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Plastidial Phosphoglucose Isomerase Is an Important Determinant of Seed Yield through Its Involvement in Gibberellin-Mediated Reproductive Development and Storage Reserve Biosynthesis in Arabidopsis

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The plastid-localized phosphoglucose isomerase isoform PGI1 is an important determinant of growth in *Arabidopsis thaliana*, likely due to its involvement in the biosynthesis of plastidial isoprenoid-derived hormones. Here, we investigated whether PGI1 also influences seed yields. *PGI1* is strongly expressed in maturing seed embryos and vascular tissues. *PGI1*-null *pgi1-2* plants had ~60% lower seed yields than wild-type plants, with reduced numbers of inflorescences and thus fewer siliques and seeds per plant. These traits were associated with low bioactive gibberellin (GA) contents. Accordingly, wild-type phenotypes were restored by exogenous GA application. *pgi1-2* seeds were lighter and accumulated ~50% less fatty acids (FAs) and ~35% less protein than wild-type seeds. Seeds of cytokinin-deficient plants overexpressing *CYTOKININ OXIDASE/DEHYDROGENASE1* (35S:AtCKX1) and GA-deficient *ga20ox1 ga20ox2* mutants did not accumulate low levels of FAs, and exogenous application of the cytokinin 6-benzylaminopurine and GAs did not rescue the reduced weight and FA content of *pgi1-2* seeds. Seeds from reciprocal crosses between *pgi1-2* and wild-type plants accumulated wild-type levels of FAs and proteins. Therefore, PGI1 is an important determinant of Arabidopsis seed yield due to its involvement in two processes: GA-mediated reproductive development and the metabolic conversion of plastidial glucose-6-phosphate to storage reserves in the embryo.

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INTRODUCTION

Oilseeds are major sources of calories for human consumption and are of a significant agricultural and industrial value. Seed number and weight are the two main components of seed yield. However, the genetic factors and the molecular and biochemical mechanisms controlling these agronomic traits are still poorly understood (Van Daele et al., 2012).

Isoprenoid hormones such as cytokinins (CKs) and gibberellins (GAs) regulate many aspects of plant growth, development, and metabolism, including shoot branching and elongation, shoot and reproductive meristem activity, the transition from vegetative growth to flowering, ovule formation, seed development, and the accumulation of seed storage compounds and, thus, seed yield (Fleet and Sun, 2005; Riefler et al., 2006; Rieu et al., 2008b; Mutasa-Göttgens and Hedden, 2009; Bartrina et al., 2011; D'Aloia et al., 2011; Chen et al., 2012a; Yamaguchi et al., 2014). In plants, these hormones are synthesized from precursors produced in the cytosol by the mevalonate pathway and in plastids by the methylerythritol 4-phosphate (MEP) pathway. The MEP pathway uses the central carbon intermediates pyruvate and glyceraldehyde 3-phosphate (GAP) to produce isopentenyl diphosphate and dimethylallyl diphosphate (DMAPP),

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IN A NUTSHELL

Background: Oilseeds are major sources of calories for human consumption and have significant agricultural and industrial value. Seed number and weight are the two main components of seed yield. In plants, plastidial isoprenoid-derived hormones (e.g. cytokinins [CKs] and gibberellins [GAs]) regulate growth and development and, thus, seed number. In oil crop species, fatty acids (FAs) and proteins are major contributors to seed dry weight. The metabolic pathways involved in the synthesis of these compounds use glycolytic intermediates as precursors. The plastid-localized phosphoglucose isomerase PGI1 is involved in early steps of glycolysis and thus might be involved in the production of precursors for plastidial isoprenoid-derived hormones and storage reserves. However, the role of PGI1 in these processes has not been clarified through genetic tests.

Question: We wanted to investigate if PGI1 is an important determinant of Arabidopsis seed yield. We tested this by investigating the expression pattern of *PGI1* and characterizing PGI1-null *pgi1-2* plants at the external phenotypic (number of inflorescences, siliques, and seeds per plant; flower and seed morphology) and metabolic (shoot GA and seed FA and protein contents) levels.

Findings: *pgi1-2* plants had much lower seed yields than wild-type plants. They produced fewer inflorescences and, thus, fewer siliques and seeds per plant. These traits were associated with reduced contents of bioactive GAs in shoots. In addition, *pgi1-2* seeds were lighter and accumulated less FAs and proteins than wild-type seeds. The expression pattern of *PGI1* was very similar to that of genes encoding GA and CK biosynthetic enzymes. Our results show that PGI1 is an important determinant of Arabidopsis seed yield due to its involvement in two very different and apparently unrelated processes: GA-mediated reproductive development and the metabolic conversion of plastidial glucose-6-phosphate to storage reserves in the embryo.

Next steps: We are investigating if primary carbohydrate metabolism and secondary isoprenoid metabolism in plastids are coregulated via PGI1-mediated mechanisms that harmonize the biosynthesis of isoprenoid-derived compounds with carbon supplies. In other words, we are investigating if PGI1 acts as a metabolic sensor and regulator of plastidial isoprenoid biosynthesis (and, thus, growth, development, and metabolism-related processes).

the universal prenyl diphosphate precursors of all isoprenoids (Pulido et al., 2012; Pokhilko et al., 2015).

In oleaginous species, lipids and proteins are major contributors to seed dry weight and are thus important determinants of yield (Baud et al., 2008). Maturing oilseed embryos convert sucrose obtained from maternal tissues to lipids and proteins, which are used to support postgerminative seedling growth and establishment. De novo synthesis of fatty acids (FAs) is an ATP and NAD(P)H-dependent process that is initiated in plastids (Baud et al., 2008). Analyses of expressed sequence tags from developing seeds (White et al., 2000) and microarrays of genes expressed in seeds (Girke et al., 2000; Ruuska et al., 2002) have suggested that the major route of FA biosynthesis in the model oilseed species *Arabidopsis thaliana* involves the glycolytic conversion of sucrose to phosphoenolpyruvate (PEP) and pyruvate in the cytosol. PEP is then transferred to plastids by the plastidial PEP translocator (PPT) and converted to pyruvate via the action of pyruvate kinase (PK). Genetic support for this view has been obtained from characterization of mutants impaired in cytosolic GAPDH, PPT, and plastid-localized PK (Baud et al., 2007; Prabhakar et al., 2010; Guo et al., 2014). Seeds of these mutants accumulate lower levels of FAs than wild-type seeds. Recently, Lee et al. (2017) reported that seed-specific overexpression of the pyruvate transporter BASS2 increases oil content in Arabidopsis seeds, suggesting that the transport of cytosolic pyruvate into the chloroplast plays an important role in FA biosynthesis. However, metabolic flux analyses have shown that, in oilseed rape (*Brassica napus*) and developing Arabidopsis embryos, large proportions of FAs are synthesized from glucose-6-phosphate (G6P) in plastids via the action of phosphoglucose isomerase (PGI) coupled with pathways involving glycolysis/oxidative pentose phosphate pathway (OPPP)/Calvin-Benson enzymes (Schwender et al., 2004; Lonien and Schwender, 2009).

PGI catalyzes the reversible isomerization of fructose-6-phosphate (F6P) and G6P. This enzyme is involved in early steps of glycolysis and is thus important in the generation of ATP, reductants, and precursors (amino acids) for protein biosynthesis. PGI is also involved in the regeneration of G6P pools in the oxidative pentose phosphate OPPP, which provides metabolic intermediates for the synthesis of RNA, DNA, phenolic compounds, aromatic amino acids, and NADPH. Arabidopsis has two PGI isozymes, one in the cytosol (cytPGI) and the other in the plastid (PGI1) (Yu et al., 2000; Bahaji et al., 2015). It is widely accepted that PGI1 plays a key role in transitory starch biosynthesis in mesophyll cells of leaves, connecting the Calvin-Benson cycle with the starch biosynthetic pathway (Yu et al., 2000). We have shown that Arabidopsis mutants impaired in PGI1 accumulate low levels of CKs derived from the MEP pathway, display reduced photosynthetic capacity and slow growth phenotypes, and accumulate low levels of transitory starch in leaves, a phenotype that can be reverted to the wild type by supplementation with exogenous CK (Bahaji et al., 2015). Stimulation of photosynthesis and synthesis of active CK forms is accompanied by enhanced growth and the accumulation of exceptionally high levels of starch in the mesophyll cells of PGI1 null *pgi1-2* plants (Sánchez-López et al., 2016). Thus, we have proposed that PGI1 is an important determinant of photosynthesis, transitory starch accumulation, and growth, likely due to its involvement in the synthesis of metabolic intermediates, e.g., GAP and pyruvate, required for the synthesis of plastidial isoprenoid-derived molecules (Bahaji et al., 2015).

While PGI1 appears to be involved in the production of precursors and reductants for plastidial isoprenoid-derived hormones

and storage reserves such as proteins and FA and could thus potentially be a determinant of yield, its role in these processes has not been clarified through direct genetic tests. The purpose of this study was to investigate the contribution of PGI1 to seed yield via its involvement in GA metabolism and protein and FA biosynthesis. To this end, we investigated the expression pattern of *PGI1* and characterized *pgi1-2* plants. Our results show that PGI1 is an important determinant of seed yield in Arabidopsis due to its involvement in two processes: GA-mediated reproductive development and the metabolic conversion of plastidial G6P to embryonic FA and proteins.

RESULTS

PGI1 Is Strongly Expressed in Vascular Tissues and Developing Embryos

We analyzed *PGI1* tissue expression profiles in wild-type (Ws-2) plants by qRT-PCR and histochemically analyzed wild-type plants transformed with constructs carrying the *PGI1* promoter region (1700 bp) fused to the GUS reporter. As shown in Figure 1A, qRT-PCR analyses revealed that *PGI1* is weakly expressed in mature leaves and silique envelopes but strongly expressed in roots, stems, flowers, and maturing embryos. Zymogram analyses of PGI activity further confirmed that PGI1 is expressed in maturing embryos (Figure 1B). Histochemical analyses of 10 independent *promPGI1:GUS*-expressing lines showed that the *PGI1* promoter is strongly active in vascular tissues of roots, cotyledons, and hypocotyls of young seedlings (Figure $1C_1$). It is also active in vascular tissues of fully expanded mature leaves (Figures $1C_2$ and $1C_3$), roots (Figures $1C_2$ and $1C_4$), and sepals (Figure $1C_5$). The *PGI1* promoter did not drive expression in anthers, pollen grains, or seed coat (Figure $1C_5$) and was weakly active in embryos at the heart developmental stage (Figure $1C_6$), but strongly active in maturing embryos (Figures $1C_7$ and $1C_8$). In keeping with findings of Tsai et al. (2009), compared with Figure 5, the *PGI1* promoter was expressed at subdetection limits in mesophyll cells of mature leaves (Figures $1C_2$ and $1C_3$). However, this was sufficient for normal starch synthesis in leaf mesophyll cells, as mature leaves of *pgi1-2* plants ectopically expressing *PGI1* under the control of its own promoter (*promPGI1:PGI1* plants) accumulated wild-type starch levels (Supplemental Figure 1).

