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Feedback inhibition of starch degradation in Arabidopsis leaves mediated by trehalose 6-phosphate

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Summary: Trehalose 6-phosphate inhibits night-time breakdown of transitory starch in leaves, potentially linking starch remobilization to sucrose demand for respiration and growth at night.

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Abstract
Many plants accumulate substantial starch reserves in their leaves during the day, and remobilise them at night to provide carbon and energy for maintenance and growth. In this paper we explore the role of a sugar signalling metabolite, trehalose-6-phosphate (Tre6P), in regulating the accumulation and turnover of transitory starch in Arabidopsis leaves. Ethanol-induced overexpression of trehalose-phosphate synthase (TPS) during the day increased Tre6P levels up to 11-fold. There was a transient increase in the rate of starch accumulation in the middle of the day, but this was not linked to reductive activation of ADP-glucose pyrophosphorylase. A 2 to 3-fold increase in Tre6P during the night led to significant inhibition of starch degradation. Maltose and maltotriose did not accumulate, suggesting that Tre6P affects an early step in the pathway of starch degradation in the chloroplasts. Starch granules isolated from induced plants had a higher P content than granules from non-induced control plants, consistent with either disruption of the phosphorylation-dephosphorylation cycle that is essential for efficient starch breakdown, or with inhibition of starch hydrolysis by β-amylase. Non-aqueous fractionation of leaves showed that Tre6P is predominantly located in the cytosol, with estimated in vivo Tre6P concentrations of 4-7 µM in the cytosol, 0.2-0.5 µM in the chloroplasts and 0.05 µM in the vacuole. It is proposed that Tre6P is a component in a signalling pathway that mediates feedback regulation of starch breakdown by sucrose, potentially linking starch turnover to demand for sucrose by growing sink organs at night.

Abbreviations
AGPase, ADP-glucose pyrophosphorylase; ED, end of day; EN, end of night; GWD, glucan, water dikinase; PWD, phosphoglucan, water dikinase; TPP, trehalose-
phosphate phosphatase; TPS, trehalose-phosphate synthase; Tre6P, trehalose 6-phosphate
INTRODUCTION

Starch and sucrose are the main products of photosynthesis in higher plants (Stitt et al., 2010). Much of the sucrose synthesised during the day is exported from the leaves to support the growth of heterotrophic sink organs, whereas starch accumulates in the leaves during the day. This starch is then broken down at night to provide substrates for leaf respiration and sucrose synthesis, allowing sucrose export to continue in the dark. In this way, the transitory starch reserves in the leaf enable the plant to meet the daily challenge of surviving and growing through the night when photosynthesis is no longer possible. The efficient management of these reserves is essential if the plant is to optimise its growth rates and achieve reproductive success (Smith and Stitt, 2007; Stitt and Zeeman, 2012; Andriotis et al., 2012). In this paper we explore the role of a sugar signalling metabolite, trehalose-6-phosphate (Tre6P), in regulating the accumulation and/or turnover of transitory starch in Arabidopsis leaves, potentially linking the management of these reserves to the availability and demand for sucrose.

In leaves, starch and sucrose are ultimately synthesised from intermediates of the Calvin-Benson cycle, and the total flux into these end products is closely coordinated with the net rate of CO₂ assimilation (Stitt et al., 2010). From the fluctuations in metabolite levels observed in leaves during the diurnal cycle and in vitro studies of enzyme properties, a model was developed to explain how various regulatory mechanisms are integrated to bring about feed-forward or feedback regulation of sucrose synthesis and photoassimilate partitioning (for a detailed review see MacRae and Lunn, 2006). This model considers starch to be an “overflow” product that is synthesised from surplus photoassimilate when photosynthesis exceeds the capacity of the leaves to export or store sucrose (Cseke et al., 1994). According to the model, rising levels of sucrose result in progressive inactivation of sucrose-phosphate synthase (SPS; Stitt et al., 1988). This triggers a cascade of events, including inhibition of the cytosolic fructose-1,6-bisphosphatase (FBPase) by rising levels of fructose-2,6-bisphosphate (Fru2,6BP), that coordinately decrease flux through the whole pathway of sucrose synthesis, and therefore the release of orthophosphate (Pi) in the cytosol. The limited availability of Pi in the cytosol restricts export of triose-phosphates from the chloroplasts via the triose-phosphate:Pi
translocator, leading to a rise in 3-phosphoglycerate (3PGA) and a fall in Pi in the stroma. These reciprocal changes in the allosteric activator (3PGA) and inhibitor (Pi) of ADP-glucose pyrophosphorylase (AGPase) strongly activate the enzyme (Preiss, 1988), increasing production of ADP-glucose (ADPG) and the synthesis of starch.

The “overflow” model is supported by various studies on wild-type and mutant plants (Neuhaus et al., 1989; 1990; Neuhaus and Stitt, 1990; Stitt and Sonnewald, 1995; Scott et al., 2000; Strand et al., 2000, Stitt et al., 2012). However, there is good evidence that other mechanisms also contribute to the control of starch synthesis. For example, many species, including Arabidopsis, increase their rate of starch synthesis when shifted from long to short days, so despite the shorter period of photosynthesis the plants still accumulate sufficient starch during the day to last through the longer night ahead (Chatterton and Silvius, 1980; Gibon et al., 2004). The circadian clock is potentially involved in this anticipatory response, providing information about the expected length of the coming night so that the plant can set an appropriate rate of starch synthesis (Stitt et al., 2007; Smith and Stitt, 2007; Stitt and Zeeman, 2012).

Another mechanism that is potentially important for controlling starch synthesis is redox regulation of AGPase (Fu et al., 1998; Ballicora et al., 2000; Tiessen et al., 2002). In the light, or in response to high sugar levels in the leaves, AGPase is activated by reduction of an intermolecular disulphide bridge between cysteine residues (Cys81 in Arabidopsis) in the two small subunits of the heterotetrameric holoenzyme (Hendriks et al., 2003; Hädrich et al., 2011). Light-dependent activation and sugar-dependent activation appear to be mediated by the ferredoxin-thioredoxin system and NADP-thioredoxin reductase C, respectively (Michalska et al., 2009; de Dios Barajas-López et al., 2012). Kolbe et al. (2005) found there was little or no change in the redox status of AGPase when isolated pea chloroplasts were incubated in the dark with dithiothreitol and sucrose, whereas incubation with dithiothreitol and 0.1-1.0 mM Tre6P led to substantial reduction of the enzyme.

Tre6P is the intermediate of trehalose synthesis; it is synthesised by trehalose-phosphate synthase (TPS) and dephosphorylated to trehalose by trehalose-phosphate phosphatase (TPP). Constitutive over-expression of the *Escherichia coli* TPS (*otsA*)
in Arabidopsis gave rise to stunted, early-flowering plants that accumulated more starch in their leaves than wild type plants (Schluepmann et al., 2003; Kolbe et al., 2005; Wingler et al., 2012). Together, these observations raised the possibility that Tre6P acts as an intermediary in the sugar-induced activation of AGPase. Lunn et al. (2006) found that the amount of Tre6P in Arabidopsis rosettes changes in parallel with diurnal fluctuations in sucrose content and the redox status of AGPase, lending correlative support to this hypothesis. However, a direct causal relationship between changes in sucrose, Tre6P and the redox status of AGPase in vivo remains to be demonstrated.

With the onset of darkness, the transitory starch reserves must be managed prudently if they are to last through the night. The pathway of starch degradation in Arabidopsis leaves has been largely resolved, mainly by studies of starch excess mutants that fail to degrade their starch effectively during the night (reviewed in Zeeman et al., 2010). The initial step involves a cycle of glucan phosphorylation and dephosphorylation to open up the crystalline structure at the surface of the starch granule (Edner et al., 2007; Hejazi et al., 2008; Blennow and Engelson, 2010). Glucose residues within the glucan chains are phosphorylated in the C6 and C3 positions by glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD), respectively (Blennow et al., 2002; Ritte et al., 2002; Baunsgaard et al., 2005; Kötting et al., 2005; Ritte et al., 2006). Dephosphorylation is catalysed by the SEX4 and LSF2 phosphoglucan phosphatases (Kötting et al., 2009; 2010; Comparot-Moss et al., 2010; Hejazi et al., 2010; Tagliabracchi and Roach, 2010; Santelia et al., 2011). Transitory starch is degraded by a combination of β-amylases (principally BAM1 and BAM3; Fulton et al., 2008) and debranching enzymes (Streb et al., 2012), in conjunction with the plastidial disproportionating enzyme (DPE1). The main products are maltose and glucose, which are exported from the chloroplast via the MALTOSE EXCESS1 (MEX1) transporter and the plastidial glucose transporter, respectively (Weber et al., 2000; Niittylä et al., 2004; Cho et al., 2011). In the cytosol, maltose is metabolised by the cytosolic disproportionating enzyme (DPE2), which catalyzes the reversible transfer of one glucosyl moiety to a soluble heteroglycan (SHG), releasing the other as free glucose, which is phosphorylated by hexokinase (Chia et al., 2004; Lu and Sharkey, 2004; Fettke et al., 2005; 2006; 2009). Cytosolic phosphorylase
(PHS2) is thought to catalyse the Pi-dependent transfer of terminal glucosyl moieties from the SHG to form glucose 1-phosphate, which enters the cytosolic hexose-phosphate pool, making it available for sucrose synthesis, respiration and other pathways (Fettke et al., 2004; 2005).

In Arabidopsis leaves, starch is degraded in a near-linear manner throughout the night, and is almost but not totally exhausted at dawn. The rate of starch degradation is regulated by the circadian clock, which presumably provides information about the expected length of the night (Lu et al., 2005; Graf et al., 2010; Graf and Smith, 2011; Yazdanbakhsh et al., 2011; Stitt and Zeeman, 2012). This is integrated with information about how much starch has been accumulated during the day, enabling the plant to set an appropriate rate of degradation (Scialdone et al., 2013). The underlying signalling pathways still need to be elucidated. The transcripts of genes involved in starch degradation show large, coordinated diurnal changes (Smith et al., 2004; Lu et al., 2005; Usadel et al., 2008), but at present there is no evidence that these lead to large changes in the levels of the encoded proteins (Smith et al., 2004). Several proteins involved in starch degradation are subject to redox modification, but the physiological significance of this is uncertain (Valerio et al., 2011; Glaring et al., 2012), and as yet there is no evidence of any connection with control of starch degradation by the circadian clock.

There is also evidence that high levels of sucrose in the leaves at night might inhibit starch degradation. When Arabidopsis plants were grown in elevated CO$_2$, they accumulated higher levels of sucrose and starch during the day than control plants in ambient CO$_2$, and did not start degrading their starch until several hours into the night when sucrose levels had begun to fall (Cheng et al., 1998). Other observations point to a potential role of trehalose metabolism in the regulation of starch turnover. Growth of wild type (WT) Arabidopsis seedlings on trehalose-containing medium without sucrose led to hyper-accumulation of starch in the cotyledons and inhibition of root growth (Wingler et al., 2000; Ramon et al., 2007). Expression of the $APL3$ gene, encoding one of the large subunits of AGPase, was induced by trehalose, whereas $SEX1$ (encoding GWD) and $BAM3$ were repressed.

The level of Tre6P in plant tissues fluctuates in parallel with endogenous changes in sucrose content, and in response to exogenously supplied sucrose, leading
to the proposal that Tre6P acts as a signal of sucrose availability in plants (Lunn et al., 2006). This strong correlation between Tre6P and sucrose has made it difficult to resolve which of the regulatory functions of sucrose are mediated by Tre6P and which are not. Therefore, we engineered Arabidopsis plants to express the *E.coli otsA* (*TPS*) gene under the control of an ethanol-inducible promoter, enabling us to bring about short-term changes in the level of Tre6P that are not driven by changes in sucrose. These plants were used to test *in vivo* the hypothesis that Tre6P mediates sucrose-linked changes in the rate of starch synthesis via redox regulation of AGPase (Kolbe et al., 2005), and to investigate the effect of Tre6P on remobilisation of leaf starch reserves. We also investigated how Tre6P might interact with the regulation of starch breakdown by the circadian clock.

**RESULTS**

**Ethanol-inducible expression of the *E. coli* TPS in Arabidopsis.**

Arabidopsis (accession Col-0) was transformed with a construct containing the *E. coli otsA* gene (encoding TPS) under the control of the *Aspergillus nidulans* AlcR/AlcA ethanol-inducible promoter system (Caddick et al., 1998). Primary transformants were selected on kanamycin and then screened by immunoblotting using antibodies against the *E. coli otsA* protein. Plants induced by spraying with 2% (v/v) ethanol contained an immunoreactive protein of the expected size (53-kDa) of the *E. coli otsA* (Supplemental Fig. S1). No expression of the protein was detected in wild-type (WT) or non-induced plants. After selfing, the T₂ and T₃ progeny were screened to select two independent homozygous lines TPS29.2 and TPS31.3 (see Materials and Methods for details). Based on Kan^R^:Kan^S^ segregation ratios in the T₂ generation, these lines each possessed a single transgenic locus (data not shown). In addition, one homozygous line (AlcR) was established that contained the 35S::AlcR::t-nos gene construct and the pAlcA::t-ocs promoter-terminator cassette with no insert, for use as a negative control.
Temporal kinetics of starch accumulation in response to induced over-expression of TPS

The AlcR and TPS29.2 lines were grown under 12-h photoperiod conditions, and sampled at 1- to 2-h intervals throughout the light period after spraying the plants with water (non-induced controls) or ethanol (induced). The results are shown in Fig. 1, with asterisks indicating time points where the induced TPS29.2 samples were significantly different from all three controls (i.e. AlcR plants sprayed with ethanol or water and TPS29.2 plants sprayed with water) using one-way analysis of variance (ANOVA; Holm-Sidak test).

The Tre6P content of the induced TPS29.2 plants was significantly higher than in the non-induced and AlcR control plants by 6 h after induction (Fig. 1A), and continued to increase up to the ED, when levels were comparable to those in the two earlier experiments (Fig. 1A; Supplemental Fig. S2A-B). The rate of starch accumulation in the induced TPS29.2 plants was the same as in the controls up to 6 h after induction, significantly higher between 6 and 8 h, and then comparable with the controls thereafter (Fig.1B). The sucrose content of the induced TPS29.2 plants was similar to the control plants throughout the day (Fig. 1C). The starch:sucrose ratio in the induced TPS29.2 plants was significantly higher than the controls at 4 h and 12 h after induction, and also tended to be higher at the intervening time points (Fig. 1D). Glucose, fructose, glucose 6-phosphate (Glc6P) and sucrose 6´-phosphate (Suc6P) tended to be lower in the induced TPS29.2 plants in the first hours after induction, but the differences were mostly small and statistically significant only for fructose at 2 h and Suc6P at 4 h after induction (Supplemental Figure S2C-F). The Tre6P:sucrose ratio was remarkably similar in all of the control plants and fairly stable throughout the day. The ratio in the induced TPS29.2 plants was significantly higher than the controls by 4 h after induction, continuing to rise up to 6 h but then changing relatively little during the second half of the day (Supplemental Fig. S2B).

The redox status of AGPase was determined by immunoblotting after SDS-polyacrylamide gel electrophoresis of leaf proteins under non-reducing conditions, which separates the reduced form of the APS1 small subunit protein (50-kDa monomer) from the oxidised form (100-kDa dimer). There were no striking differences in either the redox status of AGPase or the ADPG content of the induced
TPS29.2 plants compared to the controls, with the values from the former overlapping with at least one of the controls at any given time point (Fig. 1E-F). Retention of reduced APS1 protein during extraction and analysis was confirmed by including leaf extracts from WT Col-0 and the pgm mutant on each gel (see Supplemental Fig. S3). On average, the percentage of APS1 protein present as the 50-kDa monomer was 10 times higher in the pgm samples than in WT, comparable to the previously reported differences between these genotypes (Lunn et al., 2006).

In two further experiments, TPS29.2, TPS31.3, WT Col-0 and AlcR plants were sprayed with water or 2% (v/v) ethanol at the beginning of the day and harvested 12 h later, at the end of the day (ED). In both experiments, the WT (0.17-0.37 nmol g⁻¹FW) and AlcR (0.18-0.39 nmol g⁻¹FW) rosettes contained very similar amounts of Tre6P, with no significant differences between the two genotypes, or between water and ethanol-sprayed plants (Supplemental Fig. S4A-B). Induced TPS29.2 and TPS31.3 plants had much higher Tre6P (1.44-2.21 nmol g⁻¹FW), representing a 4 to 11-fold increase over the respective non-induced plants and control genotypes. There was no significant effect of induced over-expression of TPS on the sucrose or trehalose contents of the plants (Supplemental Fig. S4C-D, I) and only small changes in hexose sugars (Supplemental Fig. S4J-M). Ethanol spraying slightly decreased the amount of starch accumulated by WT plants by the ED and, in one experiment, increased ED starch content by about 50 % in the TPS31.3 line (Supplemental Fig. S4E-F). There were only small and mostly non-significant differences in the AlcR and TPS29.2 plants. Compared to the respective water-sprayed controls, the starch to sucrose ratio at the ED was slightly decreased in the WT plants (P<0.01) after ethanol treatment, unaffected in the AlcR plants, and 22-72% higher in the two TPS lines (Supplemental Fig. S4N-O). The percentage of APS1 protein in the 50-kDa monomeric form was not significantly affected by ethanol spraying except for a decrease in the AlcR line in one experiment (Supplemental Fig. S4G-H).

In summary, induction of TPS expression consistently increased Tre6P levels in the independent TPS lines, TPS29.2 and TPS31.3. There was a small but consistent increase in the starch:sucrose ratio in the induced TPS plants, but no reproducible effect on the redox status of AGPase or the starch content on its own.
Tobacco plants with constitutively elevated levels of Tre6P were reported to have higher rates of photosynthesis per unit leaf area than WT plants (Pellny et al., 2004). To test if a short-term rise in Tre6P could affect photosynthetic capacity, we measured net CO₂ assimilation rates in TPS29.2 and AlcR plants 12 h after spraying with water or 2% (v/v) ethanol. Induction of TPS expression had no obvious effect on photosynthetic CO₂ fixation in the TPS29.2 plants when rates were compared with those in unsprayed or water-sprayed plants, or in AlcR control plants (Supplemental Fig. S5).

Inhibition of starch degradation in Arabidopsis leaves with high Tre6P at night.

We next investigated whether Tre6P has any influence on starch remobilisation in Arabidopsis leaves at night. WT, AlcR, TPS29.2 and TPS31.3 plants were grown in soil with a 12-h photoperiod, in parallel with the plants described in Supplemental Fig. S4 (Experiment 1). The plants were sprayed with water or 2% (v/v) ethanol at the beginning of the night and harvested 12 h later, at the end of the night (EN), for metabolite and enzyme analysis.

Induction of TPS expression in the TPS29.2 and TPS31.3 plants led to a 2- to 3-fold increase in Tre6P compared to control plants, reaching levels (0.46-0.51 nmol g⁻¹FW; Fig. 2A) that were slightly higher than those seen in WT plants at the ED (Supplemental Fig. S4A-B). There were no significant changes in trehalose content in the TPS29.2 or TPS31.3 plants after induction (Fig. 2B). There was a small but significant increase in sucrose in WT plants after ethanol spraying, whereas sucrose fell slightly in all the other genotypes, with the decrease being significant in TPS29.2 and TPS31.3 (P<0.05; Fig. 2C). Ethanol treatment led to a significant increase in glucose in WT and AlcR plants but no change in the TPS lines (Fig. 2D), whereas fructose fell slightly in WT, AlcR and TPS29.2 plants (P<0.05) but not in TPS31.3 (Fig. 2E).

The WT and AlcR plants contained only a small residue of starch (5.4-8.5 µmol[Glc] g⁻¹FW) at the EN. In the parallel daytime induction experiment (Supplemental Fig. S4E), WT, AlcR and TPS control plants sprayed with water accumulated 62-79 µmol[Glc] g⁻¹FW of starch by the end of the day. This comparison
reveals that the WT and AlcR plants had degraded most, but not quite all, of their starch during the night (Fig. 2F), which is typical for Arabidopsis plants grown in a 12-h photoperiod (Gibon et al., 2004; Smith and Stitt, 2007; Hädrich et al., 2011). There was no obvious effect of ethanol on EN starch content in either of the control lines. In contrast, the induced TPS29.2 and TPS31.3 plants contained a much greater residual amount of starch at the EN (34-36 µmol[Gl] g⁻¹FW), indicating that they had degraded only half of their starch reserves (Fig. 2F).

Conceivably, resynthesis of starch from the products of starch degradation could have given the appearance of a lower net rate of starch breakdown in induced TPS29.2 and TPS31.3 plants. However, immunoblotting showed that AGPase was in the fully oxidised (i.e. low activity) form in all of the plants harvested at the EN, including the induced TPS29.2 and TPS31.3 plants (data not shown). Furthermore, there were no obvious differences in ADPG content between the induced TPS29.2 and TPS31.3 plants at the EN compared to the controls (data not shown). In fact, ADPG was barely detectable (<0.02 nmol g⁻¹FW) in any of the dark-harvested plants, in contrast to plants harvested in the light which contained 0.5-4.0 nmol g⁻¹FW ADPG (Fig. 1E). Thus resynthesis of starch appears unlikely to explain the higher EN starch content of the induced TPS plants.

In Arabidopsis leaves, the main product of starch degradation is maltose, which is exported from the chloroplasts to the cytosol and metabolised further to provide substrates for sucrose synthesis and respiration (Niittylä et al., 2004; Weise et al., 2004). Maltose levels were 40% lower in the induced TPS29.2 and TPS31.3 plants than in the mock-induced and other controls, with this decrease being significant in TPS29.1 (P<0.05) (Fig. 2G). Maltotriose appeared to be marginally but non-significantly decreased in both TPS lines (Fig. 2H).

The sugars produced by starch degradation are used for maintenance respiration and growth. The rate of dark respiration in ethanol-induced TPS29.2 plants was not significantly different from the rates in non-sprayed and water-sprayed controls, and a similar pattern was observed in AlcR control plants (Supplemental Fig. S6). Protein synthesis is essential for growth and represents one of the biggest energy demands for the plant. We used polysome loading analysis (Piques et al., 2004) as a proxy for assessing the rate of protein synthesis. During the night, the percentage of
ribosomes bound to mRNA was slightly lower in ethanol-induced TPS29.2 plants than in water-sprayed (i.e. non-induced) control plants (Supplemental Fig. S7), but the differences were not statistically significant.

These results showed that elevated levels of Tre6P led to substantial inhibition of starch degradation in Arabidopsis leaves at night. To better understand the effects of Tre6P on starch breakdown, we performed a series of time course experiments with the inducible-TPS lines, comparing the nocturnal changes in Tre6P, starch and sugars in induced versus non-induced plants.

**Temporal kinetics of induced changes in Tre6P, starch and sugar levels in the dark.**

TPS29.2 and TPS31.3 plants were grown in soil with a 12-h photoperiod and sprayed with either water or 2% (v/v) ethanol at the ED. Triplicate pools of five plants were harvested in the light before spraying (ED) and at 2-h or 4-h intervals during the night for metabolite analysis. The nocturnal changes in metabolites were qualitatively similar in both of the inducible TPS lines, therefore, for brevity results are shown only for line TPS29.2 (Fig. 3A-D).

With the onset of darkness, Tre6P fell in the non-induced plants and then began to rise after 2 h in the dark, but remained lower than the levels seen at the ED (Fig. 3A). In the induced plants, the initial fall in Tre6P was less pronounced in the induced plants, and by 2 h the induced plants contained significantly more Tre6P than the controls. Within 4 hours of induction, Tre6P exceeded the level at the ED, rising even further later in the night. These results suggest that within 2 h of induction, expression of the heterologous TPS might already be counteracting the fall in Tre6P levels seen in the first hours of the night.

In the non-induced plants, starch levels decreased at a more or less constant rate throughout the night, and by the EN the plants had remobilised almost 90% of their starch reserves (Fig. 3B). This resembles the pattern seen in WT plants in many previous experiments (Smith and Stitt, 2007; Graf et al., 2010; Pyl et al., 2012). Although the initial rate of starch degradation was similar in the ethanol-sprayed plants, it slowed down considerably from about 2 h after induction, and at the EN the plants retained about 30% of their original starch reserves.
In the non-induced plants, sucrose levels from 2-6 h into the night were about 20% lower than at the ED, and fell slightly further towards the EN (Fig. 3C). At all time points in the dark, the induced plants had less sucrose than the non-induced plants at the EN (Fig. 3C). Glucose decreased markedly at the beginning of the night in non-induced plants, and fell even further (27-43%) in the induced plants (Supplemental Fig. S8A). Fructose was also 25-46% lower in the induced plants (Supplemental Fig. S8B). Glc6P, Fru6P and Suc6P were all lower in the induced plants compared to the non-induced controls (Supplemental Fig. S8C-E). Maltose was low in the non-induced plants at the ED, increased over 4-fold during the first 2 h of darkness, and stayed more or less constant for the next 6 h before falling back again by the EN (Fig. 3D). In sharp contrast, maltose remained low throughout the night in the induced plants. These plants also had less maltotriose than the non-induced controls (Supplemental Fig. 8F).

A second time course experiment was carried out to investigate if starch breakdown was even more strongly inhibited if Tre6P levels were already elevated at the beginning of the night. Plants were sprayed with either water or ethanol in the middle of the day (i.e. 6 h before the ED), and rosettes were harvested at the ED and at 2-h or 4-h intervals during the night. At the ED and at each time point through the night, Tre6P levels were about 2-fold higher in the induced plants than the non-induced controls (Fig. 3E). In both sets of plants, Tre6P fell by about half over the first 4 h of darkness, showed a slight rise at 6 h but then fell back again towards the EN.

Starch decreased in a fairly linear manner throughout the night in the non-induced plants, and by the EN nearly 80% of the ED starch reserves had been remobilised (Fig. 3F). In the induced plants, which started the night with elevated levels of Tre6P, there appeared to be little loss of starch during the first half of the night as the plants retained almost 90% of their ED starch content after 6 h in the dark. The rate of starch degradation appeared to accelerate to some extent during the second half of the night. However, by the EN the plants still retained over 50% of their ED starch, which was twice as much as the non-induced controls. The acceleration of starch degradation in the second half of the night, while Tre6P levels stayed fairly constant, suggested that the inhibitory effect of high Tre6P on starch
breakdown had been partially overridden in some way. With the exception of fructose and Fru6P, the induced and non-induced plants showed qualitatively similar differences to those in the ED induction experiment, with the induced plants having lower sucrose and maltose levels (Fig. 3G-H), as well as less glucose, Glc6P, Suc6P and maltotriose (Supplemental Fig. S9A, C, E & F). Fructose was not significantly affected in the induced plants, and Fru6P only decreased towards the EN (Supplemental Fig. S9B & D).

To summarize, in both time course experiments the non-induced control plants showed fairly similar behaviour: (i) the rate of starch degradation was more or less constant throughout the night and by the EN most of the starch had been degraded; (ii) maltose was low at the ED and rose as the plants started to degrade their starch; and (iii) sucrose levels dropped when the lights went out, but during the middle hours of the night they tended to start rising again or at least stabilise. In comparison, the induced plants degraded less of their starch during the night, their maltose content increased to a much lesser extent or did not increase at all, they contained less maltotriose and there was a weaker recovery in sucrose levels after the initial fall at the beginning of the night.

**How does Tre6P affect starch degradation?**

Arabidopsis mutants that lack the capacity to export maltose from the chloroplasts (mex1) or metabolise it in the cytosol (dpe2) accumulate high levels of maltose (Niittylä et al., 2004; Chia et al., 2004; Lu and Sharkey, 2004). As the induced TPS plants had less maltose than the non-induced controls (Figs 2G, 3D, 3H), this suggested that neither maltose export nor its metabolism in the cytosol were restricted in the induced TPS plants. Therefore, to investigate potential targets for Tre6P inhibition, we focussed our attention mainly on upstream steps in the pathway of starch breakdown in the chloroplasts.

Immunoblotting of leaf extracts from AlcR, TPS29.2 and TPS31.3 plants showed no obvious differences in GWD, PWD or SEX4 protein abundance in ethanol versus water-sprayed plants (Supplemental Figs S10A-B and S11). Native polyacrylamide gel electrophoresis, followed by in-gel activity staining, indicated that the maximal activities of the plastidial phosphorylase (PHS1), cytosolic
phosphorylase (PHS2) and DPE2 were very similar in leaf extracts from AlcR, TPS29.1 and TPS31.3 plants, irrespective of whether the plants were sprayed with water or ethanol (Supplemental Fig. S10C-D). From these results we have no evidence of Tre6P-induced changes in the maximal activities or protein abundances of the main enzymes involved in starch phosphorylation and dephosphorylation (GWD, PWD, SEX4), the minor pathway of starch degradation via starch phosphorylase (PHS1), or the cytosolic pathway of maltose catabolism (DPE2 and PHS2).

We compared the structure and properties of starch granules from induced and non-induced TPS29.2 plants, to seek clues to the mechanism of inhibition of starch degradation in plants with elevated Tre6P. TPS29.2 plants were grown with a 12-h photoperiod, sprayed with either water or ethanol in the middle of the day, and harvested at 2-h intervals starting from the ED. Starch granules were isolated from both sets of plants and examined under the scanning electron microscope. The granules from both induced and non-induced plants appeared to be similar in size and general shape (Supplemental Fig. S12A-B), having the flat discoid structure that is typical of Arabidopsis leaf starch (Zeeman et al., 2002). However, the granules from the induced TPS plants harvested during the night, particularly at 2 and 4 h, appeared to have a more uneven surface than those from the non-induced plants (Supplemental Fig. S12C-J).

The C6-phosphate content of the granules was determined by gentle acidic hydrolysis of the α-1,4 and α-1,6 glucosidic bonds, releasing the individual glucose moieties from the glucan chains, followed by enzymatic measurement of Glc6P and glucose (Haebel et al., 2008). Total phosphate content of the starch granules, reflecting both C6 and C3 phosphorylation, was measured colorimetrically after acidic hydrolysis of the starch and treatment with alkaline phosphatase. Large numbers of plants were needed to isolate enough starch granules for these measurements, and due to growth space limitations only single determinations were possible for each time point. The results of two independent experiments are presented separately (Fig. 4). Tre6P was measured in replicate plants harvested in parallel with the samples for starch granule isolation, and was substantially higher in the induced plants compared to the non-induced controls (Fig. 4A-B). In both experiments, the C6-phosphate content tended to decrease during the night in starch.
granules from non-induced plants, but remained fairly constant in the granules from the induced TPS plants (Fig. 4C-D). The total phosphate content of the starch granules showed a similar pattern, generally decreasing through the night in the non-induced control plants, but remaining more or less constant in the induced plants with elevated Tre6P (Fig. 4E-F).

We investigated the ability of recombinant potato GWD and Arabidopsis PWD to phosphorylate starch granules isolated from induced and non-induced TPS29.2 plants. Phosphorylation was determined by measuring the incorporation of radioactivity upon incubation of isolated starch granules with the enzymes and $\beta$-$^{33}$P]ATP. Again, due to the large number of plants needed to isolate starch granules, only single measurements were possible at each time point, so the experiment was done twice and the results of each experiment are presented separately (Fig. 4G-H). While there were some differences between the two experiments, the following trends were observed in both experiments. The amount of $^{33}$P incorporated into the starch granules varied with the time of harvest of the plants, with little phosphorylation occurring with starch granules isolated from leaves harvested at the ED and more rapid phosphorylation at later times. In all of the samples harvested in the dark, less $^{33}$P was incorporated into the granules from induced plants than those from non-induced controls.

Together, these results indicate that induction of TPS affected both the phosphate content of the starch granules and their suitability as substrates for phosphorylation by GWD and PWD. This suggested that the cycle of phosphorylation by GWD and PWD and dephosphorylation by SEX4 phosphoglucan phosphatase (supplemented by LSF2) might be perturbed in plants with high night-time levels of Tre6P. As no evidence was found of Tre6P-induced changes in the abundance of the GWD, PWD or SEX4 proteins (Supplemental Figs S10-11), we investigated whether Tre6P or its derivative, trehalose, might act directly as activators or inhibitors of these enzymes.

Do Tre6P or trehalose directly affect the activities of enzymes involved in starch degradation?
The effects of Tre6P and trehalose on the activities of starch (de)phosphorylating enzymes were determined by in vitro activity assays of recombinant potato GWD, Arabidopsis PWD and Arabidopsis SEX4. Neither Tre6P (0.01-1.0 mM) nor trehalose (0.5-1.0 mM) had any consistent inhibitory or activating effect on GWD or SEX4 activity (Supplemental Table S1). The rate of phosphate incorporation by PWD was very low in the controls without Tre6P, and the addition of Tre6P did not affect the activity (data not shown). BAM3 is the main β-amylase isoform responsible for starch hydrolysis in Arabidopsis leaves (Fulton et al., 2008). Recombinant BAM3 activity was assayed colorimetrically in the absence or presence of 0.05-1.0 mM Tre6P or trehalose. Tre6P appeared to inhibit BAM3 activity very slightly, but even at the highest concentration tested (1 mM) the activity was only decreased by 12% (Supplemental Table S1). Trehalose had no obvious effect on BAM3 activity.

To summarize, the low maltose levels and altered patterns of starch phosphorylation in the induced TPS plants pointed to inhibition of an early step in the pathway of starch breakdown. However, the immunoblotting and enzyme activity experiments did not reveal any evidence of a direct effect of Tre6P on the enzymes involved in those steps, leaving open the possibility that Tre6P acts indirectly, and not necessarily within the chloroplasts, to inhibit starch degradation. Therefore, it was of interest to investigate whether Tre6P is even present in the chloroplasts.

**Subcellular compartmentation of Tre6P**

We used non-aqueous density gradient fractionation (Gerhardt et al., 1984; Stitt et al., 1989) to determine the subcellular compartmentation of Tre6P in WT Arabidopsis plants. This technique allows the chloroplastic and vacuolar pools of metabolites to be resolved from the rest of the cell, which is mostly comprised of the cytosol but also includes the mitochondria and peroxisomes.

WT Arabidopsis plants were grown in either short-day (8 h light/16 h dark) or equinoctial (12 h light/12 h dark) conditions. In both experiments, rosettes were harvested from 5-week-old plants around 4 h before the ED, and the leaf material was fractionated on three separate non-aqueous gradients (i.e. three technical replicates). The subcellular compartmentation of Tre6P was found to be similar in both sets of plants. Based on the distribution of marker enzymes for chloroplasts, cytosol and
vacuole, it was estimated that 16-22% of the Tre6P was in the chloroplasts, 7-11% in the vacuoles and 71-72% elsewhere, presumably mostly in the cytosol (Table 1).

As a cross check, we compared the distribution of Tre6P with metabolite markers for the three compartments. Suc6P, which is a cytosolic marker (MacRae and Lunn, 2006), showed an almost identical distribution to Tre6P across all three gradients from the short-day grown plants, with a Pearson’s correlation coefficient (r) of 0.981 (Supplemental Fig. S13A). Based on the distribution of marker enzymes, Suc6P was assigned predominantly (66-72%) but not exclusively to the cytosol in both experiments, although there was considerable variation between aliquots from the 12-h grown plants (Table 1). Tre6P was less well correlated (r=0.843; Supplemental Fig. S13B) with ribulose-1,5-bisphosphate (RuBP), a chloroplast metabolite marker (Raines, 2003), which was found to be almost exclusively in the chloroplasts (Table 1). Tre6P was poorly correlated with nitrate (r=0.363; Supplemental Fig. S13C), which is considered to be a vacuolar marker (Krüger et al., 2009).

The in vivo concentration of Tre6P in each compartment was estimated from the total Tre6P content of the leaf, its distribution between the chloroplasts, cytosol and vacuole, and the likely volumes of these compartments. In Arabidopsis leaves, starch is found predominantly in the palisade and spongy mesophyll cells. Therefore, to estimate the maximal in vivo concentrations likely to be found in these starch-containing cells, it was assumed that all of the Tre6P in the leaf was present in the mesophyll cells. In Arabidopsis, the palisade and mesophyll cells together represent between 72 and 89% of the total leaf thickness (Wuys et al., 2010), which is considered to be a reasonable proxy for the cell volume expressed as a percentage of the total leaf volume. However, these leaf thickness measurements do not take into account the contributions of the vascular tissue and intracellular air spaces to the total leaf volume, with the latter in particular likely to represent a substantial fraction of the total. Unpublished data (H. Poorter, FZJ, Jülich, Germany; pers. comm.) indicate that the mesophyll cells occupy 65-76% of the total leaf volume in Arabidopsis, with a mean value of 69%. We used this mean value in our calculations of the in vivo concentrations of Tre6P. Due to the lack of suitable estimates for subcellular volumes in Arabidopsis leaves, we used the percentages of total leaf volume reported for
spinach: chloroplasts 25.4%, cytosol 5.4% and vacuole 68% (Winter et al., 1994). These seem likely to be a reasonable approximation for Arabidopsis leaves, given that both species are herbaceous, non-sclerophyllous, non-succulent, C₃ eudicots. Furthermore, values reported for barley (chloroplasts 27.8%, cytosol 9.7% and vacuole 60.5%; Winter et al., 1993), a more distantly related monocot species, differ by a factor of less than two from the spinach data. The maximal in vivo concentrations of Tre6P in mesophyll cells were estimated to be between 0.18-0.46 μM in the chloroplasts, 3.81-6.71 μM in the cytosol and 0.05 μM in the vacuole (Table 1).

Responses of starch degradation, maltose levels and Tre6P after an unexpected early dusk

Finally, we investigated the relationship between circadian clock- and Tre6P-mediated regulation of starch breakdown. Starch breakdown is under the control of the circadian clock, which acts to prevent premature exhaustion of starch (Lu et al., 2005; Graf et al., 2010; Graf and Smith 2011; Yazdanbakhsh et al., 2011; Scialdone et al., 2013). If Arabidopsis plants are darkened several hours before the customary dusk, they break down their starch at a slower rate than usual so that their carbohydrate reserves are not exhausted before the expected dawn (Lu et al., 2005; Graf et al. 2010). Given the observed inhibitory effect of Tre6P on the rate of starch breakdown, we investigated if this clock-dependent regulation of starch breakdown is mediated by Tre6P, or if the clock acts independently of Tre6P, or even antagonistically to Tre6P.

WT plants were grown under equinoctial conditions for 3 weeks and harvested through two sequential diurnal cycles: the first being the control (12 h light/12 h dark), and the second the early dusk treatment, with the lights being switched off after 8 h, subjecting the plants to a 16-h night. A dense series of sampling points was included after both light-dark transitions to capture changes occurring during the period when the rate of starch degradation is likely to be set for the night ahead.

During the 12 h/12 h diurnal cycle, the plants accumulated starch in a linear manner through the day and degraded over 95% of their starch reserves during the night (Fig. 5A). The dense series of time points taken shortly after darkening the plants indicates that there may be a lag before net starch degradation begins. Maltose
levels were low during the light period but showed a significant rise between 8 h and the end of the light period, rose further but gradually over the first 2 h of the night, remained high until 6 h, and then declined (Fig. 5B). Sucrose increased in two phases during the 12 h light period, fell abruptly in the first hour of the night and then recovered (Fig. 5C). The gradual rise in maltose and the transient fall in sucrose levels provide supporting evidence that there is a delay before the plants begin to degrade their starch after being suddenly darkened (see also Pal et al., 2013). Tre6P tracked sucrose closely during the day and the first hour of the night, except that the recovery in Tre6P levels after falling at the beginning of the night was slightly delayed compared to sucrose (Fig. 5D). These transient changes were not clearly seen in the experiments shown in Fig. 3 because samples were not harvested until 2 h after dusk.

After the early dusk, starch was degraded at about half the rate seen during the preceding 12-h night (Fig. 5A), confirming the results of Graf et al. (2010). The dense time points for the starch (Fig. 5A), the slow increase of maltose (Fig. 5B) and the transient fall in sucrose (Fig. 5C) again indicate there may be a lag before starch degradation begins. After this transient, maltose levels peaked at a noticeably lower level than during the previous 12 h night and fell back more quickly, remaining at a low level throughout the second half of the night (Fig. 5B). Sucrose was also lower in the 16 h night than in the 12-h night. Tre6P levels fell in the first 30 min of darkness after the early dusk, as seen in the unperturbed light-dark cycle, but then remained low and more or less constant throughout the night (Fig. 5D). It is important to note that the levels of Tre6P in the 16-h night period following the early dusk were lower than in the 12-h night, even though the rate of starch breakdown was approximately 50% lower in the 16 h night than in the 12-h night (Fig. 5A).

Suc6P showed a similar pattern of changes to sucrose during both the 12 h/12 h and 8 h/16 h diurnal cycles (Fig. 5C and Supplemental Fig. S14A). ADPG was barely detectable during either the 12-h night or the prolonged 16-h night after the early dusk (Supplemental Fig. S14B).

DISCUSSION

Subcellular compartmentation and in vivo concentrations of Tre6P
In Arabidopsis, TPS1 is the only isoform that has been unequivocally shown to have TPS activity, based on *in vitro* activity measurements and yeast complementation assays (Blázquez et al., 1998; Harthill et al., 2006; Lunn, 2007; Ramon et al., 2009; Vandesteene et al., 2012). Although the subcellular location of the Arabidopsis TPS1 has not yet been experimentally determined, there is circumstantial evidence that it is restricted to the cytosol. The consensus of *in silico* prediction methods is that the TPS1 protein is cytosolic (http://suba.plantenergy.uwa.edu.au/; Tanz et al., 2012), and the TPS1 protein has not been detected so far in any proteomic analyses of Arabidopsis chloroplasts, mitochondria or other organelles. Furthermore, when the N-terminal domain of the orthologous *Selaginella lepidophylla* TPS1 was fused to a green fluorescent protein reporter, the fusion protein was not targeted to any recognisable organelle (van Dijck et al., 2002). In our TPS29.2 and TPS31.3 lines, the *E. coli* TPS (otsA) protein has no recognisable transit or signal peptide and so is expected to be targeted to the cytosol. Therefore, Tre6P should also be synthesised only in the cytosol after induction of these plants with ethanol.

Non-aqueous fractionation of Arabidopsis rosettes indicated that 16-22% of the Tre6P is located in the chloroplasts, 71-72% in the cytosol and 7-11% in the vacuoles (Table 1). These estimates are based on the distributions of enzyme markers for the three compartments and computer fitting of the data to a three-compartment model using the BESTFIT algorithm (Klie et al., 2011). The algorithm assigned a similar percentage (66-72%) of the Suc6P to the cytosol in both experiments (Table 1). However, there is compelling evidence that Suc6P is restricted to the cytosol. The only enzymes known to metabolise Suc6P are SPS and sucrose-phosphate phosphatase, both of which are strictly cytosolic (see MacRae and Lunn, 2006, and references therein), and we are not aware of any reports of Suc6P transporters in plants that might allow movement of Suc6P into other compartments. Thus, it seems reasonable to conclude that Suc6P is restricted to the cytosol, and that the BESTFIT assignments based on marker enzymes over-estimated the percentage of Suc6P in the chloroplasts and vacuole.

Tre6P showed an almost perfect correlation with Suc6P in the non-aqueous gradient fractions. It seems likely that the minor amounts of Tre6P assigned to the chloroplasts and vacuolar compartments by the BESTFIT algorithm reflect the technical...
limitations of the non-aqueous fractionation procedure, and fitting of the data to a three-compartment model that does not take into account the different cell types within the leaf (i.e. palisade and spongy mesophyll, epidermis, stomatal guard cells, trichomes and vascular tissue) (Klie et al. 2011).

The subcellular in vivo concentrations of Tre6P in Arabidopsis mesophyll cells were estimated to be 3.81-6.71 µM in the cytosol, 0.18-0.46 µM in the chloroplasts and 0.05 µM in the vacuole (Table 1). For the reasons outlined above, the calculated concentrations of Tre6P in the chloroplasts and vacuole are likely to be overestimates, and should be regarded as the upper limits of the Tre6P concentrations in these organelles. To our knowledge, these are the only experimentally based estimates of subcellular in vivo concentrations of Tre6P in plant cells to have been reported. We suggest that these estimates are a useful guide to the range of concentrations that should be used for in vitro experiments to investigate the targets of Tre6P signalling, and the physiological significance of any responses observed in vitro with much higher concentrations of Tre6P must be assessed with caution.

Regulation of starch synthesis by Tre6P

The rate of starch synthesis is determined by the rate of net CO₂ fixation and the partitioning of photoassimilates between starch and sucrose. These pathways are controlled by a complex network of transcriptional, allosteric and post-translational mechanisms (reviewed in MacRae and Lunn, 2006). According to the “overflow” hypothesis, starch is synthesised when the rate of photosynthesis exceeds the capacity of the leaf to export or store sucrose (Cseke et al., 1994; Stitt et al., 2010). The original model proposed that rising levels of sucrose in the leaf inhibit sucrose synthesis, leading to an increase in the ratio of 3PGA:Pi in the chloroplast stroma, which stimulates starch synthesis via allosteric activation of AGPase (Cseke et al., 1994). Kolbe et al. (2005) extended the model by proposing that Tre6P mediates sugar-induced increases in the rate of starch synthesis via redox activation of AGPase. Lunn et al. (2006) observed parallel changes in sucrose, Tre6P and the redox status of AGPase in WT and mutant Arabidopsis rosettes, which appeared to be consistent with the proposed role of Tre6P. However, experimental evidence of a causal relationship between these parameters in vivo was lacking. We tested the hypothesis by
investigating whether an induced rise in the level of Tre6P leads to reductive activation of AGPase and increased starch accumulation, in the absence of any increase in sucrose levels.

In three separate experiments, ethanol-induced overexpression of TPS led to a substantial (up to 11-fold) increase in the level of Tre6P (Fig. 1A; Supplemental Fig. S4A-B). In two out of the three experiments, the induced plants accumulated a little more starch than the non-induced controls by the end of the day (Fig. 1B; Supplemental Fig. S4C-D). The time course experiment suggested that there was a transient increase in the rate of starch accumulation from 4-6 h after induction (Fig. 1B), resulting in a higher ED starch content. The starch:sucrose ratio showed a consistent trend to be higher in the induced TPS plants, even though sucrose on its own was not significantly lower (Fig. 1C-D; Supplemental Fig. S4C-D, N-O). The rates of net CO2 assimilation in the induced TPS plants were indistinguishable from those in non-induced and AlcR control plants (Supplemental Fig. S5). Taken together, these results point towards a slight shift in photoassimilate partitioning in the induced TPS plants in favour of starch.

We found no consistent evidence of an increase in AGPase activation in the induced TPS plants. The redox status of AGPase after TPS induction was determined at the ED in three separate experiments and no reproducible differences between induced and non-induced plants were observed (Fig. 1F; Supplemental Fig. S4G-H). In one of these experiments, in which we examined the redox status of AGPase at time points throughout the day, as well as ED, there appeared to be a transient ethanol effect in both the AlcR and TPS29.2 lines in the middle of the day (Fig. 1F). However, at every sampling time, the redox status of AGPase in the induced TPS plants overlapped with at least one, usually more, of the controls. It is also worth noting that during the period from 4-6 h after induction, when there was the clearest evidence of an increased rate of starch synthesis in the induced plants (Fig. 1B), the AGPase was, if anything, less reduced (i.e. less activated) in the induced plants (Fig. 1F). Further, the induced and control plants contained very similar levels of ADPG not only at these time points, but also at the later time points (Fig. 1E). From these results, we conclude that the induced increase in Tre6P led to a slight stimulation of starch synthesis, but this was not due to reductive activation of AGPase.
There are several potential explanations why AGPase activation was not increased in the induced TPS plants. One possibility is that the basal levels of Tre6P in the plants were already saturating the putative mechanism by which Tre6P affects the redox status of AGPase. The concentration of Tre6P in the cytosol was estimated to be 7 µM in WT plants grown in a 12-h photoperiod (Table 1), presumably rising to 14-77 µM as a result of the 2- to 11-fold increase in Tre6P content observed in the induced TPS lines. However, in their isolated chloroplast experiments, Kolbe et al. (2005) reported that 100 µM Tre6P in the external medium was not quite saturating for the enzyme’s response. Furthermore, Lunn et al. (2006) reported that AGPase was considerably more reduced in the pgm mutant, which had 3-fold higher levels of Tre6P and sucrose, than in WT plants (see also Supplemental Fig. S4). Together, these observations suggest the absence of a clear AGPase response in the induced TPS plants is not due to the mechanisms involved in redox modulation of AGPase being saturated by the levels of Tre6P already present in WT plants. Another possible explanation is that redox activation of AGPase requires the concerted action of two or more signalling pathways. Tre6P and sucrose levels typically change in parallel (Lunn et al., 2006; see also Fig. 6). It is possible that a change in Tre6P levels is only effective when it is combined with other signalling events that are triggered when sucrose rises. In any case, it appears that changes in the level of Tre6P on their own do not play a dominant role in regulating the redox status of AGPase in leaves.

It is also worthwhile noting that abolition of the redox sensitivity of AGPase by site-directed mutagenesis of APS1 had surprisingly little effect on the rate of starch synthesis in plants grown in a 12-h photoperiod (Hädrich et al., 2011). Thus, even if the induced increase in Tre6P had led to redox-related activation of AGPase, this might not have had much impact on the rate of starch synthesis.

At present, we can only speculate on possible mechanisms by which Tre6P brings about this small shift in photosynthate partitioning in favour of starch. The cytosolic FBPase and SPS are considered to be the major sites for controlling sucrose synthesis (MacRae and Lunn, 2006). The former is regulated by Fru2,6BP, which is synthesised and degraded by a bifunctional kinase-phosphatase (F2KP), which in turn is regulated allosterically and by protein phosphorylation. SPS is regulated by phosphorylation at multiple sites by SnRK1 and calcium-dependent protein kinases.
SnRK1 from developing tissues is inhibited by micromolar concentrations of Tre6P (Zhang et al., 2009), but it seems unlikely that this sensitivity to Tre6P provides an explanation for a shift in photoassimilate partitioning in favour of starch synthesis. First, the SnRK1 activity in extracts from mature source leaves was found to be much less sensitive to inhibition by Tre6P (Zhang et al., 2009). Second, phosphorylation of the major SnRK1 target site (Ser158 in spinach leaf SPS) deactivates the enzyme (Huber and Huber, 1996), so any inhibition of SnRK1 activity by Tre6P would be expected to activate, not inhibit, SPS, and so favour sucrose over starch synthesis.

In conclusion, our results indicate that Tre6P does have some influence over the rate of starch synthesis and photoassimilate partitioning. The change in partitioning is, however, relatively small compared to those that occur in response to other stimuli, for example, short or long term changes in the photoperiod (Gibon et al., 2009; Hädrich et al., 2011). Furthermore, we found little evidence to support the hypothesis that Tre6P mediates sucrose-induced activation of starch synthesis via reductive activation of AGPase. We suggest that the simplest interpretation of our results is that sucrose-induced changes in the redox status of AGPase occur via a mechanism that does not involve Tre6P, and that the correlation between Tre6P and the redox status of AGPase (Lunn et al., 2006) is driven by parallel but independent responses to sucrose. Nevertheless, we cannot rule out the possibility that Tre6P can act to promote activation of AGPase, but only in the presence of further signalling events that are initiated by an increase in sucrose. Labelling experiments on the inducible TPS lines, using $^{13}$CO$_2$ or $^{14}$CO$_2$ to measure photosynthetic fluxes into sucrose and starch (Szecowka et al., 2013), combined with measurements of Fru2,6BP and the activation status of SPS would appear to be useful avenues for future research to understand how Tre6P influences photoassimilate partitioning.

**Inhibition of starch degradation mediated by Tre6P**

Induced increases in the Tre6P content of Arabidopsis rosettes at night led to a strong inhibition of starch degradation, resulting in the plants retaining up to half of their starch reserves at the EN (Fig. 2). This contrasts markedly with the relatively weak
effect on starch synthesis and points to Tre6P playing a major role in the regulation of starch breakdown.

Tre6P acts at an early step in starch breakdown. Maltose is the predominant product of starch breakdown in Arabidopsis leaves, and is exported from the chloroplasts to the cytosol where it provides substrates for sucrose synthesis, respiration and other processes (Niittylä et al., 2004; Weise et al., 2004). The mex1 and dpe2 mutants contain high levels of maltose, due to blocks in maltose export from the chloroplasts and catabolism in the cytosol, respectively (Niittylä et al., 2004; Chia et al., 2004; Lu & Sharkey, 2004). The dpe1 mutant accumulates maltotriose and maltotetraose in the chloroplasts as it is unable to metabolise these maltooligosaccharides (Critchley et al., 2001). Whilst maltose rose rapidly in the dark as starch began to be degraded in WT or non-induced control plants (Figs 3D, H; 5B), this increase was abolished or greatly attenuated in the induced plants with high Tre6P (Figs 2G; 3D, H), which contained 2- to 3-fold lower maltose levels than those in WT plants at the same time of the night. Induced TPS plants also had less maltotriose than the controls (Fig. 2H; Supplemental Figs S8F and S9F). From these results, we conclude that Tre6P must be acting, directly or indirectly, to inhibit the pathway of starch degradation at some point upstream of maltose production.

There is considerable redundancy between the main starch debranching enzymes that are involved in starch degradation – isoamylase 3 and limit dextrinase – and their functions can be at least partially replaced by the plastidial α-amylase (Streb et al., 2012). Therefore, unless Tre6P inhibits all three enzymes, it seems unlikely that the starch debranching process is the primary target of Tre6P. This leaves us with the earliest steps in starch degradation as the most likely targets for the inhibitory effect of high Tre6P: (i) the cycle of phosphorylation by GWD and PWD and dephosphorylation by SEX4 and LSF2, and (ii) glucan chain exohydrolysis by β-amylase (especially BAM3).

Starch granules isolated from non-induced TPS29.2 plants had the typical discoid structure of Arabidopsis leaf starch, with a smooth surface. In contrast, the starch granules from induced plants had an irregular surface (Supplemental Fig. S12). This might indicate that binding of the starch degrading enzymes is disrupted or
blocked over large areas of the granule surface, preventing the more even pattern of degradation usually observed in Arabidopsis leaf starch granules.

Starch granules isolated from induced TPS29.2 plants generally had a higher phosphate content than those from non-induced plants, and were found to be a poorer substrate for phosphorylation by recombinant GWD and PWD \textit{in vitro} (Fig. 4). It should be noted that our measurements of phosphate content would include phosphate groups embedded within the granule as well as those on the surface of the granule, the latter being the most likely to influence further phosphorylation by GWD and PWD and hydrolysis of the glucan chains by \( \beta \)-amylose. In the non-induced control plants, both the C6-phosphate and total phosphate content showed a tendency to decline through the night (Fig. 4). The surface area to volume ratio of the granules increases as the granules are degraded and become smaller, so the falling phosphate content indicates that the density of phosphorylated sites on the surface of the granules in the non-induced plants is likely to decrease through the night. In contrast, both the C6-phosphate and total phosphate content remained fairly constant in the starch granules from the induced TPS plants.

One explanation for both the inhibition of starch degradation and the observed differences in phosphate content could be perturbation of the cycle of starch phosphorylation (by GWD and PWD) and dephosphorylation (by SEX4 and LSF2) by high Tre6P. An alternative explanation might be inhibition of \( \beta \)-amylose by Tre6P. If the starch granules were not degraded due to inhibition of this enzyme, their surface area and volume, and by implication their phosphate content, would remain unchanged. It is also possible that a phosphorylation-dependent alteration of the structure of the granule surface could affect both the hydrolytic degradation of the starch and/or the (de)phosphorylation cycle.

We found no evidence of changes in the amounts of the GWD, PWD or SEX4 proteins in the induced TPS plants (Supplemental Figs. S10-11). \textit{In vitro} assays indicated that GWD, PWD, SEX4 and BAM3 are insensitive to Tre6P (Supplemental Table S1 and data no shown), even at concentrations (0.5-1.0 mM) that are 1000-5000 times higher than the estimated concentration of Tre6P in the chloroplasts \textit{in vivo} (Table 1). These enzymes also showed little sensitivity to trehalose.
Failure to detect changes in the *in vitro* activities of these enzymes in the presence of Tre6P must be interpreted with caution, as it is possible that factors needed for a response to Tre6P were absent from the *in vitro* assays. However, the simplest conclusion is that these enzymes are not directly sensitive to Tre6P. In this context it is worth considering whether they would even encounter Tre6P *in vivo*. We estimated the *in vivo* concentration of Tre6P in the chloroplasts to be 0.18-0.46 µM (Table 1) but, for the reasons discussed above, even these very low values might be an over-estimate and the possibility there is no Tre6P in the chloroplasts cannot be excluded. Based on the subcellular compartmentation of Tre6P, it seems more likely that Tre6P triggers a response in the cytosol or at the surface of the chloroplasts, which subsequently leads to inhibition of starch breakdown, rather than acting directly on the enzymes involved in starch degradation within the chloroplast.

In summary, we found several lines of evidence that Tre6P acts to inhibit one or more of the early steps in the starch degradation pathway within the chloroplast, probably indirectly via a signalling pathway that is initiated in the cytosol: (i) the majority, perhaps even all, of the Tre6P in Arabidopsis leaves is located in the cytosol, (ii) induced TPS plants have low maltose, pointing to inhibition of an early reaction in starch breakdown; (iii) there is no accumulation of maltotriose, ruling out inhibition of the chloroplastic DPE1; and (iv) there are changes in the visual appearance and the phosphorylation status of the starch granules. Whatever, the mechanism, we suggest that inhibition of starch degradation by Tre6P is likely to be an important factor underlying the high-starch phenotype observed in Arabidopsis plants with constitutive over-expression of TPS (Schluempmann et al., 2003; Kolbe et al., 2005), and probably more important than stimulation of starch synthesis by Tre6P.

**The inhibition of starch degradation by Tre6P results in a decrease in sucrose levels during the night.**

The inhibition of starch degradation in induced TPS plants with increased Tre6P was accompanied by a decrease in sucrose levels during the night (Figs 2C; 3C, G). These plants also had less Suc6P (Supplemental Figs S8E and S9E), which is consistent with the lower sucrose being due to decreased synthesis rather than increased use of sucrose. The decrease in sucrose was most pronounced between 4-6 h into the night.
The difference between induced and non-induced plants became smaller towards the end of the night as sucrose levels gradually declined in the control plants, coinciding with the gradual depletion of starch. In our time course experiments the first time point analysed during the night was at 2 h after dusk. At this time, Tre6P was significantly higher in the induced plants, while sucrose and maltose levels were lower than in non-induced plants (Fig. 3). The starchless \textit{pgm} mutant does not accumulate maltose at night (Niittylä et al., 2004), and \(\beta\)-amylase deficient mutants that degrade their starch more slowly than WT plants have lower maltose levels than WT during the first half of the night (Fulton et al., 2008). These observations indicate that the rise in maltose levels in WT plants after dusk is dependent on starch breakdown. Thus the low maltose in the induced TPS plants at 2 h after dusk provides indirect evidence that starch breakdown is already inhibited in these plants, even if this is not obvious from the starch measurements themselves. It should be noted that rates of starch degradation inferred from the differences in starch content at successive time points lack precision, especially during the early hours of the night when starch content is high and small differences in starch content are more difficult to observe because of the technical limitations of the starch assay. For this reason, we suggest that maltose content is a more reliable guide to the rate of starch breakdown during the early hours of the night, and the low maltose levels in the induced TPS plants at 2 h after dusk indicate that starch breakdown is already inhibited by Tre6P at this time. Inhibition of starch degradation will restrict the supply of substrates for sucrose synthesis, offering a simple explanation for the lower sucrose content of the induced TPS plants.

An alternative explanation for the low level of sucrose in the induced lines might be that high Tre6P has stimulated sucrose consumption. Tre6P has been proposed to stimulate growth by inhibiting the SnRK1 protein kinase in developing tissues (Zhang et al., 2009; Debast et al., 2011). Higher growth rates at night would be expected to increase respiration of sucrose to provide the energy and carbon skeletons needed for growth, potentially explaining the lower sucrose levels in the induced TPS plants. However, we found that induced TPS plants did not have significantly higher dark respiration rates (Supplemental Fig. S6). In fact, if anything, respiration rates showed a tendency to be lower after induction of the TPS plants. Polysome loading, a
proxy for protein synthesis, also did not increase after induction of the TPS plants (Supplemental Fig. S7), and even showed a tendency to be lower in the induced plants at 2 and 4 h after dusk. This coincides with the time when the differences in maltose and sucrose content became apparent (Fig. 3). As respiration and protein synthesis are major requirements for growth, these results argue against increased sucrose consumption, resulting from a stimulation of growth by Tre6P, being the primary cause of the low sucrose levels in the induced TPS plants at night.

Control of starch mobilization by the circadian clock also involves inhibition of an early step in the pathway of starch degradation

There is compelling evidence that the circadian clock plays a major role in control of night-time starch degradation (Lu et al., 2005; Graf et al., 2010; Graf and Smith, 2011). If WT Arabidopsis plants growing under 12 h light/12 h dark conditions are darkened several hours before the usual dusk, they not only start the night with a lower amount of starch but also face a longer night than usual. Despite this, they still manage to degrade their starch in a linear manner at a rate that avoids them running out of starch before the EN (Graf et al., 2010). This adjustment implies that the plant is able to measure the amount of starch present in the leaves and predict the length of the night, the latter being a potential readout of the circadian clock (Graf and Smith, 2011; Stitt and Zeeman, 2012). The mechanisms by which the plant measures its starch content and sets the rate of degradation are unknown, although modelling has provided a conceptual framework for understanding how the necessary calculation (i.e. starch divided by time) might be achieved (Scialdone et al., 2013).

The regulation of starch breakdown by the clock is important for optimization of plant growth. WT plants grown in abnormally short (20 h) or long (28 h) light-dark cycles mistime their starch turnover during the night (Graf et al., 2010), and grow more slowly than control plants in a 24-h light-dark cycle (Dodd et al., 2005). In contrast, the short-period clock mutant cca1/lhy exhausts its starch reserves before the end of the night, grows more slowly than WT plants when grown under 12 h light/12 h dark conditions (Graf et al., 2010), and grows better in a 10 h light/10 h dark cycle, in which the plants are reilluminated before their starch reserves run out (Dodd et al., 2005). Yazdanbakhsh et al. (2011) showed that the premature exhaustion of starch in
the *cca1/lhy* mutant under 12 h light/12 h dark conditions is accompanied by an inhibition of root growth at the end of the night, and that this inhibition can be reversed by exogenous supply of sucrose. Thus, under optimal growth conditions, it appears to be advantageous for the plant to time its remobilisation of transitory starch so that it avoids premature depletion of these reserves before the end of the night.

It is not yet known how the clock regulates starch turnover, whether it acts in a direct or indirect manner, and whether Tre6P is directly involved or not. One hypothetical example of an indirect mechanism would be for the clock to restrict growth, resulting in accumulation of sugars, an increase of Tre6P and inhibition of starch breakdown. A clock-driven increase in Tre6P leading to inhibition of starch degradation, independently of changes in sucrose, is also conceivable. To distinguish between these possibilities, we repeated the early-dusk experiment of Graf et al. (2010). The lower rate of starch degradation after an early dusk was accompanied by: (i) lower levels of Tre6P; (ii) lower levels of intermediates in the starch breakdown pathway (maltose, maltotriose); and (iii) lower levels of the products of starch breakdown and related metabolites (sucrose, Glc6P, Suc6P) (Fig. 5; Supplemental Fig. S14). These results provide evidence that the clock regulates starch breakdown by acting at an early step in the pathway, upstream of maltose. Most importantly, the lower Tre6P levels after the early dusk show that Tre6P is not an intermediate in the clock-dependent signalling pathway that sets an appropriate rate of starch degradation for the expected length of the night. If anything, the lower level of Tre6P would be expected to allow a faster rate of starch degradation due to the lifting of the inhibitory effect of this metabolite.

The clock and Tre6P interact to regulate starch breakdown

We propose that the clock and Tre6P interact to determine the rate of starch degradation (Fig. 6). The clock paces the mobilisation of starch to ensure that starch reserves are not exhausted before the end of the night. However, it is important to note that under growth conditions that allow large amounts of C to be fixed during the day (e.g. high light and long days), or which restrict growth at night, the plant might not use up all of its starch reserves by dawn (Hadrich et al., 2011; Stitt and Zeeman, 2012). The implication is that the clock sets a maximum permissible rate of starch
degradation, but does not drive a faster rate of starch degradation than is necessary to supply the sucrose needed for growth and maintenance. We propose that Tre6P plays a significant role in matching the rate of starch breakdown with sucrose use. If the environmental conditions during the night are unfavourable for growth (e.g. low temperature, limiting nutrients), lower demand for sucrose by growing sink organs may limit sucrose export from the source leaves. The resulting accumulation of sucrose will trigger a rise in the level of Tre6P, as this signal metabolite closely tracks any fluctuations in the level of sucrose (Fig. 5; Lunn et al., 2006), resulting in an inhibition of starch degradation. In this way, the plant would avoid remobilizing its starch reserves unnecessarily when the carbon is not needed to support growth. Conversely, if conditions become more favourable for growth and demand for sucrose exceeds the supply from starch breakdown, both sucrose and Tre6P levels would fall, lifting the inhibition of starch degradation. However, the rate of starch breakdown will not be allowed to rise above the maximum limit set by the clock. In summary, our model envisages the rate of starch breakdwon to be jointly regulated by the circadian clock and Tre6P, with the clock-dependent regulation preventing premature exhaustion of starch before dawn, while Tre6P links the rate of degradation to the demand for sucrose.

MATERIALS AND METHODS

Plant Material

The *E. coli otsA* gene encoding TPS (E.C. 2.4.1.15) was cloned into the SpeI site of the pSRN binary vector (Caddick et al., 1998), downstream of the AlcA promoter. The resulting construct was introduced into *Arabidopsis thaliana* (L.) Heyhn. accession Columbia-0 (Col-0) via *Agrobacterium tumefaciens* transformation using the floral dip method (Clough and Bent, 1998). A control line (AlcR) expressing only the AlcR transcription factor was generated by transforming Arabidopsis Col-0 with the empty pSRN plasmid. Primary transformants were selected on ½ MS medium (Murashige and Skoog, 1962) containing 50 mg L⁻¹ kanamycin. Kanamycin resistant pSRN/otsA
Plants were further screened for ethanol-inducible expression of the otsA protein by immunoblotting. T2 progeny from the primary transformants were screened by kanamycin selection, and lines showing segregation patterns consistent with the presence of a single transgenic locus (3:1 KanR:KanS) were re-screened in the T3 generation to identify homozygous T2 lines.

**Plant growth conditions and ethanol induction**

Seeds from Arabidopsis were sown on soil mixed with vermiculite (1:1) in 10-cm pots and placed for 1 week in a phytotron under long day conditions, 16 h (20°C) light/8 h (4°C) dark, with an irradiance of 160 μE m−2 s−1. In the second week, pots were transferred to a short day phytotron, 8 h (20°C) light/16 h (16°C) dark, at the same irradiance. At 2 weeks after sowing, seedlings were transplanted into 10-cm pots (5 plants per pot) and transferred to equinoctial conditions (12 h light/12 h dark) at constant 20°C, with an irradiance of 130 μE m−2 s−1, until harvest.

Four-week-old plants were sprayed to run-off with water (mock-induction control) or 2% (v/v) ethanol and harvested at various times from 2 to 18 h after spraying, as indicated for each individual experiment. Whole rosettes were excised under the ambient growth conditions and immediately quenched in liquid nitrogen. Plants from one or two pots (i.e. five or ten plants) were pooled to form one sample, ground to a fine powder at -70°C using a robotized ball mill (Stitt et al., 2007), and stored at -80°C until use.

**Extraction and measurement of metabolites**

Soluble sugars (glucose, fructose and sucrose) were extracted with ethanol and enzymatically assayed according to Stitt et al. (1989). Starch was determined enzymatically in the insoluble material after ethanolic extraction of soluble sugars (Hendriks et al., 2003). Tre6P and phosphorylated intermediates were extracted in chloroform-methanol and measured by high performance anion exchange chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described by Lunn et al. (2006), using a Finnigan TSQ Quantum (ThermoFinnigan, Waltham, MA, U.S.A.) or a QTrap 5500 MS-Q3 (AB Sciex, Foster City, USA) triple quadrupole mass spectrometer. Tre6P was quantified using enzymatically calibrated standards.
and a [\textsuperscript{2}H]Tre6P internal standard to correct for ion suppression and matrix effects (Lunn et al., 2006). Trehalose was quantified fluorimetrically in the same chloroform-methanol extract as described by Mollo et al. (2011).

Maltose and maltotriose were measured using a modification of the method described by Fulton et al. (2008). The extraction was performed with 1.5 N perchloric acid and the extract was neutralised with 2 N KOH/0.4 M MES/0.4 M KCl. The neutral fraction was separated and analysed by high pressure anion exchange chromatography on a CarboPac PA-100 4×250 mm column (ThermoFisher GmbH, Idstein, Germany) with pulsed amperometric detection as in Fulton et al. (2008). Maltose and maltotriose were identified and quantified by comparison with original standards (Carbosynth Limited, Berkshire, UK) using Chromeleon software (version 6.8, ThermoFisher).

**Non-aqueous fractionation**

Frozen tissue powder was lyophilized and fractionated by centrifugation on anhydrous hexane-tetrachloroethylene gradients as described by Gerhardt and Heldt (1984), with the modifications for Arabidopsis leaf material described in Krüger et al. (2009). The following markers were used in the experiments reported in Table 1 (\textsuperscript{a}experiment 1 only, \textsuperscript{b}experiment 2 only; \textsuperscript{c}both experiments): chloroplasts – AGPase\textsuperscript{a}, ribulose-1,5-bisphosphate carboxylase/oxygenase\textsuperscript{a}, transketolase\textsuperscript{b} and phosphoribulokinase\textsuperscript{b}; cytosol – UDP-glucose pyrophosphorylase\textsuperscript{c} and phosphoenolpyruvate carboxylase\textsuperscript{c}; vacuole – acid invertase\textsuperscript{c}, nitrate\textsuperscript{a} and α-glucosidase\textsuperscript{b}. The subcellular distribution of Tre6P between chloroplasts, cytosol and vacuoles was calculated using the BESTFIT program (Klie et al., 2011).

**Native polyacrylamide gel electrophoresis (PAGE)**

Soluble proteins were extracted from 200 mg of powdered frozen leaf material with 1 mL extraction buffer containing: 50 mM HEPES-NaOH buffer (pH 7.5), 1 mM EDTA, 5 mM dithioerythritol, 0.5 mM phenylmethanesulfonylfluoride and 10 % (v/v) glycerol. Following centrifugation (12,000 x g for 10 min, 4°C), aliquots of the supernatant were assayed for total protein content (Bradford, 1976). For analysis of cytosolic disproportionating enzyme (DPE2) and cytosolic/chloroplastic
phosphorylase (PHS2 and PHS1) activities, aliquots containing 20 µg protein were separated by native PAGE under non-denaturing conditions, as described by Steup (1990). Electrophoresis was performed at 4°C at constant 250 V and the separating gels were stained according Fettke et al. (2005).

**Immunoblotting**

A His₆-tagged otsA protein was overexpressed in *E. coli* and purified by metal ion affinity chromatography and gel filtration (otsA) as described in Lunn et al. (2006). Antisera were raised by immunisation of rabbits with the purified otsA protein. Antibodies were purified by affinity chromatography on an otsA-Sepharose column, generated by coupling the His₆-otsA protein to a 1-mL HiTrap NHS-activated HP Sepharose pre-packed column (GE Healthcare Biosciences, Little Chalfont, UK). SDS-PAGE and immunoblotting with NBT/BCIP colorimetric detection was carried out as described in Lunn et al. (1999), using the anti-otsA antibody at a 1:20,000 dilution. Quantification of GWD, PWD and SEX4 (Niittylä et al., 2006) proteins by immunoblotting was performed as in Fettke et al. (2004).

The redox status of AGPase was determined by immunoblotting of leaf proteins after SDS-PAGE under non-reducing conditions as described in Hendriks et al. (2003). Leaf extracts from the WT Col-0 and the starch-deficient pgm mutant were included as controls in each gel to allow correction for blot-to-blot variation. Quantitative detection of the Arabidopsis APS1 protein was performed using an Odyssey infra-red imaging system (LiCor) as described in Hädrich et al. (2011). The percentage of total APS1 protein present as the 50-kDa monomer was determined for each sample after subtraction of the background. For each individual immunoblot, the percentage monomer values from the WT Col-0 and pgm samples were compared with the averages for those samples across all the immunoblots and used to calculate a linear scaling factor for normalisation of the other samples.

**Starch granule isolation and scanning electron microscopy**

Powdered frozen leaves (15-20 g) were extracted in 50 mL of extraction buffer (20 mM HEPES-KOH, 0.4 mM EDTA, 0.5 % (v/v) Triton X-100, pH 7.5) using a 250-mL goblet blender. The homogenate was filtered sequentially through nylon nets with
mesh sizes of 100 µm, 20 µm and 15 µm, and the final filtrate was centrifuged at 1000×g (4°C) for 5 min. The supernatant was discarded and the pellet was washed twice by resuspension in 15 ml of extraction buffer and centrifugation. The resulting pellet was suspended in 500 µl H2O, layered on top of a 15-ml cushion of 95 % (v/v) Percoll (GE Healthcare Bio-Sciences) in water and centrifuged at 2000×g (4°C) for 15 min. After centrifugation, the Percoll cushion was discarded, the pelleted starch granules were washed three times by resuspension in 30 ml H2O and centrifugation at 1000×g (4°C) for 5 min. The final pellet was then frozen in liquid nitrogen and lyophilized. The surface structure of the starch granules was examined under a Quanta 200 scanning electron microscope (FEI, Eindhoven, The Netherlands) after sputter coating of the granules with gold under a low vacuum.

**Determination of starch phosphate content**

About 6 mg of isolated starch granules were hydrolysed by incubation with 300 µl of 0.7 N HCl for 4 h at 95°C. After neutralisation with 1.8 N NaOH containing 0.2 mM Tris (base), glucose and Glc6P were determined enzymatically according to Stitt et al. (1989). To determine total phosphate content, aliquots of 200 µL of the hydrolysed starch were incubated in a reaction mixture (final volume 300 µl) containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5mM MgCl2 and 20 U of alkaline phosphatase for 1 h at 37°C, and orthophosphate was measured colorimetrically using malachite green reagent (Harder et al., 1994; Worby et al., 2006).

**Incorporation of phosphate into starch by recombinant GWD and PWD**

Isolated starch granules (about 30 mg) were suspended in 1 mL H2O. Aliquots (188 µL) of the suspension were incubated in a reaction mixture (final volume 250 µl) containing: 50 mM HEPES-KOH, pH 7.5, 6 mM MgCl2, 2 mM EDTA, 2 mM dithiothreitol, 25 µM ATP, 1 µCi [β-33P]ATP, 20 µg bovine serum albumin, 0.1 µg recombinant GWD from *Solanum tuberosum* (StGWD) and 0.1 µg recombinant PWD from Arabidopsis (AtPWD). The reaction mixture was incubated at 30°C with continuous agitation for 25 min. Every five min, 50-µL samples were removed and mixed with 50 µL of 10 % (w/v) SDS to stop the reaction. The starch granules were pelleted by centrifugation at 10,000×g, at room temperature for 5 min. The
supernatant was discarded and the pellet was washed five times by resuspension in 1 ml of 5 mM HEPES-KOH, pH 7.4, containing 2 mM ATP, and centrifugation as described above. The washed granules were resuspended in 100 µL water before addition of 3 mL of scintillation cocktail and determination of radioactivity by liquid scintillation counting, using an external standard to correct for quenching (Hejazi et al., 2008).

**Enzyme assays**

*Glucan, water dikinase* – GWD activity was assayed by measuring the incorporation of $^{33}$P from [$\beta$-$^{33}$P]ATP into the B-type allomorph of crystallized maltodextrin (MD$_{\text{cryst}}$). The reaction (final volume 100 µL) contained: 50 mM HEPES-KOH (pH 7.5), 6 mM MgCl$_2$, 2 mM EDTA, 2 mM dithioerythritol, 25 µM unlabelled ATP, 1 µCi [$\beta$-$^{33}$P]ATP, 20 µg bovine serum albumin, with 4 mg of MD$_{\text{cryst}}$ as substrate and 0.5 µg recombinant StGWD. The reaction mixture was incubated at 30°C with continuous agitation. Aliquots (50 µL) were taken at 2.5, 5, 10 and 20 min, heated at 95°C for 3 min to stop the reaction and incorporation of $^{33}$P determined as described in Hejazi et al., 2008.

*Phosphoglucan, water dikinase* – PWD activity was assayed by measuring the incorporation of $^{33}$P from [$\beta$-$^{33}$P]ATP into B-type MD$_{\text{cryst}}$ that had been pre-phosphorylated by incubation with unlabelled ATP and StGWD (Hejazi et al., 2008). The basic PWD assay reaction mixture was identical to that described above for GWD above, except that 4 mg phosphorylated MD$_{\text{cryst}}$ was used as the substrate, and the StGWD was replaced by 0.5 µg recombinant AtPWD.

*SEX4 phosphoglucan phosphatase* – SEX4 activity was assayed by measuring the release of $^{33}$P from A-type MD$_{\text{cryst}}$ that had been pre-phosphorylated by incubation with [$\beta$-$^{33}$P]ATP and StGWD as described above. The reaction contained recombinant SEX4 enzyme from Arabidopsis (AtSEX4) and was carried out as described in Hejazi et al. (2010).

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LITERATURE CITED


tyrosine phosphatase β (HPTP β) using synthetic phosphopeptides. Biochem J 298: 395–401


regulatory mechanism linking starch synthesis to the sucrose supply. Plant Cell **14**: 2191–2213


FIGURE LEGENDS

Figure 1. Effect of TPS over-expression on metabolites and the redox status of ADPglucose pyrophosphorylase in Arabidopsis rosettes during the day.
Ethanol-inducible TPS line 29.2 (TPS) and plants expressing the ethanol-binding transcription factor (AlcR) were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water or 2% (v/v) ethanol at the beginning of the day and harvested at 1 or 2-h intervals after spraying for determination of: (A) Tre6P, (B) starch, (C) sucrose, (D) the starch:sucrose ratio, (E) ADPG, and (F) the redox status of ADP-glucose pyrophosphorylase (AGPase). Grey symbols at t=0 represent unsprayed AlcR (▲) or TPS (■) plants. Data are mean ± S.D. (n=3). Significant differences (one-way ANOVA, Holm-Sidak test) between the ethanol-sprayed TPS line and the three controls – AlcR (sprayed with water or ethanol) and TPS (water) – are indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Induced changes in the Tre6P content of Arabidopsis rosettes at night.
Ethanol-inducible TPS plants (TPS29.2 and TPS31.3) were grown in soil with a 12-h photoperiod. Wild-type (Col-0) and plants expressing the AlcR ethanol-binding transcription factor (AlcR) were grown as controls. Four-week-old plants were sprayed with water (white bars) or 2% (v/v) ethanol (black bars) at the beginning of the night. Pools of ten rosettes were harvested 12 h later at the end of the night for determination of: (A) Tre6P, (B) trehalose, (C) sucrose, (D) glucose, (E) fructose, (F) starch, (G) maltose and (H) maltotriose. Data are mean ± S.D. (n=3). Significant difference between ethanol and water treated plants from the same genotype are indicated by asterisks (Student’s t-test) *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Inhibition of starch degradation at night by induced high levels of Tre6P.
Ethanol-inducible TPS29.2 plants were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water (○) or 2% (v/v) ethanol (●) at the end of the day (A-D) or in the middle of the day (E-H). Pools of five rosettes were harvested at the end of the day and at 2 or 4 h intervals through the night for determination of: (A,E) Tre6P, (B,F) starch, (C,G) sucrose and (D,H) maltose. Data are mean ± S.D. (n=3). Significant differences between the water and ethanol treated plants at the same
time point are indicated by asterisks (Student’s t-test) *P<0.05, **P<0.01, ***P<0.001.

**Figure 4.** Phosphate content and phosphorylation of starch granules isolated from induced and non-induced TPS29.2 plants at night.

Inducible TPS29.2 plants were sprayed with water (open symbols) or 2% (v/v) ethanol (closed symbols) 6 h before the end of the day. Rosettes were harvested at the end of the day and at 2-12 h into the night for determination of Tre6P (A,B) and isolation of starch granules. The phosphate content of the starch granules was determined after enzymatic and acid hydrolysis. (C,D) Pi content (C₆ only). (E,F) total phosphate content (C₆ + C₃). (G,H) *In vitro* phosphorylation of the granules by recombinant potato GWD and Arabidopsis PWD was determined by incorporation of ³³P from [β-³³P]ATP. Tre6P data are mean ± S.D. (n = 3 or 4). Significant differences between ethanol and water-treated plants are indicated by asterisks (Student’s t-test): *P<0.05, **P<0.01, ***P<0.001. Starch granule data are single measurements from two independent experiments: Expt. 1 (A,C,E,G) and Expt. 2 (B,D,F,H).

**Figure 5.** Effect of an early dusk on metabolite levels in Arabidopsis leaves.

Wild type Arabidopsis Col-0 plants were grown in soil with a 12-h photoperiod for 3 weeks. Pools of five rosettes were harvested at various intervals through two sequential diurnal cycles: (i) 12 h light/12 h dark (control), and (ii) 8 h light/16 h dark (early dusk treatment) for measurement of: (A) starch, (B) maltose, (C) sucrose and (D) Tre6P. Data are mean ± S.D. (n=4).
Table 1. Subcellular compartmentation of Tre6P in Arabidopsis rosettes.

Wild type Arabidopsis Col-0 plants were grown in: (i) 8 h light/16 h night (Experiment 1), and (ii) 12 h light/12 h dark (Experiment 2) conditions for five weeks. In both experiments, rosettes were harvested around 4 h before the end of the light period, rapidly quenched in liquid N₂, ground to a fine powder at -70°C, lyophilized, and then fractionated by centrifugation on three separate non-aqueous density gradients. The amount of Tre6P in the original plant material and in the gradient fractions was measured by anion-exchange LC-MS/MS, using enzymatically calibrated standards and a [²H]Tre6P internal standard to correct for ion suppression and matrix effects (Lunn et al., 2006). Suc6P was measured by anion-exchange LC-MS/MS (Lunn et al., 2006), and RuBP by ion-pair reverse-phase LC-MS/MS (Arrivault et al., 2009). The subcellular distribution of each metabolite between chloroplasts, cytosol and vacuoles was determined by reference to marker enzymes and metabolites for each compartment, using the BESTFIT program (Klie et al., 2011). The in vivo concentrations were estimated assuming that all of the metabolites were in the mesophyll cells and that these cells occupy 69% of the total leaf volume (see text for details). For each experiment, data represent the mean ± S.D. of measurements on three aliquots of the pooled plant material harvested in the individual experiments.
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<th>Metabolite</th>
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SUPPLEMENTAL DATA FILES

**Supplemental Figure S1.** Inducible expression of E. coli otsA (TPS) in Arabidopsis.
Immunoblot of leaf proteins (20 μg per lane) extracted from: (i) WT Col-0, (ii) TPS29.2 sprayed with ethanol and (iii) TPS29.2 sprayed with water, probed with antibody raised against the E. coli otsA (TPS) protein. Leaves were harvested 24 h after spraying.

**Supplemental Figure S2.** Effect of TPS over-expression on the metabolite content of Arabidopsis rosettes during the day.
Ethanol-inducible TPS line 29.2 (TPS) and plants expressing the ethanol-binding transcription factor (AlcR) were grown in soil with a 12-h photoperiod. Thirty-day-old plants were sprayed with water or 2% (v/v) ethanol at the beginning of the day and harvested at 1 or 2-h intervals after spraying for determination of: (A) Tre6P, (B) Tre6P:sucrose ratio (C) glucose, (D) fructose, (E) Glc6P, and (F) Suc6P. Grey symbols at t=0 represent unsprayed AlcR (▲) or TPS (■) plants. Data are mean ± S.D. (n = 3). Significant differences (one-way ANOVA, Holm-Sidak test) between the ethanol-sprayed TPS line and the three controls – AlcR (sprayed with water or ethanol) and TPS (water) – are indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001.

**Supplemental Figure S3.** Immunoblotting of the APS1 protein in Arabidopsis leaf extracts to determine the redox status of AGPase.
Arabidopsis rosette leaves were extracted in trichloroacetic acid to maintain the redox status of the APS1 protein and subjected to immunoblotting after SDS-PAGE under non-reducing conditions as described in Hendriks et al. (2003). Quantitative detection of APS1 protein was performed using an Odyssey infrared imaging system as described in Hädrich et al. (2011). Extracts from WT Col-0 and the pgm mutant were included as controls on each gel to allow correction for variation between gels. The redox status of AGPase is expressed as the percentage of total APS1 protein present as the 50-kDa monomer. The values were normalised by linear scaling to the average values for the WT Col-0 and pgm values across all the blots. (M = molecular weight marker)
Supplemental Figure S4. Effect of TPS over-expression on the metabolite content of Arabidopsis rosettes during the day.

Ethanol-inducible TPS plants (TPS29.2 and TPS31.3) were grown in soil with a 12-h photoperiod. Wild-type (Col-0) and plants expressing the AlcR ethanol-binding transcription factor (AlcR) were grown as controls. Four-week-old plants were sprayed with water (white bars) or 2% (v/v) ethanol (black bars) at the beginning of the day. Data are shown from two independent experiments: Expt. 1 (left column) and Expt. 2 (right column). Pools of ten rosettes were harvested 12 h later at the end of the day for determination of: (A-B) Tre6P; (C-D) sucrose; (E-F) starch; (G-H) oligomeric status of ADP-glucose pyrophosphorylase (n.b. different y-axis scales in G and H); (I) trehalose (Expt. 1 only); (J-K) glucose; (L-M) fructose; and (N-O) starch/sucrose (based on µmol[hexose equivalents] g⁻¹FW). Data are mean ± S.D. (n=3, Expt. 1 or n=4, Expt. 2). Significant differences between ethanol and water treated plants from the same genotype are indicated by asterisks (Student’s t-test) *P<0.05, **P<0.01, ***P<0.001.

Supplemental Figure S5. Effect of induced changes in Tre6P on photosynthetic CO₂ assimilation in Arabidopsis.

AlcR and TPS29.2 plants were grown in soil with a 12-h photoperiod. Net CO₂ assimilation rates at different intercellular CO₂ concentrations were measured in intact rosettes of 3-week-old plants by infra-red gas analysis using a Li-Cor LI-6400XT Photosynthesis System (Li-Cor, Lincoln NE) (red symbols). The plants were then sprayed with either water (A,B) or 2% (v/v) ethanol (C,D) in the middle of the day, and net CO₂ assimilation rates were measured 24 h after spraying (black symbols).

Supplemental Figure S6. Effect of induced changes in Tre6P on dark respiration in Arabidopsis rosettes.

AlcR and TPS29.2 plants were grown in soil with a 12-h photoperiod. Dark respiration rates were measured in intact rosettes of unsprayed (white bars) 3 to 4-week-old plants by infra-red gas analysis using a Li-Cor LI-6400XT Photosynthesis System (Li-Cor, Lincoln NE). The plants were then sprayed with either water (grey
bars) or 2 % (v/v) ethanol (black bars) at the end of the day, and dark respiration rates were measured the following day (12-22 h after spraying). Values are mean ± S.D. (n = 7 individual plants). There were no significant differences between genotypes or treatments according to one-way ANOVA.

**Supplemental Figure S7.** Effect of Tre6P on polysome loading in Arabidopsis rosettes at night.

Ethanol-inducible TPS29.2 plants were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water or 2% (v/v) ethanol at the end of the day. Pools of five rosettes were harvested at the end of the day and at 2-h intervals through the night (samples are the same as those shown in Fig. 3A-D). Polysome loading was determined by sucrose density gradient centrifugation. Data are mean ± S.D. (n=3). There were no significant differences between the water and ethanol treated plants at the same time point according to Student’s t-test.

**Supplemental Figure S8.** Effect of inducing Tre6P synthesis at the end of the day on metabolite levels during the following night.

Ethanol-inducible TPS29.2 plants were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water (○) or 2% (v/v) ethanol (●) at the end of the day. Pools of five rosettes were harvested at the end of the day and at 2 or 4 h intervals through the night for determination of: (A) glucose, (B) fructose, (C) Glc6P, (D) Fru6P, (E) Suc6P and (F) maltotriose. Data are mean ± S.D. (n=3). Significant differences between the water and ethanol treated plants at the same time point are indicated by asterisks (Student’s t-test) *P<0.05, **P<0.01, ***P<0.001.

**Supplemental Figure S9.** Effect of inducing Tre6P synthesis in the middle of the day on metabolite levels during the following night.

Ethanol-inducible TPS29.2 plants were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water (○) or 2% (v/v) ethanol (●) 6 h before the end of the day. Pools of five rosettes were harvested at the end of the day and at 2 or 4 h intervals through the night for determination of: (A) glucose, (B) fructose, (C) Glc6P, (D) Fru6P, (E) Suc6P and (F) maltotriose. Data are mean ± S.D. (n=3).
Significant differences between the water and ethanol treated plants at the same time point are indicated by asterisks (Student’s t-test) \(^*P<0.05, \^{**}P<0.01, \^{***}P<0.001\).

**Supplemental Figure S10.** Effect of induced changes in Tre6P on enzymes involved in starch degradation in Arabidopsis rosettes.

Inducible TPS lines (TPS29.2 and TPS31.3) and AlcR (negative control) were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed at the end of the day with either water (mock-induced) or ethanol (induced) and harvested at the end of the night. In each section, the upper and lower panels show data from water and ethanol sprayed plants, respectively. Protein abundance of (A) glucan, water dikinase (GWD) and (B) phosphoglucan, water dikinase (PWD) was determined by immunoblotting. Catalytic activities of (C) cytosolic disproportionating enzyme (DPE2), and (D) plastidial glucan phosphorylase (PHO1) and cytosolic glucan phosphorylase (PHO2) were compared by in situ activity staining following gel electrophoresis under non-denaturing conditions. Three independent biological replicates are shown for each line.

**Supplemental Figure S11.** Effect of induced changes in Tre6P on the SEX4 phosphoglucan phosphatase in Arabidopsis rosettes.

Immunoblot showing the abundance of SEX4 phosphoglucan phosphatase protein in leaf extracts from inducible TPS29.2 plants sprayed with water (-) or 2% ethanol (+) 6 h before the end of the day (ED) and harvested at 2-h intervals during the night. M, prestained protein molecular weight markers.

**Supplemental Figure S12.** Scanning electron micrographs of starch granules isolated from induced and non-induced TPS29.2 plants.

Inducible TPS29.2 plants were sprayed with water (A,C,E,G,I) or 2% ethanol (B,D,F,H,J) 6 h before the end of the day (ED), and harvested at the ED (A,B) and at 2 h (C,D), 4 h (E,F), 6 h (G,H) and 8 h (I,J) into the night. Starch granules were isolated, lyophilised, coated with gold, and imaged by scanning electron microscopy. Bar = 2 \(\mu\)m.
**Supplemental Figure S13.** Subcellular compartmentation of Tre6P in Arabidopsis rosettes.

Wild type Arabidopsis Col-0 plants were grown in short day (8 h light/16 h night) conditions (see Table 1, Experiment 1). Rosettes were harvested from 5-week-old plants between 4-5 h into the light period, frozen and ground to a fine powder in liquid N₂, lyophilized and fractionation by centrifugation on non-aqueous density gradients. The amounts of Tre6P in each fraction are compared with the amounts of: (A) Suc6P (metabolite marker for the cytosol), (B) RuBP (chloroplast) and (C) nitrate (vacuole). Data are individual measurements from three gradients derived from the same batch of leaf material. Pearson’s correlation coefficient (r) is shown for each pairwise comparison.

**Supplemental Figure S14.** Effect of an early dusk on metabolite levels in Arabidopsis leaves.

Wild type Arabidopsis Col-0 plants were grown in soil with a 12-h photoperiod for 3 weeks. Pools of five rosettes were harvested at various intervals through two sequential diurnal cycles: (i) 12 h light/12 h dark (control), and (ii) 8 h light/16 h dark (early dusk treatment) for measurement of: (A) Suc6P and (B) ADPG. Data are mean ± S.D. (n=4).
Figure 1. Effect of TPS over-expression on metabolites and the redox status of ADPglucose pyrophosphorylase in Arabidopsis rosettes during the day.

Ethanol-inducible TPS line 29.2 (TPS) and plants expressing the ethanol-binding transcription factor (AclR) were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water or 2% (v/v) ethanol at the beginning of the day and harvested at 1 or 2-h intervals after spraying for determination of: (A) Tre6P, (B) starch, (C) sucrose, (D) the starch:sucrose ratio, (E) ADPG, and (F) the redox status of ADP-glucose pyrophosphorylase (AGPase). Grey symbols at t=0 represent unsprayed AclR (grey triangle) or TPS (grey square) plants. Data are mean ± S.D. (n = 3). Significant differences (one-way ANOVA, Holm-Sidak test) between the ethanol-sprayed TPS line and the three controls – AclR (sprayed with water or ethanol) and TPS (water) – are indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001.
Figure 2. Induced changes in the Tre6P content of Arabidopsis rosettes at night. Ethanol-inducible TPS plants (TPS29.2 and TPS31.3) were grown in soil with a 12-h photoperiod. Wild-type (Col-0) and plants expressing the AlcR ethanol-binding transcription factor (AlcR) were grown as controls. Four-week-old plants were sprayed with water (white bars) or 2% (v/v) ethanol (black bars) at the beginning of the night. Pools of ten rosettes were harvested 12 h later at the end of the night for determination of: (A) