Plastidial Folate Prevents Starch Biosynthesis Triggered by Sugar Influx into Non-Photosynthetic Plastids of Arabidopsis

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(Received February 28, 2017; Accepted May 16, 2017)

Regulation of sucrose–starch interconversion in plants is important to maintain energy supplies for viability and growth. Arabidopsis mutants were screened for aberrant responses to sucrose to identify candidates with a defect in the regulation of starch biosynthesis. One such mutant, fpgs1-4, accumulated substantial amounts of starch in non-photosynthetic cells. Dark-grown mutant seedlings exhibited shortened hypocotyls and accumulated starch in etioplasts when supplied with exogenous sucrose/glucose. Similar starch accumulation from exogenous sucrose was observed in mutant chloroplasts, when photosynthesis was prevented by organ culture in darkness. Molecular genetic analyses revealed that the mutant was defective in plastidial folylpolyglutamate synthetase, one of the enzymes engaged in folate biosynthesis. Active folate derivatives are important biomolecules that function as cofactors for a variety of enzymes. Exogenously supplied 5-formyl-tetrahydrofolate abrogated the mutant phenotypes, indicating that the fpgs1-4 mutant produced insufficient folate derivative levels. In addition, the antifolate agents methotrexate and 5-fluorouracil induced starch accumulation from exogenously supplied sucrose in dark-grown seedlings of wild-type Arabidopsis. These results indicate that plastidial folate suppresses starch biosynthesis triggered by sugar influx into non-photosynthetic cells, demonstrating a hitherto unsuspected link between plastidial folate and starch metabolism.

Keywords: Folate metabolism • Folylpolyglutamate synthetase • Plastid • Starch metabolism • Sucrose transport • Sugar uptake.

Abbreviations: ADPG, ADP glucose; AGPase, ADP-glucose pyrophosphorylase; DHF, dihydrofolate; FPGS, folylpolyglutamate synthetase; SFU, 5-fluorouracil; GFP, green fluorescent protein; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; PGM, phosphoglucomutase; RT–PCR, reverse transcription–PCR; RubisCO, ribulose-1,5-biphosphate carboxylase/oxygenase; SXK, starch excess 4; SS, starch synthase; TAG, triacylglycerol; THF, tetrahydrofolate; UDPG, UDP glucose.

Introduction

Oilseed plants such as Arabidopsis accumulate triacylglycerol (TAG) lipid reserves in their seeds (Hayashi and Nishimura 2006, Theodoulou and Eastmond 2012). Each cell within a seed contains numerous oil bodies, which are specialized TAG storage organelles. When the seed starts to germinate, TAG lipase on the oil body membrane degrades TAG into glycerol and fatty acids (Eastmond 2006). The fatty acids are then imported into the glyoxysome by PED3/CTS/PXA1, an ABC transporter found in the glyoxysomal membrane (Hayashi et al. 2002b), and activated to acyl-CoA by acyl-CoA synthetases (Hayashi et al. 2002a). Acyl-CoA is then catabolized into succinate by two metabolic pathways, namely fatty acid β-oxidation and the glyoxylate cycle (Mori and Nishimura 1989, Hayashi et al. 1995, Kato et al. 1995, Kato et al. 1996, Mano et al. 1996, Hayashi et al. 1998a, Kato et al. 1998, Hayashi et al. 1999, Hayashi et al. 2001). Succinate is converted to sucrose by the successive action of mitochondrial and cytosolic enzymes (Hayashi and Nishimura 2002, Hayashi and Nishimura 2003). Sucrose is utilized for long-distance energy transfer from storage sites to cells requiring energy for growth and division during the early stages of post-germinative development. The onset of photosynthesis is required for sucrose to be available for the final destination deeply affects the seedling growth. For example, mutants that have defects in glyoxysomal fatty acid β-oxidation cannot germinate in the absence of sucrose, and require exogenously supplied sucrose for their post-germinative growth (Hayashi et al. 1998b).

Non-photosynthetic cells need to catabolize sucrose rapidly to obtain sufficient energy to support ongoing growth. To investigate the mechanisms underlying sucrose metabolism in non-photosynthetic cells, we screened Arabidopsis mutants that exhibited aberrant responses to sucrose during post-germinative growth in the dark without the action of photosynthesis. One of these mutants, which abnormally accumulated starch in non-photosynthetic cells when sucrose was exogenously supplied, is described in this study. The mutant had a defect in a folate biosynthesis gene that encodes plastidial folylpolyglutamate synthetase (FPGS).
Folate, also known as vitamin B₉, is an essential cofactor for several metabolic enzymes involved in processes such as nucleotide and amino acid biosynthesis (Ravanel et al. 2011). Although a large variety of folate derivatives have been identified, the biologically active molecules are thought to be tetrahydrofolate (THF) and its C1-substituted derivatives with polyglutamate tails such as formyl-THF(Glu)n, methyl-THF(Glu)n, methenyl-THF(Glu)n and methylene-THF(Glu)n. Plants can synthesize these derivatives de novo, but animals, including humans, cannot, and require dietary sources. The folate biosynthetic pathway has been extensively studied in plants, mainly because of the importance of folate in human nutrition and health (Hanson and Gregory 2011, Gerdes et al. 2012).