Knocking out *PGI1* Decreases Seed Yield

To investigate the effects that a lack of PGI1 would have on seed yield, we compared the seed weight per plant at maturity between Ws-2 and *pgi1-2* plants. We also characterized *aps1* plants impaired in the small subunit of ADP-glucose pyrophosphorylase (Ventriglia et al., 2008), which, like *pgi1-2* plants, show a slow growth phenotype when cultured under long-day conditions and accumulate low levels of transitory starch in their leaves (Bahaji et al., 2015). As shown in Table 1, we found that seed yields of *pgi1-2* plants were only ~40% of those of wild-type plants, but wild-type yields could be restored by ectopic expression of *PGI1* under its own promoter or the 35S promoter (Table 1). In clear contrast, *aps1* plants had similar to wild-type seed yields (Table 1).

Knocking out *PGI1* Reduces the Number of Seeds per Plant

The reductions in seed yields of *pgi1-2* plants could potentially be due to reductions in seed weight or in the number of either seeds per silique or siliques per plant. To differentiate between these possibilities, we first counted the numbers of inflorescences and siliques per plant and the numbers of seeds per silique in wild-type (Ws-2) and *pgi1-2* plants. We also characterized *aps1* plants. At first sight, we detected no major differences between the external phenotypes of Ws-2 and *pgi1-2* plants at maturity (Figure 2A). Additionally, there was no significant difference in the number of seeds per silique between Ws-2 and *pgi1-2* plants (Table 1). However, many inflorescences failed to elongate in *pgi1-2* plants, and flowers developing first on the raceme exhibited short anthers and failed to produce siliques and seeds (Figure 2B). Consequently, the numbers of inflorescences with siliques and siliques per plant were lower in *pgi1-2* plants than in Ws-2 plants (Table 1), thus causing a reduction in the number of seeds per plant. This phenotype could be rescued by the ectopic expression of *PGI1* under the control of its own promoter or the 35S promoter (Table 1). Contrary to *pgi1-2*, *aps1* plants had similar numbers of inflorescences and siliques per plant to wild-type plants (Table 1). These results demonstrate that PGI1 is an important determinant of seed yield, at least partly because of its involvement in reproductive development.

The Reduced Number of Inflorescences in the *pgi1-2* Mutant Is Associated with Low Bioactive GA Content

We have reported that *pgi1-2* plants accumulate lower than wild-type levels of MEP-derived CKs and proposed that PGI1 may be involved in the production of precursors of MEP pathway-derived isoprenoid compounds (Bahaji et al., 2015). As GAs and CKs are important determinants of reproductive development (Rieu et al., 2008a, 2008b; Bartrina et al., 2011), we hypothesized that the reduction in the numbers of elongated inflorescences, well-developed flowers, siliques, and seeds in *pgi1-2* plants could be at least partly due to reduced contents of these isoprenoid hormones. To explore this possibility further, we measured levels of different GAs in shoots (all aerial parts including rosette) at bolting time in Ws-2, *pgi1-2*, and *promPGI1:PGI1* plants. We also counted the numbers of inflorescences and siliques in Ws-2 and *pgi1-2* plants following exogenous 6-benzylaminopurine (BAP) or GA_{4+7} application.

Notably, levels of GA_1, GA_3, and GA_4—the main bioactive GAs in plants (Yamaguchi, 2008; Hedden and Thomas, 2012)—were lower in *pgi1-2* shoots than in Ws-2 shoots, but wild-type levels could be restored by ectopic expression of *PGI1* (Figure 3). Also, *pgi1-2* plants accumulated higher levels of the inactive catabolites GA_{29} and GA_8 than Ws-2 plants and *promPGI1:PGI1*-expressing *pgi1-2* plants (Figure 3). Moreover, exogenous application of GA_{4+7} restored to wild type the numbers of elongated inflorescences and siliques of the *pgi1-2* mutant (Table 2). In contrast, exogenously applied BAP did not affect the number

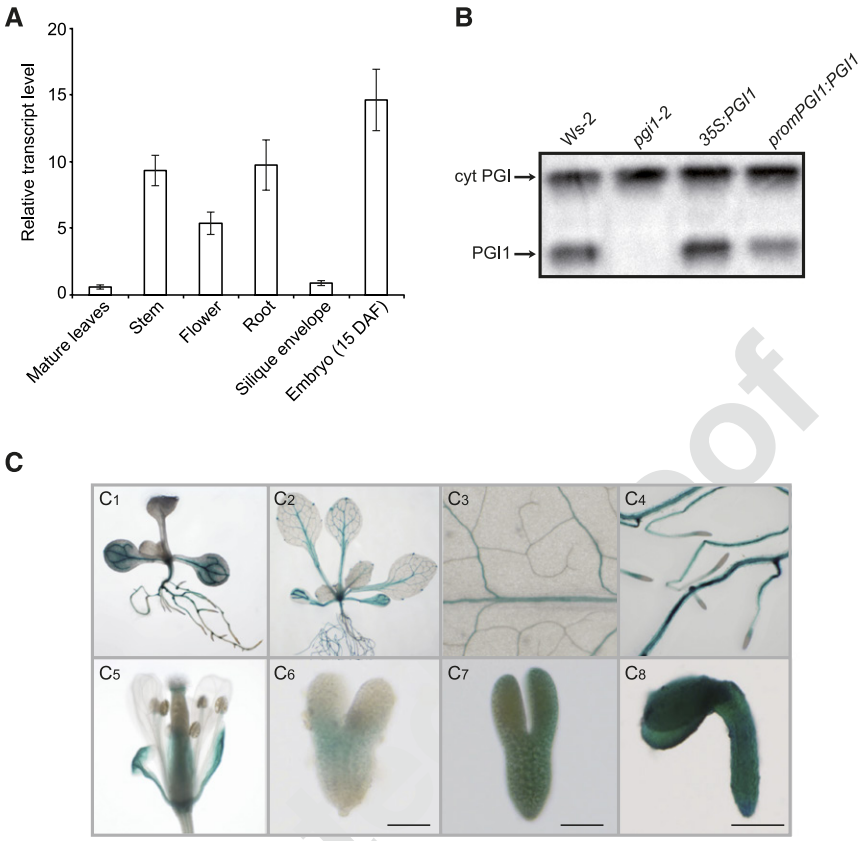

Figure 1. Expression Pattern of *PGI1*.

(A) qRT-PCR analysis of *PGI1* expression in the indicated organs of Arabidopsis. Values represent the means ± SE of four biological replicates obtained from four independent experiments, each biological replicate being a pool of the indicated organs from four plants.
(B) Zymogramic detection of PGI in proteins extracted from wild-type (Ws-2), *pgi1-2*, *promPGI1:PGI1*, and *35S:PGI1* developing seeds.
(C) Tissue-specific expression pattern of *PGI1* in transgenic *promPGI1:GUS* plants, as manifested by GUS histochemical staining of a young seedling **(C_1)**, a 3-week-old plant **(C_2)**, a magnified expanded leaf **(C_3)**, roots **(C_4)**, a flower **(C_5)**, and three stages of embryo development (**[C_6]** to **[C_8]**). Heart stage embryo **(C_6)**, embryo during transition from heart to torpedo stage **(C_7)**, mature embryo **(C_8)**. DAF, days after flowering. Bars = 50 μm in **(C_6)** and **(C_7)** and 200 μm in **(C_8)**.

Table 1. Harvest Parameters for Wild-Type (Ws-2 and Col-0), *pgi1-2*, *promPGI1:PGI1(1)*, *35S:PGI1*, and *aps1* Plants

Plant Line	No. of Elongated Inflorescences per Plant	No. of Siliques per Plant	No. of Seeds per Silique	Specific Seed Weight (μg Seed^{-1})	Seed Yield (mg Plant^{-1})
Ws-2	29.5 ± 2.5	684.1 ± 14.7	48.2 ± 0.8	18.1 ± 0.8	596.8 ± 22.1
pgi1-2	15.3 ± 1.3*	457.3 ± 22.4*	47.2 ± 0.9	11.7 ± 0.4*	252.5 ± 7.3*
promPGI1:PGI1(1)	27.5 ± 1.9	682.4 ± 18.2	46.6 ± 1.3	18.0 ± 0.5	572.4 ± 9.8
35S:PGI1	28.6 ± 0.9	662.7 ± 21.5	47.3 ± 1.2	18.2 ± 0.6	570.4 ± 19.7
Col-*0*	30.6 ± 2.6	766.1 ± 40.8	57.2 ± 0.9	17.1 ± 1.0	749.3 ± 11.9
aps1	29.6 ± 3.0	761.6 ± 22.2	58.2 ± 0.9	17.6 ± 0.7	780.1 ± 19.9

Values are means ± SE obtained from four independent experiments using 10 plants in each experiment. The asterisks indicate significant differences with respect to the wild type according to Student's *t* tests ($P < 0.05$).

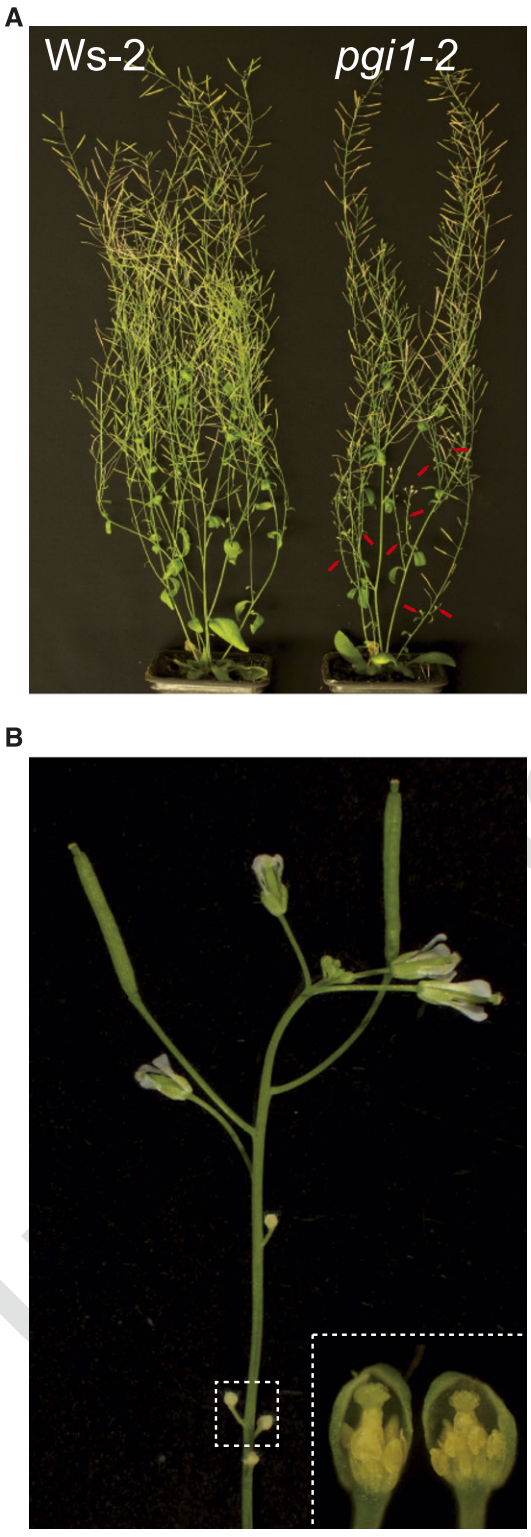

Figure 2. Knocking out *PGI1* Reduces the Number of Elongated Inflorescences and Causes Anther Development Arrest in the First Flowers of Inflorescences That Do Not Elongate.

