Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase

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Trehalose is the most widespread disaccharide in nature, occurring in bacteria, fungi, insects, and plants. Its precursor, trehalose 6-phosphate (T6P), is also indispensable for the regulation of sugar utilization and growth, but the sites of action are largely unresolved. Here we use genetic and biochemical approaches to investigate whether T6P acts to regulate starch synthesis in plastids of higher plants. Feeding of trehalose to *Arabidopsis* leaves led to stimulation of starch synthesis within 30 min, accompanied by activation of ADP-glucose pyrophosphorylase (AGPase) via posttranslational redox modification. The response resembled sucrose but not glucose feeding and depended on the expression of SNF1-related kinase. We also analyzed transgenic *Arabidopsis* plants with T6P levels increased by expression of T6P synthase or decreased by expression of T6P phosphatase (TPP) in the cytosol. Compared with wild type, leaves of T6P synthase-expressing plants had increased redox activation of AGPase and increased starch, whereas TPP-expressing plants showed the opposite. Moreover, TPP expression prevented the increase in AGPase activation in response to sucrose or trehalose feeding. Incubation of intact isolated chloroplasts with 100 μM T6P significantly and specifically increased reductive activation of AGPase within 15 min. Results provide evidence that T6P is synthesized in the cytosol and acts on plastidial metabolism by promoting thioredoxin-mediated redox transfer to AGPase in response to cytosolic sugar levels, thereby allowing starch synthesis to be regulated independently of light. The discovery informs about the evolution of plant metabolism and how chloroplasts of prokaryotic origin use an intermediate of the ancient trehalose pathway to report the metabolic status of the cytosol.

SNF1 kinase | sugar signaling | thioredoxin

Starch is the major carbon store in plants consisting of an insoluble polymer of α-1,4- and α-1,6- linked glucose units (1). In the chloroplast of leaves, starch is synthesized during the day as a transient store, which is degraded during the night to support nonphotosynthetic leaf metabolism and sucrose export. In heterotrophic storage organs such as potato tubers, most of the incoming sucrose is converted to starch as a long-term carbon store for reproductive growth. In addition to its central role in carbon metabolism of plants, starch is also of great economical importance and is used for food and feed purposes and many industrial applications (2).

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis in the plastid, converting glucose 1-phosphate and ATP to ADP-glucose and PP_i. ADP-glucose is subsequently used by starch synthases and branching enzymes to elongate the glucan chains of the starch granule. AGPase is a heterotetramer that contains two large (AGPS, 51 kDa) and two slightly smaller subunits (AGPB, 50 kDa) (3, 4). Work with *Arabidopsis* mutants (5) and potato tubers (6) showed that the enzyme catalyzes a near rate-limiting step in the pathway of starch synthesis. AGPase from leaves and potato tubers is exquisitely sensitive to allosteric regulation, with 3-phosphoglyceric acid (3PGA) acting as an activator and P_i as an inhibitor (7).

More recently, AGPase was found to be subject to posttranslational redox regulation, which involves reversible disulfide-bridge formation between the two AGPB subunits (8). The change in redox state can be detected from a modification of the electrophoretic mobility of AGPB in nonreducing SDS gels, with AGPB running as a dimer in the oxidized form and as a monomer in the reduced form. AGPase from potato tubers (9) and pea leaf chloroplasts (10) is activated by thioredoxin *f* and *m*, leading to an increase in AGPB monomerization and increased sensitivity to activation by 3PGA. There is evidence for the *in vivo* role of posttranslational redox modulation of AGPase in regulating starch synthesis in heterotrophic potato tubers (11) and photosynthetic leaves of potato, pea, and *Arabidopsis* plants (12). Posttranslational redox activation of AGPase allows the rate of starch synthesis to be increased in response to external inputs and independently of any increase in the levels of glycolytic intermediates (11).

In leaves, AGPase is redox-regulated by a light-dependent signal. This resembles the light activation of enzymes of the Calvin cycle and related photosynthetic processes. Electrons are transferred from photosystem I to ferredoxin and via ferredoxin:thioredoxin reductase to thioredoxins *f* and *m*, which activate target enzymes by reduction of regulatory disulfides (13–15). Redox activation of AGPase also responds to changes of sugars. Activation of AGPase in leaves (12) and potato tubers (11) was closely correlated with the sucrose content across a range of physiological and genetic manipulations. This indicates that redox modulation is part of a regulatory loop that channels sucrose toward synthesis of storage starch in leaves and potato tubers. More recent studies in potato tubers revealed that sucrose and glucose lead to redox activation of AGPase via two different signaling pathways involving SNF1-related protein kinase (SnRK1) and hexokinase, respectively (16). Hexokinase and SnRK1 are both implicated in a regulatory network that controls the expression and phosphorylation of cytosolic enzymes in response to sugars (17). How they are linked to reductive activation of AGPase and starch synthesis in the plastid remains unresolved.

