Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*

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Tre6P (trehalose 6-phosphate) is implicated in sugar-signalling pathways in plants, but its exact functions *in vivo* are uncertain. One of the main obstacles to discovering these functions is the difficulty of measuring the amount of Tre6P in plant tissues. We have developed a highly specific assay, using liquid chromatography coupled to MS-Q3 (triple quadrupole MS), to measure Tre6P in the femto-picomole range. The Tre6P content of sucrose-starved *Arabidopsis thaliana* seedlings in axenic culture increased from 18 to 482 pmol · g^{-1}FW (fresh weight) after adding sucrose. Leaves from soil-grown plants contained 67 pmol · g^{-1}FW at the end of the night, which rose to 108 pmol · g^{-1}FW after 4 h of illumination. Even greater changes in Tre6P content were seen after a 6 h extension of the dark period, and in the starchless mutant, *pgm*. The intracellular concentration of Tre6P in wild-type leaves was estimated to range from 1 to 15 μM. It has recently been reported that the addition of Tre6P to isolated chloroplasts leads to redox activation of AGPase (ADPglucose pyrophosphorylase) [Kolbe, Tiessen, Schluepmann, Paul, Ulrich and Geigenberger (2005) Proc. Natl. Acad. Sci. U.S.A. **102**, 11118–11123]. Using the new assay for Tre6P, we found that rising sugar levels in plants are accompanied by increases in the level of Tre6P, redox activation of AGPase and the stimulation of starch synthesis *in vivo*. These results indicate that Tre6P acts as a signalling metabolite of sugar status in plants, and support the proposal that Tre6P mediates sucrose-induced changes in the rate of starch synthesis.

Key words: liquid chromatography-MS (LC-MS), triple quadrupole MS (MS-Q3), redox regulation, starch, sucrose, trehalose 6-phosphate (Tre6P).

INTRODUCTION

Tre6P (trehalose 6-phosphate) is the intermediate in the classical pathway of trehalose biosynthesis, via TPS (trehalose-phosphate synthase) and TPP (trehalose-phosphatase) in bacteria, fungi, invertebrates and plants [1]. In yeast (*Saccharomyces cerevisiae*), Tre6P is also an essential signalling molecule, involved in regulating the entry of glucose and fructose into glycolysis via inhibition of hexokinase [2]. With the exception of a few 'resurrection' plants that use trehalose to help them survive extreme desiccation [3], most angiosperms, including *Arabidopsis thaliana*, contain only trace amounts of trehalose. However, evidence is accumulating that Tre6P has an essential signalling function in plants. Disruption of the *TPS1* gene in *A. thaliana* was found to be embryonic lethal, with growth being arrested at the torpedo stage of embryo development [4]. Although the *tps1* mutant can be rescued during embryogenesis by the inducible expression of TPS, the resulting plants show retarded vegetative growth and are unable to flower when TPS expression is no longer induced [5]. Plants expressing *TPS* or *TPP* genes from yeast or bacteria also show complex morphological and biochemical phenotypes [6–10]. The contrasting phenotypic effects of TPS and TPP overexpression in *A. thaliana* [9] indicate that changes in the level of Tre6P, rather than trehalose, are responsible for the altered phenotypes of the plants. This is corroborated by the phenotypic similarity of plants expressing TPP or phosphotrehalase, an enzyme that cleaves Tre6P to Glc6P (glucose 6-phosphate) and glucose without producing trehalose [9].

It is clear that Tre6P plays a vital role in the life of plants, but the exact nature of this role is unknown, as the complex pleiotropic phenotypes of plants with altered TPS or TPP activity make it difficult to distinguish between primary and secondary responses. Nevertheless, several lines of evidence point to a link between Tre6P and nutrient signalling, in particular sugars, in plants. For example, in *A. thaliana*, the transcript abundance of several *TPS* and *TPP* genes responds strongly to changes in sugar levels [11–14]. The expression of some of these genes is also affected by nitrate starvation and re-supply [15]. The growth of *tps1* embryos in culture can be partially rescued by decreasing the sucrose concentration in the medium [4]. Constitutive overexpression of heterologous TPS [9] or AtTPS1 [16] overcomes the inhibition of germination by high glucose or ABA (abscisic acid) concentration, and promotes growth on a high-sucrose medium [9,14]. In contrast, transformants with constitutive overexpression of TPP show decreased growth on a high-sugar medium [9,14]. Furthermore, overexpression of AtTPS1 [16] or the addition of trehalose [14] affects the expression of genes that respond to other sugars. The *A. thaliana* genome encodes 11 TPS proteins. Several of these are targets for phosphorylation by SNF1 (sucrose non-fermenting 1)-related

Abbreviations used: ADPGlc, ADPglucose; AGPase, ADPGlc pyrophosphorylase; Fru6P, fructose 6-phosphate; FW, fresh weight; Glc1P, glucose 1-phosphate; Glc6, glucose 6-phosphate; LC, liquid chromatography; MS-Q3, triple quadrupole MS; 3PGA, 3-phosphoglycerate; Pi, inorganic orthophosphate; SNF1, sucrose non-fermenting 1; SPP, sucrose-phosphatase; SPS, sucrose-phosphate synthase; Suc6P, sucrose-6^F-phosphate; TPP, trehalose-phosphatase; TPS, trehalose-phosphate synthase; Tre6P, trehalose 6-phosphate; UDPGlc, UDPglucose.

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or calcium-dependent protein kinases and binding of 14-3-3 proteins, and these processes are influenced by sugar status [17,18]. Taken together, these observations indicate that the level of Tre6P in plants is likely to be influenced by sugar-dependent transcriptional and post-translational regulation of TPS and TPP.

Little is known about the downstream targets of Tre6P signalling in plants. Unlike some hexokinases from yeast and other fungi, HXK1 and HXK2 from *A. thaliana* were found to be insensitive to Tre6P [19]. However, Tre6P has recently been implicated in the redox regulation of AGPase (ADPglucose pyrophosphorylase), which plays a major role in the control of flux through the pathway of starch synthesis [10]. Redox regulation of AGPase involves reversible formation of an intermolecular disulphide bridge between the two small subunits of the heterotetrameric holoenzyme [20]. The reduced, monomeric form has higher affinity for its substrates, and increased sensitivity to allosteric activation by 3PGA (3-phosphoglycerate), compared with the oxidized dimeric form [21]. Monomerization is triggered by sugars in potato tubers [21,22], and by light or sugars in potato, pea and *A. thaliana* leaves [23].

Several lines of evidence suggest a role for Tre6P in the signalling pathway that mediates the sucrose-dependent redox activation of AGPase [10]. First, feeding of 100 mM trehalose, which inhibits TPP, to potato tuber discs leads to an SNRK1 (SNF1-related protein kinase-1)-dependent activation of AGPase, similar to that seen with sucrose. Secondly, AGPase activation is increased in *A. thaliana* plants with constitutive overexpression of *Escherichia coli* TPS, but is decreased in TPP-overexpressors. Finally, the addition of 100 μM Tre6P to intact pea leaf chloroplasts promotes the activation of AGPase. It was proposed that rising sucrose levels in the cytosol lead to increased synthesis of Tre6P, which either enters the plastid or binds to some factor in the chloroplast envelope to trigger redox activation of AGPase [10]. Although such a signalling pathway would provide a simple explanation for sucrose-induced activation of AGPase, as yet we have no direct evidence that changes in sugar levels lead to changes in the amount of Tre6P, or that these are accompanied by redox modulation of AGPase.

One of the greatest obstacles to discovering the exact role(s) of Tre6P in plants is the lack of a reliable Tre6P assay that is sensitive and specific enough to measure the amount of this metabolite in plant tissues. None of the currently available methods for assaying the level of Tre6P is really sensitive enough to reliably measure the small amounts of Tre6P in plant cells, which appear to be several orders of magnitude lower than in yeast cells [9,14,16,24,25]. The available methods are also prone to interference by other metabolites in plant extracts, and need tedious sample preparation, limiting the number of samples that can be analysed. In the present paper we describe a simple extraction procedure for Tre6P, and the development and validation of a highly specific assay for this metabolite using LC (liquid chromatography) coupled to MS-Q3 (triple quadrupole MS). The new assay is up to 100-fold more sensitive than previously used methods, and allowed us to investigate how the level of Tre6P in *A. thaliana* responds to alterations in the sugar status of the plants, and to directly compare the levels of Tre6P with changes in the post-translational redox activation of AGPase.

EXPERIMENTAL

Materials

Tre6P and other biochemicals were obtained from Sigma–Aldrich Chemie GmbH, (Taufkirchen, Germany). Enzymes were from Roche Diagnostics GmbH (Mannheim, Germany) unless indicated otherwise. Deuterated Tre6P ([6,6-^{2}H]Tre6P) was synthesized enzymatically from [6,6-^{2}H]D-glucose. The reaction contained: 785 nmol of [6,6-^{2}H]D-glucose, 1.2 μmol of ATP, 1.2 μmol of UDPGlc (UDP glucose), 10 units of hexokinase and 5 units of TPS (from *E. coli*), in 25 mM Hepes-Na^+, 5 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.2), in a final volume of 100 μl. After incubation at 30 °C for 3 h, the reaction was stopped by heating at 99 °C for 2 min, and the precipitated proteins were removed by centrifugation at 18 000 ***g*** for 2 min. The supernatant contained 8 nmol of unreacted [6,6-^{2}H]D-glucose, 32 nmol of [6,6-^{2}H]Glc6P and 732 nmol of [6,6-^{2}H]Tre6P.

Plant growth and harvest

A. thaliana (L.) Heynh. ecotype Col-0 and the *pgm* mutant [26] were grown in soil (6 cm pots), with a 12 h photoperiod (irradiance of 130 μmol of photons $m^{-2} \cdot s^{-1}$) at 20 °C. *A. thaliana* seedlings were also grown in sterile liquid culture for 7 days, transferred to medium without sucrose for 2 days, and resupplied with sucrose (15 mM final concentration) as described in [27]. Plant material was harvested under ambient illumination, immediately frozen in liquid nitrogen and stored at − 80 °C until further use.

Cloning, overexpression and purification of enzymes

The *otsA* and *otsB* genes, encoding TPS (E.C. 2.4.1.15) and TPP (3.1.3.12) respectively, were amplified from *E. coli* genomic DNA by PCR and cloned between the NcoI and EcoRI sites of expression plasmid pETM11 (Günter Stier, EMBL, Heidelberg, Germany). Expression plasmid pSG2, containing the *Bacillus subtilis treA* gene, encoding α,α-phosphotrehalase (E.C. 3.2.1.93) [28], was obtained from Dr Michael Dahl (Department of Biology, University of Konstanz, Germany).

TPS and TPP were expressed as N-terminal His_6-tagged fusion proteins in *E. coli* strain Rosetta™ (Novagen). Protein expression was induced at A_{600} 0.5 using 0.5 mM isopropyl β-D-thiogalactoside. The induced cells were incubated at 20 °C for 16 h, harvested by centrifugation at 4000 ***g*** (4 °C) for 10 min, and extracted by single passage through an EmulsiFlex®-C3 high pressure homogenizer (Avestin Inc., Ottawa, Canada) at 110–120 MPa peak pressure. The cell lysate was centrifuged at 20 000 ***g*** (4 °C) for 10 min, and the supernatant comprised the soluble extract. All subsequent procedures were carried out at 4 °C.

TPS was extracted in buffer A [50 mM Hepes-Na^+/300 mM NaCl (pH 8.0)] containing 1 mM PMSF, and purified by immobilized metal affinity chromatography on Talon™ Co^{2+} resin (Clontech Laboratories, Inc., Mountain View, CA, U.S.A.) according to the manufacturer's instructions. The enzyme was further purified by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Biosciences Europe GmbH, Freiburg, Germany), equilibrated with buffer B [50 mM Hepes-Na^+, 150 mM NaCl, 5 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.5)]. The flow rate was 2 ml $\cdot$ min^{-1}, and 2 ml fractions were collected. TPS peak fractions were pooled, concentrated to a volume of approx. 100 μl using a Centricon Plus-20 (30 kDa cutoff) centrifugal concentrator (Millipore, Billerica, MA, U.S.A.) and stored at − 80 °C. TPS activity was assayed as described in [29] for SPS (sucrose-phosphate synthase), except that Fru6P (fructose 6-phosphate) was replaced with 10 mM Glc6P. One unit of activity represents 1 μmol of UDP produced per min.

TPP was extracted and purified as described above for TPS. Superdex 200-fractions containing TPP were pooled, diluted 5-fold with buffer C [50 mM Hepes-Na^+, 5 mM $MgCl_2$ and

0.5 mM EDTA (pH 7.5)] and applied to an HR 5/5 MonoQ® column (Amersham Biosciences), equilibrated with buffer D, at a flow rate of 1 ml · min^{-1}. TPP was eluted with a linear gradient of 0–0.5 M NaCl (30 ml) in buffer C and 0.5 ml fractions were collected. Samples (1 μl) were analysed by SDS/PAGE on 12 % (w/v) polyacrylamide gels, and fractions containing a single Coomassie Blue stained protein band (32 kDa) were pooled, concentrated to a volume of approx. 100 μl using a Centricon Plus-20 (10 kDa cutoff) centrifugal concentrator, and stored at − 80 °C. TPP activity was assayed colorimetrically by measuring Pi production as described in [30]. One unit of activity represents 1 μmol of Pi (inorganic orthophosphate) produced per min.

Phosphotrehalase was overexpressed in *E. coli* strain DH5α and purified as described in [28]. The α-glucosidase activity of the enzyme was assayed with *p*-nitrophenyl-α-D-glucopyranoside as the substrate [28] during the purification. The purified enzyme was stored as a suspension in 80 % saturated $(NH_4)_2SO_4$ at 4 °C. The *Synechocystis* sp. PCC 6803 SPP (sucrose-phosphatase) was expressed in *E. coli* and purified as described in [31].

Assay of Tre6P standards

Stock solutions of Tre6P were prepared in ultra-pure water (Elga Labwater, Celle, Germany) from unopened bottles of the dipotassium salt of Tre6P (approx. 95 % pure, containing 0.2 % methanol). The nominal concentration was calculated from the amount of weighed Tre6P, without correction for the indicated purity or solvent content. The actual concentration of Tre6P was determined spectrophotometrically using phosphotrehalase. The reaction contained: 100 mM Tricine-Na$^+$, pH 8.0, 5 mM $MgCl_2$, 0.25 mM $NADP^+$, 0.2 unit of glucose-6-phosphate dehydrogenase and 0–10 nmol of Tre6P, in a final volume of 1 ml, and was started by the addition of 0.0025 unit of phosphotrehalase (dissolved in 100 mM Tricine-Na$^+$, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.0). Glc6P production was calculated from the increase in absorbance at 334 nm ($\varepsilon = 6180\ M^{-1} \cdot cm^{-1}$), measured using a ZFP 22 dual wavelength photometer (Sigma Instruments GmbH, Berlin, Germany).

The concentration of Tre6P in standard solutions was also assayed by measuring the release of Pi after complete hydrolysis by TPP. The reaction contained: 25 mM Hepes-Na$^+$, pH 7.2, 5 mM $MgCl_2$, 0.5 mM EDTA, 0–10 nmol Tre6P and 0.15 unit of TPP in a final volume of 100 μl. After incubation at 30 °C for 10 min the amount of Pi released was assayed as described in [30].

Extraction of Tre6P

Tre6P was extracted from plant tissues by a modification of the method described in [32]. Frozen plant material was ground to a fine powder at liquid nitrogen temperature using a ball mill (Retsch Technology, Haan, Germany). Aliquots [12–13 mg FW (fresh weight)] of the frozen powder were transferred to pre-cooled, 2 ml Safe-Lock microcentrifuge tubes (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) and quenched by adding 250 μl of ice-cold $CHCl_3/CH_3OH$ (3:7, v/v). The frozen mixture was warmed to − 20 °C with vigorous shaking, and incubated at − 20 °C for 2 h with occasional mixing. Water soluble components, including Tre6P, were extracted from the $CHCl_3$ phase by adding 200 μl of water and warming to 4 °C with repeated shaking. After centrifugation at 420 ***g*** for 4 min, the upper, aqueous-CH_3OH phase was transferred to a new tube, and kept at 4 °C. The lower, $CHCl_3$ phase was re-extracted with 200 μl of cold water, centrifuged as described above, and the second aqueous-CH_3OH extract was added to the first. The combined aqueous-CH_3OH extract was evaporated to dryness using a centrifugal vacuum dryer at 20 °C and redissolved in 250 μl of water. Viscous, high molecular-mass components were removed from the samples by applying individual extracts to separate wells on a Multiscreen Ultracel-10 (Millipore) filter, and centrifugation at 2300 ***g*** for 2–3 h, 20 °C. If this step was omitted, recovery of Tre6P was decreased by approx. 30 %. Recovery experiments were carried out by the addition of known amounts of authenticated Tre6P standards to the frozen tissue slurry immediately after addition of the cold $CHCl_3/CH_3OH$.

Assay of Tre6P and Suc6P (sucrose-6^F-phosphate) by LC/MS-Q3

Tre6P and Suc6P were assayed in plant extracts by LC using a Dionex HPLC system (Sunnyvale, CA, U.S.A.), coupled to a Finnigan TSQ Quantum MS-Q3 apparatus (ThermoFinnigan, Waltham, MA, U.S.A.). Aliquots (100 μl) of the extract were passed through a 2 × 50 mm AG11-HC pre-column (Dionex), before separation of anionic compounds on a 2 × 250 mm IonPac AS11-HC column (Dionex) at 25 °C. The column was equilibrated with a mixture of 95 % solution A [5 % (v/v) methanol] and 5 % solution B [100 mM NaOH in 5 % (v/v) methanol]for 15 min before each sample run. Solutions were gassed with helium. Anionic compounds were eluted with a multi-step gradient as follows: 0–5 min, 5 % B; 5–25 min, 5–29 % B; 25–26 min, 29–100 % B; 26–31 min, 100 % B; 31–32 min, 5 % B, at a flow rate of 0.2 ml · min^{-1}. Peak detection in the eluate from the Dionex system was made with suppressed conductivity (ASRS-Ultra, 2 mm, 50 mA, external water mode), after which the eluate entered directly into the MS-Q3, which was operated in a multiple reaction monitoring mode, with an electrospray ionization source in negative ionization mode, and centroid data acquisition [33]. Nitrogen was used as a sheath and auxiliary gas, and the Q2 (quadrupole 2) collision gas (argon) was set to 1.1 mTorr. The capillary voltage ranged from − 1 to − 56 V, the capillary temperature was 320 °C and the scan time was 0.2 ms. The Q1 and Q3 peak widths were 0.5 and 0.7 *m*/*z* respectively. Tre6P and Suc6P were selected using a parent ion of 421.1 *m*/*z* in the first quadrupole, a collision energy of 46 eV, and a product ion of 79 *m*/*z* [33] in the third quadrupole. Metabolites were quantified by comparison of the integrated MS-Q3 signal peak area with a calibration curve obtained using authentic standards. The standard addition method [34] was used to correct for matrix effects in the LC/MS-Q3 analysis.

Assay of other metabolites

Glucose, fructose, sucrose, starch, UDPGlc, hexose phosphates, 3PGA and ATP were extracted and measured as described in [35]. ADPGlc (ADPglucose) was assayed by LC/MS-Q3 in the same $CHCl_3/CH_3OH$ extracts as Tre6P and Suc6P. For ADPGlc measurements the parent and product ions selected were 588.5 *m*/*z* and 346.1 *m*/*z* respectively, and the collision energy was 28 eV.

Quantification of AGPase redox status

The redox status of AGPase was assessed by monitoring the shift in electrophoretic mobility of the small subunit protein under non-reducing conditions, as described in [23]. The protein was visualized by immunoblotting and chemiluminescent detection with CDP-Star® reagent (New England Biolabs, Ipswich, MA) and BioMax XAR film (Eastman Kodak Company, Rochester, NY, U.S.A.). Exposed films were digitally scanned, and signal

intensity was quantified using Tina 2.10i software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

RESULTS

Assay of Tre6P using anion-exchange LC linked to MS-Q3

Three techniques have been used previously to measure Tre6P in plants: a yeast (*Yarrowia lipolytica*) hexokinase inhibition assay [9], HPLC [16], and enzymatic dephosphorylation and hydrolysis of Tre6P followed by determination of glucose content [14]. All three methods involve lengthy sample preparation and suffer from other drawbacks. The first method requires chemical destruction of other metabolites in the plant extracts, and the treatment of reference samples with alkaline phosphatase before the inhibition assay, and is susceptible to over-estimation of the amount of Tre6P if the plant extracts contain other phosphatase-sensitive inhibitors of the yeast hexokinase. The HPLC method also requires sample pretreatment with an alkali, and is susceptible to over-estimation of Tre6P if other compounds in the extract co-elute with the Tre6P. For the enzymatic assay, a preliminary ion-exchange chromatography step is needed to remove sugars, followed by successive incubation with alkaline phosphatase, glucose oxidase and trehalase before the final measurement of glucose content. This approach is highly dependent on the purity and specificity of the enzymes, and susceptible to over-estimation of the level of Tre6P if any of the preceding treatments to remove glucose and trehalose are not 100 % effective.

In preliminary experiments to test the hexokinase inhibition and enzymatic assays, as well as an HPLC approach linked to detection by MS-Q1 (single quadrupole MS), we were able to detect Tre6P standards at comparable sensitivities to those reported previously for these methods. However, when we applied these methods to extracts from *A. thaliana*, the Tre6P signal was barely distinguishable from background, especially for darkened or carbon-starved material (results not shown). We therefore decided to develop a more sensitive assay with stringent specificity for Tre6P. The approach we took was to couple LC to detection with MS-Q3, providing three sequential filters to separate, identify and quantify different compounds. The anion-exchange LC step removes all neutral and basic components in the extract, and separates the acidic components (phosphorylated intermediates, nucleotides and organic acids) according to their charge and molecular mass. The eluate from the LC is fed on-line to the MS-Q3 apparatus, which provides exquisite specificity and allows detection of metabolites that are present even in trace amounts.

Figure 1 shows the raw data from a typical measurement of an extract from *A. thaliana* seedlings grown in sterile liquid culture. To detect Tre6P, parent ions with a mass/charge ratio corresponding to a disaccharide-monophosphate (421.1 *m*/*z*) were selected in the first quadrupole, fragmented in the second quadrupole, and the product ion (phosphate, 79 *m*/*z*) was detected in the third quadrupole. These values were optimized using an authentic Tre6P standard, which eluted from the LC with a retention time of approx. 21 min (Figure 1A). A distinct signal was obtained at this position when an extract from 4.5 mg of plant material was injected into the LC column (Figure 1B). The area of this peak doubled when an extract from 9 mg of plant material was injected (results not shown). The signal increased in a linear fashion upon the addition of authentic Tre6P to plant extracts in the range of 0 to 40 pmol of Tre6P (results not shown). The only other disaccharide-monophosphate known to be widely present in plants is Suc6P, the intermediate of sucrose biosynthesis. Co-injection of authentic Suc6P showed that this metabolite is clearly resolved from Tre6P during the LC step, with a retention time of approx. 23 min, although it shares the same parent ion and fragment ion masses as Tre6P (Figure 1A). Peaks corresponding to Suc6P were clearly detectable in plant extracts. Their identity was confirmed by pre-incubation of the extract with excess SPP, which led to specific loss of the Suc6P signal but did not affect the Tre6P signal (Figure 1C). The identity of the Tre6P peak was confirmed by similar pre-incubation of plant extracts with a highly purified preparation of TPP, which led to specific loss of the signal attributed to Tre6P (Figure 1D). The TPP preparation was shown to be highly specific for Tre6P, having little or no ($\leqslant 1$ %) hydrolytic activity with Suc6P, Glc6P, Glc1P (glucose 1-phosphate) or Fru6P as substrates.

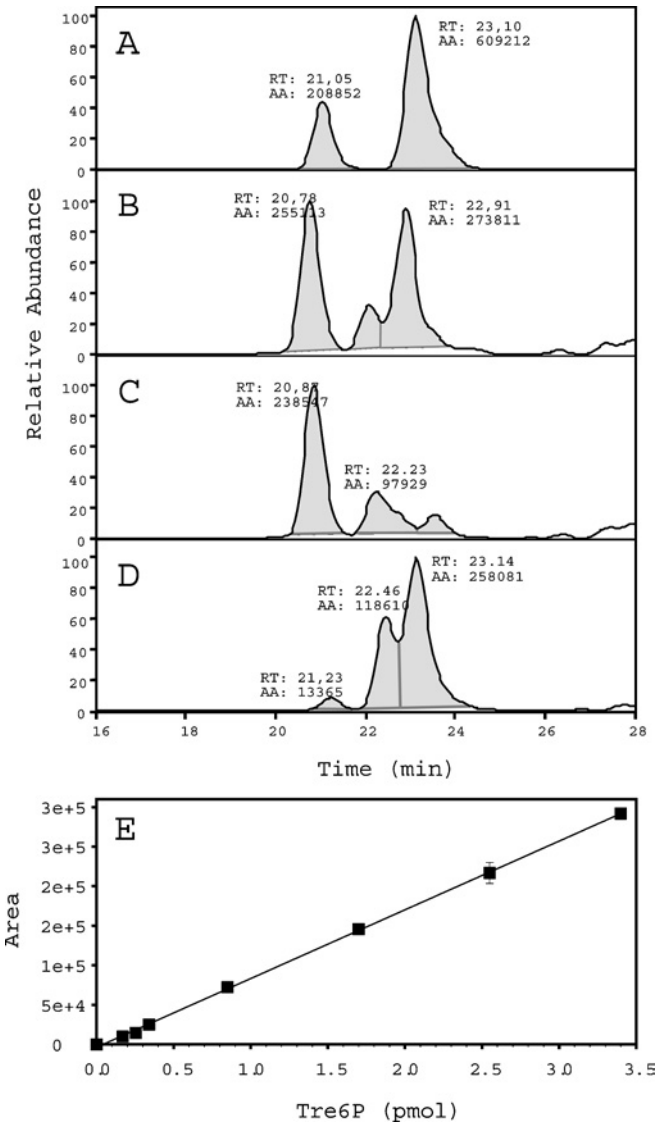

Figure 1 Tre6P assay using liquid chromatography coupled to LC/MS-Q3

(**A**) Standards containing 3.4 pmol of Tre6P and 10 pmol of Suc6P. (**B**)–(**D**) Aqueous methanol extract (100 μl) from seedlings grown in liquid culture with full nutrition. (**B**) untreated; (**C**) pre-incubated with 0.25 unit of SPP; (**D**) pre-incubated with 0.37 unit of TPP. (**E**) Calibration curve for Tre6P, values are the means $\pm$ S.D., $n = 3$. RT, retention time; AA, area.

Tre6P was quantified by comparison with authentic standard-solutions, the concentrations of which were verified by two independent enzymatic tests using either phosphotrehalase or TPP (see Experimental section). Solutions with a nominal Tre6P concentration of 1 mM were prepared from three separate batches

of Tre6P. The actual concentrations of Tre6P measured with phosphotrehalase or TPP (in parentheses) were: 0.65 mM (0.66 mM), 0.68 mM (not determined) and 0.77 mM (0.82 mM). All measurements of Tre6P in plant extracts were calibrated with standard solutions, the concentration of which had been independently verified in this way. A typical calibration curve from the LC/MS-Q3 data is shown in Figure 1(E), demonstrating the linear response ranging from 0.18 to 3.4 pmol of Tre6P.

Recovery experiments were carried out to check that Tre6P was quantitatively extracted from the plant tissue, and to test for matrix effects that might interfere with detection. Separate aliquots of the same plant material were extracted in parallel, with or without the addition of known amounts of Tre6P to the frozen plant material prior to extraction. As described in more detail below, we found endogenous levels of Tre6P in *A. thaliana* tissues ranging from 20 to 500 pmol $\cdot$ g^{-1}FW. We took samples from the low end of this range (approx. 106 pmol $\cdot$ g^{-1}FW, equivalent to 1.1 pmol per 100 μl of extract) and added 1.2, 2.4 or 6.0 pmol of authenticated Tre6P standard to the samples (22–30 mg) of the frozen plant material (containing 2.3–3.2 pmol of Tre6P) before extraction. Recoveries of the added 1.2, 2.4 and 6.0 pmol of Tre6P were 85 $\pm$ 20 %, 82 $\pm$ 11 % and 86 $\pm$ 11 % (means $\pm$ S.D., $n = 4$) respectively, demonstrating that even the smallest amounts of Tre6P were quantitatively recovered.

For routine measurements, another way to assess recovery is to spike samples with isotopically labelled Tre6P. For this purpose, Tre6P containing two deuterium atoms on C_6 of the phosphorylated glucose moiety {[6,6-^{2}H]Tre6P} was synthesized enzymatically, by incubation of [6,6-^{2}H]D-glucose with a slight stoichiometric excess of ATP and UDPGlc, plus hexokinase and TPS. The reaction resulted in a greater than 93 % conversion of glucose to Tre6P. The deuterated Tre6P was shown to have the same retention time in the LC step as unlabelled Tre6P, but the parent ion was 2 Da heavier, as expected. Recovery experiments using the deuterated Tre6P gave comparable results to those described previously (results not shown).

Changes in Tre6P level after addition of sucrose to sugar-starved *A. thaliana* seedlings

The development and validation of the LC/MS-Q3 assay for Tre6P allowed us to investigate how the level of Tre6P in *A. thaliana* responds to sugar starvation and sucrose resupply. Seedlings were grown in axenic liquid culture with 15 mM sucrose under continuous low-light [11,27], transferred to medium without sugar for 2 days, and then re-supplied with 15 mM sucrose. Sugars and starch levels decreased 5- to 8-fold in starved seedlings, compared with control seedlings maintained with full nutrition throughout the experiment (Figure 2). Sucrose re-addition to the medium led to a rapid 3-fold increase in the sucrose content of the seedlings (within 12 min), and a 6-fold increase within 30 min (Figure 2). Sucrose content peaked at 3 h, and subsequently declined (Figure 2). The level of hexose sugars (glucose and fructose) showed a marked rise from 75 min onwards (Figure 2). Starch also accumulated from 75 min after sucrose re-addition, and accounted for more than 60 % of the carbohydrate present in the seedlings by 8 h (Figure 2).

Tre6P content was 6-fold lower in carbon-starved seedlings (18 pmol $\cdot$ g^{-1}FW) than in control seedlings with full nutrition (121 pmol $\cdot$ g^{-1}FW) (Figure 3). Re-addition of sucrose led to a dramatic increase in the level of Tre6P within 30 min, which peaked at 3 h (482 pmol $\cdot$ g^{-1}FW), when the level was over 26-fold higher than before sucrose re-addition, and substantially higher than that in full nutrition status. Controls that received 15 mM

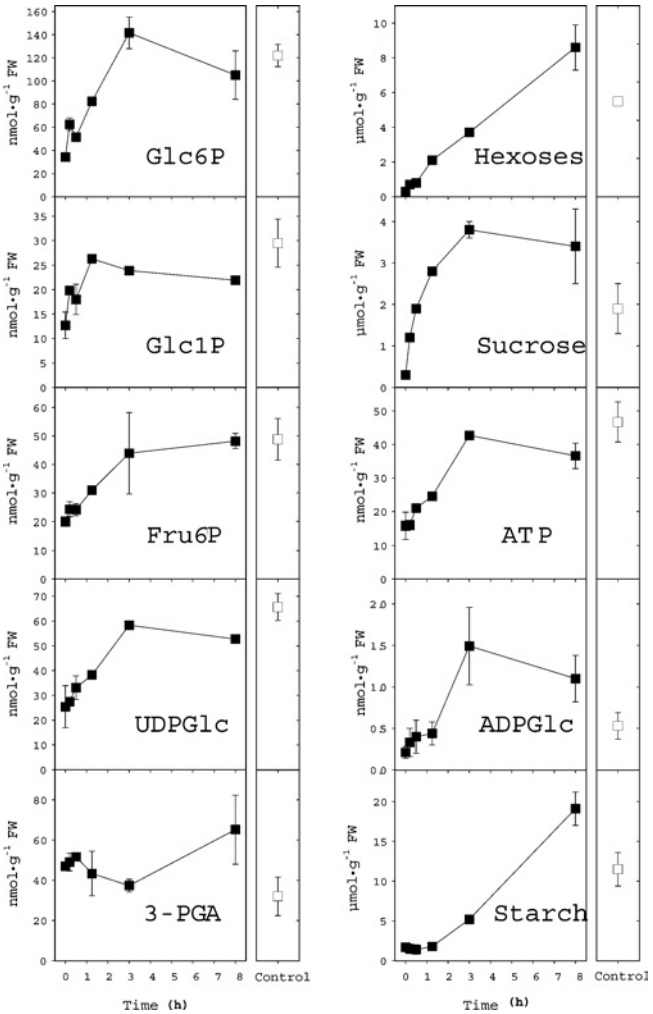

Figure 2 Effects of carbon starvation and sucrose resupply on glycolytic intermediates, ATP, sugars and starch in *A. thaliana* seedlings

Seedlings were grown axenically for 7 days under continuous light in sucrose-containing liquid medium, then starved for 2 days in sucrose-free medium. At time 0 h, sucrose (15 mM final concentration) was added to the medium, and seedlings were harvested at 0.2, 0.5, 1.25, 3 and 8 h after sucrose resupply. Control samples were grown with full nutrition for 9 days, and harvested at the time of sucrose re-addition to the starved samples. Values are the means $\pm$ S.D., $n = 2$ ($n = 4$ for ADPGlc).

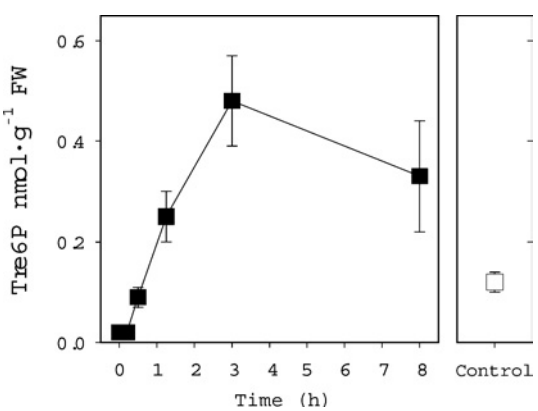

Figure 3 Sucrose starvation and resupply affect Tre6P levels in *A. thaliana* seedlings

For experimental details see legend to Figure 2. Values are the means $\pm$ S.D., $n = 3$–4.

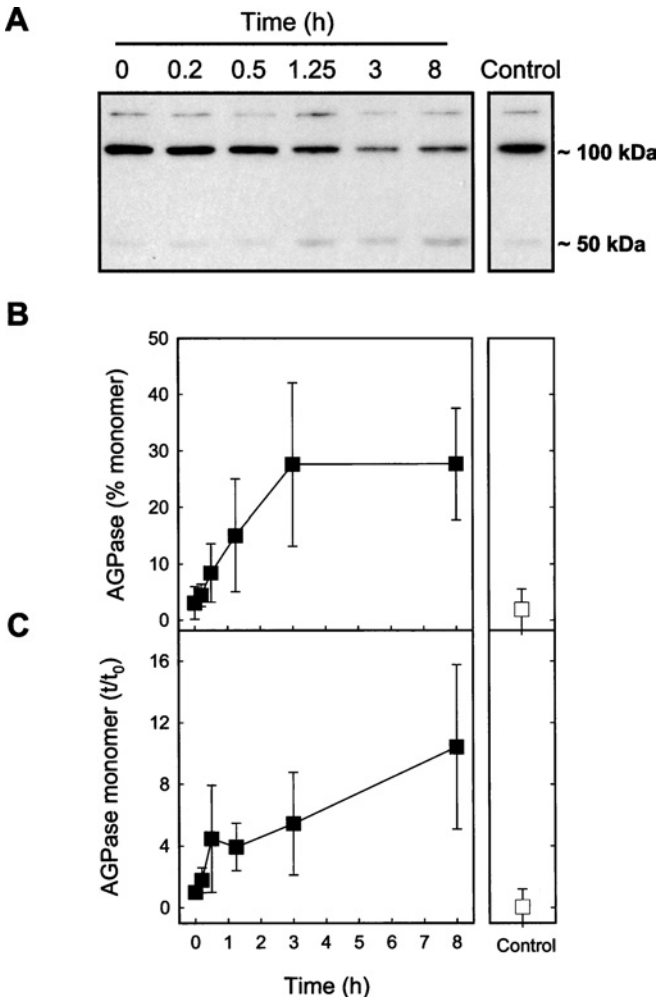

Figure 4 Sucrose starvation and resupply affect redox regulation of AGPase in *A. thaliana* seedlings

Seedlings were starved and then resupplied with sucrose as described in the legend to Figure 2. (**A**) Representative immunoblots showing the oxidized AGPase small subunit dimer (100 kDa) and reduced monomer (50 kDa), separated by non-reducing SDS/PAGE. (**B**) The degree of activation of AGPase was assessed by quantifying the signal intensity of the 50 and 100 kDa bands to determine the proportion of total AGPase in the monomeric form. (**C**) The amounts of active monomer, normalized to the zero-time value on the same immunoblot, are also shown. Values are the means ± S.D., $n = 3$–4.

mannitol instead of sucrose contained similar levels of Tre6P compared with carbon-starved seedlings (results not shown).

Glc6P, Glc1P, Fru6P, UDPGlc and ATP levels were 2- to 3-fold lower in carbon-starved seedlings than in controls, whereas the level of 3PGA was slightly higher (Figure 2). After sucrose re-addition, the level of hexose-phosphates showed a marked increase, and UDPGlc and ATP a small increase, in the first 12–30 min. There was a further and marked increase in the level of all of these metabolites between 75 min and 3 h, reaching levels close to those found in control seedlings with full nutrition (Figure 2). The level of 3PGA showed a slight decrease between 75 min and 3 h, but then increased between 3h and 8 h (Figure 2).

The redox activation state of AGPase (Figure 4) was monitored in the same samples, using non-reducing SDS/PAGE and immunoblotting to measure the amount of the AGPase small subunit protein that was present in a reduced monomeric (50 kDa) form [23]. This approach is preferred over activity measurements to assess the activation state of AGPase, because there are compounds in plant extracts that rapidly oxidize and inactivate the enzyme [23]. Representative immunoblots are shown in Figure 4(A), along with values for the proportion (Figure 4B) and absolute amount (Figure 4C) of the enzyme in the monomeric form at each time point, from three biological replicates. The proportion of AGPase in the monomeric form (Figure 4B) showed a similar time course to the changes in Tre6P (Figure 3), rising steadily during the first 3 h after sucrose re-addition, although it remained fairly constant thereafter, rather than declining slightly as observed for Tre6P. By 75 min there was substantial activation of AGPase. This matched the onset of marked increases in the level of ADPGlc and the rate of starch accumulation (Figure 2). The absolute amount of AGPase in the monomeric form also rose during the first 3 h after sucrose re-addition, but showed a further increase between 3 h and 8 h after sucrose was resupplied (Figure 4C). It should be noted that this measure of AGPase activation is likely to be less accurate than measuring the proportion of enzyme in the monomeric form, because it is more sensitive to technical variation in signal strength between different blots. The apparent differences between the proportion and absolute amounts of the AGPase monomer (Figures 4B and 4C) are explained by the decrease in the total amount of AGPase protein as the enzyme was activated. A similar tendency has been observed in previous studies [23,36], and indicates that there are changes in AGPase expression and/or turnover during this time period, in addition to changes in redox activation.

Changes in Tre6P levels during dark–light transitions in wild-type *As thaliana* Col-0 and the starchless *pgm* mutant

A second set of experiments was carried out to investigate whether endogenous changes in sugar levels during light–dark transitions are accompanied by changes in Tre6P, the activation state of AGPase and the rate of starch synthesis. Experiments were carried out using wild-type plants under normal conditions or after a prolonged dark treatment, and with the starchless *pgm* mutant. In the latter two systems there are even larger changes in the level of sugars than under normal conditions in wild-type plants.

Wild-type Col-0 plants growing in a standard 12 h/12 h light–dark diurnal cycle retain a substantial level of sugars and glycolytic intermediates in their leaves at the end of the night (Table 1). When they are re-illuminated there is a small, but marked increase in sucrose and reducing sugars, and starch begins to accumulate. When wild-type plants are subjected to an extension of the night, sugars and glycolytic intermediates are depleted to very low levels by the end of the dark period.

Re-illumination after an extended night leads to a larger increase in the sugars, and a stronger stimulation of starch synthesis than normal (Table 1) [36]. An even more extreme situation occurs in the *pgm* mutant, which is unable to synthesize starch because it lacks plastidial phosphoglucomutase [26]. The level of sugars in the *pgm* mutant is very low at the end of the night, but increases dramatically after illumination (Table 1), as reported previously [26,36]. In the *pgm* mutant, or after an extension of the night in wild-type plants, the rapid accumulation of sugars in the presence of light occurs because the preceding period of low carbon conditions leads to a temporary inhibition of carbohydrate utilization when the plants are re-illuminated [36].

Figure 5(A) shows the accompanying changes in the amount of Tre6P. In wild-type Col-0, Tre6P is present at low levels at the end of the night, and increases 2-fold during the first 4 h of the light period. Extension of the night for 6 h leads to a 3-fold decrease in Tre6P. When the plants are re-illuminated for 4 h there is a 12-fold increase in Tre6P, which reaches levels that are 2-fold higher than after illumination at the end of the normal dark period.

Table 1 Effects of light and extended darkness on sugars, starch and Glc6P levels in *Arabidopsis thaliana* rosette leaves

Arabidopsis thaliana ecotype Col-0 and starchless *pgm* mutant plants were grown in soil with a 12 h/12 h light–dark cycle for 5 weeks. Rosette leaves were harvested at the end of the dark period (EN) and after 4 h of illumination for measurement of carbohydrates and Glc6P. Wild-type (WT) plants were also subjected to a single 6 h extension of the night before re-illumination, and rosette leaves were harvested at the end of the extended night (EN) and 4 h after re-illumination. Values are the means ± S.D., $n = 4$–5 independent biological samples, each containing leaves from 3 plants. hex. eq., hexose equivalents.

	Leaf content [μmol of (hex. eq.) $\cdot$ g^{-1}FW]					
	Col-0		Col-0 extended night		*pgm*	
Compound	EN	4 h light	EN	4 h light	EN	4 h light
Sucrose	2.1 ± 0.2	3.5 ± 0.7	1.0 ± 0.2	4.8 ± 0.5	1.4 ± 0.2	9.0 ± 0.8
Glucose	0.1 ± 0.1	1.0 ± 0.2	0.1 ± 0.2	2.2 ± 0.3	0.3 ± 0.1	5.2 ± 1.3
Fructose	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	1.4 ± 0.2	0.5 ± 0.1	3.8 ± 0.6
Starch	3.1 ± 0.5	16 ± 1	0.5 ± 0.1	25 ± 2	0.1 ± 0.1	0.0 ± 0.1
Glc6P	0.081 ± 0.025	0.186 ± 0.010	0.075 ± 0.011	0.242 ± 0.004	0.094 ± 0.018	0.474 ± 0.069

In the starchless *pgm* mutant, Tre6P content is very low at the end of the night, and increases 20-fold after 4 h illumination, reaching levels that are more than 3-fold higher than in wild-type plants in the light (Figure 5A).

In wild-type Col-0, AGPase is almost completely inactivated in the dark (Figures 5B and 5C), and is only moderately activated after 4 h of illumination. Re-illumination after a 6 h extension of the normal night leads to a much greater activation of AGPase. An even more pronounced effect is observed in the *pgm* mutant, where AGPase is almost fully activated in the light. These changes in the activation state of AGPase show a strikingly similar pattern to those observed for the levels of Tre6P (Figures 5A–5C), and are consistent with differences in the accumulation of starch in the wild-type plants (Figure 5D).

DISCUSSION

Measurement of Tre6P content by LC/MS-Q3 compared with other methods

We have developed a highly sensitive and specific assay for the measurement of the amount of Tre6P in plant extracts, based on the separation of anionic compounds by LC coupled to detection with LC/MS-Q3. Using this method we can reliably measure Tre6P in the femto-picomole range, which allows for measurements from as little as 5 mg of plant tissue. The reliability of the extraction and analysis procedures was confirmed by recovery experiments, which showed that > 80 % of Tre6P added to plant tissues before extraction was recovered and measured in the final extract. The specificity of the assay was clearly demonstrated by incubation of plant extracts with TPP and SPP to selectively remove Tre6P and Suc6P respectively (Figures 1C and 1D). Although we have only reported LC/MS-Q3 data for Tre6P, Suc6P and ADPGlc, this procedure has wider application and can be used to measure many other anionic metabolites simultaneously.

With a detection limit in the femtomole range, the LC/MS-Q3 method is much more sensitive (>100-fold) than the hexokinase inhibition and enzymatic assay methods, which have a detection limit of between 50–100 pmol of Tre6P [9,14]. The relatively simple sample preparation is an added advantage, and allows higher throughput. The LC/MS-Q3 method is also much less susceptible to interference from other compounds in the plant extracts. The HPLC method described in [16] avoids some of these problems, but still involves pre-treatment of samples with a strong alkali, and is less sensitive than our LC/MS-Q3 method, requiring 20 times more plant tissue (100 mg) for reliable measurements.

In wild-type *A. thaliana* Col-0 rosette leaves, we found Tre6P levels ranging from 23 to 298 pmol $\cdot$ g^{-1}FW, with values of up to 380 pmol $\cdot$ g^{-1}FW in the *pgm* mutant. Previously reported values for plants of a similar age grown in soil (16 h light/8 h dark) were much higher – up to 12 nmol $\cdot$ g^{-1}FW [9]. These values were obtained using the hexokinase inhibition assay, and one possible explanation for this discrepancy is that inhibition of the hexokinase by other compounds in the plant extract gave rise to an overestimation of the level of Tre6P. Another possibility is that plants developing under long day-conditions have higher levels of Tre6P than our plants, which were grown with a 12 h photoperiod. In 9-day-old seedlings grown in sterile liquid culture, we found levels of Tre6P ranging from 18 pmol $\cdot$ g^{-1}FW in sugar-starved plants to 482 pmol $\cdot$ g^{-1}FW after sucrose re-addition. These levels are also lower than previously reported values from 7-day-old seedlings grown in liquid culture (2–11 nmol $\cdot$ g^{-1}FW [14]), or in 7-day-old seedlings grown on solid media (1.8 nmol $\cdot$ g^{-1}FW [16]). The former values were obtained using the enzymatic assay, and the latter by HPLC. All of these previously reported values would have been calibrated with Tre6P standards, but none of the reports describe verification of the Tre6P standards, as we did using the two independent enzymatic assays. Our data suggest that the standards used for these other studies were likely to contain less Tre6P than expected, and this could have led to significant overestimation of the amount of Tre6P in the plant extracts. At present, we are unable to fully account for the significantly higher levels of Tre6P reported in these previous studies. However, it is worth noting that none of the previous studies reported use of TPP to specifically remove the Tre6P from plant extracts to check the specificity of the assay. We believe that this is an essential test for the reliability of any measurement of Tre6P in plant extracts.

In spinach leaves, mesophyll cells occupy 58 % of the total leaf volume and the cytosol comprises 3.4 % of the total volume of mesophyll cells, or approx. 2 % of total leaf volume [37]. If we assume that most of the Tre6P in *A. thaliana* leaves is present in the cytosol of mesophyll cells, and that the leaves of this species have a similar cellular composition to spinach, we estimate that the intracellular concentration of Tre6P ranges from approx. 1 to 15 μM in wild-type *A. thaliana* Col-0 leaves. These estimates are somewhat lower than the lowest concentration (100 μM) of Tre6P supplied to isolated pea leaf chloroplasts [10]; therefore, it will be important to repeat the isolated chloroplast experiments with lower concentrations of Tre6P, to see if they also lead to redox activation of AGPase.

