeds (Fig. 7). To investigate if exogenous carbohydrate supply could overcome this lethality, plants were transferred from selective plates either to soil or to half-strength MS medium supplemented with 2% sucrose in subsequent experiments. Three weeks after sowing, antibiotic-resistant plants transferred to MS medium containing sucrose without antibiotics had developed rosette leaves and had considerably increased in size since transfer (Fig. 7). Starch-free amiR-cpgi x adg1-1 or amiR-cpgi x pgi1-1 plants transferred to sucrose-containing medium eventually flowered and developed a small number of seeds. In contrast, plants transferred to soil instead had developed only very tiny leaves that displayed almost no increase in size and died before producing seeds (Fig. 7).
Analyses of cpgi T-DNA mutants reveal reduced transmission efficiency for mutant male and female gametophytes

Loss of cPGI activity in mature leaves of amiR-cpgi plants led to viable though size-reduced plants. However, no homozygous cpgi T-DNA insertion mutants could be isolated and genotyping of the progeny of self-pollinated heterozygous mutants revealed a segregation ratio of approximately 1:1 (cpgi/cPGI : cPGL;cPGI) clearly diverting from the expected 2:1 ratio (Supplemental Table S1). Thus, we investigated the mutant allele transmission efficiency performing reciprocal crosses either using pollen from heterozygous cPGI/cpgi-1 or cPGI/cpgi-2 to pollinate wild-type pistils or pollinating pistils of heterozygous cPGI/cpgi-1 or cPGI/cpgi-2 plants with wild-type pollen. Genotyping of the progeny of these reciprocal crosses revealed that the transmission efficiency of the mutant allele (ratio of number of heterozygous over wild-type plants; Howden et al., 1998) was strongly decreased through both the male and female gametophyte. While an evenly split genotype distribution in the progeny of crosses would be expected at a transmission efficiency of 100 %, transmission efficiency was in fact reduced to at least 32 % for male and 49 % for female cpgi gametophytes (Table I). However, when testing the vitality of pollen from heterozygous cPGI/cpgi-1 and cPGI/cpgi-2 plants we did not detect an increased number of non-vital pollen grains by Alexander staining (Fig. 8A).

Expression of cPGI is higher in developing seeds/embryos compared to other tissues according to publicly available databases (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi: Winter et al. (2007)). Therefore, to assess the viability of the early embryo, we scored the occurrence of empty spots in self-pollinated siliques from heterozygous cPGI/cpgi-1 and cPGI/cpgi-2 plants. With an observed percentage of 21 % (cpgi-1) and 11 % (cpgi-2) aborted seeds per cPGI/cpgi siliques a significantly higher percentage of empty spots occurred in siliques of both cPGI/cpgi T-DNA insertion lines compared to wild-type siliques (Fig. 8B and C). To gain insight into the stage of gametophyte or embryo development depending on cPGI function we transformed the cPGI cDNA into heterozygous cPGI/cpgi plants and screened the progeny for complementation, i.e. homozygous cpgi T-DNA plants. Expression of the cPGI cDNA was driven by three different promoters, the ubiquitously active ubiquitin 10 promoter (UBQ10), the cauliflower mosaic virus 35S promoter (CaMV35S) active throughout most developmental stages and the unknown seed protein promoter (USP, Bäumlein et al., 1991) mainly active during early embryo development in seeds. We could isolate primary transformants (T1) for all three promoter:cPGI
complementation constructs that were homozygous for the \textit{cpgi} T-DNA insertion. However, only UBQ10:\textit{cPGI} plants were able to set seed. In contrast siliques of CaMV35S:\textit{cPGI} and pUSP:\textit{cPGI} plants remained short and dried back without producing seeds (Supplemental Fig. S5A). Closer inspection of flowers of these plants revealed that pollen grains were absent from their anthers (Supplemental Fig. S5B) explaining the inability to produce seeds by self fertilization. Notably pollination of stigmas of homozygous \textit{cpgi-1} mutants, expressing the CaMV35:\textit{cPGI} construct with wild-type pollen led to elongating siliques (Supplemental Fig. S5) that harbored a uniformly heterozygous progeny.

**Discussion**

A balanced carbohydrate turnover in the cytosol that is well integrated into overall metabolism is of major importance for plant fitness and viability (Gibon et al., 2006; Usadel et al., 2008). Thus, maintenance and proper adjustment of the cytosolic hexose phosphate pool is an essential process ensured by \textit{cPGI} and \textit{cPGM} activities interconverting Fru6P, Glc6P and Glc1P respectively. In this study we have investigated \textit{cPGI} knock-down mutants since viable, homozygous \textit{cpgi} T-DNA mutants could not be identified. We provide evidence that \textit{cPGI} activity impacts photosynthesis and starch and sucrose metabolism but its absence can be compensated in adult plants at least during the light period.