Recently, trehalose metabolism has been implicated in the regulation of sugar utilization in yeast and plants (18–20). Trehalose, an ancient sugar consisting of two α-1,1-linked glucose molecules, is synthesized via the phosphorylated precursor

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Abbreviations: AGPase, ADP-glucose pyrophosphorylase; T6P, trehalose 6-phosphate; TPS, trehalose phosphate synthase; TPP, trehalose phosphate phosphatase; SnRK1, SNF1-related protein kinase; 3PGA, 3-phosphoglyceric acid; DTT, dithiothreitol.

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trehalose 6-phosphate (T6P), with a trehalose phosphate synthase (TPS) and a trehalose phosphate phosphatase (TPP). Disruption of At*TPS1* in *Arabidopsis* mutants led to an arrest in embryo development early in the phase of cell expansion and storage-reserve deposition (21). Studies on transgenic lines overexpressing TPS or TPP identified T6P as essential for sugar utilization and growth in *Arabidopsis* plants (22). External feeding of trehalose or sucrose to wild-type *Arabidopsis* led to an increase in T6P level within 30 min or 2 h, respectively, indicating a link between sugars and T6P that responds within a short time frame (23). However, the sites of T6P action remain largely unknown.

To investigate a role of T6P in the regulation of starch synthesis, we (*i*) measured posttranslational redox activation of AGPase and starch accumulation after short-term feeding of trehalose to wild-type potato tubers and *Arabidopsis* leaves, (*ii*) analyzed the same parameters in leaves of transgenic *Arabidopsis* plants with altered T6P levels due to overexpression of heterologous TPS or TPP in their cytosol, and (*iii*) provided T6P directly to purified chloroplasts to investigate whether it affects redox activation of AGPase. The results provide genetic and biochemical evidence that T6P reports the cytosolic sucrose status to the plastid, leading to a stimulation of starch synthesis in response to sucrose by promoting reductive activation of AGPase.

Methods

Plant Material. *Arabidopsis* var. Col0, wild type, and transgenic plants overexpressing *otsA* encoding for an *Escherichia coli* TPS (line A19.3) or *otsB* encoding for an *E. coli* TPP (line B12.1) (22) were grown in a growth chamber with a 16-h day of 180 microeinstein, 21/19°C (day/night), and 50% humidity as in ref. 12. Lines A19.3 and B12.1 have been documented (22) as representative among several lines, showing high and stable expression of *otsA* and *otsB* over five generations, respectively. Wild-type and transgenic potato plants with reduced expression of SnRK1 (24) were propagated in tissue culture and grown in a greenhouse during the summer as in ref. 11. Pea plants were grown as in ref. 12.

Incubation Experiments. Incubation experiments with *Arabidopsis* leaves were performed as in ref. 12 by feeding sugars via the cut petiole. Short-term incubation experiments up to 3 h were performed with discs cut from leaves at the end of the dark period, which were floated on buffer solutions containing 2 mM Mes-KOH, pH 6.5, and different sugars, as indicated in the figure legends. Chloroplasts were prepared from pea leaves and Percoll purified as described in ref. 12, diluted 1:5 with buffer containing 330 mM sorbitol; 50 mM Hepes-KOH, pH 7.5; 2 mM EDTA; 1 mM $MgCl_2$; 4 mM $NaHCO_3$; 1 mM 3PGA; and 0.3 mM dithiothreitol (DTT), and then incubated for 15 min with additional compounds as indicated in the figure legends. Intactness of chloroplasts was determined routinely by microscopy and chlorophyll content measured as in ref. 12. Incubation experiments with potato tuber discs were performed as in ref. 16.

Analysis of AGPase Redox Activation. In leaves and isolated chloroplasts, redox activation of AGPase was measured by analyzing the monomerization degree of AGPB and the activity of the enzyme under nonreducing assay conditions as in ref. 12. In discs of growing potato tubers, redox activation of AGPase was measured enzymatically as described in ref. 11.

Metabolite Analysis. The levels of starch, soluble sugars, and hexose phosphates were determined in ethanol extracts as in ref. 12. To measure the levels of NADPH and $NADP^+$, 100 mg of plant tissue was rapidly frozen and homogenized in liquid nitrogen and extracted with 1 ml of either alkaline (50 mM NaOH) or acid (50 mM HCl) 80% (vol/vol) ethanol/water to measure NADPH or $NADP^+$, respectively. Extracts were heated at 90°C for 2 min, centrifuged at 14,000×*g* for 2 min at 4°C, and 10 μl of the supernatant added to 200 μl of assay mix [200 mM Tricine, pH 9/10 mM $MgCl_2$/2 mM EDTA/6 μg of phenazine methosulfate/41 μg of dimethylthiazolyl diphenyltetrazolium bromide/152 μg of glucose-6-P/3 units of glucose-6-P dehydrogenase from yeast (Roche, Mannheim, Germany, Grade II)]. The linear rate of absorbance increase at 570 nm of the alkaline or acidic extract minus the blank value was used to calculate the NADPH/$NADP^+$ ratio directly. Extraction and measurement were completed within 15 min.

