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Molecular characterisation of a new mutant allele of the plastid phosphoglucomutase in *Arabidopsis*, and complementation of the mutant with the wild-type cDNA

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Abstract Screening of transposon-associated mutants of *Arabidopsis thaliana* for altered starch metabolism resulted in the isolation of a mutant that did not accumulate starch in any tissue or at any developmental stage (starch-free mutant, *stf1*). Allelism tests with known mutants showed that *stf1* represents a new mutant allele of the plastid isoform of the enzyme phosphoglucomutase (PGMp). The mutation was mapped to chromosome 5. An *Arabidopsis* EST that showed significant homology to the cytosolic isoform of phosphoglucomutase (PGM) from maize was able to complement the mutant phenotype. The *Arabidopsis* EST was transcribed and translated in vitro and the protein product was efficiently imported into isolated chloroplasts and processed to its mature form. The lack of starch biosynthesis in *stf1* is accompanied by the accumulation of soluble sugars. The rate of CO_2 assimilation measured in individual leaves was substantially diminished only under conditions of high CO_2 and low O_2. Remarkably, *stf1* exhibits an increase rather than a decrease in total leaf PGM activity, suggesting an induction of the cytosolic isoform(s) in the mutant. The substrate for PGM, glucose 6-phosphate, accumulated in *stf1* during the day, resulting in 10-fold higher content than in the wild type at the end of the photoperiod.

Key words *Arabidopsis thaliana* · Phosphoglucomutase · Plastid Starch biosynthesis · Starch-free mutant

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Introduction

During photosynthesis, most higher plants accumulate starch transiently in the chloroplasts as a temporary deposit of photoassimilates that can be mobilised during the following dark period (Preiss 1982; Beck and Ziegler 1989). The rest of the photoassimilates is exported to the cytosol in the form of triose phosphates, as precursors for sucrose biosynthesis. Only when sucrose biosynthesis is limited (e.g. by restriction of phloem loading), does the rate of starch biosynthesis in the chloroplasts reach a maximum due to the allosteric regulatory properties of ADPglucose pyrophosphorylase (AGPase), which is most active in the presence of high 3-phosphoglycerate (3-PGA)/P_i ratios in the stroma (Preiss 1991). In principle, the precursor for the synthesis of transitory starch is the Calvin-Benson cycle intermediate fructose-6-phosphate (Fru6P), which is converted to glucose-1-phosphate (Glc1P), the substrate of AGPase, by the combined action of phosphoglucoisomerase (PGI) and phosphoglucomutase. ADPglucose (ADP-Glc) is then used by soluble and/or starch granule-bound starch synthases for the formation of transitory starch.

Mutants in starch metabolism provide an excellent tool for dissecting the reaction pathways and studying regulatory mechanisms of starch biosynthesis and breakdown. Mutants in the biosynthesis and breakdown of starch have been isolated from EMS-mutagenised populations of *Arabidopsis thaliana*. Mutations have been obtained in the plastid isoform of PGM (Caspar et al. 1985) and AGPase (Lin et al. 1988a, 1988b), and a putative mutant for the plastid glucose transporter (Caspar et al. 1991; Trethewey and ap Rees 1994a, 1994b) and a mutant deficient in chloroplast α-amylase have been reported (Zeeman et al. 1998).

To date, none of these EMS mutants has been characterised at the molecular level. We therefore screened mutant populations of *A. thaliana* generated by T-DNA and transposon insertion for altered starch metabolism.

A starch-free mutant was identified that was defective in the plastid isoform of PGM.

Materials and methods

Reagents and enzymes

If not stated otherwise, reagents were obtained from Roche Molecular Biochemicals (Mannheim, Germany), Sigma (Deisenhofen, Germany) or Promega (Heidelberg, Germany).

Plant material and culture conditions

Seeds of *A. thaliana* wild types (ecotypes Wassilewskija, Columbia and Landsberg *erecta*) and mutant lines used in this paper [CS2361–CS2410 (Forsthoefel et al. 1992) and a collection of *Ds* transposon insertion lines including N4351 (Bancroft et al. 1992), as well as the starch-defective lines N210, N3092, N3094 and N3095] were kindly provided by the Nottingham Arabidopsis Stock Centre (NASC).

Plants screened for altered starch contents were grown in the greenhouse on a 3:1 mixture of soil and vermiculite on a 12 h light/dark regime. Three days before the screen, plants were transferred to a photon flux density of 70 μmol per m^2/s.

Plants used for DNA or RNA extraction and metabolite measurements were grown in a cabinet at 19–23 °C and 40% humidity. A photon flux density at the level of the plant of approximately 200 μmol per m^2/s was provided on a 12 h light/dark regime.

Recombinant DNA techniques

RNA was isolated from rosette leaves of *stf1* and Landsberg *erecta* plants according to Eggermont et al. (1996). For synthesis of the first-strand cDNA the SuperscriptII RNase H^- Reverse Transcriptase kit (GIBCO-BRL) was used. The protocol provided by the manufacturer was modified as follows: 2 μg of leaf RNA was incubated with 10 U of RNase-free DNase I (Boehringer Mannheim). DNase was inactivated by the addition of EDTA (2.27 mM final concentration) followed by heat denaturation. The reaction mixture contained oligo $(dT)_{12–18}$ (500 ng), dNTPs (0.5 mM each), DTT (10 mM) and reverse transcriptase (200 U) in a total volume of 50 μl. After incubation for 1 h at 42 °C, the mixture was heat-inactivated for 15 min at 70 °C. One-tenth of this mixture was used for PCR.

The following primers were used for PCRs: Gene primer-fw (5′-TTAACGGACACTTTTCACTG-3′), Gene primer-rev (5′-ACAGATGAAGATGAGGAGGATGC-3′), Actin-fw (5′-GAAGCAAGAATGGAACCACCG-3′), and Actin-rev (5′-GGGCAAGTCATCACGATTGG-3′). The PCR products were size fractionated on 3% agarose gels and detected by ethidium bromide staining.

Genetic analysis

The genetic basis for the lack of starch formation in the line *stf1* was analysed by crossing the mutant to the wild type and other already characterised starch-free mutants. The F1 progeny was scored for the absence or presence of leaf starch following a 12-h light period. Iodine staining was used as a qualitative assay.

Two lines carry a defect in the gene for the plastid isoform of PGM (*pgm-1* = N210 and N3092), whereas the other lines harbour mutations in different subunits of the AGPase (*adg1-1* = N3094 and *adg2-1* = N3095).

Mapping population

For the mapping analysis a segregating F2 population was set up by crossing the line *stf1* to the Columbia wild type. The F1 generation was allowed to self-pollinate. F2 plants were screened for starch-free phenotypes by staining leaves with iodine. Starch-free F2 plants were used for mapping.

CAPS (cleaved amplified polymorphic sequence) mapping

Genomic DNA of 93 starch-free F2 mapping plants was prepared according to Edwards et al. (1991). The CAPS method was carried out according to the protocol of Konieczny and Ausubel (1993).

Carbohydrate measurements

For the determination of starch (Lin et al. 1988a) and soluble sugars (Caspar et al. 1985; Jones et al. 1977), individual *Arabidopsis* leaves (0.5 cm^2) or whole rosettes (0.04–0.12 g) were heated twice in 1 ml of 80% ethanol for 15–30 min until all pigments were solubilised. The water/ethanol-soluble fractions were pooled and dried in a vacuum concentrator (Bachofer, Reutlingen, Germany). The residues were dissolved in 0.2 ml of water. Insoluble material was precipitated by centrifugation (20,000 × *g* for 5 min) and discarded. For the quantitative solubilisation of starch, leaf material was homogenised in 0.25 ml of 0.2 M KOH, followed by the addition of another 0.25 ml of 0.2 M KOH and incubation at 95 °C for 45 min. The pH of the extract was adjusted to pH 5.5 by the addition of 1 M acetic acid. Starch was degraded to glucose by overnight incubation with 17 U of α-amylase and 2.5 U of amyloglucosidase (in sodium acetate buffer, pH 4.6). This procedure yielded 100% recovery of starch. The levels of glucose, fructose, and sucrose and maltose in the water/ethanol soluble fraction were determined according to Bergmeyer (1970).

Determination of phosphorylated metabolic intermediates

For the determination of phosphorylated intermediates, leaf rosettes were cut, rapidly transferred into liquid nitrogen in the light and subsequently extracted with perchloric acid. The contents of phosphorylated intermediates in the neutralised perchloric acid extracts were measured enzymatically based on methods described by Stitt et al. (1989) and Bergmeyer (1970). All metabolite determinations were performed in a total volume of 200 μl, including 10–30 μl of extract, on microtitre plates using the Spectrofluor Plus microtitre plate reader (TECAN, Salzburg, Austria) in the fluorescence mode. In order to follow the reaction kinetics for 5–10 min, the activities of the coupling enzymes were kept low. For the determination of 3-phosphoglycerate (3PGA), the reaction mixture contained 50 mM HEPES-NaOH (pH 7.5), 1.0 mM $MgCl_2$, 1.0 mM ATP and 10 μM NADH. The reaction was initiated by the addition of 0.45 U of phosphoglycerate kinase (PGK) and 0.08 U of glyceraldehyde 3-P dehydrogenase (GAPDH). For the determination of triose phosphates as well as conversion of fructose-1,6-bisphosphate (Fru1,6P_2) in the direction of glycerol-3-phosphate (Gly3P) formation, the reaction mixture contained 50 mM HEPES-NaOH (pH 7.5), 1 mM $MgCl_2$ and 10 μM NADH. Measurements of dihydroxyacetone phosphate (DAP), glyceraldehyde 3-phosphate (GAP) and Fru1,6P_2 were initiated by sequential addition of 0.032 U of GAPDH, 0.08 U of triose phosphate isomerase and 0.009 U of aldolase. Hexose phosphates were determined in a reaction mixture containing 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 0.8 mM NADP, and 0.1 μM glucose 1,6-bisphosphate (Glc1,6P_2). The individual hexose phosphates were determined by sequential addition of 0.1 U of glucose 6-phosphate dehydrogenase (for Glc6P), 0.35 U PGI (Fru6P) and 0.04 U PGM (for Glc1P). The reliability of the metabolite measurements was assessed by determination of known concentrations of metabolites in the presence or absence of extract.

Determination of enzyme activities

Frozen *A. thaliana* leaves (1–3 cm^2; 20–40 mg of fresh weight) were extracted in 1 ml of ice-cold extraction medium (see below) using a

mortar and pestle. The extracts were clarified by centrifugation (10,000 × g, 30 s).

PGM and PGI. *A. thaliana* leaves were extracted in medium A [100 mM HEPES-KOH (pH 7.4), 10% (v/v) glycerol, 0.1% (v/v) Triton-X-100 and 1 mM PMSF]. PGM activity was measured according to Bergmeyer (1974). PGI activity was assayed in the same reaction mixture as used for PGM, except that Glc1P was replaced by 3.3 mM Fru6P and Glc1,6P_2 was omitted.

AGPase. Leaf material was extracted in 100 mM Tricine-NaOH (pH 8.0), 10 mM NaH_2PO_4, 1 mM EDTA (pH 8.0), 5 mM dithiothreitol, 10% (v/v) glycerol, 0.1% (v/v) Triton-X-100, and 1 mM PMSF. The supernatants were desalted by gel filtration on Sephadex G-25 (NAP5, Pharmacia Biotech) equilibrated with the appropriate extraction medium. The activity of AGPase in the direction of Glc1P formation was assayed according to Bergmeyer (1970).

Inorganic pyrophosphatase (PPase). For the determination of PPase, the leaf material was extracted in medium A and the PPase activity was measured in a stopped assay. The incubation mixture contained 50 mM HEPES-NaOH (pH 7.7), 2.5 mM $MgCl_2$, 1.0 mM $Na_4P_2O_7$ and 10 µl of extract (in a total volume of 1.5 ml). Aliquots (0.5 ml) were mixed at time 0, after 5 min and after 30 min with 0.5 ml of 1 N H_2SO_4. The phosphate content in the reaction mixture was determined according to Bartlett (1959).

Starch synthase. The activities of starch synthases were determined radiometrically using ADP-*U*[^{14}C]glucose as a substrate (modified after Tyson and ap Rees 1988). Six to ten *A. thaliana* leaves were pulverised in liquid nitrogen and extracted in 1 ml of 100 mM HEPES-NaOH (pH 7.5), 5 mM DTT, and 1 mM EDTA. Aliquots (20 µl) of the extracts were incubated in 100 mM Tricine-KOH (pH 8.0), 25 mM potassium acetate, 500 mM sodium citrate and 5 mM ADP-*U*[^{14}C]glucose (specific activity 4500 Bq/µmol) and 1.6 mg amylopectin, in a total volume of 200 µl for 2 h at 25 °C. As a control, 20 µl of heat-denatured (5 min at 95 °C) extract was incubated as described above. The incubation was stopped by heating at 95 °C for 1 min followed by the addition of 1.5 ml of 1% (w/v) KCl in 75% (v/v) methanol and subsequent centrifugation at 1700 × *g* for 1 min. From this supernatant, a 750-µl sample was taken for liquid scintillation counting (to determine the specific activity of the ADP-*U*[^{14}C]glucose in the assay). The pellet was washed four times by resuspension in 300 µl of water, followed by the addition of 1.5 ml 1% (w/v) KCl in 75% (v/v) methanol. The polyglucan sediment was resuspended in 1 ml of 100 mM sodium acetate (pH 4.8) and boiled for 30 min. After cooling, the polyglucan was digested with 6 U each of α-amylase and amyloglucosidase in 100 mM sodium acetate (pH 4.8) for 16 h at 37 °C. The assay mixture was cleared by centrifugation (11,000 × *g*, 5 min) and the incorporated ^{14}C was determined by scintillation counting of 0.5 ml of the supernatant.

Native gel electrophoresis and PGM activity staining

Native discontinuous polyacrylamide gel electrophoresis was performed according to Davis et al. (1964) in 7.5% separation gels. PGM activity was stained by incubating gel slabs in the standard PGM assay mixture supplemented with 33 µM PMS and 0.25 mM NBT. As a control, Glc1P was omitted.

Protein determination

Protein contents were determined using a modified Bradford assay (Bradford 1976; Zor and Selinger 1996).

Photosynthesis measurements

The gas exchange characteristics of *A. thaliana* leaves were measured with an LCA-4 open gas analyser (Analytical Development, Hodderson, Herts., UK) in a leaf chamber at a leaf temperature of 20–22 °C. The leaf area inside the chamber was approximately 0.8–1.2 cm^2. Fibre optic illumination was provided by a Schott KL 1500 projector. The humidity was maintained at approximately 30%. Photosynthesis parameters were measured on individual leaves rather than on whole plants; this provides the opportunity to estimate CO_2 assimilation rates per unit leaf area, as well as intercellular CO_2 concentrations. For this purpose a leaf chamber was designed that allowed gas exchange measurements to be made on individual *A. thaliana* leaves. The rates of CO_2 assimilation (A) and transpiration (E), as well as the intercellular CO_2 concentrations (C_i) were calculated according to von Caemmerer and Farquhar (1981). Gas exchange parameters of fully developed rosette leaves of 4-week-old *A. thaliana* plants were measured either in air or in a special gas mixture containing 1490 µl of CO_2/l and 2% O_2, with the balance (N_2) supplied by a gas cylinder. Modulated Chl *a* fluorescence emission from the upper surface of the leaf was measured with a pulse amplitude modulation fluorometer (Schreiber et al. 1986) PAM-2000 (Walz, Effeltrich, Germany). The rate of linear photosynthetic electron transport was calculated from the quantum efficiency of photosystem II electron flux (ϕ PSII) according to Genty et al. (1989) using the ratio of $(F'_m - F_s)/F'_m$ (F'_m = maximum fluorescence; F_s = steady state fluorescence). The ground fluorescence (F_o) was measured by exposing dark-adapted leaves (30–60 min) to a weak, modulated measuring beam. For the determination of maximal fluorescence (F_m), a flash of saturating light (approximately 5000 µmol per m^2/s; duration 800 ms) was applied.

In vitro protein import experiments

DNA of the EST 99H14T7 was transcribed and translated in vitro in the cell-free TNT Coupled Reticulocyte System according to the instructions given by the manufacturer (Promega). The in vitro protein import experiments and the subsequent subcellular fractionation of chloroplasts were carried out according to Flügge et al. (1989). Envelope membranes, the stroma fraction and thylakoid membranes were analysed by SDS-PAGE (Laemmli 1970) and phosphoimaging.