Analyses of \textit{cpgi} T-DNA mutants indicate an essential role of \textit{cPGI} activity for reproduction and embryo development

Despite of screening more than 300 individual plants from the progeny of two independent heterozygous \textit{cPGI/cpgi} T-DNA insertion lines, we could not identify plants homozygous for the T-DNA insertion (Fig. 2B, Supplemental Table SI). Attempts to complement T-DNA lines by \textit{cPGI} overexpression led to plants homozygous for the T-DNA insertion. These complemented lines were unable to produce pollen and self fertilize when \textit{cPGI} expression was driven by either the CaMV35S or the USP promoter. However, when \textit{cPGI} expression was driven by the constitutive UBQ10 promoter (Krebs et al., 2012), complemented homozygous lines were able to set seed and produce a homozygous progeny.

The initial study describing the USP promoter, reported its activity mainly in developing embryos of \textit{Arabidopsis} and seeds of tobacco (Bäumlein et al., 1991). In addition Zakharov et al.
(2004) detected USP promoter activity in mature pollen of tobacco and also weakly in developing flowers by testing for transcript of a USP promoter-driven reporter gene. Congruent with the initial report by Bäumlein et al. (1991) we could detect USP promoter-driven β-glucuronidase activity in Arabidopsis only in developing embryos but not in pollen or flowers (Supplemental Figure S7) indicating that the USP promoter induces only weak or no expression during pollen development in Arabidopsis.

As with the USP promoter, varying results regarding the activity of the CaMV 35S promoter during male gametophyte development have been observed. Early studies reported the absence of CaMV 35S promoter activity in mature Arabidopsis pollen or during pollen development (Wilkinson et al., 1997; Custers et al., 1999) that were later corroborated by studies unsuccessfully using the CaMV 35S promoter to complement Arabidopsis mutants defective in pollen development (Harrison-Lowe and Olsen, 2008), induce a pollen lethal phenotype (Zhang et al., 2009) or label pollen peroxisomes with a GFP reporter gene fusion (Footitt et al., 2007). However, other studies suggest some activity of the CaMV 35S promoter during pollen maturation in Arabidopsis since pollen callose deposition can be altered by over expressing callose synthase 5 (Xie et al., 2010) or male sterility can be induced by expressing an RNA interference construct hampering meiosis (Wang et al., 2012). Low activity or even inactivity of the CaMV 35S promoter may also be the reason for the fact that we could isolate viable and fully fertile amiR-cpgi plants since the artificial micro RNA would not be sufficiently expressed during cPGI-critical stages of plant development.

Thus available data on CaMV 35S and USP promoter activity in Arabidopsis and our own data essentially suggest that cPGI expression from both promoters is insufficient to complement cPGI loss during pollen development and indicate that cPGI activity is essential during this phase of male gametophyte development. Activity of cPGI in mature pollen appears to be of minor importance since vitality of pollen from heterozygous cpgi mutants was indistinguishable from wild-type pollen (Fig. 8A) and homozygous cpgi T-DNA mutants could be identified in the T1 generation of complementation lines which requires viable mutant cpgi pollen.

In spite of unaltered pollen vitality we detected strongly decreased transmission efficiency of the mutant allele through both male and female gametophytes (Fig. 8, Table I). Calculating the expected genotype distribution by taking the decreased transmission efficiency into account (Howden et al., 1998) revealed that the found distribution differs from the expected distribution
Homozygous cpgi plants that would be expected according to these data but that were not identified (Supplemental Table I, Supplemental Figure S6), are likely among the aborted seeds observed at a higher percentage in cPGI/cpgi compared to wild-type silique (Figure 8). Thus aborted seeds/ovules observed in silique of cPGI/cpgi plants most likely result from both, embryo lethality and reduced transmission through the female gametophyte. Because both effects overlay the observed percentage of aborted seeds/ovules diverts from expected numbers (e.g. 25% if only embryo lethality would be considered). Notably the impact on cpgi mutant fertility observed in this study is in line with earlier results from cpgm mutants, where homozygous cpgm2/3 double mutants could not be identified due to impaired gametophyte fertilization ability (Egli et al., 2010). In essence analyses of cpgi T-DNA mutants support the conclusion that cPGI activity is important for mutant allele transmission through male and female gametophytes and essential for pollen and embryo development. However, elucidation of the exact processes for which cPGI activity is necessary during these developmental stages requires further investigation.