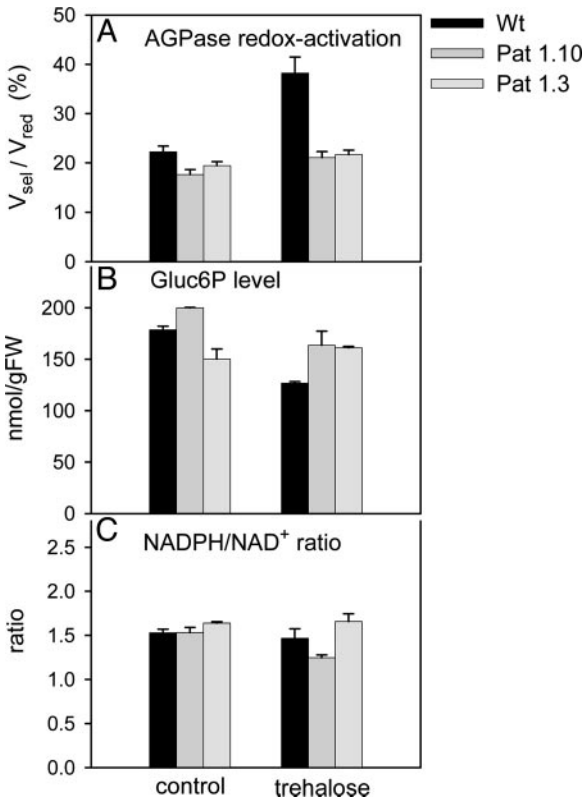

Fig. 1. Short-term feeding of trehalose to discs of growing potato tubers leads to SnRK1-dependent redox activation of AGPase independent of cellular redox-state. Freshly prepared tuber discs of wild-type and SnRK1 antisense lines (pat 1.10 and pat 1.3) were incubated for 1 h in 100 mM trehalose before AGPase redox activation (*A*), glucose-6-phosphate level (*B*), and NADPH/$NADP^+$ ratio (*C*) were measured. AGPase redox activation was determined as the ratio of activities measured under selective (V_{sel}; lacking DTT) and reductive (V_{red}; including DTT) assay conditions (11). Results are means ± SE (n = 3).

Results

Short-Term Feeding of Trehalose to Discs of Heterotrophic Potato Tubers or *Arabidopsis* Leaves Leads to Posttranslational Redox Activation of AGPase. In heterotrophic potato tubers, two different sugar-sensing systems have been identified leading to posttranslational redox activation of AGPase in response to sucrose and glucose (16). Although the sucrose-related response required SnRK1 and was independent of changes in metabolites, glucose-related activation of AGPase was independent of SnRK1 and involved a strong increase in hexose-P levels (16). To investigate a possible role of trehalose metabolism in these signaling events, tuber slices from wild-type and SnRK1 antisense lines were incubated with 100 mM trehalose for 1 h. It has been shown in *Arabidopsis* that feeding trehalose leads to an increase in its precursor T6P (23). Redox activation of AGPase was assessed

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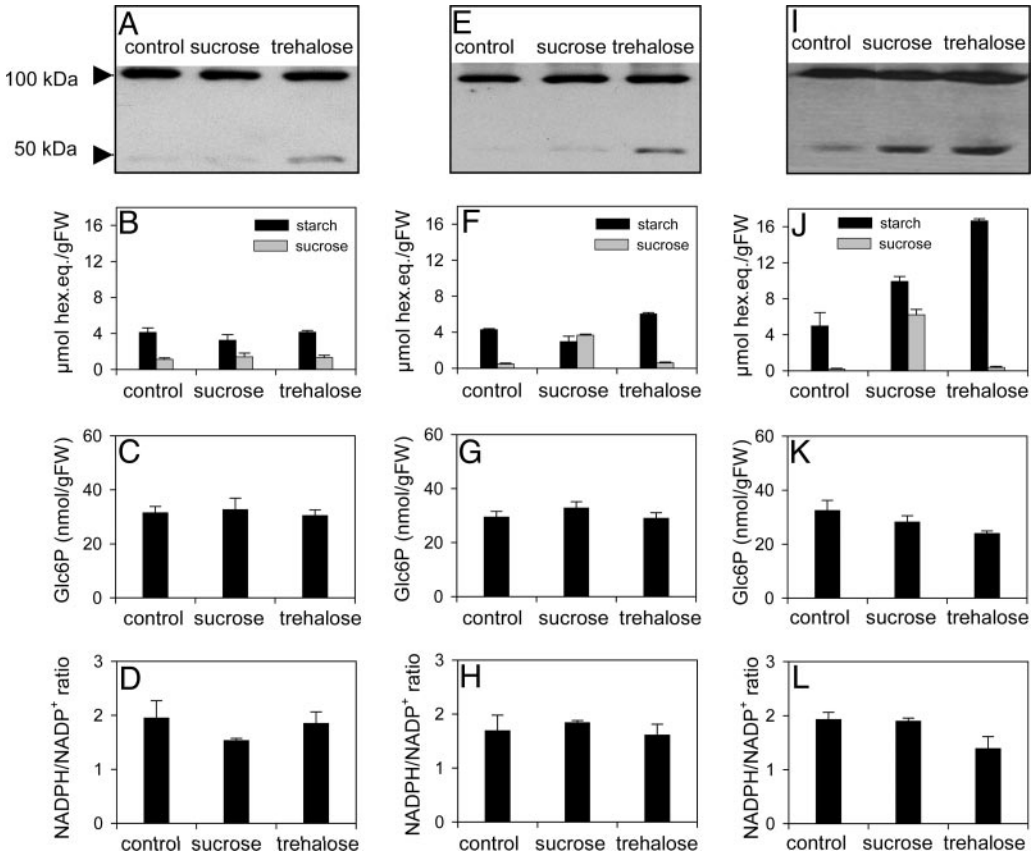

