SUGAR-DEPENDENT6 Encodes a Mitochondrial Flavin Adenine Dinucleotide-Dependent Glycerol-3-P Dehydrogenase, Which Is Required for Glycerol Catabolism and Postgerminative Seedling Growth in Arabidopsis[1][C]

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The aim of this study was to clone and characterize the *SUGAR-DEPENDENT6* (*SDP6*) gene, which is essential for postgerminative growth in Arabidopsis (*Arabidopsis thaliana*). Mutant alleles of *sdp6* were able to break down triacylglycerol following seed germination but failed to accumulate soluble sugars, suggesting that they had a defect in gluconeogenesis. Map-based cloning of *SDP6* revealed that it encodes a mitochondrial flavin adenine dinucleotide (FAD)-dependent glycerol-3-P (G3P) dehydrogenase:ubiquinone oxidoreductase called FAD-GPDH. This gene has previously been proposed to play a role both in the break down of glycerol (derived from triacylglycerol) and in NAD^+/NADH homeostasis. Germinated seeds of *sdp6* were severely impaired in the metabolism of [U-^{14}C]glycerol to CO_2 and accumulated high levels of G3P. These data suggest that *SDP6* is essential for glycerol catabolism. The activity of the glycolytic enzyme phosphoglucose isomerase is competitively inhibited by G3P in vitro. We show that phosphoglucose isomerase is likely to be inhibited in vivo because there is a 6-fold reduction in the transfer of ^{14}C-label into the opposing hexosyl moiety of sucrose when [U-^{14}C]glucose or [U-^{14}C]fructose is fed to *sdp6* seedlings. A block in gluconeogenesis, at the level of hexose phosphate isomerization, would account for the arrested seedling growth phenotype of *sdp6* and explain its rescue by sucrose and glucose but not by fructose. Measurements of NAD^+ and NADH levels in *sdp6* seedlings also suggest that NAD^+/NADH homeostasis is altered, and this observation is consistent with the hypothesis that SDP6 participates in a mitochondrial G3P shuttle by cooperating with the cytosolic NAD-dependent GPDH protein GPDHC1.

Plant cells contain a FAD-dependent glycerol-3-P (G3P) dehydrogenase:ubiquinone oxidoreductase (FAD-GPDH; EC 1.1.99.5), which is associated with the outer surface of the inner membrane of their mitochondria (Stumpf, 1955; Huang, 1975; Shen et al., 2003, 2006). This enzyme catalyzes the oxidation of G3P to dihydroxyacetone phosphate (DHAP) and donates the electrons to the mitochondrial ubiquinone pool. FAD-GPDH is believed to play a direct role in at least two important metabolic processes.

FAD-GPDH has been proposed to participate in the pathway of glycerol breakdown in plants (Huang, 1975). This pathway is particularly important in oilseeds, such as Arabidopsis (*Arabidopsis thaliana*), because the hydrolysis of triacylglycerol (TAG) releases free fatty acids and glycerol, which are both converted to sugars to support seedling growth. It has been established that the catabolism of glycerol in Arabidopsis is initiated by the glycerol kinase NHO1/GLI1, and that a GPDH must therefore catalyze the second step in the pathway to produce the glycolytic intermediate DHAP (Eastmond, 2004). Although plants contain both cytosolic and plastidial isoforms of NAD^+-dependent GPDH, it can be argued that FAD-GPDH is more likely to play the predominant role in glycerol dissimilation. This is because it is substantially more active than NAD-GPDH in germinated oilseed tissue (Huang, 1975) and it is a flavoprotein, whose oxidation is strongly exergonic, driving the conversion of G3P to DHAP (Shen et al., 2006). In contrast, the small equilibrium constant of NAD-GPDH strongly favors the reverse reaction (Aubert et al., 1994).

FAD-GPDH is also thought to participate in a mitochondrial G3P shuttle by combining with cytosolic NAD-GPDH (Shen et al., 2006). NAD-GPDH consumes NADH to generate G3P from DHAP, and the resulting G3P passes through the permeable mitochondrial outer membrane. G3P is reoxidized to DHAP at the outer surface of the inner mitochondrial membrane by FAD-GPDH. The DHAP is subsequently channeled back to the cytosolic compartment. The shuttle transfers reducing equivalents from the cytosol to mitochondria

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without utilizing a metabolite transporter. Shen et al. (2006) have recently shown that disrupting cytosolic NAD-GPDH (*GPDHC1*) in Arabidopsis perturbs the redox state of the cytosolic NAD(H) pool and can impair plant growth under conditions of abiotic stress.

Here, we describe the phenotype of an Arabidopsis mutant in the single copy *FAD-GPDH* gene At3g10370 (Shen et al., 2003). Our data show that FAD-GPDH is required for glycerol dissimilation in plants and are also consistent with a role for the enzyme in normal redox homeostasis. Furthermore, the mutant has pleiotropic growth phenotypes including complete postgerminative growth arrest, which can be rescued by providing exogenous Suc. This phenotype can probably be explained by the overaccumulation of G3P in mutant seedlings leading to inhibition of the glycolytic enzyme P-Glc isomerase (PGI).

RESULTS

sugar-dependent6 Is Impaired in Gluconeogenesis during Postgerminative Growth

Immediately following germination, Arabidopsis seedlings rely on stored TAG to fuel growth until they can achieve photosynthetic competence. The recessive *sugar-dependent6* (*sdp6*) mutant was selected in a genetic screen designed to identify ethyl methanesulfonate (EMS)-mutagenized lines that are unable to utilize TAG and therefore cannot make this transition without the provision of exogenous Suc (Eastmond, 2006). The *sdp6* mutant alleles that were isolated from this screen are able to germinate. However, 100% of the seedlings exhibit growth arrest shortly after germination, even in the presence of normal levels of photosynthetically active radiation (photosynthetic photon flux density [PPFD] = 150 μmol m^{-2} s^{-1}). The mutant alleles are therefore conditionally seedling lethal (Fig. 1A). The growth of *sdp6* seedlings in the dark is also severely inhibited (Fig. 1B), suggesting that growth arrest cannot be attributed to a defect in photosynthesis. Seedlings that are rescued with Suc are able to grow and once they develop photosynthetic capacity in the light they can complete their life cycle without exogenous Suc.

TAG measurements performed on *sdp6* seeds and seedlings grown in the presence of Suc showed that the mutant is capable of storage oil breakdown, despite the strong sugar-dependent phenotype (Fig. 1C). This contrasts with mutants that are impaired in TAG hydrolysis or fatty acid β-oxidation (Hayashi et al., 1998; Eastmond, 2006). In wild-type Arabidopsis seedlings the decline in TAG levels that occurs during postgerminative growth is mirrored by a corresponding increase in sugars (predominantly Glc), which are derived from the breakdown products of TAG via gluconeogenesis (Footitt et al., 2002; Pritchard et al., 2002). In 3-d-old *sdp6* seedlings the levels of Suc, Glc, and Fru are approximately 2-, 4- and 3-fold less than the wild type (Fig. 1D). These data suggest that *sdp6*

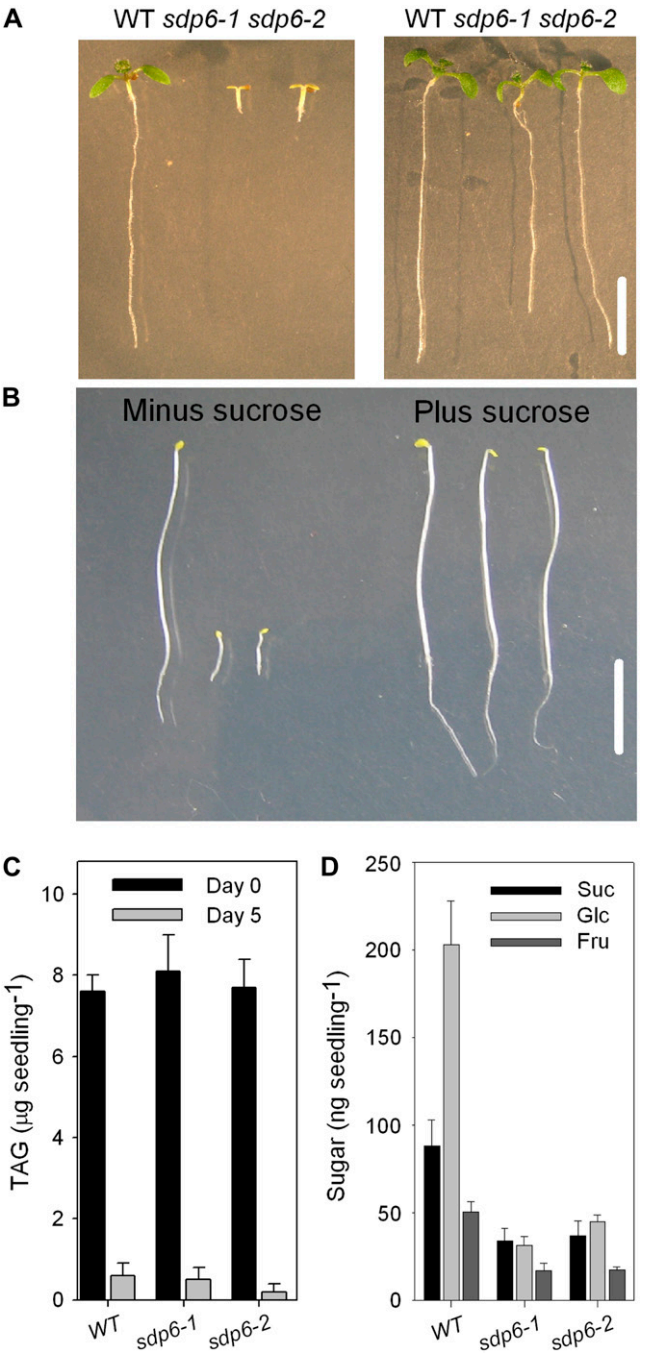

Figure 1. Postgerminative growth, TAG breakdown, and sugar content in *sdp6* seedlings. A and B, Five-d-old *sdp6* and wild-type seedlings grown in the light (A) or in the dark (B) on agar plates containing half-strength MS salts plus or minus 1% (w/v) Suc. Bar = 0.5 cm. C, TAG content of imbibed seeds and 5-d-old *sdp6* and wild-type seedlings grown in the light on agar plates containing half-strength MS salts plus 1% (w/v) Suc. Values are the mean ± SE of measurements on five batches of 20 seeds or seedlings. D, Sugar content of 3-d-old *sdp6* and wild-type seedlings grown in the light on agar plates containing half-strength MS salts. Values are the mean ± SE of measurements on four batches of 500 seedlings. [See online article for color version of this figure.]

is impaired in gluconeogenesis, rather than TAG or fatty acid catabolism (Eastmond et al., 2000; Pritchard et al., 2002).

SDP6 Encodes the Mitochondrial FAD-G3P Dehydrogenase

High-resolution mapping was used to locate the *SDP6* locus. The *sdp6-1* mutant was out-crossed to wild-type Landsberg *erecta*, and 768 *sdp6-1* lines were selected from the F_2 progeny. PCR-based polymorphic markers were used to localize *SDP6* to a 35-kb region on chromosome 3, situated near marker nga126 (Fig. 2A). This region contains nine genes. Sequencing candidate genes in two independent *sdp6* alleles revealed that there were point mutations in locus At3g10370 (Fig. 2B). This gene encodes a FAD-dependent G3P dehydrogenase (FAD-GPDH) that is situated on the mitochondrial inner membrane (Shen et al., 2003). In *sdp6-1*, a G-to-A mutation at positions +2,272 bp after the ATG causes Glu-546 to become Gly, and in *sdp6-2*, a C-to-T mutation at +257 bp causes Ser-86 to become Phe. Both amino acid substitutions occur in regions of the polypeptide that are highly conserved, and in *sdp6-2* it falls within the putative FAD-binding domain (Guerra et al., 2006).

A T-DNA insertion allele of *sdp6* was also obtained from the Salk Institute Genomic Analysis Laboratory's T-DNA Express Arabidopsis gene mapping tool (Alonso et al., 2003). The *sdp6-3* allele (SALK_080169) contains a T-DNA inserted in the fourth intron of At3g10370 (+1,856 bp). The *sdp6-3* mutant exhibits a recessive seedling growth arrest phenotype that cosegregates with the T-DNA insertion in At3g10370. However, the phenotype is more severe than the two EMS alleles. Seed germination in *sdp6-3* is delayed and seedlings require several weeks to establish photosynthetic competence, even when provided with exogenous Suc (Fig. 2C). PCR performed on cDNA from homozygous *sdp6-3* seedlings, using primers that flank the T-DNA insertion site, showed that *SDP6* mRNA is either lacking, or that the presence of the T-DNA in the fourth intron prevents it from being spliced out (Fig. 2D).

FAD-GPDH appears to be a single copy gene in Arabidopsis (Shen et al., 2003). To confirm that *sdp6* seedlings are defective in FAD-GPDH activity enzyme assays were performed on whole extracts from 2-d-old seedlings (Eastmond, 2004). No activity was detectable in the *sdp6-3* allele, while in *sdp6-1* and *sdp6-2* activity was reduced by more than 90% (Table I). Because the germination and growth of *sdp6-1* and *sdp6-2* seedlings were comparable to the wild type when Suc was provided, the majority of our biochemical analysis was performed on these lines rather than on *sdp6-3*.

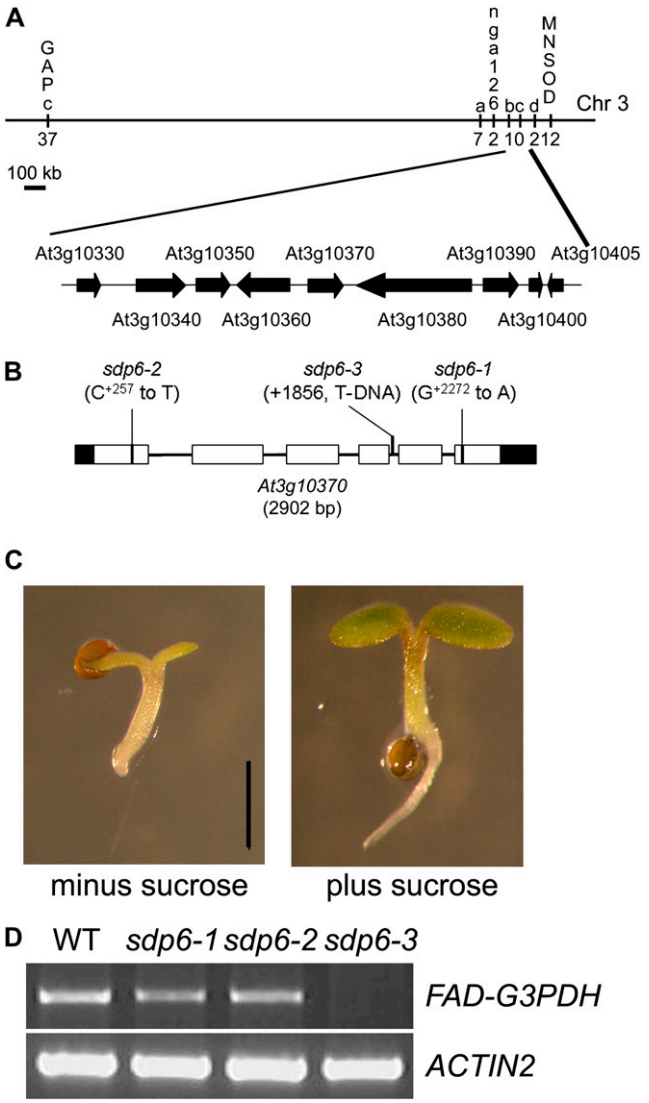

Figure 2. Positional cloning of *SDP6* and characterization of a T-DNA allele. A, A diagram to show how PCR-based SSLP and CAPS markers were used to map SDP6 to a 35-kb region on chromosome 3 that contains nine genes. The positions of markers a to d are marked by bars, and the number of recombination events/total number of chromosomes (1,536) is listed below each. B, A diagram of the *SDP6* locus (At3g10370) showing the positions of mutations that were identified in three independent *sdp6* alleles. Insertion/substitution positions are numbered relative to the initiation codon. White bars are exons, and the black bars indicate the 5′ and 3′ untranslated regions. C, Phenotype of *sdp6-3* seedlings. Five-d-old *sdp6-3* and wild-type seedlings grown in the light on agar plates containing half-strength MS salts plus or minus 1% (w/v) Suc. Bar = 0.1 cm. D, Detection of *SDP6* transcript in seedlings from the wild type and *sdp6* alleles. PCR was performed on cDNA made from total RNA from 2-d-old seedlings. The actin gene *ACT2* is shown as a control. [See online article for color version of this figure.]

FAD-GPDH Is Required for Glycerol Catabolism

FAD-GPDH has been proposed to play a key role in the pathway of glycerol breakdown in plants (Huang, 1975). This pathway is highly active in oilseeds following germination because the hydrolysis of TAG releases free fatty acids and glycerol, which are both converted to sugars to support seedling growth. The postgerminative growth of many Arabidopsis sugar-dependent mutants, such as the glyoxylate cycle

Table I. *Glycerol kinase and FAD-GPDH activity in gli1-3 and sdp6 seedlings*

Enzyme assays were performed on extracts from 2-d-old seedlings grown on medium containing 1% (w/v) Suc. Values are the mean ± SE of assays on three extracts and are expressed as nmol mg^{-1} protein min^{-1}. ND, Not detected.

Line	Glycerol Kinase	FAD-GPDH
Wild type	0.7 ± 0.1	7.1 ± 0.6
gli1-3	ND	6.4 ± 0.5
sdp6-1	0.8 ± 0.1	0.3 ± 0.1
sdp6-2	0.7 ± 0.2	0.4 ± 0.1
sdp6-3	0.9 ± 0.2	ND

mutant *isocitrate lyase-2* (*icl-2*), can also be rescued using glycerol as a carbon source (Eastmond, 2004). However, glycerol cannot rescue *sdp6* alleles (Fig. 3A). Furthermore, radiolabel feeding experiments show that *sdp6* seedlings metabolize at least 10-fold less [U-^{14}C]glycerol to CO_2 than the wild type (Fig. 3C). These data strongly suggest that FAD-GPDH/SDP6 is required for glycerol catabolism in Arabidopsis.

sdp6 Seedlings Accumulate G3P

Glycerol only represents a small proportion (approximately 5%) of the carbon present in TAG, and failure to degrade glycerol alone cannot account for the sugar-dependent phenotype of *sdp6* because the glycerol kinase mutant *gli1* is not sugar dependent (Eastmond, 2004). Measurements of glycerol and G3P levels in *gli1-3* and *sdp6* mutant seedlings grown in the presence of Suc showed that while *gli1* dramatically overaccumulates glycerol (Eastmond, 2004), *sdp6* alleles overaccumulate G3P (Table II). Studies have previously shown that high concentrations of glycerol are toxic to plants (with the apparent exception of germinated oilseeds) and that this toxicity is likely to be caused by glycerol metabolism rather than glycerol itself (Leegood et al., 1988; Aubert et al., 1994; Eastmond, 2004). The *gli1* mutant was originally selected based on its insensitivity to the inhibitory effect of glycerol on seedling root growth (Eastmond, 2004). Growing *sdp6* seedlings on medium containing glycerol revealed that germination is strongly inhibited by glycerol (Fig. 3B). Analysis of a *sdp6-1 gli1-3* double mutant also showed that *gli1* is a suppressor of the *sdp6* growth arrest phenotype (Fig. 3A). The hypersensitivity of *sdp6* to glycerol, and suppression by *gli1*, provide genetic evidence that seedling growth arrest is linked to G3P overaccumulation (and/or ATP consumption/phosphate sequestration by GLI1) and not to further downstream metabolism of G3P.