Because homozygous cpgi T-DNA insertion mutants were not available to investigate loss of cPGI function in adult plants, cPGI activity was decreased by employing an artificial micro RNA based approach. Analyses of cpgi mutants had indicated that cPGI activity might be important for proper pollen development and the CaMV 35S promoter does not induce expression throughout the entire microspore and pollen development (Custers et al., 1999; Munoz-Bertomeu et al., 2010; Krebs et al., 2012). This feature was used to generate plants that expressed amiRNAs targeted against cPGI and did not display any detectable cPGI activity in protein extracts of mature leaves (Fig. 4A, Supplemental Fig. S2B). Hence these plants allowed us to investigate the impact of strongly decreased cPGI activity in somatic tissue even though homozygous T-DNA mutants were non-viable.

**Lack of cPGI activity in leaves results in starch excess and increased non-photochemical quenching in photosynthesis**

Expression of artificial micro RNAs targeted against cPGI led to plants without detectable cytosolic PGI activity (Fig. 4A, Supplemental Fig. S2) that displayed growth reduction (Fig. 3) but apart from that appeared healthy. This seems to be a common feature of mutants impaired in primary carbohydrate metabolism such as pgi1, pgm1 and adg1 (Caspar et al., 1985; Lin et al.,
1988; Yu et al., 2000) or sugar transport such as suc2 and sweet1 sweet2 (Gottwald et al., 2000; Chen et al., 2012) which results from the inability to properly allocate and distribute fixed carbon as energy or carbon skeletons to growing tissues (Ludewig and Flügge, 2013).

Additionally, amiR-cpgi plants over-accumulated starch, most significantly at the end of the night but also throughout the entire day/night cycle (Fig. 5, Supplemental Fig. S3B and C). Starch excess in mutants with an impaired cytosolic enzyme activity that is not directly involved in starch biosynthesis or degradation may seem unexpected at first sight. However, this has also been described for other processes such as sucrose export from source-tissue mesophyll cells (Gottwald et al., 2000; Srivastava et al., 2008) or transient integration of maltose-derived glucose into heteroglycans mediated by DPE2 (Chia et al., 2004; Lu and Sharkey, 2004). In the process of nocturnal starch mobilization cPGI acts downstream of DPE2, hence loss of cPGI activity apparently phenocopies the starch-excess phenotype of dpe2 plants (Supplemental Fig. S1A). DPE2-defective plants are also characterized by a strongly decreased or even absent turnover of glucosyl residues in their cytosolic heteroglycans (Fettke et al., 2006; Malinova et al., 2013). Thus, we analyzed the monomer composition of cytosolic heteroglycans and found that their glucose content was significantly increased in amiR-cpgi plants compared to wild-type plants at the end of the night (Fig. 6B). Accumulation of glucose moieties in cytosolic heteroglycans of amiR-cpgi plants indicates decreased glucose turnover and as a consequence leads to decreased export of starch degradation products from chloroplasts. In turn this argues for a feedback mechanism originating in the cytosol and inhibiting cytosolic metabolism of starch degradation products but also the chloroplast-localized starch degradation itself.

However, impairment of cPGI activity not only affected carbohydrate metabolism in chloroplasts and the cytosol, but also photosynthesis performance. After dark incubation, light induction curve measurements revealed a significant increase in qN concomitant with a decrease in qP in both amiR-cpgi lines (Fig. 4C, Supplemental Fig. S3A). In contrast, comparison between dpe2 mutants and corresponding wild-type plants (Wassilewskija) did not reveal any increase in qN (Supplemental Fig. S4).

Chloroplasts have to protect themselves from energy overload and accompanied damage. This is achieved by dissipation of excess energy in the form of heat as a way to cope with such unfavorable situations. In the light the diminished formation of sucrose from exported triose phosphates feeds back directly on photophosphorylation in the chloroplasts (i.e., by stromal Pi
depletion which occurs at least temporarily until starch biosynthesis is fully induced) and thereby inhibits photosynthetic electron transport (Fig. 4C, Supplemental Fig. S3A). Hence, our data indicate a role for sucrose synthesis as a short term energy buffer which underpins the complex, fast and well-coordinated reactions of two compartments involved in proper photosynthesis.