Results

Screening for high-starch and starch-free phenotypes

From the T-DNA knockout *Arabidopsis* population described by Forsthoefel et al. (1992), we screened 920 individual lines (5 plants per line, or 4600 plants in total) for altered starch contents at the end of both the light and dark periods. In addition, we screened a randomly chosen selection of the transposon insertion lines described by Bancroft and Dean (1993). We were not able to isolate plants that reproducibly contained starch (as judged by iodine staining) at the end of the dark period (high-starch phenotype). In the transposon population, however, we found one plant that did not contain starch at the end of the light period (starch-free phenotype). This starch-free phenotype was confirmed in the next generation and the corresponding mutant line was termed *stf1*. The mutant *stf1* does not accumulate starch in any tissue or at any developmental stage. Growth on a light/dark cycle of 12 h/12 h is retarded by about 2 weeks. The rosette leaves display a slightly epinastic phenotype and have fewer trichomes than the wild type. Growth retardation is relieved under long-day conditions (18 h light) or in continuous light.

Genetic analysis of *stf1*

The *stf1* line was crossed to the wild type, and the starch content of the F1 plants was analysed. All F1 plants contained starch at the end of the light period. In the F2 generation, the starch-free phenotype segregated in a 3:1 manner, indicating a single, recessive, nuclear mutation.

Using PCR primers derived from the hygromycin phosphotransferase gene (HPT, a marker for the presence of the *Ds* element) and the neomycin phosphotransferase gene (NPT, a marker for the presence of the T-DNA), we checked 138 starch-free plants from the F2 generation for co-segregation of the *Ds* element and the T-DNA with the starch-free phenotype. Twenty-eight starch-free plants contained neither the *Ds* element nor the T-DNA, indicating that the site of the mutation causing the *stf1* phenotype is not linked to the insertion site of the *Ds* element or the T-DNA.

The *Ds* element co-segregated with the T-DNA (donor site), indicating that the *Ds* element remains close to its original donor site on chromosome 3 (Bancroft and Dean 1993). In addition, we checked the parental *Ds* line N4534 (Bancroft et al. 1992) for the presence of starch. This line displayed a normal starch content at the end of the light period, suggesting that the mutation in *stf1* might represent a second-site mutation.

Complementation analysis with known mutants in the starch biosynthetic pathway and mapping of the mutation

In order to analyse whether the mutation in *stf1* is allelic to one of the already known starch-free mutants, we crossed *stf1* to mutants defective in the large and small subunits of the plastid AGPase (*adg1-1*, N3094; *adg2-1*, N3095) and to two mutants defective in the plastid isoform of PGM (*pgm-1*, N210, N3902; Caspar et al. 1985). Crosses to the AGPase mutants resulted in a F1 generation that contained starch in all tissues. Crosses to the *pgm-1* mutants resulted in a starch-free F1 generation, indicating that *stf1* represents a new mutant allele of the gene for PGMp.

Using CAPS markers (Konieczny and Ausubel 1993) we mapped the mutation in *stf1* in a segregating population obtained from a cross of *stf1* (ecotype Landsberg *erecta*) to the ecotype Columbia. The mutation is located between the molecular markers DFR (position 91 cM) and EG7F2 (position 118 cM) on the lower arm of chromosome 5, which differs from the position indicated by the use of classical markers [66.8 cM (Patton et al. 1991) and 73.3 cM (Hauge et al. 1993)].

The plastid PGM is missing in the *stf1* line

We measured the extractable activities of enzymes involved in starch biosynthesis in fully developed rosette leaves of *stf1*. Compared to the wild type, no major differences in the extractable activities of AGPase, starch synthase and PPase were found, whereas the average activities of PGI and PGM appeared to be slightly increased in the *stf1* mutants (Table 1). Hence, from total activity determinations it appeared unlikely that the starch-free phenotype was due to a mutation in the plastid form of PGM, particularly as previously reported total PGM activities in *pgmP* null mutants (Caspar et al. 1985) were 25% lower than in the wild type (Neuhaus and Stitt 1990). It has to be stressed that the *pgmP* null and the *stf1* mutants were obtained in the background of the ecotypes Columbia and Landsberg *erecta*, respectively. PGI and PGM exist as isoforms, one being localised in the stroma, the other(s) in the cytosol. Activity staining after fractionation of leaf extract on native polyacrylamide gels revealed that the chloroplast isoenzyme of PGM was missing in the mutant (Fig. 1, lanes 2 and 3). It can therefore be assumed that the observed increase in the specific PGM activity in the mutant reflects an increase in the catalytic activities of the cytosolic isoform(s) of this enzyme. Thus, at both the genetic and biochemical levels, the *stf1* line was shown to be defective in the plastid isoform of PGM.

Table 1 Activities of enzymes involved in the biosynthesis of starch measured in leaf extracts of wild-type *Arabidopsis* (L.*e*.) or the starch-free mutant *stf1*

Enzymes	Enzyme activities (mU/mg)[a]	
	Landsberg *erecta*	*stf1*
AGPase	75 ± 32	97 ± 34
PGI	333 ± 34	395 ± 68
PGM	380 ± 130	400 ± 40
PPase	13 ± 2	16 ± 2
Total starch synthase	7.8 ± 0.6	6.9 ± 0.6

[a] Enzyme activities are expressed per mg of total leaf protein. The data are the means ± SD of 3–5 measurements

Fig. 1 Fractionation of extracts from *Arabidopsis* leaves by native discontinuous polyacrylamide gel electrophoresis (PAGE) and staining for PGM activity. Lanes 1–4 show PGM activities after fractionation of leaf extracts of wild-type *Arabidopsis*, ecotype *Columbia* (lane 1), the mutant *stf1* (lane 2), the mutant *stf1* transformed with an empty binary vector (lane 3) and the complemented mutant N80 (lane 4). PGM isoforms in the cytosol (c1, c2) and chloroplasts (p) are indicated

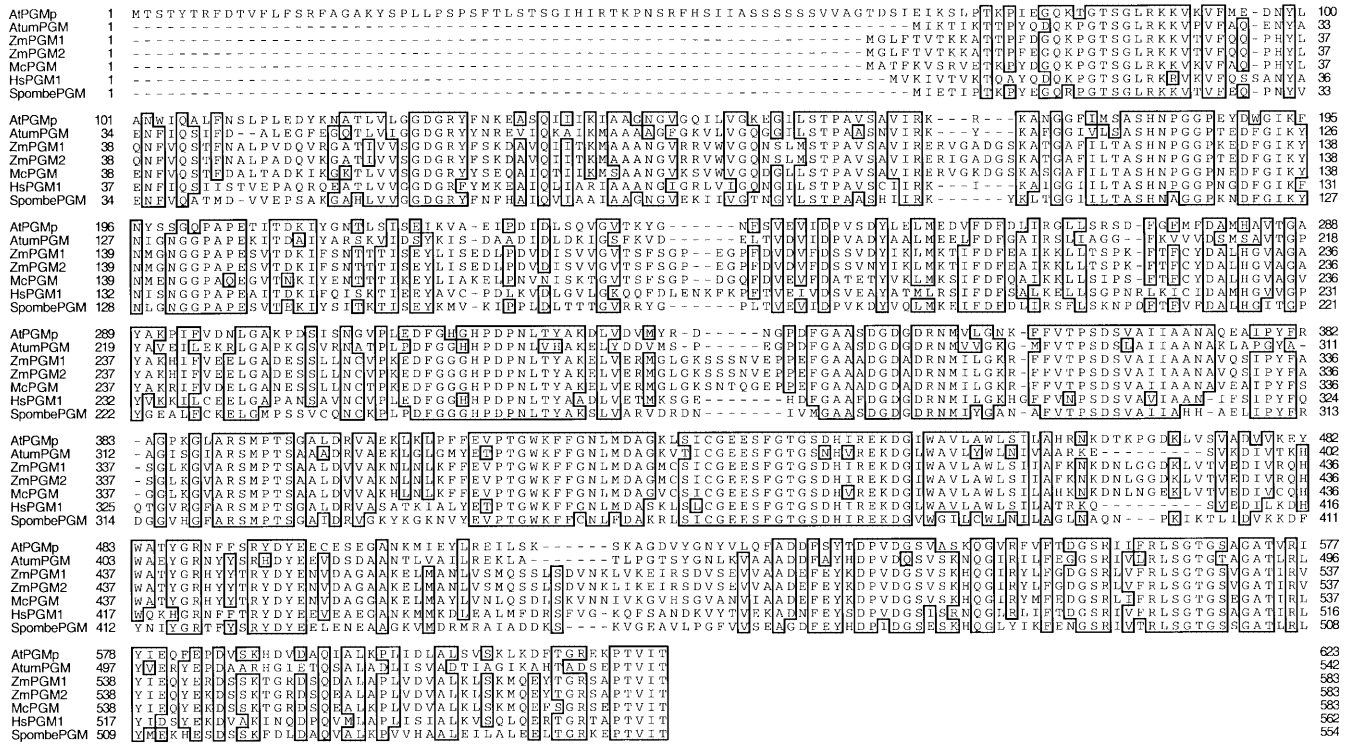

Fig. 2 Amino acid sequence alignment of PGMs from different species. AtPGMp, plastid isoform of PGM from *A. thaliana*; AtumPGM, PGM from *Agrobacterium tumefaciens*; ZmPGM1 and ZmPGM2, cytosolic isoforms of PGM from maize; McPGM, cytosolic isoform of PGM from *Mesembryanthemum crystallinum;* HsPGM1, isoform 1 of PGM from human; SpombePGM, PGM from *Schizosaccharomyces pombe*. Identical amino acid residues are *boxed*

Isolation of the PGMp cDNA sequence and the corresponding genomic DNA sequence

We used the cDNA sequence of a known cytosolic isoform of PGM from *Mesembryanthemum crystallinum* (Accession No. U84888) to search *A. thaliana* sequences from chromosome 5. A sequence showing significant homology to PGM was detected between the markers DFR and EG7F2. Compared to known sequences of cytosolic PGM isoforms, this sequence contained an N-terminal extension which displays features of plastid targeting sequences. A corresponding *Arabidopsis* EST (99H14T7) was identified and sequenced. This EST turned out to be full-length, encoding a protein of 623 amino acids with a calculated molecular mass of 67,946 kDa. The deduced amino acid sequence shows significant homology to PGMs from plants (*Arabidopsis*: 58% identity, 74% similarity in the mature segment of the protein), fungi and mammals (human: 55% identity, 74% similarity in the mature protein) (Fig. 2). No significant homology was detected with a putative plastid PGM (Accession No. X75898) that was described by Penger et al. (1994).

The structure of the genomic DNA (GenBank Accession No. AB010074, nucleotides 14377–18776) was analysed using the PGM cDNA sequence (Accession No. AF216580). We detected 20 introns and 21 exons. Using PCR, we amplified the mutant allele from genomic DNA and found a 55-bp deletion. Sequencing of the mutant DNA showed that this deletion is located in the 5′ region of the mutant gene. This deletion includes the ATG start codon of the corresponding cDNA; thus, a truncated mRNA should be produced, leading to the observed lack of PGMp biosynthesis (Fig. 3).

