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 silique 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.

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).

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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; Riefer 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),...
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 phosphoglucomerase 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).
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 plastidal 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 strongly expressed in vascular tissues of 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 hypocots of young seedlings (Figure 1C). It is also active in vascular tissues of fully expanded mature leaves (Figures 1C and 1D), roots (Figures 1C and 1D), and sepals (Figure 1C). The PGI1 promoter did not drive expression in anthers, pollen grains, or seed coat (Figure 1C) and was weakly active in embryos at the heart developmental stage (Figure 1C), but strongly active in maturing embryos (Figures 1C and 1D). 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 and 1D). 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 3SS 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 (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 3SS 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 GA47 application.

Notably, levels of GA4, GA5, and GA7—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 GA20 and GA3 than Ws-2 plants and promPGI1:PGI1-expressing pgi1-2 plants (Figure 3). Moreover, exogenous application of GA47 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...
Table 1. Harvest Parameters for Wild-Type (Ws-2 and Col-0), pg1-2, promPG1:PGI1, 35S:PGI1, and aps1 Plants

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>No. of Elongated Inflorescences per Plant</th>
<th>No. of Siliques per Plant</th>
<th>No. of Seeds per Silique</th>
<th>Specific Seed Weight (μg Seed⁻¹)</th>
<th>Seed Yield (mg Plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws-2</td>
<td>29.5 ± 2.5</td>
<td>684.1 ± 14.7</td>
<td>48.2 ± 0.8</td>
<td>18.1 ± 0.8</td>
<td>596.8 ± 22.1</td>
</tr>
<tr>
<td>pg1-2</td>
<td>15.3 ± 1.3*</td>
<td>457.3 ± 22.4*</td>
<td>47.2 ± 0.9</td>
<td>11.7 ± 0.4*</td>
<td>252.5 ± 7.3*</td>
</tr>
<tr>
<td>promPG1:PGI1</td>
<td>27.5 ± 1.9</td>
<td>682.4 ± 18.2</td>
<td>46.6 ± 1.3</td>
<td>18.0 ± 0.5</td>
<td>572.4 ± 9.8</td>
</tr>
<tr>
<td>PGI1(1)</td>
<td>28.6 ± 0.9</td>
<td>662.7 ± 21.5</td>
<td>47.3 ± 1.2</td>
<td>18.2 ± 0.6</td>
<td>570.4 ± 19.7</td>
</tr>
<tr>
<td>35S:PGI1</td>
<td>30.6 ± 2.6</td>
<td>766.1 ± 40.8</td>
<td>57.2 ± 0.9</td>
<td>17.1 ± 1.0</td>
<td>749.3 ± 11.9</td>
</tr>
<tr>
<td>Col-0</td>
<td>30.6 ± 2.6</td>
<td>768.6 ± 22.2</td>
<td>58.2 ± 0.9</td>
<td>17.6 ± 0.7</td>
<td>780.1 ± 19.9</td>
</tr>
<tr>
<td>aps1</td>
<td>29.6 ± 3.0</td>
<td>768.6 ± 22.2</td>
<td>58.2 ± 0.9</td>
<td>17.6 ± 0.7</td>
<td>780.1 ± 19.9</td>
</tr>
</tbody>
</table>

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).
PGI1 Contributes to Seed Yield

5

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 pg11-2 plants is at least partly due to lower levels of active GA forms.

GA1 and GA4 are produced by the GA3-oxidase (GA3ox)-mediated oxidation of GA20 and GA9, respectively (Figure 3). These bioactive GAs can be deactivated by GA2-oxidase (GA2ox), yielding GA8 and GA34, respectively (Yamaguchi, 2008; Hedden and Thomas, 2012) (Figure 3). GA2ox can also metabolize GA20 to the inactive GA29 (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 pg11-2 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 pg11-2 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 pg11-2 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 pg11-2 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 pg11-2 plants could also be related to reductions in seed weight. As shown in Table 1, pg11-2 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 pg11-2 seeds and Ws-2 seeds (Supplemental Figure 3), excluding the possibility that the reduced weight of pg11-2 seeds could be due to aberrant embryo growth and development. Dry pg11-2 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 pg11-2 seeds, we performed time-course analyses of sucrose levels in developing Ws-2 and pg11-2 seeds. As shown in Supplemental Figure 4, there were

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 pg11-2 plants.

(B) External phenotype of a representative inflorescence that does not elongate in pg11-2 plants and morphology of two dissected early flowers (inset) from the same inflorescence. In (A), red arrows indicate inflorescences that do not elongate in pg11-2 plants.
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

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::P-GI1(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).
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 as *APETALA2*, which are involved in the development of vascular tissues and the production of hormones such as GA and CKs.

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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.
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-
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., GA1, GA3, and GA4) 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

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 GA₄₊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).
PGI1 contributes to seed yield through participation in the generation of precursors for embryonic fatty acid and protein biosynthesis, as schematically illustrated in Figure 9B. The hypothesis is strongly supported by the following findings: pg1-2 seeds accumulate ∼35% less protein and 50% less FAs than Ws-2.

Figure 7. The Phenotype of pg1-2 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 pg1-2 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) or 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).

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: pg1-2 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 3SS:AtCKX1 and GA-deficient ga20ox1 ga20ox2 do not accumulate low levels of FA (Figure 6C), and exogenous application of BAP and GA4,7 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 reductant 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 13C 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.utoronto.ca/epf/cgi-bin/epfWeb.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.
METHODS

Plants, Growth Conditions, and Sampling

The work was performed using Arabidopsis thaliana wild-type plants (ecotypes Col-0 andWs-2), pgI1-2 knockout mutants (Bahai et al., 2015),Ws-2 plants expressing GUS under the control of the 1700-bp Ws-2 PG1 promoter (promPG1:GUS), pgI1-2 plants expressing PG1 under the control of the 1700-bp PG1 promoter (promPG1:PG1), pgI1-2 plants expressing PG1 under the control of the cauliflower mosaic virus 3S promoter (35SPG1), the ADP-glucose pyrophosphorylase null aps1 mutant (SALK_040155), CK OXIDASE/DEHYDROGENASE1-overexpressing 35S:CKX1 plants (Werner et al., 2003), the GA-deficient ga20ox1 ga2ox2 double mutant (Rieu et al., 2008b), and the pffk4 and pffk5 T-DNA insertional knockout mutants (SALK_012602 and SAIL_297_F05, respectively) obtained from the European Arabidopsis Stock Center (NASC). By crossing pffk4 with pffk5, self-pollinating the resulting heterozygous mutants, and PCR screening for homozygous progeny using the oligonucleotide primers listed in Supplemental Table 1, we produced pffk4 pffk5 double mutants. The promPG1:PG1, 35SPG1, and promPG1:GUS plasmid constructs were produced using Gateway technology as illustrated in Supplemental Figure 6 and confirmed by sequencing. Primers used for PCR amplification of PG1 cDNA, GUS, and the PG1 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 promPG1:PG1, 35SPG1, and promPG1: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⁻²s⁻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, 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 Taq Mix (Takara RR420A) according to the manufacturer’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-1α RNA internal control and compared with obtain relative expression levels. Primers used for qRT-PCR amplification of PG1 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 10,000g. 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 MgCl₂, 5 mM DTT, 1 mM PSMF, 10% [v/v] ethylene glycol [pH 7.5], 1 mM EDTA, and 1% Polyclar), sonicated, and centrifuged at 10,000g. The supernatant thus obtained was kept on ice and the pellet was treated with 60 µL of 1 M NaOH and centrifuged at 10,000g. 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 13,000g 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 amylo-glucosidase-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, pgil-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 MgCl2, 0.2 mM methylothiazolyldiphenyl-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 ([2H2]GA1, [2H2]GA3, [2H2]GA4, [2H2]GA5, [2H2]GA6, [2H2]GA7, [2H2]GA8, [2H2]GA9, [2H2]GA15, [2H2]GA19, [2H2]GA20, [2H2]GA21, [2H2]GA22, [2H2]GA23, [2H2]GA28, [2H2]GA29, [2H2]GA30, and [2H2]GA31; 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 (At2g41680), GA3ox4 (At1g98330), GA2ox1 (At4g25420), GA2ox2 (At4g51810), GA2ox3 (At5g07200), GA2ox4 (At1g60980), and GA2ox5 (At1g44090).

Supplemental Data

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

Supplemental Figure 2. Expression levels of different GA2ox, GA3ox, and GA20ox genes in wild-type (Ws-2) and pgil-1-2 shoots.

Supplemental Figure 3. Embryo development in wild-type (Ws-2) and pgil-1-2 embryos at different developmental stages.

Supplemental Figure 4. Time course of sucrose content in developing Ws-2 and pgil-1-2 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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