The decrease in \(q_p\), which reflects an increased reduction state of \(Q_A\) (the primary quinone electron acceptor of PSII), points at a limitation of ETR at the site of PSII (Fig. 4C, Supplemental Fig. S3A). In addition, the increase in \(q_N\) would be consistent with a steeper proton gradient across the thylakoid membrane suggesting a diminished ATP synthesis. In contrast, in the \(dpe2\) mutant the induction of photosynthesis is not severely impaired because the DPE2 protein fulfills its major function in the dark, when maltose deriving from starch degradation is further processed after export in the cytosol. In the light maltose production from starch turnover ceases.

**Sucrose synthesis is impaired but not abolished in amiR-cpgi plants**

Although cytosolic PGI activity was virtually absent from amiR-cpgi leaf extracts (Supplemental Fig. S2), sucrose could still be detected in leaves (Fig. 6A). While at most time points during the day leaf sucrose contents in amiR-cpgi plants were comparable to those in wild-type plants, sucrose content was significantly reduced at the end of the night in amiR-cpgi plants (Fig. 6A, Supplemental Fig. S3D). These data led to the conclusion that sucrose synthesis during the day is apparently operating without cPGI-mediated conversion of Fru6P to Glc6P which serves as substrate for UDPglucose synthesis via cytosolic PGM and UDPglucose pyrophosphorylase. Fru6P is easily generated during the day from chloroplast-exported triose phosphates independent from cPGI activity. Flexibility of plant carbohydrate metabolism could enable amiR-cpgi plants to generate the SPS substrate UDPglucose from Glc1P originating from transitory starch degradation during the day (Fig. 9). amiR-cpgi plants may be able to use the night-path of photoassimilate export from chloroplasts already during the day. This includes the export of the starch breakdown products glucose (via the chloroplastic glucose transporter pGlcT; Weber et al., 2000) and maltose (via the maltose exporter MEX1; Niittylä et al., 2004) from chloroplasts. Glucose can subsequently be converted to Glc1P via hexokinase and cPGM. Alternatively, cytosolic glucose may originate from DPE2-catalyzed maltose turnover (Chia et al., 2004; Lu and Sharkey, 2004) involving soluble heteroglycans (SHG; Fettke et al., 2006) and...
the cytosolic glucan phosphorylase PHS2 (Fig. 9). Recently, the possibility of direct export of Glc1P from chloroplasts has been discussed (Stitt and Zeeman, 2012) in face of apparent Glc1P import into isolated Arabidopsis protoplasts and chloroplasts (Fettke et al., 2011). This could avoid the necessity for partial starch degradation during the day since Glc1P could be channeled directly from the chloroplast to the cytosol and into sucrose biosynthesis instead of plastidic starch synthesis. However, the molecular entity mediating Glc1P transport has not been identified yet.

Starch breakdown during the day as compensatory mechanism has clearly been demonstrated in mutant plants defective in TPT, the chloroplast envelope triose phosphate/phosphate translocator which mediates the day-path of photoassimilate export from chloroplasts (Schneider et al., 2002; Walters et al., 2004). The tpt-1 mutant only showed severe growth retardation with simultaneously defective starch synthesis and less pronounced with starch degradation (Schmitz et al., 2012). In agreement with this, amiR-cpgi mutants in the starch-free genetic background of the pgil-1 or adg1-1 mutation displayed massive growth retardation which was rescued to some extent by exogenous sucrose supply (Fig. 7).

In contrast to the situation in the light, the lack of cPGI activity during the dark period appears to be less well compensated since sucrose was strongly decreased at the end of the night (Fig. 6A). During darkness, the cytosolic pathway which uses exported triose phosphates from the chloroplast to yield Fru6P throughout the day, is mainly blocked because of the nocturnal inactivation of the key enzyme fructose 1,6-bisphosphatase (Cseke et al., 1982; Stitt, 1990). Fructose 1,6-bisphosphatase activity is tightly regulated by fructose 2,6-bisphosphate (Fru2,6BP) and strongly repressed in presence of the low triose phosphate and high Fru2,6BP concentrations found in leaf tissue during the night (Stitt et al., 1985). Hence, the growth depression observed in amiR-cpgi plants (Fig. 3) may predominantly be explained by the inability to provide sufficient energy in the form of sucrose at night (Fig. 9) as has been demonstrated for starch metabolism mutants such as sex1 through metabolic modeling (Rasse and Tocquin, 2006; Streb et al., 2012). This energy shortage of metabolism in amiR-cpgi plants may further be aggravated by the elevated dissipation of incident light energy in the form of heat as shown in increased qN (Fig. 4B-C). This argument is in line with the more dramatic growth retardation observed in plants grown in longer nights, i.e. in short-day growth conditions (Supplemental Fig. S1B-C).
In summary our data provide evidence for an essential role of cytosolic PGI that cannot be compensated by gametophyte and early embryo metabolism. However, in mature somatic tissue, cytosolic hexose phosphate metabolism revealed a much higher adaptive ability. Here, cPGI activity-free amiR-cpgi plants performed well although plant growth was generally reduced, mainly due to the inability to generate sucrose during the night. Moreover, the starch excess phenotype of amiR-cpgi plants observed in this study and also previously identified for other cytosolic enzymes such as DPE2 (Chia et al., 2004; Lu and Sharkey, 2004) supports the existence of a yet unidentified feedback mechanism which originates in the cytosol and affects transitory starch turnover.