Fig. 2. Short-term feeding of trehalose to leaf discs of *Arabidopsis* plants at the end of the night leads to redox activation of AGPase and increased starch levels without changing the NADPH/NADP$^+$ ratio. Leaf discs were incubated for 15 min (*A–D*), 30 min (*E–H*), and 3 h (*I–L*) in 100 mM sorbitol (control), 100 mM sucrose or 100 mM trehalose before samples were frozen to analyze (*A*, *E*, and *I*) AGPase redox activation by measuring AGPB monomerization with nonreducing SDS gels, (*B*, *F*, and *J*) starch and sucrose levels, (*C*, *G*, and *K*) glucose-6-phosphate levels, and (*D*, *H*, and *L*) NADPH/NADP$^+$ ratios. Results are means ± SE (n = 3–4). For comparison, the NADPH/NADP$^+$ ratios measured in intact leaves at the end of the night were 1.44 ± 0.35 (mean ± SE, n = 3), whereas leaves that were sampled upon illumination had a 2-fold higher NADPH/NADP$^+$ ratio (3.3 ± 0.2, mean ± SE, n = 3).

via enzymatic assays. In the wild type, trehalose feeding resulted in a doubling in reductive activation of AGPase (Fig. 1*A*), hexose-phosphate levels decreased (Fig. 1*B*), and sucrose and glucose levels were not significantly different from control incubations (data not shown). The response of AGPase redox activation to trehalose was almost completely abolished in tuber slices of SnRK1 antisense lines, resembling the response to sucrose but not glucose (see Introduction). This makes it unlikely that the response to trehalose was due to conversion of trehalose into glucose (see below for more data).

To investigate whether redox activation of AGPase was due to an increase in the reduction state of the NADPH/NADP$^+$ system in the tuber tissue, we used parallel samples to measure the levels of NADPH and NADP$^+$. Irrespective of the genetic background, feeding trehalose did not lead to any increase in the NADPH/NADP$^+$ ratio in tuber discs (Fig. 1*C*). NADPH/NADP$^+$ ratios were also measured after incubation of wild-type tuber slices for 1 h with different sugars. NADPH/NADP$^+$ ratios were 1.53 ± 0.05, 1.47 ± 0.11, 1.51 ± 0.19, and 3.15 ± 0.20 in tuber discs incubated with buffer, 100 mM trehalose, 100 mM sucrose, or 100 mM glucose, respectively (mean ± SE, n = 3). The response to trehalose again resembled that to sucrose, not glucose.

To investigate whether trehalose feeding leads to redox activation of AGPase in leaves, discs of *Arabidopsis* leaves were incubated for 15 min, 30 min, or 3 h with 100 mM trehalose, 100 mM sucrose or 100 mM sorbitol before analysis of the reduction state of AGPase as AGPB monomerization by using nonreductive SDS/PAGE. In discs from leaves sampled at the end of the night and incubated with sorbitol as osmotic control, AGPB was completely dimerized (Fig. 2*A*), and sucrose and starch levels were relatively low (Fig. 2*B*). This resembles previous results with *Arabidopsis* leaves (see ref. 12). Incubation with trehalose led to rapid appearance of AGPB monomers visible within 15 min and increased further after 30 min and 3 h (Fig. 2 *A*, *E*, and *I*). Trehalose led to a significant increase in starch levels after 30 min and a 2.5-fold increase by 3 h (Fig. 2 *B*, *F*, and *J*). This was not accompanied by any increase in the levels of sucrose (Fig. 2 *B*, *F*, and *J*), other soluble sugars including glucose (data not shown), Glc6P (Fig. 2 *C*, *G*, and *K*), or the reduction state of the NADPH/NADP$^+$ system (Fig. 2 *D*, *H*, and *L*). A similar but delayed response was observed after sucrose feeding, with sucrose leading to increased AGPB monomerization and increased starch synthesis after 30 min and 3 h, respectively (Figs. 2 *A*, *B*, *E*, *F*, *I*, and *J*).

Manipulation of T6P Metabolism in Transgenic *Arabidopsis* Plants Leads to Redox Modification of AGPase and Changes in Starch Content. To provide genetic evidence for a role of T6P in redox activation of AGPase, we analyzed transgenic *Arabidopsis* plants with T6P levels increased by overexpression of TPS or decreased by overexpression of TPP from *E. coli* targeted to the cytosol but with no changes in trehalose content (22). Compared with wild type, TPS-expressing plants had a higher redox activation of AGPase, as shown by increased monomerization of the AGPB subunit (Fig. 3*A*), which correlated with increased starch accumulation in leaves during the day (Fig. 3*B*), whereas TPP plants showed the opposite.

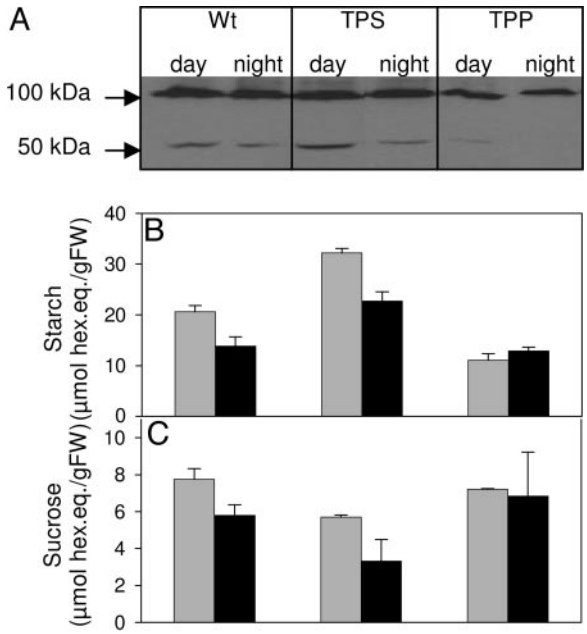

Fig. 3. Transgenic *Arabidopsis* plants show a crucial role of T6P in regulating starch synthesis via posttranslational redox activation of AGPase. Leaves of wild-type (Wt) and of transgenic *Arabidopsis* plants overexpressing TPS or TPP in their cytosol were sampled in the middle of the day and in the middle of the night to analyze redox activation of AGPase by measuring AGPB monomerization with nonreductive SDS gels (*A*), starch levels (*B*), and sucrose levels (*C*). Results are means ± SE ($n = 4$).

This shows that the level of T6P rather than trehalose is involved in activation of AGPase and starch synthesis. Changes in redox activation of AGPase occurred independently of changes in the levels of sugars (Fig. 3*C*), sugar phosphates, or the NADPH/$NADP^+$ ratio (data not shown), indicating that T6P acts downstream of light-dependent changes in redox status and metabolite levels. Analysis of tobacco transformants overexpressing *E. coli* TPP revealed a similar decrease in redox activation of AGPase and starch synthesis compared with wild type, as observed in the TPP-expressing *Arabidopsis* lines (data not shown).

Sucrose- and Trehalose-Dependent Redox Activation of AGPase Is Strongly Attenuated in Transgenic *Arabidopsis* Plants Expressing a T6P Phosphatase from *E. coli*. AGPB was converted from dimer to monomer after feeding 100 mM sucrose for 13 h in the dark to leaves of wild-type plants via the petiole (Fig. 4*A*), resembling earlier studies (12). A high concentration of sucrose was fed, because the rate of transpiration is low in the dark (not shown). AGPB monomerization was higher in the absence of sucrose in TPS overexpressors and increased still further when sucrose was supplied. Monomerization was lower in TPP-expressing plants and increased only slightly when sucrose was supplied (Fig. 4*A*). Redox activation of AGPase remained low in TPP-expressing plants despite a larger increase in internal sucrose levels, than in wild-type or TPS plants (Fig. 4*B*). The changes of sucrose levels in these feeding experiments resemble those found in leaves during the day (compare Fig. 4 *B* and *C*). This indicates that T6P is a crucial component in the sucrose-dependent redox activation of AGPase in leaves. TPP expression also prevented AGPB monomerization in response to short-term (1-h) feeding of sucrose or trehalose to leaf discs at the end of the night (Fig. 4*C*) in a similar experiment as in Fig. 2, providing additional evidence that redox activation of AGPase is attributable to T6P rather than trehalose. Also in this experiment, AGPB monomerization remained low in TPP overexpressors despite a strong increase in internal sucrose levels after supplying sucrose (Fig. 4*D*).