G3P Inhibits PGI Activity

Aubert et al. (1994) have previously used ^{31}P NMR to show that when heterotrophic plant cell cultures are fed glycerol, they accumulate high levels of G3P and are unable to convert it to sugars, although they can use it as a substrate for respiration. Aubert et al. (1994) also discovered that G3P is a competitive inhibitor of the glycolytic enzyme PGI (EC 5.3.1.9) in vitro and proposed that glycerol feeding is likely to lead to PGI inhibition, thereby preventing gluconeogenic flux to sugars by blocking Fru-6-P (F6P) conversion to Glc-6-P (G6P). This model could potentially explain the glycerol catabolism-dependent gluconeogenic defect observed in *sdp6* seedlings.

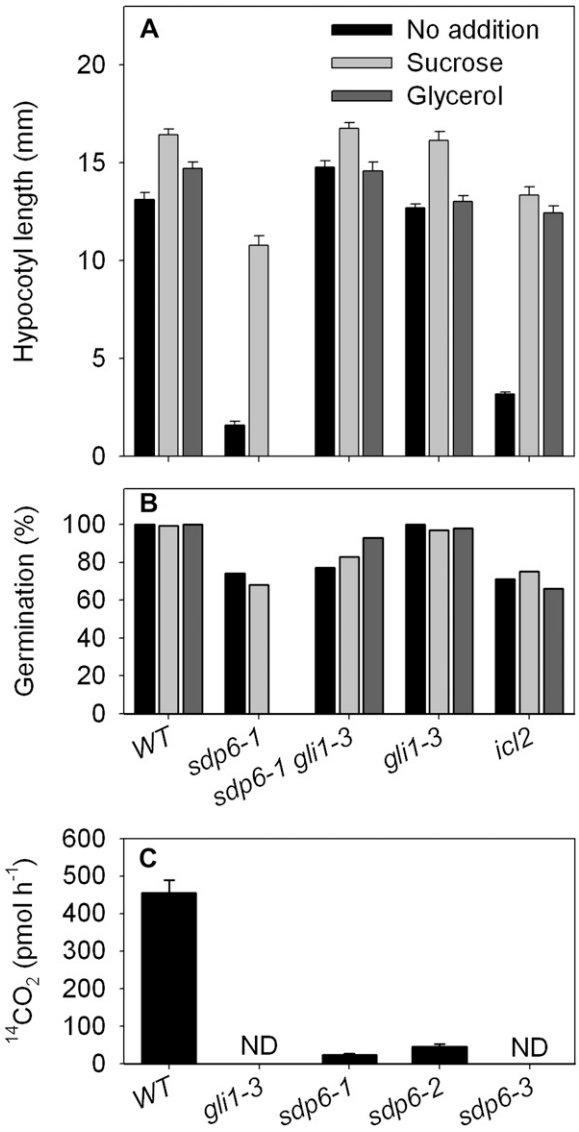

Figure 3. Utilization of glycerol by *sdp6* seedlings. A, Hypocotyl length of 5-d-old etiolated wild type, *sdp6-1*, *sdp6-1 gli1-3*, *gli1-3*, and *icl-2* seedlings grown in the dark on agar plates containing half-strength MS salts alone or plus 1% (w/v) Suc or 0.2% (w/v) glycerol. Values are the mean ± SE of measurements on three batches of 20 seedlings. B, Germination frequency of wild-type, *sdp6-1*, *sdp6-1 gli1-3*, *gli1-3*, and *icl-2* seeds grown on agar plates containing half-strength MS salts alone or plus 1% (w/v) Suc or 0.2% (w/v) glycerol. Percent of germination was scored after 3 d using batches of 200 seeds. C, Release of $^{14}CO_2$ by 2-d-old wild-type, *gli1-3*, *sdp6-1*, *sdp6-2*, and *sdp6-3* seedlings supplied with [U-^{14}C]glycerol. Values are the mean ± SE of measurements on four batches of 50 seedlings. ND, Not detected.

Table II. *Glycerol, G3P, NAD^+, and NADH levels in sdp6 seedlings*

Seedlings were grown for 5 d on agar plates containing half-strength MS salts plus 1% (w/v) Suc. Values are the mean ± SE of assays on four or five extracts. nd, Not determined.

Metabolite	Wild Type	*sdp6-1*	*sdp6-2*	*gli1-3*
		ng seedling^{-1}		
Glycerol	25.3 ± 7.1	76.0 ± 11.5	59.6 ± 18.0	234.7 ± 38.2
G3P	19.6 ± 4.2	159.6 ± 12.0	171.7 ± 9.4	4.6 ± 2.9
		nmol g^{-1} FW		
NAD^+	74.8 ± 9.3	69.7 ± 4.8	57.0 ± 11.5	nd
NADH	6.7 ± 0.6	14.6 ± 2.1	12.5 ± 1.9	nd
NADH/NAD^+ ratio	0.09	0.21	0.22	nd

Arabidopsis contains a cytosolic and a plastidial isozyme of PGI, whose activities can be discriminated based on their different temperature stability (Yu et al., 2000). Assays performed on extracts from seedlings show that approximately 60% of the activity is present in the cytosol and that there is no significant difference in total activity between the wild type and *sdp6-1* (Table III). To determine whether G3P is an inhibitor of Arabidopsis, PGI activity enzyme assays were performed on extracts from wild-type seedlings, using F6P as a substrate (Aubert et al., 1994). Total PGI activity was competitively inhibited by G3P with a K_i value of 4.7 mM (Table III). Because a substantial proportion of PGI activity is present in both the cytosol and plastid, G3P must be capable of inhibiting both isozymes. The estimated concentration of G3P in 2-d-old *sdp6* seedlings is approximately 60 mM (Table II), assuming that G3P can access about 20% of the tissue volume. This concentration of G3P would be sufficient to inhibit almost completely PGI activity in *sdp6* seedlings, if both isozymes were exposed to it. Because G3P can be transported across the chloroplast inner membrane (Fliege et al., 1978), inhibition of both isozymes is probable.