(A) External phenotype of mature Ws-2 and *pgi1-2* plants.

(B) External phenotype of a representative inflorescence that does not elongate in *pgi1-2* plants and morphology of two dissected early flowers (inset) from the same inflorescence. In **(A)**, red arrows indicate inflorescences that do not elongate in *pgi1-2* plants.

of inflorescences and siliques (Table 2). The overall data provide strong evidence that PGI1 is an important determinant of bioactive GA content, and they indicate that the low number of inflorescences, siliques, and seeds in *pgi1-2* plants is at least partly due to lower levels of active GA forms.

GA_1 and GA_4 are produced by the GA3-oxidase (GA3ox)-mediated oxidation of GA_{20} and GA_9, respectively (Figure 3). These bioactive GAs can be deactivated by GA2-oxidase (GA2ox), yielding GA_8 and GA_{34}, respectively (Yamaguchi, 2008; Hedden and Thomas, 2012) (Figure 3). GA2ox can also metabolize GA_{20} to the inactive GA_{29} (Hedden and Thomas, 2012). In Arabidopsis shoots, the most strongly expressed GA2ox- and GA3ox-encoding genes are *GA2ox6* and *GA3ox1*, respectively (Mitchum et al., 2006; Rieu et al., 2008a). Whereas *GA3ox1* is upregulated by CKs, *GA2ox6* is downregulated by these hormones (Kiba et al., 2005; Bhargava et al., 2013; Brenner and Schmülling, 2015). This and the finding that *pgi1-2* plants accumulate low levels of CKs (Bahaji et al., 2015) would suggest that the reduced contents of bioactive GAs and enhanced levels of inactive GAs in *pgi1-2* plants could be due to reductions in *GA3ox1* and/or increases in *GA2ox6* expression caused by the reduced CK content. To investigate this hypothesis, we conducted qRT-PCR analyses of *GA2ox* and *GA3ox* genes in shoots at bolting time in Ws-2 and *pgi1-2* plants. We also analyzed the expression levels of *GA20ox* genes. As shown in Supplemental Figure 2, no significant differences in the expression levels of these genes were found between wild-type and *pgi1-2* shoots.

Knocking out *PGI1* Decreases Seed Weight and the Contents of Proteins and Fatty Acids

We next addressed the possibility that the low seed yields of *pgi1-2* plants could also be related to reductions in seed weight. As shown in Table 1, *pgi1-2* seeds were 35% lighter than Ws-2 seeds, but wild-type weights could be restored by ectopic expression of *PGI1*. Analyses of embryonic phenotypes in a series of developing pollinated pistils revealed no major differences between *pgi1-2* seeds and Ws-2 seeds (Supplemental Figure 3), excluding the possibility that the reduced weight of *pgi1-2* seeds could be due to aberrant embryo growth and development. Dry *pgi1-2* seeds displayed a wrinkled phenotype (Figures 4A and 4B), accumulated 35% less dry matter than Ws-2 seeds (Figure 4C), and had lower establishment rates on MS agar with no supplemental sugar than wild-type seeds (Figure 5). These phenotypes could be restored by ectopic expression of *PGI1* (Figures 4 and 5).

Focks and Benning (1998) suggested that the wrinkled appearance of oil-deficient *wri1-1* seeds could be due to an increased sucrose content during seed development. To determine whether this might be the case in *pgi1-2* seeds, we performed time-course analyses of sucrose levels in developing Ws-2 and *pgi1-2* seeds. As shown in Supplemental Figure 4, there were

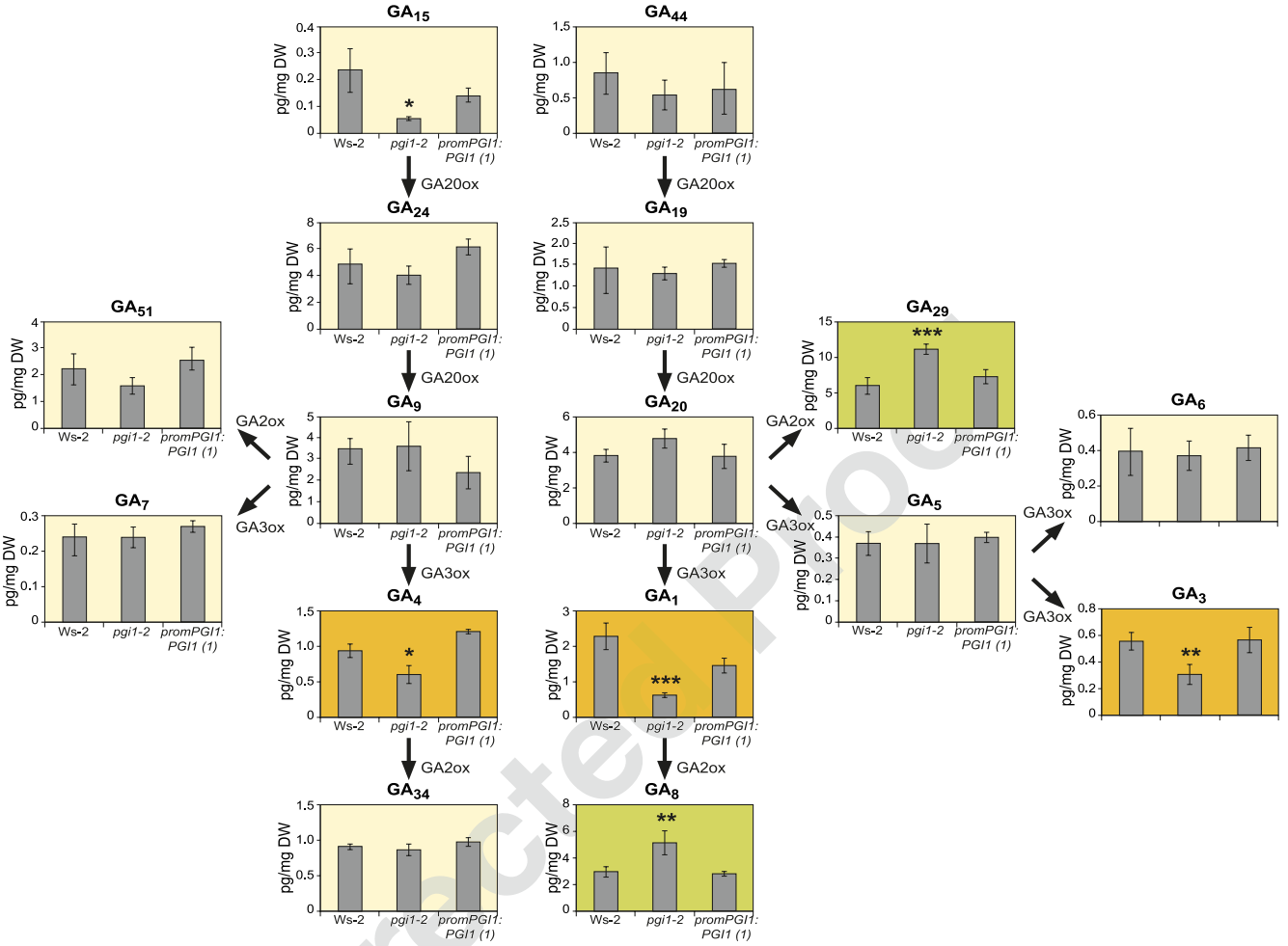

Figure 3. Knocking out *PGI1* Decreases Levels of Bioactive GAs and Increases Levels of Inactive GAs.

Contents of the indicated GAs (pg mg^{-1} DW) in shoots (20 d after sowing) of Ws-2 and *pgi1-2* plants, and *pgi1-2* plants transformed with *promPGI1:PGI1(1)*. Plants were harvested at the end of the light phase. Active GAs (significantly less abundant in *pgi1-2* plants than in Ws-2 plants) and inactive GAs (significantly more abundant in *pgi1-2* plants than in Ws-2 plants) are highlighted in orange and green, respectively. Values represent the means ± SE of two independent experiments, each consisting of four biological replicates corresponding to a pool of shoots from four plants. Asterisks indicate significant differences with respect to Ws-2 according to ANOVA (*P < 0.05, **P < 0.01, and ***P < 0.001).

no detectable differences between Ws-2 and *pgi1-2* seeds with respect to their sucrose contents during development.

Oil and proteins are major components of dry Arabidopsis seeds (Baud et al., 2008), and seeds with reduced FA content show a wrinkled phenotype (Focks and Benning, 1998; Baud et al., 2007; Chen et al., 2015). Furthermore, seedlings of mutants with defects in the synthesis or postgerminative mobilization of seed storage lipids have reduced establishment rates in the absence of an exogenous sugar source (Hayashi et al., 1998; Cernac et al., 2006; Baud et al., 2007). Thus, these observations suggested that *pgi1-2* seeds have reduced protein and lipid contents. This inference was corroborated by analysis of protein and FA levels (following methylesterification) in mature seeds. As shown in Figures 4D and 4E, *pgi1-2* seeds accumulated ~35% less protein and ~50% less FA than Ws-2 seeds at maturity, but wild-type levels could be restored by ectopic expression of *PGI1*. On a dry weight (DW) basis, *pgi1-2* seeds accumulated ~8% less protein and 23% less FA than Ws-2 seeds (Figures 4F and 4G).