Short-Term Feeding of T6P to Intact Purified Chloroplasts Leads to a Specific Increase in Redox Activation of AGPase Without Changing the Plastidial Redox State. To provide direct biochemical evidence that T6P promotes redox activation of AGPase independently of cytosolic elements, it was investigated whether addition of T6P to isolated chloroplasts leads to activation of AGPase. Chloroplasts were prepared from pea leaves and purified by centrifugation in Percoll gradients. Chloroplast intactness, verified by microscopy and by comparing the rate of ferricyanide-dependent uncoupled electron transport in preparations before and after osmotic shock, was >90%.

The chloroplasts were incubated in the dark in the presence of dilute reductant DTT (0.3 mM) to allow partial reduction of the ferredoxin/NADPH and ferredoxin/thioredoxin systems. Feeding down to 100 μM T6P significantly increased AGPB monomerization within 15 min (Fig. 5 *A* and *B*). No significant changes were observed after feeding trehalose, sucrose, sucrose 6-phosphate, P_i, or trehalose together with P_i to chloroplasts as controls (Fig. 5*A*). Similar concentrations of T6P also led to an increase in AGPase activity measured as an increase in ADPGlc production in the presence of 3 mM 3PGA (Fig. 5*C*). In a further control experiment, T6P was added to chloroplasts that had been disrupted by osmotic shock. Although adding T6P to intact chloroplasts resulted in a 2-fold increase in AGPase activity assayed in the presence of 3PGA, disruption of chloroplasts

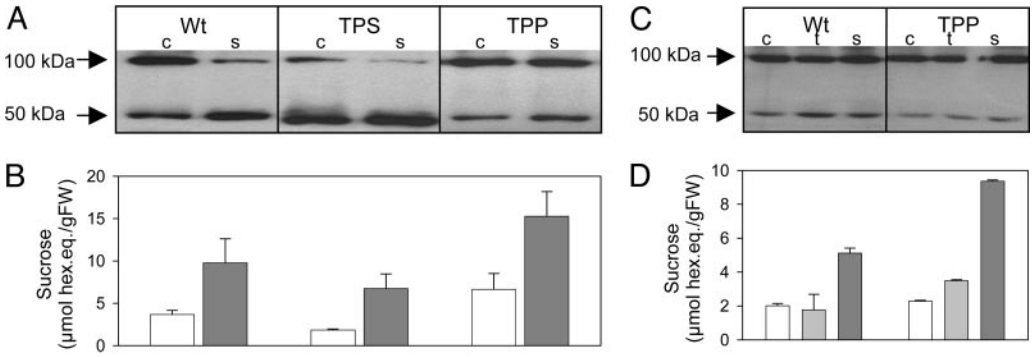

Fig. 4. Sucrose- and trehalose-dependent redox activation of AGPase is strongly attenuated in leaves of TPP overexpressing *Arabidopsis* plants. Leaves of wild-type, TPS-, or TPP-expressing plants were incubated via the transpiration stream with 100 mM sucrose (s) or osmotic control (c) for 13 h, before sampling to measure AGPB monomerization (*A*) and internal sucrose levels (*B*). In an analogous experiment with wild-type and TPP-expressing plants, AGPB monomerization (*C*) and internal sucrose levels (*D*) were measured after feeding 100 mM trehalose (t) or 100 mM sucrose (s) for 1 h to leaf discs during the end of the night (*C*). Results are means ± SE ($n = 4$).

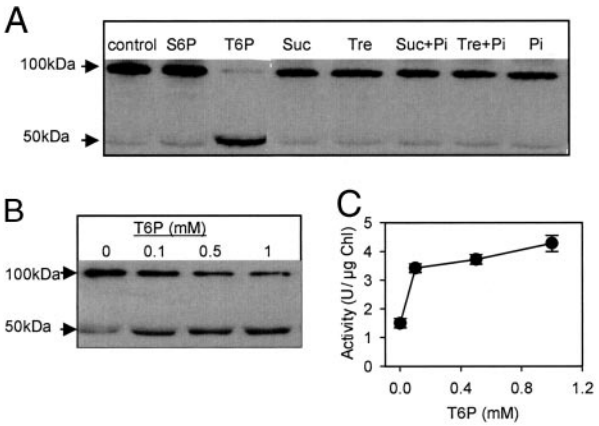

Fig. 5. Feeding T6P to isolated chloroplasts leads to redox activation of AGPase. (*A*) Changes in AGPB monomerization after incubating intact isolated pea chloroplasts for 15 min with 10 mM sucrose-6-phosphate (S6P), 10 mM T6P, 10 mM sucrose (Suc), 10 mM trehalose (Tre), 1 mM P_i or combinations of sucrose or trehalose with P_i. In an additional experiment, AGPB monomerization (*B*) and AGPase activity (*C*) were measured after feeding different concentrations of T6P to intact purified pea chloroplasts for 15 min. Quantification of the immunoblot signals shows that AGPB monomers represented 30.0%, 47.4%, 57.2%, or 62.5% of the total signal after feeding 0, 0.1, 0.5, or 1 mM T6P to the chloroplasts, respectively. AGPase activity was measured in the presence of 3 mM 3PGA and is shown as mean ± SE ($n = 2$).

prevented T6P activation (see Fig. 6, which is published as supporting information on the PNAS web site).