Evidence That PGI Is Inactive in *sdp6* Seedlings

To investigate whether the interconversion of G6P and F6P, catalyzed by PGI, is blocked in *sdp6* seedlings, feeding experiments were performed with [U-^{14}C]Glc or [U-^{14}C]Fru (Fig. 4), and the proportion of radiolabel incorporated into the two hexosyl moieties of Suc was determined (Geigenberger and Stitt, 1993; Fernie et al., 2001). In 2-d-old wild-type seedlings about 30% of the radiolabel was redistributed from Glc to the fructosyl moiety of Suc and, in reciprocal experiments, from Fru to the glucosyl moiety of Suc (Fig. 4B). Preferential labeling of the fructosyl moiety of Suc by Fru has been observed in many plant tissues and is largely attributed to some direct incorporation via Suc synthase, which bypasses hexose phosphate isomerization (Geigenberger and Stitt, 1993). In contrast to the wild type, less than 5% of the radiolabel was redistributed to the opposing hexosyl moiety when Glc or Fru was supplied to *sdp6-1* seedlings (Fig. 4B). These data suggest that *sdp6-1* seedlings exhibit a 6-fold reduction in their capacity to interconvert G6P and F6P and therefore PGI is very likely to be inhibited in vivo.

The same experiment also showed that *sdp6-1* seedlings are able to incorporate radiolabel from Glc into Suc without the addition of unlabeled Fru, whereas the incorporation of radiolabel from Fru into Suc requires the supply of unlabeled Glc (Fig. 4A). One interpretation of these data is that endogenous metabolism in germinated *sdp6-1* seedlings is able to provide F6P (and/or Fru) for Suc synthesis, but not G6P. If this is so, the reaction catalyzed by PGI is the only step in gluconeogenesis that is severely compromised in *sdp6-1* seedlings. G6P can also be converted to F6P via the oxidative pentose phosphate pathway (Hatzfeld and Stitt, 1990). However, the 6-fold reduction in the transfer of radiolabel from Glc into the fructosyl moiety of Suc in *sdp6-1* seedlings suggests that the oxidative pentose phosphate pathway is unlikely to supply sufficient F6P to account for the level of incorporation of radiolabel into Suc when the mutant is supplied with Glc alone (Fig. 4).

The different abilities of various exogenous carbon sources to rescue postgerminative growth of *sdp6-1* (measured as hypocotyl elongation in the dark) also provide corroborating evidence to support the hypothesis that gluconeogenesis is blocked at the level of PGI in this mutant. Suc and Glc can rescue *sdp6-1* growth, but Fru cannot (Fig. 5). In contrast, all three sugars can rescue postgerminative growth of the glyoxylate cycle mutant *icl-2* (Fig. 5). Although Fru can be converted to Suc by plants (Fig. 4; Geigenberger and Stitt, 1993; Fernie et al., 2001), our data suggest that in *sdp6* seedlings the accumulation of G3P leads to PGI inhibition and that this

Table III. *The effect of G3P on Arabidopsis PGI activity*

Enzyme assays were performed on extracts from 2-d-old wild-type and *sdp6-1* seedlings grown on medium containing 1% (w/v) Suc. Values are the mean ± SE of assays on three extracts and are expressed as μmol min^{-1} g^{-1} FW. The kinetics values of the enzyme were derived from assays in the presence of various concentrations of G3P and F6P, and were calculated by nonlinear regression with Prism software (GraphPad Software). nd, Not determined.

Line	Wild Type	*sdp6-1*	*sdp6-2*
V_{max}	2.98 ± 0.51	2.70 ± 0.36	2.66 ± 0.39
K_m	0.60 mM	nd	nd
K_i (G3P)	4.73 mM	nd	nd
% in cytosol	59.1 ± 3.5	61.8 ± 7.2	62.8 ± 6.0

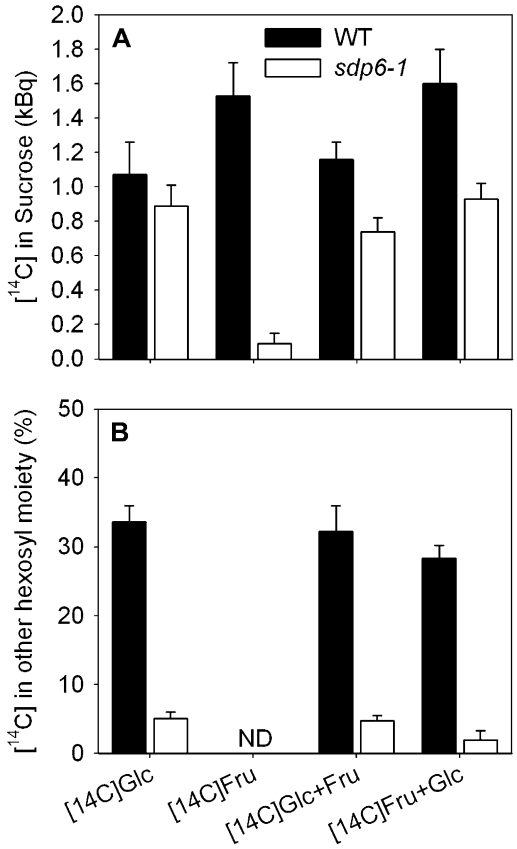

Figure 4. Incorporation of ^{14}C-label from Glc and Fru into the hexosyl moieties of Suc in *sdp6* seedlings. A and B, Two-day-old wild-type and *sdp6-1* seedlings were incubated with [U-^{14}C]-labeled Glc or Fru, either alone or with unlabeled Glc or Fru and the amount of total label in Suc (A), and percentage redistribution to the opposite hexosyl moiety of Suc (B) were determined. Values are the mean ± SE of measurements on three separate incubations.

impairs the synthesis of G6P, which is also required for Suc synthesis.

sdp6 Seedlings Have an Altered NADH/NAD$^+$ Ratio and Seed Germination Is Hypersensitive to Abscisic Acid and Salt Stress

In addition to a role in glycerol catabolism, it has been proposed that GPDH also participates in a G3P shuttle that channels reducing equivalents from the cytosol to the mitochondrion (Shen et al., 2003, 2006). Arabidopsis *gpdhc1* mutants, which are deficient in the other proposed component of this shuttle (a cytosolic G3P dehydrogenase), exhibit an altered redox state of their NAD(H) pool (Shen et al., 2006). Measurement of total NADH and NAD$^+$ levels in 5-d-old *sdp6-1* and *sdp6-2* seedlings grown in the presence of Suc show that NADH content is doubled, while NAD$^+$ content is decreased slightly (Table II). These data suggest that the cytosolic NADH/NAD$^+$ ratio is likely to be higher in *sdp6* seedlings than in the wild type. Shen et al. (2006) reported a similar shift in the NADH/NAD$^+$ ratio in leaves from *gpdhc1*, which they further corroborated by estimating the ratio of free NADH/NAD$^+$ in the cytosol according to the concentrations of metabolites considered to be in near equilibrium with the NADH/NAD$^+$ couple in the cytosolic compartment. The total NADH/NAD$^+$ ratio in 5-d-old wild-type Arabidopsis seedlings was found to be approximately 0.09 (Table II), which is lower than has been reported for photosynthetically active leaf tissue in the light (Queval and Noctor, 2007; Wang and Pichersky, 2007). However, Wang and Pichersky (2007) have previously shown that NADH/NAD$^+$ ratios are lower in Arabidopsis seedlings (approximately 0.09) and roots (approximately 0.03) than in leaves (approximately 0.4).