We also analyzed the FA composition in mature *pgi1-2* seeds and, as shown in Figure 4H, found no significant differences in relative contents of the saturated FAs palmitic acid (16:0) and stearic acid (18:0) between Ws-2 and *pgi1-2* seeds. In contrast, relative amounts of the desaturation intermediates, oleic (18:1), and linoleic (18:2) acids, were lower in *pgi1-2* seeds than in Ws-2 seeds, while the relative amounts of the end products of desaturation and elongation, linolenic (18:3) and erucic (22:1) acids, were higher (Figure 4H). The FA composition profile of *pgi1-2* seeds was similar to that of seeds of the low seed oil *wri1* mutant and plants lacking the plastid-localized PK (Focks and Benning, 1998; Baud et al., 2007). These mutations impair FA biosynthesis by reducing precursor supplies. These findings

Table 2. Number of Inflorescences and Siliques Produced by Wild-Type and *pgi1-2* Plants with or without Exogenous 100 μM BAP or GA_{4+7} Treatment

Plant Line	No. of Elongated Inflorescences per Plant	No. of Siliques per Plant
Ws-2	29.5 ± 2.5	684.1 ± 14.7
pgi1-2	15.3 ± 1.3*	457.3 ± 22.4*
Ws-2 + GA	27.3 ± 1.5	729.1 ± 34.2
pgi1-2 + GA	27.1 ± 2.2	715.6 ± 74.1
Ws-2 + BAP	26.4 ± 1.8	657.3 ± 19.3
pgi1-2 + BAP	14.4 ± 1.2*	417.3 ± 26.4*

Values are means ± SE obtained from four independent experiments using 10 plants in each experiment. The asterisks indicate significant differences with respect to the wild type according to Student's *t* tests ($P < 0.05$).

indicate that PGI1 is an important determinant of seed oil accumulation through its involvement in the provision of precursors for FA synthesis.

The Low Fatty Acid Content of *pgi1-2* Seeds Is Not Due to Reduced Active GA or CK Contents

pgi1-2 plants accumulate low levels of active forms of CKs (Bahaji et al., 2015) and GAs (this work). To determine whether the reduced weight and FA content of *pgi1-2* seeds could be due to low levels of active CKs or GAs, we characterized seeds of CK-deficient plants overexpressing *CYTOKININ OXIDASE/DEHYDROGENASE1* (35S:AtCKX1 plants) (Werner et al., 2003) and the GA-deficient *ga20ox1 ga20ox2* double mutant (Rieu et al., 2008b). Furthermore, we characterized seeds of *pgi1-2* plants treated with exogenous BAP or GA_{4+7}. In keeping with the findings of Werner et al. (2003), 35S:AtCKX1 seeds were enlarged (Figure 6A) and had almost twice the mass of wild-type seeds (Figure 6B) due to the role of CKs in controlling the cell division rate in maturing embryos (Werner et al., 2003). Additionally, the FA content of 35S:AtCKX1 seeds was twice that of wild-type seeds (Figure 6C). Conversely, the size, weight, and FA content of *ga20ox1 ga20ox2* seeds were comparable to those of wild-type seeds (Figures 6A to 6C). Moreover, treatment with exogenous BAP and GA_{4+7} did not rescue the reduced weight and FA content phenotypes of *pgi1-2* seeds (Figures 6D and 6E), showing that the low FA content of *pgi1-2* seeds is not due to reduced active GA or CK contents.

PGI1 Zygotically Controls FA and Protein Accumulation

Protein and FA contents in Arabidopsis seeds can be influenced by diverse maternal factors. For example, reductions in maternal carbon supplies may cause reductions in FA accumulation in seeds (Chen et al., 2015). Proteins involved in regulating flavonoid biosynthesis in the seed coat can affect seed oil deposition by downregulating embryonic FA biosynthetic genes (Chen et al., 2012b, 2014). In addition, the homeotic regulatory gene *APETALA2* can affect seed oil and protein accumulation through its action in the maternal sporophyte and endosperm genomes (Jofuku et al., 2005). *pgi1-2* plants have reduced photosynthesis rates (Bahaji et al., 2015), so their low seed protein and FA contents could potentially be due to maternal factors such as limited photosynthate supplies or signals from maternal tissues. To test this possibility, we performed reciprocal crosses between *pgi1-2* and Ws-2 plants and analyzed the percentages of wrinkled seeds, protein, and fatty acid methylester (FAME) content in seeds of the F1 progeny. As shown in Figure 7, there was no significant difference in the percentage of wrinkled seeds and seed FA and protein contents between the progeny of Ws-2 plants and *pgi1-2* and Ws-2 crosses, showing that the low FA and protein contents of *pgi1-2* seeds is dependent on the embryonic genotype but not on factors from the maternal tissues.

Plastidial Phosphofructokinase-Mediated Metabolism of F6P Produced by PGI1 Is Not Absolutely Required for Normal Fatty Acid Biosynthesis in Arabidopsis

Schwender et al. (2004) comprehensively delineated every possible conversion of G6P entering the plastid into FAs in oil seed embryos using a network model consisting of glycolysis, the OPPP, the Calvin-Benson cycle, and FA synthesis enzymes. The study revealed seven flux modes that include Rubisco and that have higher carbon use efficiencies than glycolysis, two of which (modes 16 and 18) involve ATP-dependent phosphofructokinase (PFK) and bypass the glycolytic steps catalyzed by GAPDH and phosphoglycerate kinase (PGK). Two other modes (24 and 25) also have zero flux through PFK (Schwender et al., 2004). To determine whether plastidial PFK-mediated metabolism of F6P produced by PGI1 is absolutely required for normal seed FA synthesis, we measured the FA contents in seeds of the *pfk4 pfk5* double knockout mutant of Arabidopsis, which has impairments in the two plastidial PFK isoforms expressed in Arabidopsis (one active, PFK4, and the other, PFK5, having weak activity when transiently expressed in wild tobacco [*Nicotiana benthamiana*] plants; Supplemental Figure 5). We reasoned that if the plastid-localized PFK is absolutely required for the G6P-to-FA conversion, *pfk4 pfk5* seeds should accumulate low levels of FAs. Conversely, if the plastid-localized PFK-mediated metabolism of F6P produced by PGI1 is not absolutely required for normal FA biosynthesis, *pfk4 pfk5* seeds should not accumulate low levels of FAs. As shown in Figure 8, the latter prediction was verified: *pfk4 pfk5* seeds displayed a wild-type external phenotype and did not accumulate low levels of FA.

DISCUSSION

PGI1 Is Expressed in Organs That Produce Active GAs and MEP Pathway-Derived CKs

We have proposed that PGI1 could be involved in the glycolytic conversion of plastidial G6P into GAP and pyruvate necessary for the synthesis of plastidial isoprenoid-derived hormones, as schematically illustrated in Figure 9A (Bahaji et al., 2015). The results presented here consistently indicate that *PGI1* is expressed in organs expressing genes associated with the biosynthesis of GA and MEP pathway-derived CKs. In particular, this gene is expressed in vascular tissues (Figure 1), like genes such

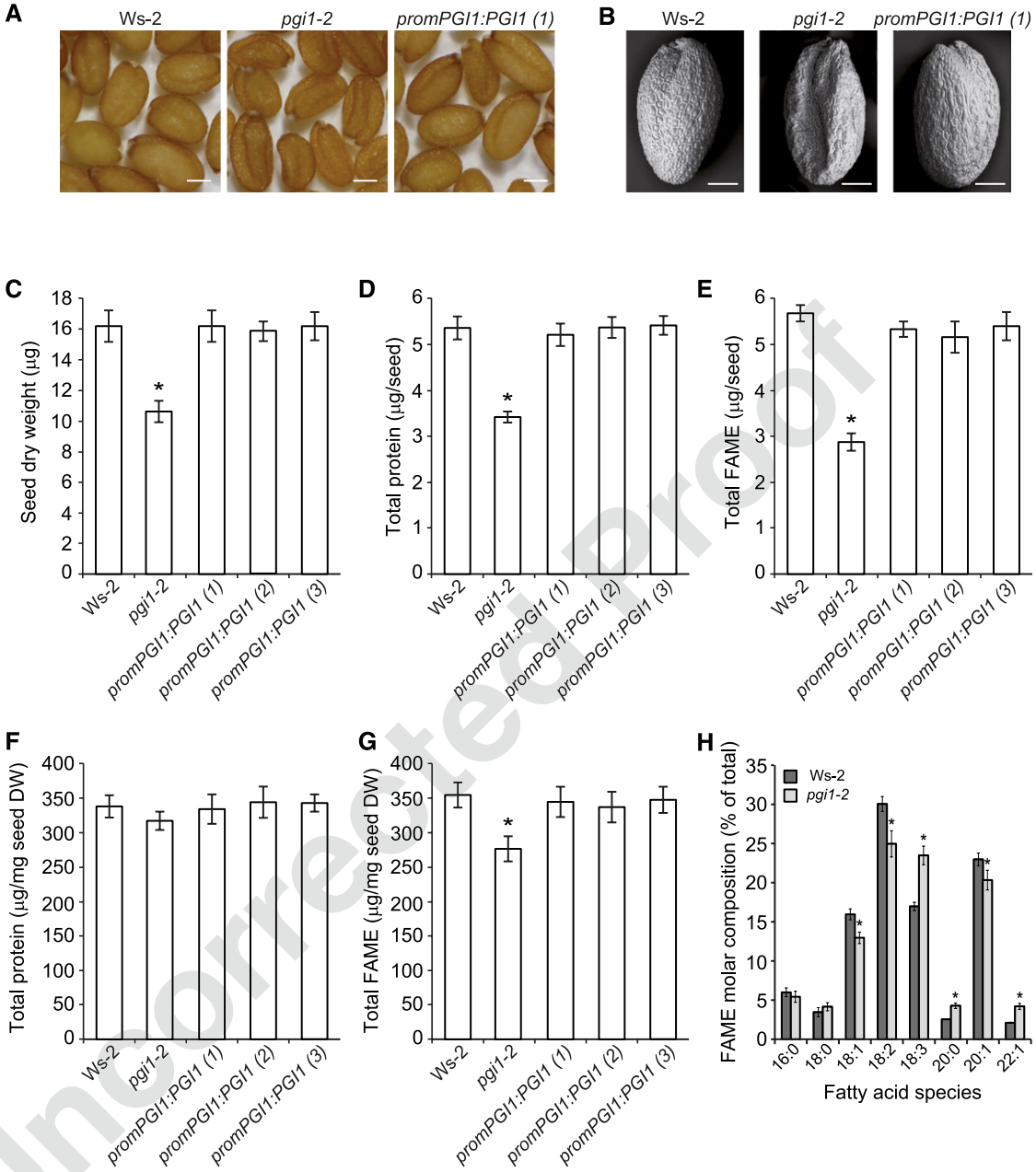

Figure 4. Protein and FA Contents Are Diminished in *pgi1-2* Seeds.

(A) and **(B)** External phenotype **(A)** and scanning electron micrographs **(B)** of seeds from wild-type (Ws-2) and *pgi1-2* plants, and one line of *pgi1-2* plants ectopically expressing *PGI1* under the control of the *PGI1* promoter [*promPGI1:PGI1(1)*].

(C) to **(G)** Seed dry weight **(C)**, total protein content per seed **(D)**, total FAME contents per seed **(E)**, total protein content on DW basis **(F)**, and total FAME content **(G)** on DW basis of dry seeds of the wild type, *pgi1-2*, and three independent lines of *pgi1-2* plants ectopically expressing *PGI1* under the control of the *PGI1* promoter [*promPGI1:PGI1(1-3)*].

(H) FA profile of dry mature wild-type and *pgi1-2* seeds.

Values in **(C)** to **(H)** represent the means ± SE of four biological replicates obtained from four independent experiments, each biological replicate being a pool of 300 seeds **(C)**, 200 seeds (**[D]** and **[F]**), or 20 seeds (**[E]**, **[G]**, and **[H]**). Asterisks indicate significant differences from wild-type seeds according to Student's *t* tests ($P < 0.05$). Bars in **(A)** and **(B)** = 200 and 100 µm, respectively.

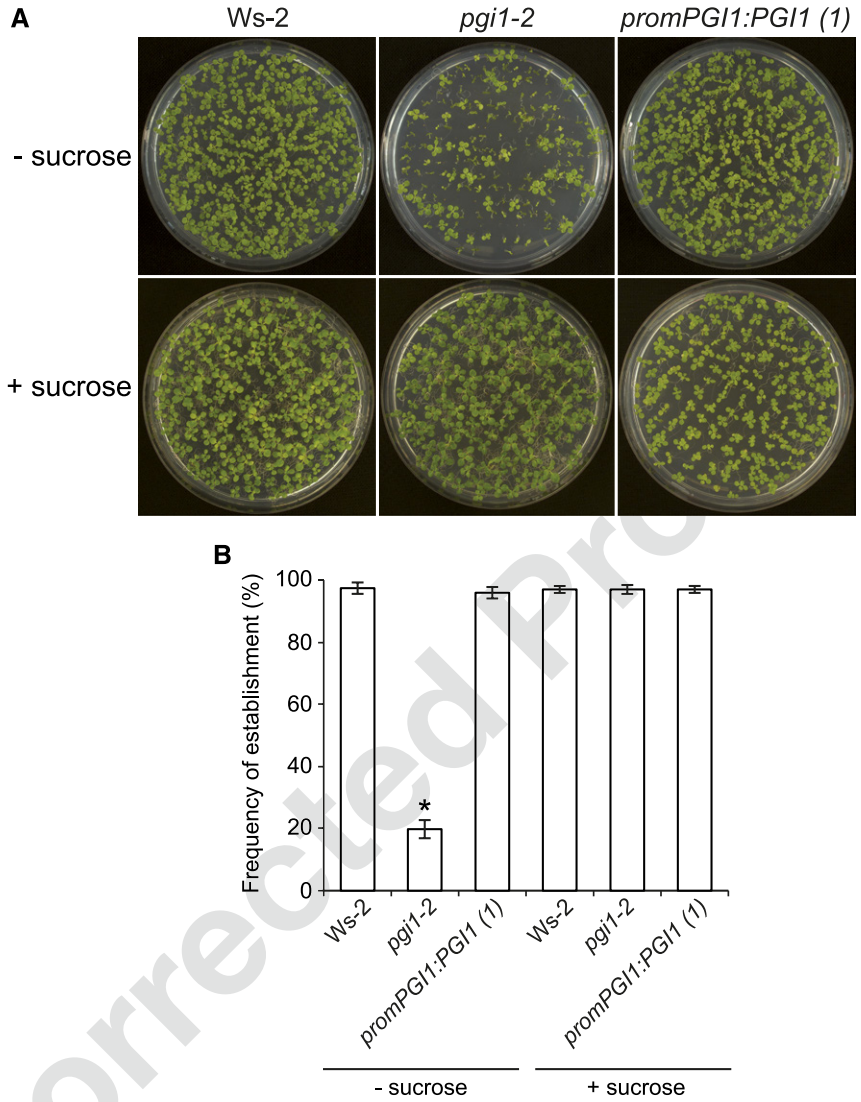

Figure 5. *pgi1-2* Seedlings Have Reduced Establishment Rates.

Photographs **(A)** and establishment rates **(B)** 12 d after sowing of wild-type (Ws-2), *pgi1-2*, and *promPGI1:PGI1(1)* plants cultured in MS with or without sucrose supplementation. Values in **(B)** are means ± SE of four biological replicates obtained from four independent experiments, each biological replicate being ~100 seeds. The asterisk indicates significant differences in establishment rates between wild-type and *pgi1-2* plants according to Student's *t* tests ($P < 0.05$).

as *GA1* (which encodes the enzyme that catalyzes the first committed step of GA biosynthesis, the *ent*-copalyl diphosphate synthase), *GA3ox1* and *GA3ox2* (which encode enzymes that catalyze the production of bioactive GAs), and several genes encoding enzymes involved in rate-limiting steps of plastidial CK biosynthesis (Silverstone et al., 1997; Miyawaki et al., 2004; Mitchum et al., 2006). The strikingly similar expression patterns of *PGI1* and these genes involved in the synthesis of active GAs and CKs would suggest that primary carbohydrate metabolism and secondary isoprenoid metabolism in plastids are transcriptionally coregulated via mechanisms that harmonize GA and CK biosynthesis rates (and, thus, growth and development-related processes) with carbon supplies. Accordingly, like *PGI1* and some genes involved in the synthesis of plastidial isoprenoid-derived hormones, *PPT1* and *PKp2* (both encoding enzymes that function in the provision of pyruvate to the plastid) are expressed in vascular tissues of adult plants and seedlings (Knappe et al., 2003; Baud et al., 2007).

Environmental stimuli such as light, temperature, and salt stress specifically regulate the expression of GA biosynthetic enzymes, allowing GAs to translate these extrinsic signals into developmental changes according to environmental conditions (Yamaguchi, 2008). Oxidative modification of redox-sensitive proteins plays important roles in modulating rapid physiologi-

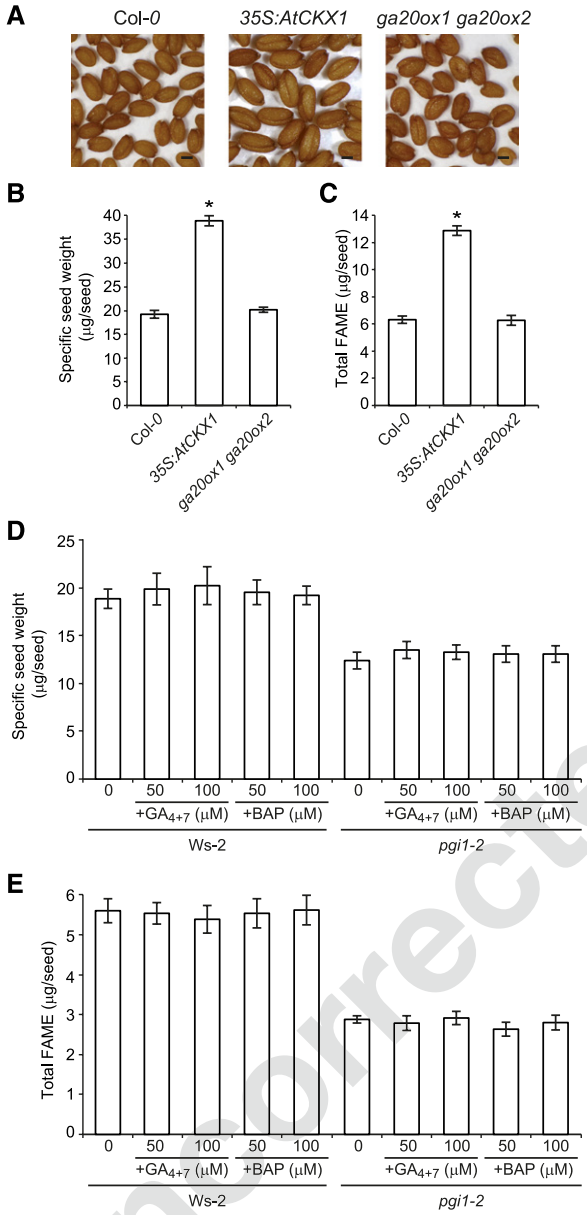

Figure 6. The Low FA Content Phenotype of *pgi1-2* Seeds Is Not Due to Reduced Active CK or GA Contents.

(A) to **(C)** External phenotype **(A)**, specific weight **(B)**, and total FAME contents **(C)** of wild-type (Col-0), *35S:AtCKX1*, and *ga20ox1 ga20ox2* dry seeds.

(D) and **(E)** Specific weight **(D)** and total FAME contents **(E)** of dry seeds of Ws-2 and *pgi1-2* plants following exogenous CK and GA_{4+7} application.

Bar in **(A)** = 200 µm. Values in **(B)** to **(E)**, represent the means ± SE of four biological replicates obtained from three independent experiments, each biological replicate being a pool of 300 seeds (**[B]** and **[D]**) or 20 seeds (**[C]** and **[E]**). In **(B)** and **(C)**, asterisks indicate significant differences from wild-type seeds according to Student's *t* tests ($P < 0.05$).

cal and developmental responses of plants to changing environmental conditions (Foyer and Noctor, 2005). Notably, recent high-throughput analyses of proteins whose thiols undergo reversible oxidative modifications in Arabidopsis have revealed that PGI1 is a redox-sensitive protein that responds to agents that perturb cellular redox homeostasis and inhibit growth (Liu et al., 2014; Yin et al., 2017). It is thus tempting to speculate that PGI1 could play an important role in fine regulatory tuning of plastidial isoprenoid hormone-driven growth to a pace that is compatible with the plant's carbon status under prevailing environmental conditions.

PGI1 Is an Important Determinant of Seed Yield Due to Its Involvement in GA-Mediated Reproductive Development

pgi1-2 plants produce fewer elongated inflorescences than Ws-2 plants, and flowers developing first on the short inflorescences fail to produce siliques and seeds (Figure 2B). Consequently, *pgi1-2* plants produce fewer siliques and seeds than Ws-2 plants (Table 1). This phenotype cannot be ascribed to slow plant growth or low leaf starch content, since the number of inflorescences and the seed yield of starch-deficient *aps1* plants were comparable to those of wild-type plants (Table 1).