Materials and Methods

Plant material and growth conditions

For all investigations Arabidopsis thaliana ecotypes Columbia (Col-0) or Wassilewskija (WS) were used as wild-type control and mutant alleles of pgil-1 (Yu et al., 2000) and adgl-1 (Lin et al., 1988). T-DNA insertion lines cpgi-1 (Salk_064423) and cpgi-2 (Salk_016862) of the SALK collection were ordered from the Nottingham Arabidopsis Stock Center (NASC) (Alonso et al., 2003). Both plant lines were screened for homozygous individuals carrying a T-DNA insertion using primers: cPGI fwd5 GAGTCTGCTAAAGGACGCCAG, cPGI rev 10 GCAAGATTAGTGCTGACAGCAAC and Salk left border primer LBaI TGGTTCACGTAGTGCCGCAAC. Although cpgi-1 (Salk_064423) was annotated as a Salk homozygous line, no homozygous individuals could be identified.

All plant lines were cultivated on ½ strength MS medium (Murashige and Skoog, 1962) plus 0.8% (w/v) phyto agar, 2% (w/v) sucrose. Seeds were stratified for two days at 4°C. Plants were grown in a growth chamber at a 16/8 h, 22°C/20°C day/night cycle, a photosynthetic photon flux density of 120 µmol • m⁻² • s⁻¹ and 60 % humidity. Eleven day-old seedlings were transferred to soil. Selection of transformed plants was performed on hygromycin and/or kanamycin (50 µg/ml each) containing plates.
Leaf area and fresh weight determination

At the time of harvest pots with individual plants were photographed and then whole rosettes harvested the fresh weight measured. Leaf area was determined from images using ImageJ 1.47m (http://imagej.nih.gov/ij/).

Pulse amplitude modulated (PAM) fluorometry

In vivo chlorophyll a fluorescence assay was performed by using a pulse amplitude modulation (PAM) fluorometer of the IMAGING-PAM M-Series (Heinz Walz GmbH, Effeltrich, Germany). Intact, three-week-old plants were dark adapted for 30 minutes prior to fluorescence measurements. To investigate the photosynthetic apparatus, a dark-light induction curve was recorded by using the standard settings of the manufacturer’s software i.e., actinic light 8 (186 µmol quanta m\(^{-2}\) s\(^{-1}\)), slow induction parameters: delay 40 s, clock 20 s, duration-time 315 s. At the start of every measurement F\(_{v}/F\(_{m}\) was calculated. Photosynthetic (q\(_{p}\)) and non-photosynthetic (q\(_{N}\)) quench coefficients at photosystem II according to Schreiber et al. (1986) and ETR according to Genty et al. (1989) as summarized by Maxwell and Johnson (2000).