Shen et al. (2006) have shown that *gpdhc1* mutant seedlings can grow normally under optimal growth conditions but exhibit hypersensitivity to abscisic acid (ABA) and salt stress. The transcript abundance of both *GPDHC1* and *FAD-GPDH* are increased by certain stress treatments (Shen et al., 2003, 2006). To investigate whether *sdp6* is also stress intolerant, seeds of the wild type and the null *sdp6-3* allele were germinated on medium containing a range of concentrations of ABA or NaCl. In the absence of exogenous ABA or NaCl, the germination of *sdp6-3* already shows a delay relative to the wild type, but after 7 d the majority of the seeds have germinated (Fig. 6). The inclusion of ABA or NaCl in the growth medium was found to have a more pronounced inhibitory effect on *sdp6-3* germination than on the wild type (Fig. 6).

sdp6 Seeds Have Reduced Weight and Fatty Acid Content

Increasing G3P levels by glycerol feeding or overexpression of NAD-GPDH has recently been shown to

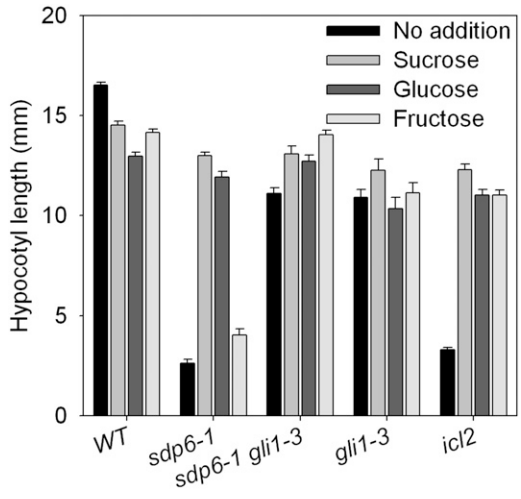

Figure 5. Rescue of *sdp6* seedling growth by various exogenous carbon sources. Hypocotyl length of 5-d-old etiolated wild-type, *sdp6-1*, *sdp6-1 gli1-3*, *gli1-3*, and *icl-2* seedlings grown in the dark on agar plates containing half-strength MS salts alone or plus 1% (w/v) Suc, 1% (w/v) Glc, or 1% (w/v) Fru. Values are the mean ± SE of measurements on three batches of 20 seedlings.

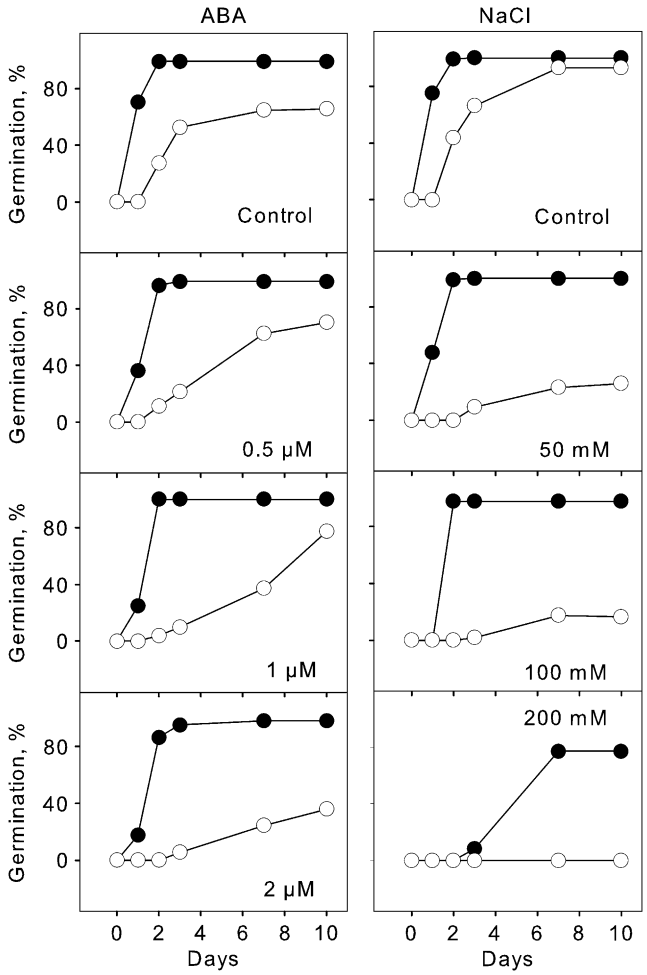

Figure 6. The effect of ABA and salt stress on *sdp6-3* (white circles) and wild-type (black circles) seed germination. Seeds were germinated on agar plates containing half-strength MS salts and various concentrations of ABA or NaCl. The percent of germination frequency was recorded over the course of 10 d.

promote TAG synthesis in developing embryos of oilseed rape (*Brassica napus*) by driving G3P acyltransferase activity (Vigeolas and Geigenberger, 2004; Vigeolas et al., 2007). To determine whether disrupting FAD-GPDH activity significantly influences seed oil content in Arabidopsis, between eight and 10 wild-type, *sdp6-1*, *sdp6-2*, and *sdp6-3* plants were grown in the glasshouse under the same growth conditions (Li et al., 2006). Seed fresh weight and fatty acid measurements performed on separate batches of seeds from each plant suggest that *sdp6* mutants produce significantly smaller seeds with less total fatty acids per seed than the wild type (Table IV).