In Arabidopsis, bioactive GAs stimulate elongation of vegetative stem internodes upon bolting and exert a positive effect on inflorescence length and flower formation, thus acting as major determinants of reproductive development and yield (Rieu et al., 2008b; Yamaguchi et al., 2014). Here, we have shown that levels of the main bioactive GAs (e.g., GA_1, GA_3, and GA_4) are lower in *pgi1-2* plants than in Ws-2 plants and *pgi1-2* plants ectopically expressing *PGI1* (Figure 3). These findings strongly indicate that the reduced numbers of elongated inflorescences, siliques, and seeds per *pgi1-2* plant are partly due to their diminished content of active GAs. Accordingly, exogenous GA application restored to wild type the numbers of elongated inflorescences and siliques in *pgi1-2* plants (Table 2). Therefore, we propose that PGI1 is a major determinant of seed yield, at least in part, due to its regulatory action on GA homeostasis and thus reproductive development.

To maintain optimum growth rates, plants have evolved homeostatic regulatory mechanisms for modulating GA levels involving both negative feedback and positive feed-forward transcriptional regulation of GA metabolism genes (Hedden and Thomas, 2012). Plants have also evolved less well-defined mechanisms for posttranscriptional regulation of GA metabolism that may involve microRNA-mediated destabilization and/or reductions in the efficiency of translation of several *GA2ox*, *GA3ox*, and *GA20ox* transcripts (http://sundarlab.ucdavis.edu/mirna/) (Barker, 2011) and modification of the stability of GA metabolism enzymes (Magome et al., 2004; Lee and Zeevaart, 2007; Barker, 2011). The qRT-PCR analyses conducted in this study revealed no major differences in the expression of GA metabolism genes between Ws-2 and *pgi1-2* plants that could account for the observed differences in the GA contents of these genotypes (Supplemental Figure 2). This finding suggests that there are further complexities in GA homeostasis due to mechanisms in which PGI1 plays an important role in regulating the [AQ2]

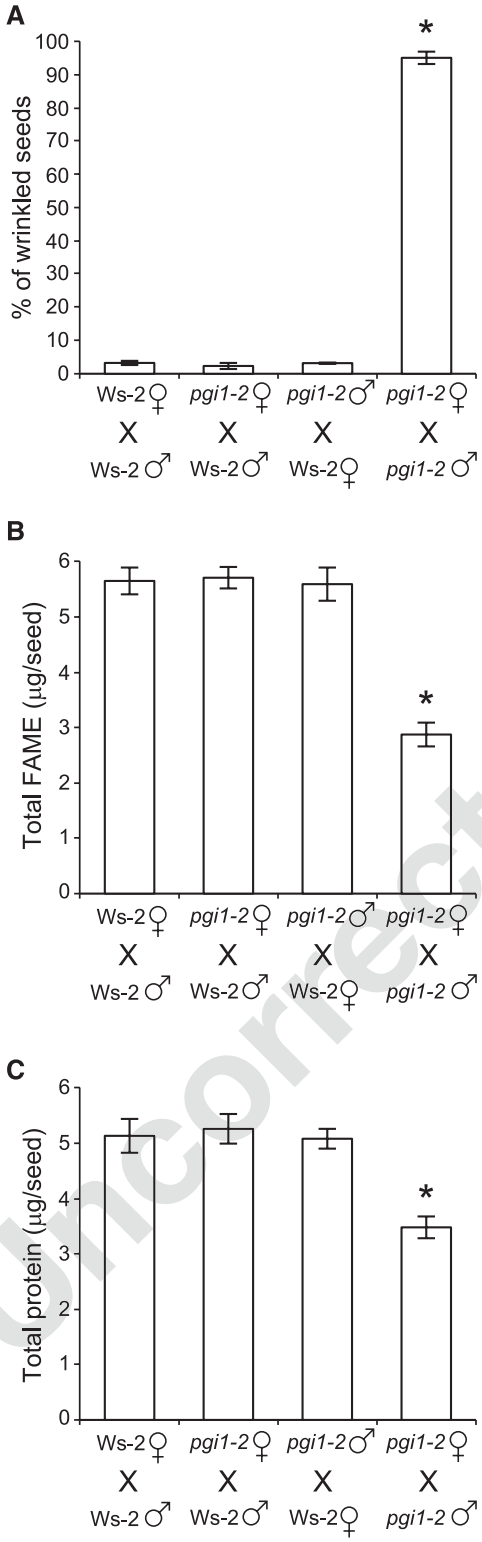

Figure 7. The Phenotype of *pgi1-2* Seeds Is Embryonically Controlled.

Percentage of wrinkled seeds **(A)**, total FAME **(B)**, and protein contents **(C)** in the F1 progeny of the indicated wild-type (Ws-2) and *pgi1-2* crosses. Values represent the means ± SE of three biological replicates obtained from three independent experiments, each biological replicate being a pool of 300 seeds **(A)**, 20 seeds **(B)**, or 200 seeds **(C)**. Asterisks indicate significant differences from Ws-2 × Ws-2 cross according to Student's *t* tests ($P < 0.05$).

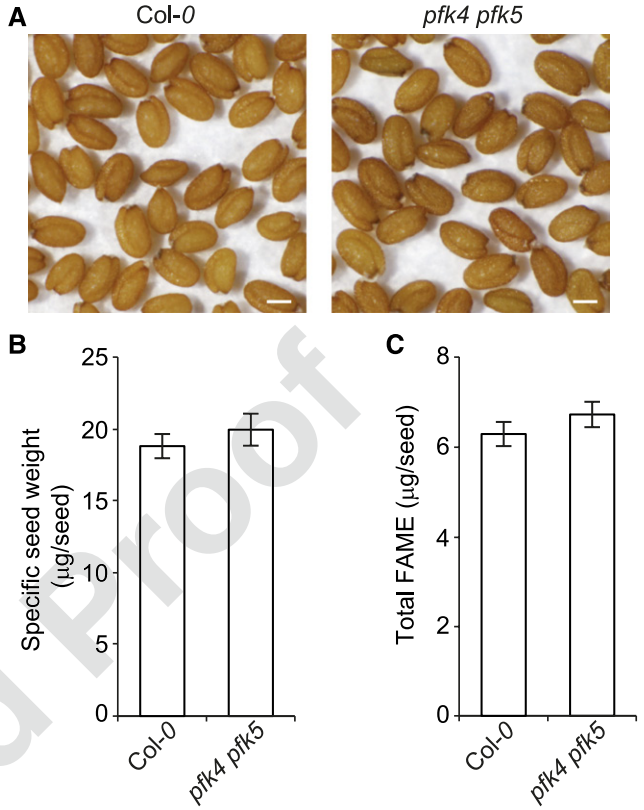

Figure 8. Plastidial PFK-Mediated Metabolism of F6P Produced by PGI1 Is Not Absolutely Required for Normal FA Biosynthesis in Arabidopsis.

External phenotype **(A)**, specific weight **(B)**, and total FAME contents **(C)** of wild-type (Col-0) and *pfk4 pfk5* dry seeds. Bar in **(A)** = 200 µm. In **(B)** and **(C)**, values represent the means ± SE of three biological replicates obtained from five independent experiments, each biological replicate being a pool of 300 seeds **(B)** or 20 seeds **(C)**.

activity of GA metabolism enzymes and/or providing precursors for active GA biosynthesis.

PGI1 Is an Important Determinant of Seed Yield Due to Its Involvement in Embryonic Fatty Acid and Protein Biosynthesis

PGI1's roles in seed FA and protein production have previously been obscure. Here, we have provided evidence that PGI1 contributes to seed yield through participation in the generation of precursors for embryonic FA and protein biosynthesis, as schematically illustrated in Figure 9B. The hypothesis is strongly supported by the following findings: *pgi1-2* seeds accumulate ~35% less protein and 50% less FAs than Ws-2

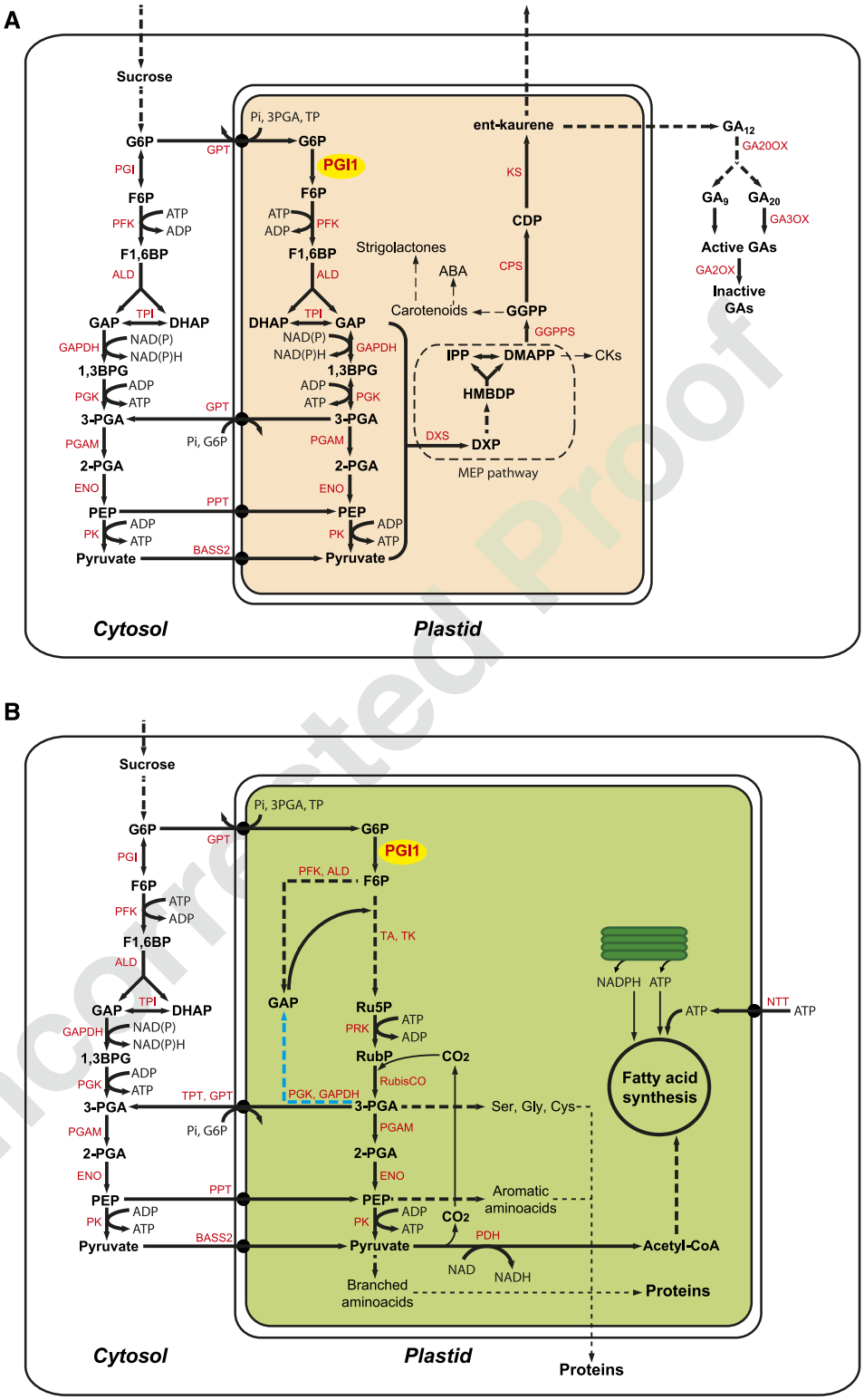

Figure 9. Suggested Schemes for PGI1-Mediated Biosynthesis of MEP Pathway Isoprenoid-Derived CKs and GAs in Cells of Vascular Tissues, and FAs and Proteins in Mixotrophic Maturing Embryos of Arabidopsis.