Changes in sugar levels lead to large and rapid changes in Tre6P content

Two complementary approaches were used to alter the levels of sugars in *A. thaliana* depletion and re-addition of exogenous

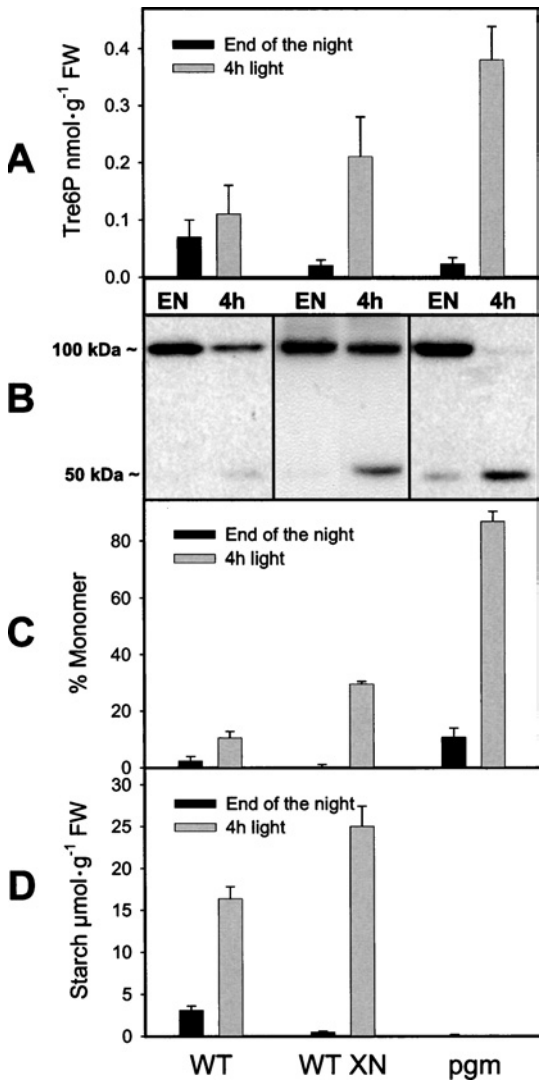

Figure 5 Effects of light and extended darkness on Tre6P and redox regulation of AGPase in rosette leaves from *A. thaliana* Col-0 (WT) and the starchless *pgm* mutant

WT and *pgm* plants were grown for 5 weeks in soil with a 12 h/12 h light–dark cycle, and rosette leaves were harvested at the end of the night (EN) and after 4 h of illumination. WT plants were also subjected to a single 6 h extension of the night (WT XN), and leaves were harvested at the end of this extension and 4 h after re-illumination. (**A**) Tre6P level, values are the means ± S.D., $n = 4$. (**B**) Representative immunoblots showing the 100 kDa dimeric (inactive) and 50 kDa monomeric (active) forms of the AGPase small subunit. (**C**) Degree of activation of AGPase, values are the means ± S.D., $n = 5$. (**D**) Starch content (μmol of hexose equivalents · g^{-1}FW), values are the means ± S.D., $n = 4$–5.

sucrose to seedlings, and perturbation of the endogenous sugar level in the leaves of older plants by changing the duration of the diurnal cycle, or by using a starchless mutant. In both experimental systems, the level of Tre6P was low when sugars were low, and rose when sugars increased, with the relative changes in Tre6P being approx. 10-fold greater than those of the phosphorylated glycolytic intermediates. The large and dynamic fluctuations in the level of Tre6P that paralleled the changes in sugars strongly support the hypothesis that Tre6P acts as a signal of sugar status in plants.

Inspection of the temporal dynamics revealed that Tre6P content correlates better with the level of sucrose than it does with glucose or fructose (Figures 2, 3 and 5; Table 1). This could mean that Tre6P is either a sucrose-specific signal or that its concentration reflects only cytosolic sugar level, because most of the free hexoses are probably sequestered in the vacuole [38]. The levels of Tre6P and sucrose remained above those found in the control seedlings even 8 h after sucrose re-addition, whereas those of the hexose-phosphates, UDPGlc and ATP, were close to control levels after 3 h, and changed relatively little thereafter (Figures 2 and 3). The increase in UDPGlc and Glc6P levels after sucrose resupply to starving tissue might have contributed to the increase in Tre6P, by stimulating TPS activity. However, the relative increase in Tre6P was much greater than the changes in UDPGlc and Glc6P, suggesting that other factors, such as sucrose-induced changes in expression or phosphorylation of TPS and TPP [15–18], also contributed to the increase in Tre6P. Athough further work is needed to understand in detail the control of Tre6P levels, our results provide clear evidence that Tre6P acts as a signal of sugar status in plants.

Changes in the level of Tre6P are correlated with changes in redox activation of AGPase and the rate of starch synthesis

In both of our experimental systems, increasing levels of sugars and Tre6P were accompanied by an increase in the redox activation state of AGPase, higher levels of ADPGlc and the stimulation of starch synthesis. These observations are consistent with the proposal that sucrose acts via changes in the level of Tre6P to influence redox activation of AGPase and the rate of starch synthesis. Within 3 h of feeding sucrose to starved seedlings, the amount of glycolytic intermediates and ATP rose to levels similar to those found in control seedlings but then remained more or less constant (Figure 2). This indicates that central metabolism was restored to a full-nutrition-like state during the first 3 h of sucrose resupply. In contrast, sucrose and Tre6P levels were both higher than in control seedlings at 3 h and 8 h after sucrose resupply (Figures 2 and 3). The high Tre6P level might account for the maintenance of AGPase in the activated state (Figure 4), and could act as a signal to divert some of the excess sucrose towards starch synthesis. Some of the excess sucrose also appears to be hydrolysed to free hexoses (Figure 2), probably by acid invertase in the vacuole, but at present there is no evidence of a role for Tre6P in this process.

Transitory starch reserves in leaves buffer plants against diurnal fluctuations in their carbon balance, and both the rate and amount of starch accumulation are adjusted to the prevailing growth conditions [12,27,36]. Current models for the regulation of photosynthate partitioning explain how starch and sucrose synthesis are co-ordinated to avoid withdrawing fixed carbon too rapidly from the Calvin cycle. They also provide a framework with which to understand how sucrose synthesis is decreased, and starch synthesis increased, when sugars accumulate in the leaf. Feedback inhibition or inactivation of SPS leads to a higher level of fructose-2,6-bisphosphate in the cytosol, which inhibits cytosolic fructose-1,6-bisphosphatase and restricts the export of triose-phosphates from the chloroplast. This leads to an increase in the ratio of 3PGA/Pi in the stroma, which activates AGPase and increases the rate of starch synthesis [39]. The recently discovered redox regulation of AGPase provides a second and complementary way to regulate starch synthesis in response to light [23] and the accumulation of sugars [10,23], and may help to explain how starch synthesis is co-ordinated with carbon utilization over the 24 h diurnal cycle, or when unfavourable conditions decrease the amount of carbon fixed in a diurnal cycle. Even a few hours of low sugar levels towards the end of the night period lead to massive changes in gene expression, and an inhibition of metabolism

and growth [12,36]. These effects are only gradually reversed when plants are re-illuminated, so that sugars accumulate in the leaves and roots when photosynthesis starts up again (Table 1) [36]. This is followed by an increase in the level of Tre6P in the leaves, redox activation of AGPase and stimulation of starch synthesis (Figure 5). From these observations, we propose that sugar-induced changes in the amount of Tre6P play a central role in co-ordinating carbohydrate utilization and storage in plants. Further studies are needed to reveal exactly how sugars affect Tre6P levels, and how Tre6P acts within the plastid to promote redox activation of AGPase, as well as to identify other processes and targets that are regulated by Tre6P in plants.

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