The deletion in the PGMp gene is detectable at the mRNA level

RNA samples from the starch-free mutant were analysed for the presence of the predicted mutant RNA by RT-PCR with gene-specific primers flanking the deletion. With both mutant and wild-type cDNA templates, we were able to detect PCR amplification products. The product amplified from the mutant cDNA was about 50 bp shorter (Fig. 4, lanes 1 and 3), which reflects the size of the deletion in the corresponding gene. The RT-

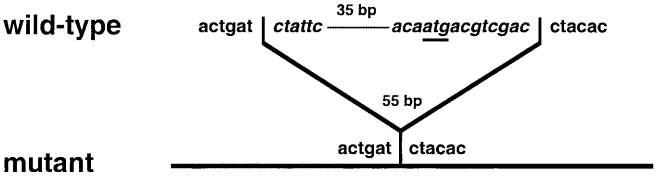

Fig. 3 Schematic representation of the structure of the mutated PGMp allele. The 55-bp deletion is indicated

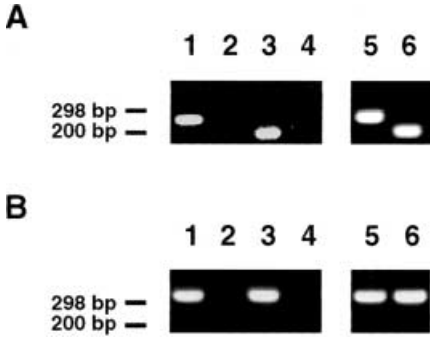

Fig. 4A, B Detection of the deletion in the PGMp gene at the mRNA level by RT-PCR. The analysis of steady-state levels of PGMp mRNA by RT-PCR was performed with RNA isolated from leaves of the wild type (ecotype *Landsberg erecta*) and the starch-free mutant *stf1* using primer pairs specific for PGMp (**A**) or the actin control (**B**). The sample numbers indicate PCRs with cDNA from wild-type leaves (lanes 1 and 2) and the starch-free mutant (lanes 3 and 4) either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of reverse transcriptase. As a control, PCR amplification was conducted with PGMp-specific primers on genomic DNA from the wild type (lane 5) and the mutant (lane 6)

PCR products were sequenced and the deletion was verified (data not shown). These results indicate that the deletion indeed resides in a transcribed region of the PGMp gene. No significant diurnal variation in the steady-state level of the PGMp mRNA was detectable (not shown).

Complementation of the *stf1* mutant phenotype

In order to verify the function of EST 99H14T7 as the coding sequence for the plastid isoform of PGM, we expressed the corresponding cDNA under the control of the CaMV 35S promoter in the genetic background of the *stf1* mutant. For the transformation we used starch-free F2 plants from the cross between *stf1* and the Columbia wild type, which contained neither the T-DNA nor the *Ds* element. By in planta transformation (Bechtold et al. 1993) of the mutant, we obtained 34 independent, hygromycin-resistant transgenics. Some 44% of these transformants accumulated starch in amounts comparable to the wild type, whereas 38% showed somewhat lower, but significant, accumulation of starch. In contrast to the *stf1* mutant, all three isoforms of PGM detectable in the wild type, including that of the plastid isoform, could be visualised in the transformants by native PAGE and PGM activity staining (Fig. 1, lane 4). These findings unambiguously demonstrate that EST 99H14T7 codes for the plastid isoform of PGM.

Protein import experiments

In order to analyse whether the PGMp precursor protein is actually directed to the chloroplasts, we studied its uptake into spinach chloroplasts. The protein was synthesised by coupled transcription-translation of the cDNA in the presence of [^{35}S]methionine and added to intact chloroplasts. The chloroplasts were subsequently fractionated into a soluble fraction (stroma) and fractions containing thylakoids and envelope membranes, respectively (Flügge et al. 1989). Without post-treatment with protease, the imported and processed mature protein (apparent molecular mass, 62 kDa) was mainly found in the stroma, only a small portion of the precursor protein (apparent molecular mass, 66 kDa) was bound to the envelope membrane (Fig. 5, lanes 1–3). Subsequent treatment of the chloroplasts with the protease thermolysin revealed that the processed mature form was resistant to protease, having been translocated into the stroma; no signals were detectable in the membrane fractions (Fig. 5, lanes 4–6). These findings indicate that the N-terminal extension of the PGMp is able efficiently to direct the protein to the stroma, where it is processed to its mature form.

Carbohydrate partitioning in wild-type *Arabidopsis*, *stf1* and a segregating complemented line

We investigated carbohydrate partitioning in wild-type *Arabidopsis*, *stf1* and a segregating complemented line at the end of the light period (9 h in the light). As is shown in Table 2, the starch content was drastically reduced in source leaves of *stf1*. The apparent absence of leaf starch was accompanied by an increase in the content of soluble sugars: the levels of sucrose, glucose and fructose were on average 3.3-, 14.8-, and 35-fold higher in *stf1* compared to the wild type. Moreover, the glucose/fructose ratios were reduced from about 4.88 in the wild type to 2.06 in the mutant, suggesting cycling of sucrose via invertase(s). The ratio of sucrose/(glucose + fructose) declined from 6 in the wild type to 1.1 in the mutant, in agreement with the above assumption. Total carbohydrate content (i.e. soluble sugars + starch) in the mutant leaves was diminished by 66% after 4 h (data not shown) and by 73% at the end of the light period, compared to the wild type. This reflects the lack of starch biosynthesis rather than a limitation on sucrose biosynthesis in the absence of PGMp. The levels of

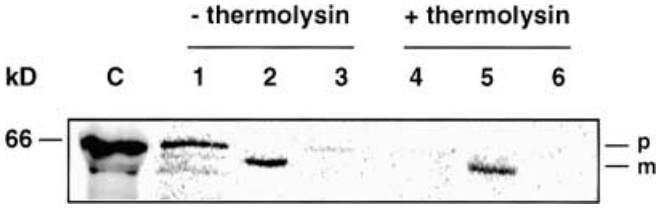

Fig. 5 In vitro protein import of PGMp from *A. thaliana* into isolated spinach chloroplasts. Lanes 1 and 4, envelope fractions; lanes 2 and 5, stroma fractions; lanes 3 and 6, thylakoid fractions. C, translation control. (+) thermolysin indicates treatment of chloroplasts with the protease thermolysin after the import step. Positions of the precursor (p) and the processed mature protein (m) are indicated

Table 2 Physiological and biochemical parameters of source leaves of wild-type *Arabidopsis* (Landsberg *erecta*), the starchless mutant *stf1* and the complemented *stf1* line, after 9 h in the light

Parameter[a]	Landsberg *erecta*	*stf1*	Complemented *stf1* line
Total fresh weight of leaf rosettes (mg)	51 ± 13	26 ± 13	38 ± 8.9
Total PGM activity (mU/mg protein)	360 ± 21	435 ± 71	409 ± 76
Metabolite levels			
Starch[b]	64.9 ± 28.4	0.09 ± 0.27	43.8 ± 2.4
Sucrose[b]	2.82 ± 1.21	9.25 ± 2.01	1.91 ± 0.27
Glucose[b]	0.39 ± 0.24	5.76 ± 2.79	0.48 ± 0.25
Fructose[b]	0.08 ± 0.04	2.79 ± 0.78	0.22 ± 0.11
Maltose[b]	0.02 ± 0.06	0.48 ± 0.78	0.015 ± 0.1
Glc6P[c]	46.0 ± 20.0	419.2 ± 136.0	123.7 ± 42.6
Glc1P[c]	22.1 ± 2.0	28.7 ± 1.2	23.5 ± 7.6
Fru6P[c]	35.4 ± 18.7	79.3 ± 32.7	44.3 ± 16.1
Fru1,6P_2[c]	23.1 ± 4.9	31.7 ± 8.2	18.9 ± 7.3
3-PGA[c]	406.2 ± 86.6	285.2 ± 67.2	291.7 ± 86.0
DHAP[c]	76.3 ± 13.0	89.3 ± 29	120.9 ± 21.0
GAP[c]	34.1 ± 8.2	50.0 ± 13.3	31.9 ± 9.7
[Glc6P]/[Glc1P]	2.08	14.6	5.26
[Fru6P]/[Glc6P]	0.77	0.18	0.35

[a] The data represent the means ± SD of six measurements
[b] Metabolite levels were measured after 9 h in the light, and are expressed as μmol per g fw
[c] Metabolite levels were measured after 9 h in the light, and are expressed as nmol per g fw

starch and soluble sugars in the complemented line resembled those of the wild type (Table 2). On regaining the ability to synthesise starch, free sugar levels fell to values similar to those in the wild type.

Effects on phosphorylated metabolic intermediates and on photosynthesis

PGM catalyses the reversible conversion of Glc1P to Glc6P. A deficiency in this conversion in one compartment of the cell might have profound effects on the levels of hexose phosphates and other phosphorylated metabolic intermediates, as well as on their concentration ratios in the stroma and the cytosol. As shown in Table 2, *stf1* contained almost ten times as much Glc6P at the end of the light period as the wild type. Moreover, Glc6P accumulated significantly during the light period only in the mutant. From 4 h to 9 h in the light, the Glc6P content in the mutant increased from 240 to 420 nmol/g fresh weight (fw), whereas the Glc6P content in the wild type remained apparently constant at about 40 nmol/g fw (data not shown). Levels of Fru6P were increased only twofold in the mutant compared to the wild type and Glc1P contents remained unchanged. It ought to be stressed that the subcellular site of Glc6P accumulation has not been determined. 3-PGA levels (most probably in the stroma) fell by 30%, whereas Fru1,6P_2 and triose phosphate contents were not significantly altered in the mutant compared to the wild type.

We also measured the effect of the lack of PGMp on CO_2 assimilation, transpiration and photosynthetic electron transport, using individual rosette leaves of *stf1*. In ambient air, these parameters were not affected; however, at high CO_2 and low O_2 concentrations (i.e. under non-photorespiratory conditions), CO_2 assimilation rates decreased by 50% from 22.9 μmol per m^2/s in the wild type to 11.2 μmol per m^2/s in *stf1*. This inhibition was evident only in plants that had been kept in the light for at least 4 h before the measurements, whereas plants kept in the dark prior to the assay showed only a small decline in CO_2 assimilation rates and in the maximum rate of photosynthetic electron transport, indicating a temporal influence on the inhibition of photosynthesis in this mutant.

Discussion

From a transposon-tagged *Arabidopsis* insertion population, we have isolated a starch-free mutant, *stf1*. Since the mutation did not co-segregate with the molecular marker, it was not possible to identify and isolate the corresponding gene directly. However, *stf1* was clearly shown to be defective in the plastid isoform of PGM: (1) crosses of *stf1* to *pgm-1* mutants resulted in starch-free F1 plants; (2) *stf1* did not contain the plastid isoform of PGM; and (3) the mutant phenotype could be complemented by an EST that was shown to encode a plastid isoform of PGM.