DISCUSSION

Here we show that mitochondrial FAD-GPDH is essential for postgerminative growth and seedling establishment in Arabidopsis plants. FAD-GPDH has a proposed dual role in glycerol catabolism and NADH/NAD^+ homeostasis (Huang, 1975; Shen et al., 2006; Fig. 7). Our data are consistent with the hypothesis that FAD-GPDH is required for both processes. However, Arabidopsis *gpdhc1* mutants that lack the other proposed component of the mitochondrial G3P shuttle do not exhibit an arrested seedling growth phenotype under normal growth conditions (Shen et al., 2006). Therefore, the failure of *sdp6* seedlings to establish is most likely to be caused by the defect in glycerol catabolism. A consequence of this defect is that young seedlings of *sdp6* accumulate very high levels of G3P, which can inhibit several important enzymes of plant primary metabolism in vitro including the central glycolytic enzyme PGI (Aubert et al., 1994). In theory the inactivation of this enzyme alone would be sufficient to block glycolysis/gluconeogenesis and stall plant growth because, without the capacity to synthesize G6P, plant metabolism cannot produce a wide array of compounds that are essential (Aubert et al., 1994). The fact that *sdp6* seedlings exhibit a greater than 6-fold reduction in their capacity to redistribute radiolabel from Glc into the fructosyl moiety of Suc, or radiolabel from Fru into the glucosyl moiety of Suc, provides strong evidence to support the hypothesis that PGI is inactive in vivo.

It is probable that G3P accumulation in *sdp6* seedlings also causes other detrimental effects, which could contribute to the arrested growth phenotype. For example, Aubert et al. (1994) showed that G3P also inhibits Met and Thr synthesis, while Leegood et al. (1988) have provided evidence to suggest that G3P accumulation

Table IV. *sdp6 seed weight and total fatty acid content*

Values are the mean ± SE of measurements on batches of seeds from eight to 10 separate plants. In Experiment 1 the batches each consisted of 1,000 seeds and in Experiment 2 they consisted of 500 seeds.

Line	Seed Weight	Fatty Acid Content	Fatty Acid Content
	μg	*μg seed*$^{-1}$	*%*
Experiment 1			
Wild type	21.07 ± 0.37	7.68 ± 0.13	36.45
sdp6-1	18.44 ± 0.21[a]	6.05 ± 0.20[a]	32.81
sdp6-2	18.02 ± 0.49[a]	5.26 ± 0.19[a]	29.19
Experiment 2			
Wild type	18.89 ± 0.48	6.70 ± 0.24	35.49
sdp6-3	16.98 ± 0.56[a]	5.45 ± 0.21[a]	32.09

[a]Significantly different from wild type ($P < 0.05$).

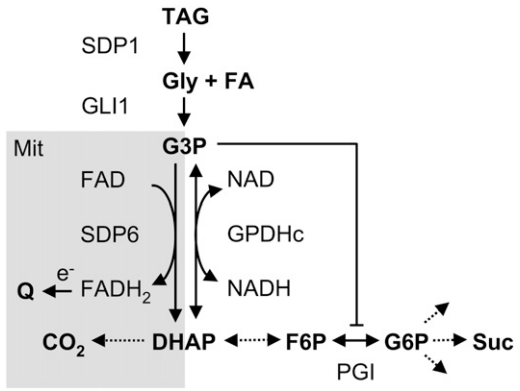

Figure 7. A model describing the role of SDP6 in glycerol catabolism and NADH/NAD^+ homeostasis in Arabidopsis seedlings. Gly, Glycerol; FA, fatty acids; Q, mitochondrial ubiquinone pool; SDP1, TAG lipase; GLI1, glycerol kinase; GPDHc, is cytosolic NAD-dependent GPDH. The gray box labeled Mit indicates steps associated with the mitochondrion. Dotted arrows represent multistep pathways.

and/or phosphate sequestration can impair photosynthetic CO_2 assimilation. Indeed, *Clarkia xantiana* mutants with reduced PGI activities have impaired Suc synthesis, starch synthesis, and photosynthetic CO_2 assimilation (Kruckeberg et al., 1989). However, neither a defect in amino acid synthesis, nor photosynthetic CO_2 assimilation can fully explain the *sdp6* phenotype. If impaired amino acid synthesis was responsible, then Suc would not be expected to rescue *sdp6* seedling growth. Furthermore, the phenotype cannot be attributable to a defect in photosynthesis because growth arrest in *sdp6* seedlings is observed in the dark and, even in the light, it is apparent before the cotyledons expand and green (Fig. 1).

The NADH/NAD^+ ratio in *sdp6* seedlings is altered in a similar fashion to that reported in *gpdhc1* mutants, which are deficient in the main cytosolic isoform of NAD-GPDH (Shen et al., 2006). These data are consistent with the hypothesis that normal NADH/NAD^+ homeostasis requires both enzymes to cooperate in a G3P shuttle that transfers reducing equivalents from the cytosol to the mitochondria (Shen et al., 2006). Shen et al. (2006) have reported that *gpdhc1* mutant seed germination and seedling growth are more sensitive to inhibition by abiotic stress, emphasizing a role for the shuttle in maintaining redox homeostasis under changing growth conditions. Germination of the null *sdp6-3* allele is hypersensitive to the inhibitory effects of ABA and salt. However, it should be noted that unlike *gpdhc1*, changes in cellular redox status in *sdp6* seedlings could also be attributable to the pleiotrophic effects of G3P accumulation. On this basis, *sdp6* does not provide a straightforward tool to investigate the role of the mitochondrial G3P shuttle.

Elevated G3P levels in plants have also recently been shown to increase seed oil content (Vigeolas and Geigenberger, 2004; Vigeolas et al., 2007) and enhance pathogen resistance/pathogen-related gene expression (Kachroo et al., 2004; Nandi et al., 2004; Chandra-Shekara et al., 2007). In both cases high G3P levels have been hypothesized to promote glycerolipid synthesis by fueling G3P acyltransferases. However, we observed a small decrease, rather than an increase, in fatty acid content in *sdp6* seeds. Reverse transcriptase PCR experiments also suggested that the transcript level of the salicylic acid-inducible systemic acquired resistance marker gene *PR-1* is not increased in *sdp6* plants (data not shown). This gene has previously been shown to be induced by prolonged glycerol feeding in Arabidopsis (Kachroo et al., 2004). One possible explanation for these observations is that G3P levels might only become dramatically increased in *sdp6* when large quantities of glycerol are rapidly released from lipids such as during early postgerminative growth (Eastmond, 2004). Indeed, high levels of FAD-GPDH activity have only been reported in tissues from germinated oilseeds (Huang, 1975). At other stages in plant development, and in other tissues, the bulk of G3P is more likely to be synthesized from DHAP by NAD-GPDH (Shen et al., 2006; Vigeolas et al., 2007). This enzyme has been reported to be sensitive to product inhibition (Bell and Cronan, 1975), which would provide a homeostatic mechanism to prevent significant G3P overaccumulation. Why seed weight and seed fatty acid content is reduced in *sdp6* plants is yet to be determined, but it is possible that this phenotype is caused by altered NADH/NAD^+ homeostasis. Alternatively, lipid breakdown in senescing maternal tissue might lead to G3P accumulation, which could interfere with the reallocation of resources from these tissues to the developing seeds. It would be interesting to determine whether the fatty acid content and the weight of *gpdhc1* mutant seeds is altered (Shen et al., 2006).

Finally, the provision of exogenous glycerol to Suc-starved tobacco cell culture has recently been shown to repress the catabolism of the major nonplastidial phospholipid phosphatidylcholine and promote its synthesis (Inoue and Moriyasu, 2006). The precise mechanism by which glycerol regulates phosphatidylcholine metabolism is not known. However, the broad-ranging effects of G3P overaccumulation on processes such as G3P acyltransferase activity and glycolytic flux could be implicated and the Arabidopsis *gli1* and *sdp6* mutants might provide a useful tool to investigate this phenomenon.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia and Landsberg *erecta*) were obtained from the European Arabidopsis Stock Centre (University of Nottingham, UK). The isolation of *sdp6-1* and *sdp6-2* is described in Eastmond (2006), and the isolation of *gli1-3* in Eastmond (2004). The *sdp6-3* mutant (SALK_080169) was obtained from T-DNA express (Alonso et al., 2003). Seeds were surface sterilized, applied to agar plates containing half-strength Murashige and Skoog (MS) salts (Sigma-Aldrich), and imbibed in the dark for 4 d at 4°C. The plates were then transferred to a growth chamber set to 21°C (16 h light/8 h dark; PPFD = 150 μmol m^{-2} s^{-1}). For experiments on etiolated seedlings, the plates

were kept in darkness in the growth chamber after 1 h of light. In some experiments Suc, Glc, Fru, glycerol, ABA, or NaCl were added to the agar medium. For measurements of seed weight and total fatty acid content, plants were grown on soil in the glasshouse with supplemental lighting (16 h light/8 h dark; PPFD = 150 μmol m^{-2} s^{-1}).

Metabolite Analysis and Radiolabel Feeding

The fatty acid and TAG content of seeds and seedlings were measured using the methods described in Eastmond (2006). Glycerol and G3P were measured as described in Eastmond (2004) and Wei et al. (2004), respectively. Sugars were determined by a sequential series of assays linked to NADPH production (Pritchard et al., 2002). Pyridine nucleotides were extracted and the concentration of NAD^+ and NADH determined as described by Shen et al. (2006). Sugars, glycerol, G3P, and pyridine nucleotide recoveries were determined to be within the range of 82% to 105% for all experiments.

For ^{14}C-radiolabel feeding experiments, seedlings were incubated in 50 mM MES/NaOH (pH 5.2) plus 0.1 mM [U-^{14}C]glycerol (20 MBq $mmol^{-1}$) or 50 mM [U-^{14}C]Glc or [U-^{14}C]Fru (1.25 MBq $mmol^{-1}$) for 4 h at 22°C. Incubations using glycerol were performed with 50 seedlings in 0.2-mL reactions and were stopped by the addition of 0.5 mL of 6 M formic acid. Each was conducted in a sealed 2-mL vial and $^{14}CO_2$ was collected in a well within the vial containing 0.1 mL of 15% (w/v) KOH. The ^{14}C content of the KOH was determined by liquid scintillation counting. Incubations using Glc and Fru were performed with 100 mg fresh weight of seedlings in 2-mL reactions. Suc was extracted and the proportion of ^{14}C-label in glucosyl and fructosyl moieties was determined according to the methods described in Fernie et al. (2001). In some experiments, unlabeled 50 mM Glc or Fru was also included in the incubation medium.

Mapping

The *sdp6-1* mutant was out-crossed to the wild-type ecotype Landsberg *erecta*. F_1 plants were allowed to self-fertilize and the F_2 progeny were screened for the sugar-dependent phenotype. Genomic DNA was isolated from 768 F_2 *sdp6-1* lines using the Extract-N-Amp plant PCR kit (Sigma-Aldrich). Mapping was carried out using simple sequence polymorphisms (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993) utilizing information from the Monsanto Arabidopsis polymorphism collection (Jander et al., 2002). New markers used in this study (Fig. 2A, a–d) are: SSLP, 5′-TGCCCAATGGTATTCAGGTTAG-3′, 5′-GCAATGAATATAAACACCAAGGACA-3′; CAPS, 5′-GGATCCGAATCATTTTGCAC-3′, 5′-CCTCTGAGCTTGTGCCTTCT-3′, *Hin*dIII; dCAPS, 5′-GTTTCTCGATATTCTTTTCTCTCTAGATCTA-3′, 5′-CATGAAAAATTCAACTGATGACG-3′, *Cla*I; and dCAPS, 5′-TCACCCATCAACTGCCTAAGTTT-3′, 5′-GTGGATGGTAAGCCGGTTG-3′, *Hpa*I. Candidate genes within the mapping interval were amplified from *sdp6-1* and *sdp6-2* genomic DNA by PCR and sequenced to identify mutations.

Transcript Analysis

DNAse-treated total RNA was isolated from Arabidopsis seedlings using the RNeasy kit from QIAGEN. The synthesis of single-stranded cDNA was carried out using SuperScript II RNase H− reverse transcriptase from Invitrogen. *SDP6* and the actin (*ACT2*) transcripts were detected by PCR using primers SDP6F (5′-TCTTAACATTAAGGTTCGACGTAC-3′), SDP6R (5′-TCACAACAAATCATCCGAGAAACATGAG-3′), ACT2F (5′-GTTGGGATGAACCAGAAGGA-3′), and ACT2R (5′-CTTACAATTTCCCGCTCTGC-3′). The product sizes are 878 and 500 bp, respectively.

Enzyme Assays

Glycerol kinase and FAD-GPDH activity were assayed using the methods described in Eastmond (2004). PGI activity was assayed by spectrophotometry at 340 nm by coupling to the reduction of NAD (Aubert et al., 1994). Leaves of ecotype Columbia of Arabidopsis plants were homogenized in 10× (w/v) of extraction buffer (100 mM Tris-HCl [pH8.3], 10 mM $MgCl_2$, 10% [w/v] glycerol, 5 mM DTT, 1 mM EDTA) and centrifuged at 15,000 rpm for 10 min at 4°C. Fifty microliters of the supernatant were used in 1 mL of reaction buffer containing 100 mM Tris-HCl (pH8.3), 10 mM $MgCl_2$, 0.75 mM NAD, 3 units of G6P dehydrogenase, and 0.05 to 10 mM F6P. The inhibitory effect of G3P was tested by adding 2 to 20 mM to the reaction buffer. Plastidial PGI was inactivated by heating the extract at 50°C for 10 min (Yu et al., 2000).

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