Although feeding DTT to chloroplasts led to an increase in the NADPH/$NADP^+$ ratio, the response to T6P did not involve changes in the reduction state of the chloroplast NADP system. The NADPH/$NADP^+$ ratio was 0.702 ± 0.01, 0.723 ± 0.06, or 1.055 ± 0.044 for chloroplasts incubated for 15 min with buffer, 5 mM T6P, or 5 mM DTT, respectively (mean ± SE, $n = 3$). This is consistent with T6P modulating redox transfer from ferredoxin or NADPH via thioredoxins to AGPase rather than increasing the plastidial redox state.

Discussion

Trehalose is an ancient disaccharide ubiquitous in bacteria, fungi, insects, and plants, functioning as a reserve carbohydrate in bacteria, component of the haemolymph system of insects, and as a general stress protectant. Recent studies show that the precursor of trehalose, T6P, is an indispensable regulator of sugar utilization in eukaryotic organisms as different as yeasts and plants (18, 20, 22). However, apart from regulating glycolysis by inhibiting hexokinase in some yeast species, other sites of T6P action have until now not been known (20, 25–27). No target of T6P has been found in plants, where hexokinases are insensitive to T6P (19). Here we provide independent lines of genetic and biochemical evidence that T6P acts indirectly on AGPase to promote posttranslational redox activation of the enzyme in response to sucrose, thereby controlling starch synthesis independently of light signals.

In *Arabidopsis*, trehalose feeding leads to an increase in T6P levels within 30 min, possibly by feedback inhibition of TPP (23). In the present experiments, trehalose feeding to potato tubers and *Arabidopsis* leaves led to an increase in reductive activation of AGPase and increased starch synthesis within a similar time frame. The response depended on SnRK1 (Fig. 1) and did not lead to changes in hexose-P levels or the NADPH/$NADP^+$ ratio in the tissue (see Figs. 1 and 2 and data in the text), which resembled sucrose feeding but was opposite to glucose feeding (for details, see ref. 16). It is therefore unlikely that the effect of trehalose was due to its metabolism to glucose. Trehalose led to reductive activation of AGPase more rapidly than sucrose (30 min compared with 3 h; see Fig. 2), which resembled the difference in time course of the increase in T6P after feeding trehalose (30 min) or sucrose (2 h) in the studies of ref. 23. This tentatively indicated a link between T6P and reductive activation of AGPase.

To provide genetic evidence for a role of T6P in reductive activation of AGPase, we analyzed transgenic *Arabidopsis* lines with T6P levels increased by overexpression of TPS or decreased by overexpression of TPP, but with no changes in trehalose content (22). TPS expression led to an increase in redox activation of AGPase and in starch, whereas TPP expression showed the opposite (Fig. 3). Moreover, TPP expression strongly attenuated the increase in reductive activation of AGPase after feeding sucrose or trehalose to leaves (Fig. 4), providing evidence for T6P linking redox activation of AGPase to sucrose. Feeding experiments with isolated chloroplasts provided direct biochemical evidence that T6P promotes redox activation of AGPase independently of cytosolic elements. Incubation down to 100 μM T6P significantly and specifically increased AGPB monomerization and AGPase activity within 15 min, whereas no significant changes were observed after feeding trehalose or sucrose to the chloroplasts (Figs. 5 and 6).

These different approaches provide cumulative evidence that T6P promotes posttranslational redox activation of AGPase in response to sucrose, reporting on metabolite status between cytosol and chloroplast. The mechanistic details of this response still have to be elucidated, but it is highly likely for two reasons that it involves synthesis of T6P in the cytosol and its subsequent perception at the chloroplast membrane or within the chloroplast. First, because expression of TPS and TPP in the transgenic plants was targeted to the cytosol, this should lead to changes in the cytosolic levels of T6P. Second, feeding experiments show that T6P concentrations down to 100 μM can act on isolated chloroplasts leading to redox activation of AGPase. The T6P content in plants has been reported to be 3–12 nmol/g of fresh weight (22, 23). Assuming T6P is in the cytosol and plastids, representing ≈10% of the cell volume, 100 μM is within the physiological range of cytosolic T6P concentrations in plants. For comparison, internal T6P concentrations in yeast cells have been determined to be in a similar range between 150 and 200 μM and to transiently rise to 1,500 μM in response to sugars (20, 28, 29). T6P had no effect when fed to chloroplasts that had been disrupted by hypoosmotic shock, showing that intact chloroplast structures are needed for T6P action. This might be attributable to specific transport characteristics that allow accumulation of T6P in intact chloroplasts, to T6P interacting factors being present at an intact chloroplast envelope, or to interacting proteins such as thioredoxins being present in concentrations usually found in intact chloroplast stroma. More experiments are also needed to define the transport mechanism of T6P in intact chloroplasts.