THF is first synthesized as dehydrofolate (DHF) through assembly of pterin, para-aminobenzoic acid (pABA) and glutamate in mitochondria. THF is produced from DHF by the action of dehydrofolate reductase, and is then distributed to the plastids and cytosol as well as the mitochondria. Each cellular compartment contains a set of enzymes that adds a polyglutamate tail and a C1 unit to THF. Addition of a polyglutamate tail to THF is catalyzed by FPGS. FPGS enzymes in different cellular compartments were identified in Arabidopsis, namely FPGS1 (At5g05980), FPGS2 (Atg310160) and FPGS3 (At3g55630) for the plastidial, cytosolic and mitochondrial isozymes, respectively (Ravanel et al. 2001).

Chemicals that inhibit enzymes involved in folate biosynthesis or that inhibit enzymes requiring folate derivatives as cofactors are called antifolate agents. An example of the former is methotrexate, which inhibits dihydrofolate reductase, and an example of the latter is 5-fluorouracil (5FU), which inhibits thymidylate synthetase (Zhao and Goldman 2003, Kremer 2004). Folate metabolism is an established target for human cancer therapy, and antifolate agents are often used as anticancer drugs. However, despite the importance of folate in animal cells, the physiological roles of folate derivatives in plants remain under investigation (Miret and Munne-Bosch 2014, Colinas and Fitzpatrick 2015).

Here, we present evidence demonstrating a previously unidentified link between folate and starch metabolism, and discuss the physiological functions of plastidial folate in sucrose–starch interconversion in non-photosynthetic cells.

Results

Isolation of a mutant with exogenous-sucrose-dependent starch accumulation

In the course of a screen of ethyl methanesulfonate-mutagenized Arabidopsis to identify mutants with an aberrant response to sucrose, we identified a mutant that exhibited a shortened hypocotyl when grown on sucrose-containing medium in darkness. This mutant was designated fpgs1-4 (see below for details). The hypocotyl length of the dark-grown seedlings depended on the sucrose concentration in the medium (Fig. 1A). When sucrose was absent, the fpgs1-4 hypocotyl was of similar length to that of wild-type Arabidopsis. However, when fpgs1-4 was grown on a medium containing >15 mM sucrose, hypocotyl length was significantly shortened and the root did not elongate. In contrast, hypocotyl length in dark-grown wild-type Arabidopsis remained unchanged at all concentrations of sucrose tested (Fig. 1B).

Detailed analysis of the phenotype revealed that the dark-grown seedlings of fpgs1-4 accumulated starch in the presence of sucrose. Starch in seedlings was visualized by iodine staining, as shown in Fig. 1. Accumulation of starch in fpgs1-4 seedlings became apparent in above-ground plant parts (cotyledons and hypocotyl) when seedlings were grown on medium containing >30 mM sucrose (Fig. 1A). In contrast, none of the sucrose concentrations tested triggered starch accumulation in wild-type Arabidopsis (Fig. 1B). To confirm accumulation of starch in the mutant, starch content was measured in the etiolated seedling and dry seed. The fpgs1-4 mutant accumulated 1.96 ± 0.14 μg of starch per seedling when grown on medium containing 60 mM sucrose in darkness, whereas wild-type Arabidopsis accumulated 0.25 ± 0.05 μg of starch per seedling in the same conditions. However, when seedlings were grown without sucrose in darkness, both fpgs1-4 and wild-type Arabidopsis contained very low amounts of starch: 1.6 ± 0.9 and 2.1 ± 0.5 ng of starch per seedling, respectively. Starch content was also determined in dry seed. The fpgs1-4 mutant and wild-type Arabidopsis contained 1.56 ± 0.05 and 1.03 ± 0.11 ng per seed, respectively, indicating that both the mutant and wild type contained very low amounts of starch. It should be noted that the mutant roots also did not accumulate starch (Fig. 1A).

Fig. 1 Etiolated fpgs1-4 seedlings accumulate starch in a sucrose-dependent manner. Mutant fpgs1-4 (A) and wild-type (B) Arabidopsis were grown for 5 d in darkness on growth medium containing sucrose. Starch accumulated in seedings was visualized by iodine staining. Photographs were taken after seedlings were rearranged on agar plates. Sucrose concentrations used for seedling growth are indicated underneath the panel. Scale bar = 5 mm.
Etioplasts of the mutant can produce starch granules

Cells of dark-grown seedlings usually contain etioplasts, a type of differentiated plastid that is not thought to accumulate starch. To analyze the morphology of etioplasts in mutant hypocotyls, fpgs1-4 was crossed with cp-GFP, an Arabidopsis line that expressed an N-terminal transient peptide of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit plus green fluorescent protein, to produce plastid-localized GFP (Mano et al. 2009). The plant homozygous for both fpgs1-4 and the transgene was designated as fpgs1-4 (cp-GFP). As shown in Fig. 2A, plastids of up to 10 μm in diameter were detected in fpgs1-4 (cp-GFP) cells grown in the presence of 60 mM sucrose. These plastids were significantly larger than the etioplasts found in wild-type Arabidopsis, which were approximately 1 μm in diameter (Fig. 2B). All the enlarged plastids contained regions that had no GFP fluorescence (see superimposed image in Fig. 2A). These regions may correspond to starch granules, which would not be penetrated by cp-GFP.

Thin and ultrathin sections were prepared from fpgs1-4 and wild-type Arabidopsis seedlings grown on 60 mM sucrose in darkness. The thin sections were stained with iodine and toluidine blue solutions and analyzed using light microscopy. Purple staining, which indicated the presence of starch, was detected in organelles of fpgs1-4 cells but not wild-type cells (Fig. 2C, D). The stained fpgs1-4 organelles were similar in size to the enlarged plastids detected in fpgs1-4 (cp-GFP) (Fig. 2A). Ultrathin sections were analyzed by electron microscopy. Starch granules were found in enlarged etioplasts in fpgs1-4 cells (Fig. 2E), but not in etioplasts in wild-type Arabidopsis cells (Fig. 2F).

Identification of FPGS1

The mutant fpgs1-4, which had a Columbia background, was outcrossed to wild-type Arabidopsis accession Landsberg erecta. F2 progeny (277 progeny; 554 chromosomes) exhibiting

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Fig. 2 Morphology of etioplasts in fpgs1-4 cells. Etiolated seedlings of fpgs1-4 (cp-GFP) (A), Cp-GFP (B), fpgs1-4 (C and E) and wild-type Arabidopsis (D and F) were grown for 5 d in darkness on growth medium containing 60 mM sucrose. GFP fluorescence in hypocotyl cells was detected using laser confocal microscopy (A and B). (A) A 2.5-fold enlarged image showing etioplasts, as indicated by the arrow. Thin sections of hypocotyl cells stained with iodine and toluidine blue solutions were observed under light microscopy (C and D). Ultrathin sections were analyzed by electron microscopy (E and F). Arrows indicate plastids. Magnifications in (A–D) and (E–F) are the same. Scale bars in (D) and (F) represent 20 and 0.2 μm, respectively.
the mutant phenotype were identified for gene mapping. Progeny were scored according to genetic background using a series of molecular genetic markers. As summarized in Fig. 3A, gene mapping revealed that the mutation was located close to At5g06250, between At5g05780 and At5g06440.

The Arabidopsis genome sequence was used to design oligonucleotide primers to amplify the At5g05980 gene. Amplified DNA fragments from genomic DNA of fpgs1-4 and wild-type Arabidopsis were fully sequenced. As indicated in Fig. 3B, fpgs1-4 contained a single nucleotide substitution that changed guanine to adenine at position 639. The substitution occurred in the conserved adenine–guanine motif found at the end of the third intron, suggesting that the substitution had caused alternative transcript splicing to occur. Abnormal splicing was confirmed by comparing cDNA sequences from fpgs1-4 and wild-type Arabidopsis. The fpgs1-4 cDNA had a 19 bp deletion at a region corresponding to the start of the fourth exon (red box in Fig. 3B). The deletion produced a frameshift of the open reading frame and a premature stop codon in the mutant transcript (Fig. 3C).

At5g05980, annotated as FPGS1, encodes a plastidial isoform of FPGS, which catalyzes the addition of a polyglutamate tail to THF at a late stage of folate metabolism in plastids (Ravanel et al. 2001). Several mutant alleles of this gene were identified previously and designated as fpgs1-1-3, atdfb-1-3 and mko2 (Mehrshahi et al. 2010, Srivastava et al. 2011, Reyes-Hernandez et al. 2014). For example, atdfb-3 contains a T-DNA insertion within the eighth exon (Fig. 3B) (Srivastava et al. 2011). None of the previous studies described a relationship between FPGS1 and starch metabolism. As our mutant constituted a new allele, it was designated fpgs1-4.

Starch accumulation in etioplasts is triggered only by plastidial FPGS

Three isogenes encoding FPGS, FPGS1, FPGS2 (At3g10160) and FPGS3 (At3g55630), were identified previously (Mehrshahi et al. 2010). FPGS1 encodes the plastidial isozyme, whereas FPGS2 and FPGS3 encode the mitochondrial and cytosolic isozymes, respectively. Mutants fpgs1-4 and atdfb-3 harbored defects in the FPGS1 gene (Fig. 3B). As shown in Fig. 4, seedlings of not only fpgs1-4 but also atdfb-3 exhibited a dwarf phenotype and accumulated starch in above-ground plant parts when grown on 60 mM sucrose in darkness. Starch accumulation was also

Starch accumulation in mutants defective for folylpolyglutamate synthetase isozymes. The fpgs1-4 and atdfb-3 mutants harbor defects in the same gene, At5g05980, which encodes plastidial folylpolyglutamate synthetase. The fpgs2 and fpgs3 mutants are defective in the genes encoding cytosolic (At3g10160) and mitochondrial (At3g55630) isozymes, respectively. Mutants were grown for 5 d in darkness on growth medium containing 60 mM sucrose. Starch accumulation in the seedlings was visualized by iodine staining. Photographs were taken after the seedlings were rearranged on agar plates. Scale bar = 5 mm.
examined in fpgs2 and fpgs3, which lacked mitochondrial and cytosolic isozymes, respectively. As shown in Fig. 4, neither mutant had a phenotype similar to that of the fpgs1-deficient mutants, suggesting that the loss of plastidial FPGS, but not of mitochondrial or cytosolic FPGS, triggered sucrose-dependent starch accumulation in plastids.

**Etioplast starch accumulation can be suppressed by the addition of active folate derivatives**

The At5g05980 mutation suggested that fpgs1-4 contained reduced amounts of active folate derivatives. Folate derivatives are known to function as coenzymes for several enzymes such as serine hydroxymethyltransferase, which catalyzes interconversion between glycine and serine. Our metabolomics analysis revealed that serine levels were substantially higher in fpgs1-4 than in wild-type Arabidopsis, while the amount of glycine was lower in fpgs1-4 than in wild-type Arabidopsis (Table 1). These results suggested that serine hydroxymethyltransferase was inactive in fpgs1-4 as a result of the loss of active folate derivatives.

If the fpgs1-4 starch accumulation phenotype occurred as a consequence of lack of folate derivatives, then exogenously supplied folate would be expected to rescue the phenotype. To examine this, 200 μM each of several folate derivatives were supplied: folate, THF, 5-formyl-THF and 5-methyl-THF (Fig. 5). Of these, exogenously supplied 5-formyl-THF efficiently suppressed the mutant phenotypes of starch accumulation and dwarfism when grown on medium containing sucrose in darkness. A weaker suppression effect on both phenotypes was observed with 5-methyl-THF. No effect was observed when folate or THF were supplied.

**Antifolate agents and adenine can trigger starch accumulation in wild-type Arabidopsis**

The effects of the antifolate agents methotrexate and SFU on starch accumulation in wild-type Arabidopsis were assessed (Fig. 6). Methotrexate inhibits dihydrofolate reductase, which is involved in folate biosynthesis (Kremer 2004), and SFU inhibits thymidylate synthase, which requires a folate derivative as a cofactor (Zhao and Goldman 2003). As described above, dark-grown wild-type Arabidopsis did not accumulate starch in darkness even in the presence of exogenously supplied sucrose. However, starch accumulation was apparent upon the addition of 0.1 μM methotrexate. Methotrexate also induced a dwarf phenotype in wild-type Arabidopsis. Dwarfism and starch accumulation were also observed when 0.1 mM SFU was supplied to wild-type Arabidopsis. No such phenotypes were observed when 1 mM uracil was supplied instead of 0.1 mM SFU (Fig. 6). These results suggested that inhibition of thymidylate synthase activity by SFU was likely to be responsible for the induction of starch accumulation and dwarfism in wild-type plants, and that thymidylate synthase was inactivated in fpgs1-4 due to loss of its cofactor. Thymidylate synthase is essential for nucleotide biosynthesis, and we therefore reasoned that nucleobase homoeostasis might be disordered in fpgs1-4. To investigate this possibility, wild-type Arabidopsis was grown in the presence of 1 mM adenine. As shown in Fig. 6, dwarfism and starch accumulation were observed in wild-type Arabidopsis supplemented with 1 mM adenine. The phenotypic similarity to fpgs1-4 suggested a strong link between addition of adenine in wild-type Arabidopsis and loss of active folate in fpgs1-4 (compare Fig. 6 with Fig. 1). Indeed, our metabolomics analysis revealed that adenine levels were significantly higher in fpgs1-4 than in wild-type Arabidopsis (Table 1).

**Folate biosynthesis and starch metabolism are linked**

Numerous genes involved in starch metabolism have been identified in Arabidopsis (Streb and Zeeman 2012, Pfister and Zeeman 2016). Of these, PGM (At5g1820) encodes plastidial phosphoglucosemutase, which is involved in starch biosynthesis, and SEX4 (starch exess 4, At3g52180) encodes plastidial phosphoglucan phosphatase, which is involved in starch degradation (Caspar et al. 1985, Zeeman et al. 1998). To determine whether FPGS1 regulated starch biosynthesis or degradation,
was crossed to the corresponding single mutants, pgm-1 (Streb et al. 2009) and sex4-3 (Niittyla et al. 2006), to create two double mutants, fpgs1pgm and fpgs1sex4. Starch accumulation was examined in the double mutants after cultivation on medium containing 60 mM sucrose (Fig. 7A), 120 mM glucose (Fig. 7B) or 120 mM fructose (Fig. 7C). The single fpgs1-4 mutant accumulated starch in the presence of glucose as well as sucrose. No starch accumulation was triggered in the pgm-1 and sex4-3 mutants under the conditions tested. The double mutant fpgs1pgm exhibited similar dwarfism to fpgs1-4 but did not accumulate starch in the presence of sucrose or glucose (Fig. 7A, B). In contrast, fpgs1sex4 had the same starch accumulation and dwarf phenotypes as fpgs1-4 (Fig. 7A, B). In the presence of fructose (Fig. 7C) and mannitol (Supplementary Fig. S1), none of the mutants accumulated starch, and seedlings exhibited elongated hypocotyls similar to those observed in wild-type Arabidopsis. Absorption and utilization of exogenously supplied sugars (sucrose, glucose and fructose) was confirmed by the growth of ped1-1. The ped1-1 mutant is defective in a gene encoding glyoxysomal 3-ketoacyl-CoA thiolase, germinates only when sugar is supplied exogenously (Hayashi et al. 1998b). Indeed, the mutant could germinate on the media containing sucrose, glucose and fructose (Fig. 7), but not on the medium containing mannitol (Supplementary Fig. S1), indicating that osmotic stress does not contribute to starch accumulation in fpgs1-4.

**FPGS1 loss triggers starch accumulation in chloroplasts under darkness in the presence of exogenous sucrose.**

Chloroplasts are one type of differentiated plastid found in leaf cells. Starch accumulation was assessed in chloroplasts of fpgs1-4, sex4-3, fpgs1sex4 and wild-type Arabidopsis in the presence of exogenous sucrose in the dark (Fig. 8). All plants accumulated starch in their leaves after cultivation for 2 weeks under constant illumination (Fig. 8, 1). Under these conditions, photosynthetically active chloroplasts synthesized transient starch from photoassimilates. In the dark, however, chloroplasts stopped synthesizing starch due to the loss of photoassimilates, and the transient starch was degraded by SEX4-encoded plastidial phosphoglucan phosphatase. Starch that had accumulated in chloroplasts of wild-type Arabidopsis and fpgs1-4 was no longer apparent after 12 h incubation in darkness (Fig. 8, 2; WT and fpgs1-4). In contrast, similarly treated sex4-3 and fpgs1sex4 plants retained substantial amounts of starch in their leaves (Fig. 8, 2; sex4-3 and fpgs1sex4), as would be expected in the absence of plastidial phosphoglucan.
phosphorylase activity. After dark treatment, leaves were removed from plants and cultured for 72 h in liquid medium in the absence or presence of 60 mM sucrose under dark, aerobic conditions. None of the leaves accumulated starch in the absence of sucrose (Fig. 8, 3). However, leaves cultured in the presence of sucrose accumulated starch (Fig. 8, 4), in a mutation-dependent fashion. Leaves of wild-type Arabidopsis did not accumulate starch, whereas starch biosynthesis was triggered in chloroplasts under dark conditions of both single and double fpgs1-4 mutants (Fig. 8, 4; fpgs1-4 and fpgs1-4sex4). The sex4-5 single mutant contained starch in the apical leaf region (Fig. 8, 4; sex4); however, the exogenous sucrose may have inhibited degradation of transient starch.

**Discussion**

**Relationship between plastidial folate and starch biosynthesis**

Analyses of the mutant fpgs1-4 uncovered evidence showing a link between starch metabolism and plastidial FPGS1 (Figs. 1, 3). The fpgs1-4 mutation resulted in loss of active folate derivatives such as 5-formyl-THF (Fig. 5), and induced accumulation of starch granules in etioplasts (Figs. 1, 2) and non-photosynthetic chloroplasts (Fig. 8) when plants were cultured in the darkness in the presence of exogenous sucrose. Similar starch accumulation was seen in atdfb-3, another allele of fpgs1, further supporting the role of plastidial FPGS in starch metabolism (Fig. 4). Methotrexate is an antifolate agent that inhibits dihydrofolate reductase, one of the enzymes involved in folate biosynthesis. Inhibition of dihydrofolate reductase would be expected to decrease the amount of active folate derivatives in wild-type seedlings and induce phenotypes identical to fpgs1-4 (Fig. 9). As predicted, exogenously supplied methotrexate induced starch accumulation in etioplasts of wild-type Arabidopsis grown in the presence of sucrose in the dark (Fig. 6). Active folate derivatives are synthesized in three different subcellular components: the plastid, mitochondrion and cytosol. However, no starch accumulation was observed in mutants of the mitochondrial and cytosolic isozymes (Fig. 4), demonstrating that starch accumulation was driven exclusively by the plastidial folate derivatives. Overall, the results clearly indicated that loss of plastidial folate derivatives induced starch accumulation in non-photosynthetic chloroplasts and etioplasts in the presence of exogenously supplied sucrose in darkness. The relationship between folate and starch accumulation has not been investigated to date (Miret and Munne-Bosch 2014, Colinas and Fitzpatrick 2015). Indeed, several previous studies examined phenotypes of fpgs1 alleles, but none described starch accumulation in plastids (Mehrshahi et al. 2010, Srivastava et al. 2011, Meng et al. 2014, Reyes-Hernandez et al. 2014, Srivastava et al. 2015). In this study, starch accumulation in non-photosynthetic plastids became apparent only when sucrose was supplied to the plants under darkness. Addition of sucrose provided an unlimited source of precursor for starch biosynthesis, whereas growth in darkness prevented differentiation of etioplasts into chloroplasts and prevented biosynthesis of starch from photoassimilates in chloroplasts.

**Non-photosynthetic plastids contain a complete set of enzymes necessary for starch biosynthesis**

Plants accumulate two different types of starch: reserved starch and transient starch. Amyloplasts, a type of functionally differentiated plastid, store reserved starch. Amyloplasts are found mainly in cells of sink organs such as cotyledons/endosperm of starchy seeds, tubers and tuberous roots (Tuncel and Okita 2013), all of which contain numerous amyloplasts. Starch biosynthesis in amyloplasts is triggered by the influx of sucrose transported from photosynthetically active source organs, i.e. leaves, into the cells of these sink organs (Comparot-Moss and Denyer 2009). Sucrose is converted to glucose-6-phosphate (G6P) in the cytosol. G6P is then imported into plastids and converted into glucose-1-phosphate (G1P) by phosphoglucomutase (PGM) (see Fig. 9). G1P is then conjugated with ATP to make ADP-glucose (ADPG) by the action of ADP-glucose pyrophosphorylase (AGPase). Multiple processes performed by enzymes such as starch synthases (SSs) then polymerize ADPG to produce...
Fig. 9 Schematic illustration showing the relationship between folate and starch biosynthesis in a non-photosynthetic cell. A non-photosynthetic plastid in the cell contains a complete set of enzymes necessary for starch biosynthesis, such as phosphoglucomutase (PGM), ADP-glucose pyrophosphorylase (AGPase) and starch synthase (SS). Glucose-6-phosphate (G6P) imported into the plastid is converted to glucose-1-phosphate (G1P). G1P, however, cannot be metabolized into ADP-glucose (ADPG), since these plastids do not contain enough ATP. ATP production in the non-photosynthetic plastids is suppressed by an enzyme (most probably thymidylate synthase (TS)) that requires active folate as a cofactor. The suppression is released when the enzyme activity is inhibited by 5-fluorouracil (5FU), or when folate biosynthesis is blocked by methotrexate (MTX), or by loss of FPGS1. Exogenously supplied adenine may also induce production of ATP and starch biosynthesis in the presence of G1P. Lines and characters shown in black represent reactions, enzymes and substrates that are present in non-photosynthetic plastids, whereas red represents those that are absent. Solid lines represent a single enzymatic reaction, whereas dotted lines represent multiple reactions.

reserved starch. In contrast, transient starch is formed in chloroplasts of photosynthetically active source organs. Transient starch is synthesized from photoassimilates, i.e. triose phosphate, and is accumulated during the daytime in the presence of sufficiently strong illumination. Reserved starch and transient starch are synthesized from a common precursor, ADPG (Murata et al. 1963, Murata and Akazawa 1964). However, transient starch is degraded at night (Graf and Smith 2011) and non-photosynthetic chloroplasts under dark conditions do not accumulate starch, even in the presence of sucrose (Fig. 8). Other plastids, including etioplasts, are not thought to accumulate starch granules. Indeed, etioplasts in dark-grown seedlings of wild-type Arabidopsis never accumulated starch (Fig. 1). In this study, however, both etioplasts and non-photosynthetic chloroplasts demonstrated a latent ability to accumulate starch in the darkness that was triggered (as in amyloplasts) by an influx of sucrose into the cells. This ability became visible only when Arabidopsis contained reduced amounts of plastidial folate derivatives, indicating that plastidial folate derivatives suppressed starch accumulation in the non-photosynthetic plastids of wild-type Arabidopsis. It should be noted, however, that no starch accumulation was observed in the roots (Fig. 1) or seeds of fpgs1-4 (see the Results), indicating that the mechanism was not universal for all plastid types.

To accumulate significant amounts of starch granules in plastids without folate, either induction of starch biosynthesis or reduction of starch degradation is required. Three lines of evidence suggested that the former is the case. (i) Seeds of fpgs1-4 did not contain starch, indicating that starch in seedlings was synthesized de novo. (ii) There was no starch accumulation in sex4-3 (Fig. 7). SEX4 is one of the enzymes involved in starch degradation and, if fpgs1-4 had reduced the activity of starch degradation, sex4-3 would be expected to accumulate starch in a similar manner to fpgs1-4. (iii) There was no starch accumulation in fpgs1pgm (Fig. 7), indicating that starch was synthesized by PGM, an enzyme engaged in starch biosynthesis. The involvement of PGM was also supported by the observation that starch biosynthesis occurred with glucose but not fructose (Fig. 7B, C).

The lack of starch synthesis in the fpgs1pgm double mutant indicated that non-photosynthetic plastids, including etioplasts and chloroplasts under dark conditions, have the potential to synthesize starch using a complete set of starch biosynthesis enzymes involving PGM, AGPase and SS (Fig. 9). These non-photosynthetic plastids may import G6P from the cytosol. It has been reported that amyloplasts in cereal endosperm can import ADPG directly from the cytosol via an ADPG transporter (Pozueta-Romero et al. 1999, Cakir et al. 2016). However, this was not the case for the fpgs1 mutant. Complete loss of starch accumulation in the fpgs1pgm double mutant indicated that G6P, rather than ADPG, was transported into these plastids. G6P was then converted into G1P by the action of PGM. G1P, one of two substrates for AGPase, however, cannot be further metabolized into starch despite the existence of AGPase in the non-photosynthetic plastids.

Suppression mechanism of starch biosynthesis by folate in non-photosynthetic plastids

Phenotypes of fpgs1 mutants were examined in several previous studies, which indicated that plastidial folate played a pivotal role in a variety of biological processes such as development, seed reserve accumulation and lignin biosynthesis (Srivastava et al. 2011, Meng et al. 2014, Reyes-Hernandez et al. 2014, Srivastava et al. 2015). Here, we showed that dark-grown fpgs1-4 seedlings in the presence of sucrose exhibited shortened hypocotyl/root lengths (Fig. 1). A dwarf phenotype was also reported when wild-type Arabidopsis was grown with sucrose together with sulfonamide, an inhibitor for folate biosynthesis, although the authors did not describe the starch content (Stokes et al. 2013). The fpgs1pgm double mutant exhibited dwarfism despite not accumulating starch, indicating that the dwarf phenotype of fpgs1-4 was independent of sugar metabolism involving PGM.
(Figs. 7, 9). Pleiotropic phenotypes in the mutant are not unexpected as folate derivatives act as cofactors for a variety of enzymes, each of which might induce a different phenotype. Our data suggest that one such enzyme may regulate starch biosynthesis in non-photosynthetic plastids.

Evidence of a starch biosynthetic regulatory enzyme in non-photosynthetic plastids is provided by the observation that SFU induced starch biosynthesis in wild-type Arabidopsis (Figs. 6, 9). SFU is an antifolate agent used for cancer therapy that predominantly inhibits thymidylate synthase in animal cells (Zhao and Goldman 2003). Thymidylate synthase requires 5,10-methylene-THF as a cofactor and converts dUMP into dTMP (Ravelan et al. 2011). Arabidopsis has bifunctional enzymes with thymidylate synthase and dihydrofolate reductase activity (Lazar et al. 1993), and it is possible that SFU inhibits the plastidial isozyme (Fig. 9). Loss of the enzyme cofactor in the fpgs1-4 mutant or in wild-type Arabidopsis supplied with methotrexate might also reduce the enzyme activity.

Since thymidylate synthase is involved in nucleotide metabolism, we assumed that reduction of the enzyme activity might disrupt nucleotide homeostasis within the plastids. The idea is supported by the observation that exogenously supplied adenine induced starch biosynthesis in etioplasts of wild-type Arabidopsis (Fig. 6). As discussed above, ADPG is a direct precursor of starch biosynthesis. Increased amounts of adenine might induce excess ATP production, which might lead to accelerated production of ADPG via conjugation of ATP with G1P, a reaction catalyzed by AGPase (Fig. 9). Excess ADPG would then up-regulate starch biosynthesis. Non-photosynthetic plastids of fpgs1-4 probably accumulated starch because loss of plastidial folate derivatives activated biosynthesis of ATP by a pathway that was independent from photosynthesis. Although plastidial thymidylate synthase is a potential candidate for an enzymatic link between folate derivatives and starch biosynthesis, any link between the enzyme and ATP remains to be elucidated (Fig. 9). Irrespective of the mechanisms involved, overall our results suggest that plastidial folate derivatives in wild-type Arabidopsis may activate enzymes, such as plastidial thymidylate synthase, whose activity reduces ATP levels, thereby suppressing starch biosynthesis in the non-photosynthetic plastids (Fig. 9). Thus, although non-photosynthetic plastids have the latent ability to produce G1P, another substrate for AGPase, from sucrose entering cells, starch accumulation is suppressed in wild-type Arabidopsis.

Sucrose is an important biomolecule that allows long-distance transfer of energy from photosynthetic source organs to distal non-photosynthetic cells. Non-photosynthetic cells then catabolize sucrose as an energy source. Sucrose is also metabolized for storage as starch. Our results showed that exogenous sucrose can be converted and stored as starch in etioplasts and non-photosynthetic chloroplasts when folate is absent. However, cells that convert sucrose to starch fail to obtain energy from the sugar. Suppression of starch biosynthesis is thus needed to maintain carbon homeostasis in cells receiving sucrose from source organs. Our results provide a breakthrough in understanding how non-photosynthetic plastids positively suppress starch biosynthesis from imported sugar.

Materials and Methods

Plant materials and chemicals

Arabidopsis thaliana was grown as described previously (Hayashi et al. 1998b, Hayashi et al. 2000). Seeds were surface-sterilized in a solution containing 2% NaClO and 0.05% Triton X-100, and were sown on growth medium (2.3 mg ml⁻¹ Murashige and Skoog (MS) salts (Wako Pure Chemical Industries Ltd.), 100 μg ml⁻¹ myo-inositol, 1 μg ml⁻¹ thiamine·HCl, 0.5 μg ml⁻¹ pyridoxine, 0.5 μg ml⁻¹ nicotinic acid, 0.5 mg ml⁻¹ MES·KOH (pH 5.8) ± 60 mM sucrose) containing 0.8% agar under axenic conditions. Germination was activated by incubation in the dark for 48 h at 4°C, followed by irradiation with white light (100 μE m⁻² s⁻¹) at 22°C for 6 h. Seedlings were grown at 22°C for 5 d in darkness.

In some experiments, the composition of the growth medium and cultivation conditions were modified as described in the text. For example, when starch accumulation in leaves was examined, plants were grown for 2 weeks under constant illumination, and were then transferred to the dark. After 12 h of dark treatment, leaves were excised from the plants and then vacuum·infiltrated with liquid growth medium (see above) without agar. The leaves were then incubated for 72 h in liquid medium containing 0.02% Silwet L-77 (Bio Medical Science) under vigorous aeration. The same conditions were used for liquid culture of dark-grown seedlings in some experiments.

Ethyl methanesulfonate-mutagenized M₂ seeds of A. thaliana accession Columbia were used for mutant screening. Seeds of atdfb-3 (SALK_015472), pgn-1 (CS201) and sex4-3 (SALK_102567C) were provided by the Arabidopsis Biological Resource Center, Ohio State University. fpgs2 (SALK_008883) and fpgs3 (SAIL_580_H10) were provided by the Nottingham Arabidopsis Stock Center. cp-GFP, a transgenic Arabidopsis line expressing rbcS–GFP (a fusion protein consisting of an N-terminal transient peptide of Rubisco small subunit plus GFP) was provided by Dr. Niwa at the University of Shizuoka.

Folate, THF, 5-formyl-THF (folinic acid), methotrexate and SFU were purchased from Wako Pure Chemical Industries Ltd. and 5-methyl-THF was obtained from Sigma-Aldrich.

Visualization of starch by iodine staining

Starch accumulated in etiolated seedlings and leaves was visualized by iodine staining. Etiolated seedlings grown on agar plates were incubated with 1% iodine solution [1% I₂, 1% KI] for approximately 1 min.

Leaves were also incubated with 1% iodine solution for 5 min, and excess iodine was removed by washing with water. Chl in stained leaves was removed by washing with a solution containing 35% chloral hydrate and 6.7% glycerol.

Quantification of starch

Starch content was assayed in seed and seedlings as previously described (Kanai et al. 2007) with minor modifications. Plant tissues were ground with a mortar and pestle in liquid nitrogen, and 5–10 mg of the resulting powder was washed twice with 1 ml of ethanol and twice with 1 ml of distilled water. The precipitate was suspended in 0.2 ml of distilled water and heated in a boiling water bath for 1 h. The mixture was then centrifuged at 10,000 × g for 10 min, and 100 μl of the supernatant was mixed with 400 μl of an extraction buffer (pH 7.4), 5 mM MgCl₂, 2 mM NADP and 25 mM ATP. The amount of glucose in the solution was measured by the increase in absorbance at 340 nm after the addition of 1 μl each of hexokinase (2.8 nkat; Sigma-Aldrich) and G6P dehydrogenase (2.3 nkat; Sigma-Aldrich).

Metabolite analysis

Metabolites accumulated in etiolated seedlings were analyzed by capillary electrophoresis–mass spectrometry as previously described (Anegawa et al. 2015). Wild-type and fpgs1-4 seedlings (30 mg FW) grown under darkness in the presence of 60 mM sucrose was homogenized in extraction buffer (MeOH·chloroform·H₂O = 1:1:0.4) and the aqueous layer collected after centrifugation. The aqueous sample was then fractionated by capillary
electrophoresis (Agilent Technologies) and the metabolites in each fraction were analyzed by mass spectrometry (TOF-MS; Agilent Technologies). The migration time and molecular mass of each metabolite were compared with standards. Metabolite amounts were calculated from spectral peak areas.

**Cytological analyses**

Five-day-old etiolated seedlings were mounted on glass slides. GFP fluorescence was examined using an LSM510 laser scanning confocal microscope equipped with a 488 nm argon laser and a BP 505−550 emission filter (Carl Zeiss). Some of the seedlings were placed in fixative solution [4% (v/v) paraformaldehyde, 1% (v/v) glutaraldehyde, 10% dimethylsulfoxide and 0.06 M sucrose in 0.05 M cacodylate buffer (pH 7.4)]. The samples were then embedded in Epon (Epon 812 resin, TAAB laboratories). Thin sections were stained with 0.05% toluidine solution (citrate-phosphate buffer, pH 7.0) and then with iodine solution, and then analyzed by light microscopy. Ultrathin sections were also prepared from the same specimen and analyzed by electron microscopy as described previously (Hayashi et al. 2002b).

**Genetic analyses and map-based cloning**

All genetic analyses were conducted using progeny that had been backcrossed twice with wild-type Arabidopsis. For mapping, crosses were made between mutant and wild-type (accession Landsberg erecta) plants. Homozygous F₂ seedlings were scored according to their dwarf phenotype. Map-based cloning was conducted as described previously (Hayashi et al. 2000). Primer pairs for two simple sequence length polymorphism (SSLP) markers (at5g05780 and at5g06230) were as follows: CAAAACAAAGGGCTAACG and CATAATGC ATGTCAGTAACGTG, and CAAAACAAAGGGCTAACG and CATAATGTC ATGTCAGTAACGTG, respectively. A CAPS (cleaved amplified polymorphic sequence) marker (at5g06440) was amplified with GGTATCGTTAAACGCTACTG and AAGCAGTGAAGGTACCA TGGC and then digested with Ddel.

**DNA sequencing analyses**

Genomic DNA was extracted from etiolated seedlings using a DNaseasy Plant Mini Kit (Qiagen). TAIR10 [Arabidopsis genome sequence data at the Arabidopsis Information Resource (www.arabidopsis.org)] was used to design primer pairs for amplification of genes at mapping positions. Amplified fragments were sequenced directly as described previously (Hayashi et al. 1998b).

**Reverse transcription–PCR (RT–PCR)**

RT–PCR was performed as described previously (Kanai et al. 2010). Total RNA was extracted from etiolated seedlings using an RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 2 μg of total RNA using Ready-to-GO RT-PCR beads (GE Healthcare). Amplification and sequence determination of cDNA fragments from wild-type Arabidopsis and fgs1-4 were performed using the A CACGCCCTCCACCTCAGT and ATGGCCTCTACGTTGGG primer pair, as described above.

**Supplementary data**

Supplementary data are available at PCP online.

**Funding**

This work was supported by the Ministry of Education, Science, and Culture of Japan [grants-in-aid for scientific research grant No. 15K07117].

**Acknowledgments**

We thank the Arabidopsis Biological Resource Center, Nottingham Arabidopsis Stock Center and Dr. Y. Niwa at the University of Shizuoka for providing seeds. We also thank Ms. M. Kondo and all the staff members at Spectrography and Bioimaging Facility, NIBB Core Research Facilities for their support with cytological analysis (No. 15-340), and Ms. Y. Yoshinori at the National Institute for Basic Biology, and Mrs. R. Hirayama and S. Yamamoto at the Nagahama Institute of Bioscience and Technology for their technical support. We dedicate this work to the late Dr. Takashi Akazawa, Professor Emeritus of Nagoya University, who made great contributions to the field of starch metabolism and who supported and encouraged us in our research over many years.

**Disclosures**

The authors have no conflicts of interest to declare.

**References**