In both cases, cytosolic G6P can be glycolytically converted to PEP and pyruvate, which can enter plastids via the PPT and BASS2 transporters, respectively. According to the scheme in **(A)**, some of the cytosolic G6P can be incorporated into plastids through the hexose-P transporter (GPT) and

seeds, respectively (Figure 4), *PGI1* is strongly expressed in the embryo (Figure 1), and *PGI1* zygotically controls FA and protein accumulation (Figure 7). Two findings strongly indicate that the low FA contents of *pgi1-2* seeds are not due to reduced active CK and GA contents: Seeds of CK-deficient 35S:AtCKX1 and GA-deficient *ga20ox1 ga20ox2* do not accumulate low levels of FA (Figure 6C), and exogenous application of BAP and GA_{4+7} did not rescue the reduced weight and FA content of *pgi1-2* seeds (Figures 6D and 6E). The observation that *pgi1-2* seeds have elevated relative contents of linolenic and erucic acids (Figure 4H) indicates that no feedback regulation occurs in this mutant to adjust the degree of unsaturation and elongation of the FAs to the decrease of carbon flow into the FA biosynthetic pathway and that different regulators control the activities of enzymes involved in lipid metabolism during seed development.

PGI may be involved in regenerating the G6P pool in the OPPP or early steps of glycolysis. The plastidial OPPP has been regarded as one of the major sources of NADPH linked to FA biosynthesis in oilseeds (Kang and Rawsthorne, 1996; Eastmond and Rawsthorne, 1998; Hutchings et al., 2005). An objection to this hypothesis is that plastid-localized G6P dehydrogenase (which catalyzes G6P's entry into the plastidial OPPP) is subject to redox inactivation during illumination (Née et al., 2009), so plastidial G6P dehydrogenase is likely inactive during FA accumulation in green oilseeds. Furthermore, metabolic flux analyses have indicated that the reductant produced by the plastidial OPPP accounts for only 20% of the total reductant needed for FA synthesis in oilseed embryos (Schwender et al., 2003). The glycolytic capacity in plastids of lipid-storing seeds considerably exceeds requirements for maximum rates of lipid synthesis (Eastmond and Rawsthorne, 2000). Thus, the glycolytic pathway could play an important role in the conversion of photosynthate to oil. However, previous biochemical analyses with oilseed rape and Arabidopsis embryos have shown that up to 80% of the 3PGA linked to FA biosynthesis is generated from G6P through pathways involving glycolysis/OPPP/Calvin-Benson enzymes that have higher carbon use efficiencies than glycolysis (Schwender et al., 2004; Lonien and Schwender, 2009). Some of these pathways involve plastidial PFK. Others have zero flux through PFK and derive the GAP needed for Ru5P production via 3PGA metabolism (Schwender et al., 2004; Lonien and Schwender, 2009). In this work, we found that *pfk4 pfk5* seeds do not accumulate low levels of FA (Figure 8C), strongly indicating that plastidial PFK is not absolutely required for the metabolic conversion of F6P produced by PGI1 into FAs in Arabidopsis seeds. To explain why the *pgi1-2* mutation reduces seed FA content but the *pfk4 pfk5* mutation does not, we propose that *pgi1-2* completely blocks the entry of carbon into the 3PGA-generating glycolysis/OPPP/Rubisco network, whereas *pfk4 pfk5* can be compensated for by Rubisco shunt flux modes that cause some 3PGA formed in the plastids to be converted into the GAP needed for Ru5P production by plastidial GAPDH and PGK enzymes (Figure 9B). The remainder of the 3PGA produced in the plastids may be metabolized in plastids and/or exported to the cytosol, where it may be converted to PEP and pyruvate, which may move back into plastids and subsequently be converted to FAs (Figure 9B).

According to a flux map of central carbon metabolism established in steady state ^{13}C labeling experiments by Schwender et al. (2003), PEP is preferentially transported from the cytosol to the stroma as a substrate for de novo FA synthesis in developing rapeseed embryos. The Arabidopsis genome contains two PPT-encoding genes: *PPT1* and *PPT2* (Knappe et al., 2003). Unlike *PPT1*, *PPT2* is not expressed in seeds (Knappe et al., 2003) (see also http://bar.utotonto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007). Although the overall information indicates that PPT1 participates in embryonic FA biosynthesis, Prabhakar et al. (2010) showed that seeds of Arabidopsis *cue1* plants with defective PPT1 can accumulate up to 85% of wild-type FA contents. This indicates that PEP may not be the major form of photosynthate that enters the plastid for subsequent conversion to FAs in Arabidopsis seeds. Moreover, the findings that *pgi1-2* seeds and seeds of *pkp2* plants impaired in plastidial PK accumulate 50% and 60% less FA than wild-type seeds, respectively (Baud et al., 2007; this work) strongly indicate that a sizable pool of the photosynthate linked to Arabidopsis seed FA production enters the plastid as G6P and pyruvate, as shown schematically in Figure 9B.

Figure 9. (continued).

subsequently glycolytically converted to GAP and pyruvate. Plastidial GAP and pyruvate are then metabolized by 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS) to products that enter the MEP pathway and fuel synthesis of isoprenoid hormones (Pulido et al., 2012; Pokhilko et al., 2015). DMAPP can be converted to *ent*-kaurene in a three-step process catalyzed by geranylgeranyl diphosphate (GGPP) synthase (GGPPS), *ent*-copalyl diphosphate synthase (CPS), and ent-kaurene synthase (KS). *ent*-Kaurene can be then transported to other parts of the plant or oxidized to GA_{12}, which is converted to bioactive GA in the cytosol by GA20ox and GA3ox. DMAPP can also be converted to CKs (Spíchal, 2012). In heterotrophic cells lacking plastid-localized phosphoglyceromutase (PGAM), enolase (ENO), and the triose-P translocator (TPT), 3PGA produced in the plastid via PGI1 can be exported to the cytosol via GPT or other nonspecific transporters then glycolytically converted into PEP and pyruvate, which can then enter plastids through the PPT and the BASS2 pyruvate transporter, respectively. According to the scheme in **(B)**, some of the cytosolic G6P can be incorporated into plastids through GPT and converted to 3PGA by the Rubisco shunt, which involves reactions of the early steps of glycolysis (PFK and aldolase), reactions of the nonoxidative pentose phosphate pathway, phosphoribulokinase (PRK), and Rubisco. In plants lacking plastidial PFK, part of the 3PGA generated can be converted to GAP necessary for Ru5P production by means of plastidial GAPDH and PGK (highlighted in blue). 3PGA can leave plastids via the TPT, the GPT, or other nonspecific transporters and glycolytically converted to PEP and pyruvate in the cytosol, which can enter plastids, as indicated above. Alternatively, and/or additionally, plastidial 3PGA can be converted to pyruvate in plastids. The ATP required for FA synthesis can be generated from photosynthesis and the PK reaction in the plastidial compartment or obtained from the cytosol via the NTT transporter (Reiser et al., 2004). NADPH and NADH can be generated in plastids from the light photosynthetic and pyruvate dehydrogenase reactions, respectively. ALD, aldolase; TA, transaldolase; TK, transketolase; TPI, triose-phosphate isomerase; PDH, pyruvate dehydrogenase; HMBDP, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate; IPP, isopentenyl diphosphate; CDP, *ent*-copalyl diphosphate. Enzymes are highlighted in red.

METHODS

Plants, Growth Conditions, and Sampling

The work was performed using *Arabidopsis thaliana* wild-type plants (ecotypes Col-0 and Ws-2), *pgi1-2* knockout mutants (Bahaji et al., 2015), Ws-2 plants expressing *GUS* under the control of the 1700-bp Ws-2 *PGI1* promoter (*promPGI1:GUS*), *pgi1-2* plants expressing *PGI1* under the control of the 1700-bp *PGI1* promoter (*promPGI1:PGI1*), *pgi1-2* plants expressing *PGI1* under the control of the cauliflower mosaic virus 35S promoter (*35S:PGI1*), the ADP-glucose pyrophosphorylase null *aps1* mutant (SALK_040155), *CK OXIDASE/DEHYDROGENASE1*-overexpressing 35S:CKX1 plants (Werner et al., 2003), the GA-deficient *ga20ox1 ga20ox2* double mutant (Rieu et al., 2008b), and the *pfk4* and *pfk5* T-DNA insertional knockout mutants (SALK_012602 and SAIL_297_F05, respectively) obtained from the European Arabidopsis Stock Center (NASC). By crossing *pfk4* with *pfk5*, self-pollinating the resulting heterozygous mutants, and PCR screening for homozygous progeny using the oligonucleotide primers listed in Supplemental Table 1, we produced *pfk4 pfk5* double mutants. The *promPGI1:PGI1*, *35S:PGI1*, and *promPGI1:GUS* plasmid constructs were produced using Gateway technology as illustrated in Supplemental Figure 6 and confirmed by sequencing. Primers used for PCR amplification of *PGI1* cDNA, *GUS*, and the *PGI1* promoter are listed in Supplemental Table 2. The plasmid constructs were transferred into *Agrobacterium tumefaciens* EHA105 cells by electroporation and utilized to transform Arabidopsis plants as described by Clough and Bent (1998). Transgenic *promPGI1:PGI1*, *35S:PGI1*, and *promPGI1:GUS* plants were selected on medium containing antibiotics.

Plants were cultured on soil in growth chambers under a long-day photoperiod with (16 h light 22°C/8 h dark 18°C) at 90 μmol photons m^{-2} s^{-1} light intensity using a mix of Sylvania 215-W cool white fluorescent tubes and 60-W mate bulbs. For exogenous application of CKs and GAs, 1 week after bolting, *pgi1-2* plants were sprayed every 2 d with the indicated concentrations of BAP or GA_{4+7}.

Confirmation of the Knockout Status of *pfk5*

PFK5 contains 13 exons (Supplemental Figure 5A) and encodes a protein of 537 amino acids (Supplemental Figure 5B) with weak activity. The *pfk5* allele has a T-DNA inserted in exon 12 and encodes a truncated protein (designated PFK5*) that lacks 85 amino acids at the C terminus (Supplemental Figure 5B). To explore whether PFK5* is enzymatically active, we produced *PFK5*, *PFK5**, and *PFK4* expression vectors (Supplemental Figure 7). Primers used for PCR amplification of *PFK4* and *PFK5* cDNA are listed in Supplemental Table 2. The plasmids were transferred to Agrobacterium GV3101, which were used for transient expression in agroinfiltrated *Nicotiana benthamiana* leaves. For coinfiltration of the RNA-silencing inhibitor P19, a bacterial suspension harboring pBin61-P19 (Voinnet et al., 2003) was added. The bacteria were infiltrated into the abaxial sides of leaves using a 1-mL syringe with no needle. *N. benthamiana* leaf samples were taken 2 d after infiltration, immediately frozen in liquid nitrogen, and subjected to ATP-PFK activity measurement analyses as described by Mustroph et al. (2007).

qRT-PCR Analyses

Total RNA was extracted from frozen organs using the TRIzol method according to Meng and Feldman (2010) and treated with RNase-free DNase (Takara). Then, 1.5 μg portions were reverse-transcribed using a mixture of oligo(dT) and random primers and an Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. RT-PCR amplifications were performed using a 7900HT sequence detector system (Applied Biosystems) with Premix Ex Tag Mix (Takara RR420A) according to the manufacture's protocol. Each amplification was performed in triplicate with 0.4 μL of the first-strand cDNA in a total volume of 20 μL. The specificity of the PCR amplicons was checked by acquiring heat dissociation curves (from 60°C to 95°C). Comparative threshold values were normalized to an *EF-1*α RNA internal control and compared with obtain relative expression levels. Primers used for qRT-PCR amplification of *PGI1* and different *GA2ox*, *GA3ox*, and *GA20ox* genes are listed in Supplemental Table 3.

GUS Expression Analysis

Expression of the GUS reporter gene was monitored using the histochemical staining assay described by Jefferson et al. (1987).

Morphological Analysis of Seeds

Seeds were observed and photographed using an Olympus MVX10 stereomicroscope. Scanning electron microscopy analyses of gold-coated dry seeds were performed using a JEOL JSM-5610LV scanning electron microscope.

Metabolite Analysis

Seed Fatty Acid Content Analysis

Sets of 20 dry seeds were prepared in Teflon-lined screw-capped glass tubes and incubated at 90°C for 90 min in 1 mL of 1 M HCl in methanol, with 20 μg of heptadecanoic acid (C17:0) as an internal standard, and 300 μL of toluene. After cooling to room temperature, FAMEs were extracted in 1 mL hexane following the addition of 1.5 mL of 0.9% (w/v) NaCl. The hexane phase was transferred to a gas chromatography vial after vigorous vortex mixing and centrifuged for 3 min at 10,000*g*. Portions (1 μL) of the samples were analyzed using a gas chromatography-mass spectrometry system consisting of a 7890A GC equipped with an Agilent J&W DB-WAX column (diameter, 0.25 mm; film thickness, 0.25 μm; length, 30 m) coupled to a 5975C Inert XL MSD mass selective detector (Agilent Technologies). The GC settings were as follows: carrier gas, helium (1 mL/min); injection mode, split (1:50); injector temperature, 250°C; GC temperature program, 1 min at 50°C then 25°C/min rise to 200°C followed by 3°C/min rise to 230°C and finally 18 min hold at 230°C. The MSD settings were: solvent delay, 5 min; ion source temperature, 230°C; fragmentation energy, 70 eV; scanning rate, 20 scans/min (40 to 500 *m/z*). Data were acquired with ChemStation software (Agilent).

Seed Protein Content Analysis

Sets of 200 dry seeds were ground in a mortar and pestle. The powder was resuspended in 60 μL of extraction buffer (50 mM HEPES, 5 mM $MgCL_2$, 5 mM DTT, 1 mM PSMF, 10% [v/v] ethylene glycol [pH 7.5], 1 mM EDTA, and 1% Polyclar), sonicated, and centrifuged at 10,000*g*. The supernatant thus obtained was kept on ice and the pellet was treated with 60 μL of 1 M NaOH and centrifuged at 10,000*g*. The supernatants were used to quantify protein levels using a Bio-Rad DC protein assay kit.

Seed Sucrose Content Analysis

For sucrose extraction, sets of 50 seeds were homogenized in 500 μL of 90% (v/v) ethanol, left at 70°C for 90 min, and centrifuged at 13,000*g* for 10 min. Sucrose content from the supernatants was determined by HPLC with pulsed amperometric detection on a DX-500 Dionex system by gradient separation with a CarboPac 10 column according to the application method suggested by the supplier (100 mM NaOH/100 mM sodium acetate to 100 mM NaOH/500 mM sodium acetate in 40 min).

Leaf Starch Content Analysis

Fully expanded source leaves of plants were harvested at the end of the light period, freeze-clamped, and ground to a fine powder in liquid nitrogen with a pestle and mortar. Starch was measured using an amyloglucosidase-based test kit (Boehringer Mannheim).

Native Gel PGI Activity Assays

PGI zymograms were acquired as described by Bahaji et al. (2015). Briefly, protein extracts of wild-type, *pgi1-2*, *promPGI1:PGI1*, and *35S:PGI1* seeds collected 15 d after flowering were loaded onto a 7.5% (w/v) polyacrylamide gel. After electrophoresis, the gels were stained by incubating in darkness at room temperature with 0.1 M Tris-HCl (pH 8.0), 5 mM F6P, 1 mM NAD^+, 4 mM $MgCl_2$, 0.2 mM methylthiazolyldiphenyl-tetrazolium bromide (Sigma-Aldrich M5655), 0.25 mM phenazine methosulfate (Sigma-Aldrich P9625), and 1 unit/mL of G6P dehydrogenase from *Leuconostoc mesenteroides* (Sigma-Aldrich G8404).

Quantitative Analysis of Endogenous GAs

GA samples were prepared and analyzed as described by Urbanová et al. (2013), with some modifications. Briefly, freeze-dried plant tissue samples (5–10 mg) were ground to a fine consistency using 3-mm zirconium oxide beads (Retsch) and a MM 301 mill (Retsch) vibrating at 30 Hz for 3 min, with 1 mL of ice-cold 80% acetonitrile containing 5% formic acid as extraction solution. The samples were then extracted overnight at 4°C using a Stuart SB3 benchtop laboratory rotator (Bibby Scientific) after adding 17 internal GAs standards ($[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_2]GA_5$, $[^2H_2]GA_6$, $[^2H_2]GA_7$, $[^2H_2]GA_8$, $[^2H_2]GA_9$, $[^2H_2]GA_{15}$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{20}$, $[^2H_2]GA_{24}$, $[^2H_2]GA_{29}$, $[^2H_2]GA_{34}$, $[^2H_2]GA_{44}$, $[^2H_2]GA_{51}$, and $[^2H_2]GA_{53}$; purchased from OlChemIm). The homogenates were centrifuged at 36,670*g* and 4°C for 10 min, and the corresponding supernatants were further purified using reversed-phase and mixed mode SPE cartridges (Waters) and analyzed by ultra-high-performance chromatography-tandem mass spectrometry (UHPLC-MS/MS; Micromass). GAs were detected using the multiple-reaction monitoring mode of the transition of the ion $[M–H]^-$ to the appropriate product ion. Masslynx 4.1 software (Waters) was used to analyze the data, and the standard isotope dilution method (Rittenberg and Foster, 1940) was used to quantify the GAs.

Statistical Analysis

The data presented are the means (±SE) from at least three independent experiments, with three to five biological replicates for each experiment. The significance of differences between control and transgenic lines was statistically evaluated with Student's *t* test using SPSS software. Differences were considered significant if $P < 0.05$. In GA analyses, significance was determined by one-way univariate ANOVA for parametric data and Kruskal Wallis tests for nonparametric data, using open source R 2.15.1 software (http://cran.r-project.org/). Tukey's HSD post-hoc tests were applied for multiple comparisons after ANOVA.

Accession Numbers

Sequence data from the article can be found in the GenBank/EMBL libraries under the following accession numbers: PGI1 (At4g24620), PKK4 (At5g61580), PFK5 (At2g22480), GA2ox1 (At1g78440), GA2ox2 (At1g30040), GA2ox3 (At2g34555), GA2ox4 (At1g47990), GA2ox6 (At1g02400), GA2ox7 (At1g50960), GA2ox8 (At4g21200), GA3ox1 (At1g15550), GA3ox2 (At1g80340), GA3ox3 (At4g21690), GA3ox4 (At1g80330), GA20ox1 (At4g25420), GA20ox2 (At5g51810), GA20ox3 (At5g07200), GA20ox4 (At1g60980), and GA20ox5 (At1g44090).

Supplemental Data

Supplemental Figure 1. Starch content in mature leaves of Ws-2, *pgi1-2* and *promPGI1:PGI1* plants.

Supplemental Figure 2. Expression levels of different *GA2ox*, *GA3ox*, and *GA20ox* genes in wild-type (Ws-2) and *pgi1-2* shoots.

Supplemental Figure 3. Embryo development in wild-type (Ws-2) and *pgi1-2* embryos at different developmental stages.

Supplemental Figure 4. Time course of sucrose content in developing Ws-2 and *pgi1-2* seeds.

Supplemental Figure 5. Confirmation of the knockout status of the *pfk4 pfk5* mutant.

Supplemental Figure 6. Stages in construction of the promPGI1:-GUS, 35S:PGI1, and promPGI1:PGI1 plasmids used to produce *promPGI1:GUS-*, *35S:PGI1-*, and *promPGI1:PGI1*-expressing plants.

Supplemental Figure 7. Stages in construction of the 35S:PFK4, 35S:PFK5, and 35S:PFK5* plasmids used for transient expression in *N. benthamiana* leaves.

Supplemental Table 1. Primers used for PCR screening of the *pfk4 pfk5* mutant.

Supplemental Table 2. Primers used to produce the promPGI1:GUS, promPGI1:PGI1 35S:PGI1, 35S:PFK4, 35S:PFK5, and 35S:PFK5* plasmids.

Supplemental Table 3. Primers used in qRT-PCR analyses.

Supplemental Table 4. ANOVA table.

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AUTHOR CONTRIBUTIONS

A.B., G.A., I.E., A.M.S.-L., F.J.M., N.D.D., L.S., K.D., and J.P.-R. designed the experiments and analyzed the data. A.B., G.A., I.E., S.G.-A., R.J.B., M.C.S., D.T., E.C., M.A.M., and E.B.-F. performed most of the experiments. A.B., I.E., K.D., and J.P.-R. supervised the experiments. A.B., F.J.M., and J.P.-R. wrote the article with contributions from all the authors. J.P.-R. conceived the project and research plans.

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