Interestingly, changes in the chloroplast redox state were not involved in the reductive activation of AGPase in response to T6P. Neither feeding T6P to chloroplasts nor changes in T6P levels in transgenic lines led to significant changes in the NADPH/$NADP^+$ ratio. This indicates that T6P promotes redox transfer from NADPH or ferredoxin to AGPase rather than leading to an increase in the reduction state of these redox systems. It has been reported that light-dependent redox activation of photosynthetic enzymes by thioredoxin is modulated by metabolites, which modify the midredox potential of the cysteines in the target proteins (14, 30). Activation of several Calvin cycle enzymes is promoted by high substrate concentrations and activation of NADP malate dehydrogenase, by a high NADPH/$NADP^+$ ratio. This allows increased reduction of the target enzyme, even though the plastidial redox state does not increase.

Two findings show that the signal transduction chain linking AGPase redox activation to sucrose involves synthesis of T6P in the cytosol as a crucial component. First, sucrose led to redox activation of AGPase when fed to intact leaves but not when fed to isolated chloroplasts, indicating that this sugar needs further cytosolic components to trigger this response. In contrast, feeding T6P to chloroplasts led to a very sensitive and marked activation of AGPase (Fig. 5). Second, redox activation of AGPase in response to sucrose feeding to leaves was strongly attenuated in plants overexpressing TPP in the cytosol (Fig. 4), indicating the effect of sucrose strongly depends on the level of T6P in the cytosol. Although these results indicate T6P as a signal component in the response of starch synthesis to an accumulation of sucrose during the day, it might also be involved to signal an increase in sugars deriving from starch degradation during the night. Studies with *Arabidopsis* mutants (A.T., S. Zeeman, A. M. Smith, and P.G., unpublished results) revealed an increase in AGPase redox activation when maltose metabolism was impaired at a step in the cytosol (*dpe2*$^{-}$ mutant, ref. 31), but not when export of maltose from the chloroplasts was blocked (*mex-* mutant, ref. 32), indicating that also the sensing of sugars deriving from starch degradation occurred in the cytosol rather than in the plastid.

The link between sucrose and T6P has not yet been fully resolved but may involve regulation of enzymes involved in T6P metabolism at the transcriptional or posttranscriptional levels. *Arabidopsis* contains 11 genes for TPS and 8 for TPP, and recent studies using transcript arrays indicate large diurnal fluctuations in the expression of these genes that are probably due to changes in the carbon status of the leaves (refs. 23 and 33; A.K., A. R. Fernie, J. T. van Dongen, and P.G., unpublished results). Microarrays also showed that increased T6P levels in transgenic *Arabidopsis* lines and wild-type plants fed with sugars correlate with increased expression of SnRK1 (23). Furthermore, decreased SnRK1 expression in transgenic potato lines prevented the increase in posttranslational activation of AGPase in response to trehalose (Fig. 1) or sucrose (16). Whether SnRK1 is acting upstream or downstream of TPS cannot be fully resolved at the moment. The presence of SnRK1 phosphorylation motives in TPS sequences (see ref. 34) and the finding that T6P acts on isolated chloroplasts without requiring further cytosolic elements (Fig. 5) are consistent with SnRK1 being an upstream component in this signaling pathway.

It will be interesting to determine whether T6P also regulates other thioredoxin-dependent processes in the plastid, such as fatty acid (35) or amino acid synthesis (36). This may allow global control of diverse pathways of carbon utilization in plants in response to short-term changes in the carbon status. A similar regulatory network could also regulate carbon utilization in other organisms, where thioredoxins are involved in regulating mitochondrial respiration (15). Recently, a thioredoxin-interacting protein has been identified in mammals that inhibits thioredoxin-mediated redox transfer from NADPH to sulfhydryl groups and affects the metabolic response to feeding and nutritional signals (37–39).

Conclusion

Our results provide evidence that T6P regulates utilization of sugars for storage starch synthesis by promoting reductive activation of AGPase in the plastid. During the evolution of plants, chloroplasts that are of prokaryotic origin have used this intermediate of the ancient trehalose pathway to regulate their own metabolism relative to the metabolic requirements of the cytosol. The discovery helps us to understand the evolution of plant metabolism and how trehalose metabolism has been adapted as an indispensable regulatory component of carbon utilization for growth, storage, and survival in a diverse range of organisms.

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