A sequence ostensibly coding for a plastid PGM has been described by Penger et al. (1994). However, a BLAST search with this sequence revealed about 30% amino acid identity with phosphomannomutases from bacteria and, hence, this most probably represents a phosphomannomutase, as already suggested by Manjunath et al. (1998), rather than a PGM. The PGMp described in this paper displays significant homology to PGMs from plants, fungi, worms, insects and mammals. The mature part of the PGMp protein shows 54% sequence identity to the PGM from man, the corresponding cytosolic isoform from *Arabidopsis* is 58% identical. Cytosolic isoforms of PGM in plants show about 80%

amino acid identity inter se (Manjunath et al. 1998), and about 56% identity to the PGM from humans.

An analysis of an unrooted phylogenetic tree generated from an alignment of PGM protein sequences revealed that PGMp represents a new class of PGM isoenzymes that is equidistant from PGMs from mammals, bacteria, yeast and cytosolic plant PGM isoforms (Fig. 6). Obviously, the plastid isoform diverged from other PGMs relatively early in evolution and then evolved independently, because the evolutionary distances to PGMs from the different kingdoms are about equal.

The *Arabidopsis* PGMp contains many regions that are highly conserved in PGMs from all kingdoms. The T-A-S-H-N motif that was identified as the catalytic site of rat PGM (Milstein and Sanger 1961) is found at positions 179–183 of the *Arabidopsis* PGMp, except that the threonine residue is replaced by serine in PGMp. During the catalytic process, the hydroxyl group of the S-116 in the human PGM serves as the phosphate acceptor and donor (Ray and Peck 1972). This residue is most likely to correspond to S-181 of PGMp. In the crystal structure of the muscle PGM, Dai et al. (1992) described a metal binding loop that has a D-G-D-G-D motif. This motif is highly conserved in PGMs from different kingdoms and is located at residues 346–350 of the Arabidopsis PGMp. Interestingly, the second G residue is replaced by an A in plant cytosolic PGMs (Manjunath et al. 1998).

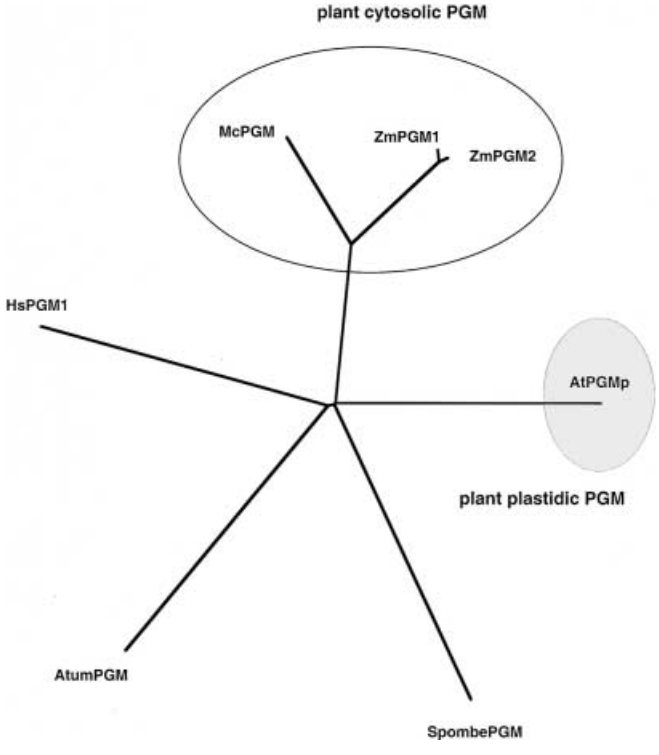

Fig. 6 Unrooted phylogenetic tree of PGMs from different species. An optimal amino acid alignment was created with the program ClustalX and a phylogenetic tree was constructed using the distance matrix-neighbor joining method. The tree was displayed with the program Treeview. Acronyms for the different species are given in the legend to Fig. 2

The physiological properties of the starch-free mutant *stf1* resemble in many respects those of the mutants described by Caspar et al. (1985), Neuhaus and Stitt (1990) and Schulze et al. (1991). Most of the differences observed in our studies may be due to differences in growth conditions and in the genetic backgrounds of the mutants. In addition, our data indicate that a lack of starch formation has an impact on CO_2 assimilation only under non-photorespiratory conditions (compare Lascève et al. 1997). This suggests that the lack of starch formation limits photosynthesis in the mutant, such that the rates of CO_2 assimilation and photosynthetic electron transport adapt to the maximum rate of sucrose synthesis.

We also determined the levels of phosphorylated intermediates in *stf1*, the wild type and the complemented *stf1* mutant. In the *stf1* mutant, Glc6P contents increased during the course of the day, resulting in a 10-fold higher Glc6P content in the mutant at the end of the photoperiod. Hence, a lack of PGMp has the most pronounced long-term effect on Glc6P contents. Neuhaus and Stitt (1990) have described similar, but less marked short-term effects on Glc6P content. During the light period, an accumulation of Glc6P results in changes in the [Glc6P]/[Glc1P] and [Fru6P]/[Glc6P] ratios which reflect the steady-state levels of hexose phosphates at the sites of PGM and PGI, respectively (the reported equilibrium constants for the reactions catalysed by PGM and PGI are 17.2 and 0.29, respectively at pH 7.0 and 25 °C; Bergmeyer 1970). Hence, only in the mutant are the reactions catalysed by PGM and PGI close to thermodynamic equilibrium (Table 2). It appears likely that the accumulation of Glc6P observed here occurs in the cytosol due to an increase in cytosolic PGM activity, and is probably facilitated by higher levels of soluble hexoses, which are substrates for hexokinase(s) and/or glucokinases.

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