Cloning and YFP localization studies

PGI and cPGI open reading frames without stop codons were amplified from *A. thaliana* Col-0 leaf tissue cDNA adding XbaI/XmaI (PGI fwd XbaI TCTAGAAAATGGCCTCTCTCTCTCAGGC, PGI rev XmaI CCCGGGTGCGTACAGGTCATCCACATT) and BamHI/XmaI (cPGI fwd BamHI GGATCCAAAATGGCGTCATCAACCGC, cPGI rev XmaI CCCGGGCATCTGGGGCTCGGAAC) restriction sites to the PGI or cPGI reading frame, respectively. PCR products were subcloned into pJet1.2 vector (Thermo Scientific Life Science), sequenced and subsequently cloned in frame into either SpeI/XmaI or BamHI/XmaI opened pHygII-UT-c-term-Venus. The pHygII-UT-c-term-Venus consists of the pUBQ10 promoter (Norris et al., 1993; Krebs et al., 2012) with mutated SacI restriction site-MCS-Venus-HSP18.2 terminator (Nagaya et al., 2010) cassette and was kindly provided by Rainer Waadt and cloned via HindIII/EcoRI into hygII-MCS plasmid (Walter et al. (2004), kindly provided by Jörg Kudla’s laboratory). Constructs were transformed into *Agrobacterium tumefaciens* GV3101, and infiltrated into *Nicotiana benthamiana* leaves. After four days, subcellular localization of PGI, cPGI and an empty vector control in leaf cells was analyzed in leaf epidermal cell by spinning-disk confocal microscopy (QLC100 confocal scanning unit from Solamere Technology Group, Salt Lake City,
UT attached to a NIKON Eclipse TE 2000-U bright field microscope) using an argon laser (500M Select, Laserphysics Inc., West Jordan, UT, excitation wavelength filter at 514 nm and emission filter 500-550nm). Images were captured by a CCD-camera (CoolSnap-HQ, Photometrics, Tucson, AZ) using the Metamorph software (Universal Imaging Corporation, Downingtown, PA).

The two \textit{cPGI}-specific amiRNAs (amiRNA1 TGTACTGTTAATATGCTCCCG, amiRNA2 TATCTAGAAGTTCCAGACTT) were identified and appropriate oligos designed with the online tool at http://wmd3.weigelworld.org (Schwab et al., 2006). Neither of the two amiRNA sequences returned PGI1 as a hit when the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi, database: Refseq_rna, organism: \textit{Arabidopsis thaliana}) was used. The closest hits covered a maximum of 61% and 66% of the 21 nucleotides at E-values of 4.7 and 1.2 respectively indicating that the amiRNA sequences are specific for \textit{cPGI} transcript. The \textit{CaMV35S:amiRNA cPGI1} and 2 constructs were cloned using the TOPO- and Gateway system (Life Technologies). The final PCR product including a \textit{cPGI}-specific amiRNA was cloned into pENTR/D-TOPO vector to give the entry clones. Subsequently, pGWB2 (Nakagawa et al., 2007) was used in L/R reactions to obtain destination clones. Constructs were transformed into \textit{Agrobacterium tumefaciens} GV3101 and used to transform Col-0 plants with the floral dip method (Clough and Bent, 1998). Positive T1 individuals were selected based on kanamycin and hygromycin resistance conferred by pGWB2. After transfer to soil and another three weeks of growth in long-day conditions \textit{cPGI} enzyme activity was determined compared to a wild-type control. In total five amiRNA lines were analyzed in detail that showed almost no detectable \textit{cPGI} activity in leaf extracts. For amiRNA1 two lines designated amiR-\textit{cpgi 6} and amiR-\textit{cpgi 10} and for amiRNA2 three lines designated amiR2-\textit{cpgi 8}, amiR2-\textit{cpgi 9} and amiR2-\textit{cpgi10} were selected.

Attempts to isolate homozygous amiR-\textit{cpgi} plants revealed that despite selection on antibiotic medium, the high NPQ phenotype was lost in subsequent generations indicating that amiRNA expression might be silenced in later generations. Hence, for all analyses amiR-\textit{cpgi} plants were selected from a segregating T3 population based on occurrence of the high NPQ phenotype (Fig. 4B and C).
**Plant protein extraction and PGI activity assay**

Total protein was extracted by grinding 100 mg of leaf tissue in liquid nitrogen, resuspending the material in extraction buffer containing 100 mM Hepes-NaOH (pH 7.4), 1 mM EDTA, 5 mM β-mercaptoethanol (Carl Roth GmbH & Co KG, Karlsruhe) and 50 µg/ml PMSF in 100% EtOH (AppliChem GmbH, Darmstadt, Germany) followed by a 10 min incubation on ice. Total protein of the centrifugation-cleared solution was determined by standard Bradford assay (Roti-Quant, Carl Roth GmbH, Karlsruhe, Germany).

PGI activity was measured in a TECAN Infinite M200 multi-plate reader (Tecan Austria GmbH, Groedig, Austria) as described previously (Kunz et al., 2010). Briefly, 190 µl of assay buffer (50 mM Hepes-NaOH (pH 7.4), 1 mM EDTA, 3 mM MgCl₂, 3 mM fructose 6-phosphate, 1 mM NAD⁺ and 0.4 U/ml glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, Roche Applied Science, Mannheim, Germany) per sample were added to each well and absorption was measured at 366 nm. Then 10 µl of protein extract (10 µg total protein per well) were added and absorption at 366 nm was monitored. PGI activity was calculated from the slope of NADH production and normalized to total protein. Inactivation of the plastidic PGI isoform by heating an aliquot of each extract to 50 °C for 10 min (Jones et al., 1986b) was used to distinguish between cytosolic and plastidic PGI contribution to total PGI activity.

**Carbohydrate measurements**

Starch and sucrose leaf content were determined as described previously (Kunz et al., 2010) with minor modifications. Briefly, liquid nitrogen-ground, frozen leaf material (80-100 mg) was used for extraction in 900 µl of ethanol (80% (v/v); 60 min. at 75°C). Sucrose and starch were determined from supernatant and pellet of the same extract after centrifugation (5 min at 14,000 rpm), respectively. Isolation and measurement of water-soluble heteroglycans (SHG) were performed as described in Fettke et al. (2004).

For whole leaf starch staining chlorophyll of freshly harvested leaf rosettes was first removed in 70% ethanol (v/v) at 60°C for 20 minutes and subsequently stained in iodine-potassium iodide solution (Lugol's solution, AppliChem). Water-rinsed leaves were mounted and photographed.
**Vitality staining of pollen grains**

The viability of the pollen grains was assessed using the staining method according to Alexander (1969). Whole filaments were mounted on glass slides in staining solution and incubated for 10 min. Viable pollen grains were able to absorb the dye and would stain red.

**Accession numbers**

Sequence data of loci investigated in this study can be found in the Arabidopsis Genome Initiative under the following accession numbers: At4g24620 (PGII), At5g42740 (cPGI), At5g48300 (ADG1), At5g50950 (FUM2), At2g40840 (DPE2)

**Supplemental Material**

Supplemental Table SI: Observed and expected distribution of genotypes from the progeny of heterozygous cPGI T-DNA mutants

Supplemental Table SII: Germination rate [%] of segregating seeds collected from heterozygous cPGI/cpgi plants and Col-0 wild type.

Supplemental Figure S1: Plant growth of mutants and corresponding wild types in long and short-day conditions.

Supplemental Figure S2: Reduced PGI activity in amiR-cpgi plants.

Supplemental Figure S3: Photosynthetic and metabolic data of three independent lines expressing the second amiRNA targeting cPGI transcript (amiR2-cpgi).

Supplemental Figure S4: Non-photochemical (qN) and photochemical (qP) quench coefficients in amiR-cpgi, dpe2 and wild-type plants.

Supplemental Figure S5: Phenotype of a homozygous cpgi-1 plant expressing cPGI driven by the CaMV35 promoter

Supplemental Figure S6: Diagram illustrating the calculation of the expected genotype distribution in the self progeny of heterozygous cPGI/cpgi plants

Supplemental Figure S7: USP promoter activity monitored histochemically as β-glucuronidase activity and analyzed microscopically in flowers (A-C) and developing seeds (D-I) of transgenic pUSP:uidA plants
Acknowledgments

We thank Dr. Frank Ludewig (University of Cologne) for critical reading and comments on the manuscript, Dr. Rainer Waadt (University of California, San Diego) for providing the UBQ10-MCS-Venus-HSP18.2 cassette and Dr. Joerg Kudla (WWU-MS) for hygII-MCS plasmid, Drs. Thomas Sharkey (University of Wisconsin, Madison) and Junshi Yazaki (The Salk Institute, La Jolla) for providing dpe2 lines and SALK_149466, respectively, Sonja Lott and Sophie Wolf for excellent technical assistance.
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Figure Legends

Figure 1: Subcellular localization of PGI1 and cPGI.

PGI1 (A) and cPGI (B) Venus fusion proteins (yellow) localize to chloroplasts (red) and the cytosol, respectively. An unfused Venus protein expressed from the empty vector was used as cytosolic control (C). Constructs were transiently expressed in *Nicotiana benthamiana* leaves. All images are merged with the corresponding chlorophyll autofluorescence image (red). Scale bar = 20 µm.

Figure 2: Localization and identification of T-DNA insertion and artificial microRNA target sites for cPGI.

A: Two independent cPGI insertion lines (*cpgi-1, cpgi-2*) were isolated and the insertion site confirmed at the positions indicated.

B: Confirmation of T-DNA insertions in cPGII/cpgi-1 and cPGII/cpgi-2 by genomic DNA PCR using primers indicated in A. No homozygous plants could be identified.

C: Position of target sites of two independent artificial micro RNAs (amiRNA) designed against cPGI mRNA. Two independent lines of amiRNA1 (amiR-cpgi6, amiR-cpgi10) and three lines of amiRNA2 (amiR2-cpgi8, amiR2-cpgi9, amiR2-cpgi10) were analyzed.

Figure 3: Growth phenotype of amiR-cpgi and pgi1-1 mutants.

A: Col-0, amiR-cpgi 6, amiR-cpgi 10 and pgi1-1 grown on soil in long-day conditions for 21 days. Scale bar = 1 cm.

B: Relative leaf area and rosette leaf fresh weight of wild-type and mutant plants grown on soil in long-day conditions for 21 days. Bars represent average ± SD from three independent experiments (n = 15-30). Asterisks indicate significant difference to wild type (student’s t-test, α ≤ 0.01).

Figure 4: amiR-cpgi mutants display strongly reduced PGI activity in leaves and increased non-photochemical quenching.

A: Total enzymatic PGI (cPGI and PGI) activity in leaf extracts of *pgi1-1*, amiR-cpgi 6 and amiR-cpgi 10 plants relative to Col-0 wild-type leaves. Average ± SEM. n=4.
B: Pulse Amplitude Modulated (PAM) fluorescence imaging displaying the variable over maximal fluorescence ratio (Fv/Fm). Blue-purple false colors in leaves of all plants indicate an Fv/Fm of about 0.8 which is typical for plants with intact PSII.

C: Non-photochemical (qN) and photochemical (qP) quench coefficients and photosynthetic electron transport rates (ETR) during light induction curves determined by PAM fluorescence imaging in rosette leaves of wild type, amiR-cpgi 6, amiR-cpgi 10 and pgi1-1 plants. Average ± SD (n = 15).

Figure 5: Starch excess in leaves of amiR-cpgi plants.

A: Starch staining of whole leaf rosettes harvested at the end of the night period
B: Starch content of wild-type, amiR-cpgi 6, amiR-cpgi 10 and pgi1-1 leaves at different time points during the light period. Average ± SEM. n=3. Asterisks indicate significant difference to wild type of the same time point (student’s t-test, α ≤ 0.05).

Figure 6:Sucrose concentration and relative monosaccharide composition of cytosolic heteroglycans in leaves.

A: Sucrose concentration in wild type, amiR-cpgi 6, amiR-cpgi 10 and pgi1-1 leaves at different time points during the light period. Average ± SEM. n=3.
B: Monomer composition of low molecular weight (<10 kDa) cytosolic heteroglycans (SHGs) in leaves of wild-type, amiR-cpgi 6, amiR-cpgi 10 and pgi1-1 plants at the end of the night. Data were normalized to the major heteroglycan-monomer galactose. Average ± SEM. n = 6.

Figure 7: Phenotype of plants expressing cpgi amiRNA1 in the virtually starch-free mutant background of pgi1-1 or adg1-1 after 21 days of growth in long-day conditions on soil or sucrose-supplemented (2%, w/v) solidified ½ strength MS medium.

Figure 8: Effect of T-DNA insertion in cPGI on pollen viability and ovule/seed development.

A: Bright field image of Alexander-stained pollen grains from whole-mount filaments. Viable pollen accumulates red stain. Scale bar = 100 µm.
B: Representative siliques from wild-type, cPGI/cpgi-1 and cPGI/cpgi-2 plants. Asterisks mark empty spots in opened siliques.
C: Rate of seed abortion/development in cPGI/cpgi T-DNA mutants and wild type. Average ±SD, n = 30. Asterisks indicate significant difference to wild type (student’s t-test, α ≤ 0.01).
Figure 9: Model of cPGI involvement in sucrose and starch metabolism in wild-type and amiR-cpgi plants. Light gray arrows and labels indicate inactive steps and pathways during the respective period. MEX1 maltose exporter 1, pGlcT plastid glucose transporter, TPT triosephosphate phosphate translocator, DPE2 disproportionating enzyme 2, PHS2 cytosolic phosphorylase 2, cPGM cytosolic phosphoglucone mutase, SHG soluble heteroglycans.
Tables

Table I: Reciprocal crosses between wild type and heterozygous *cpgi* T-DNA mutants. The F1 progeny of crosses between wild type and heterozygous *cpgi* T-DNA mutants was genotyped by PCR and transmission efficiency (TE) calculated as the ratio of number of heterozygous over number of wild-type plants. Transmission efficiency of the *cpgi* allele is strongly reduced through both, male and female gametes.

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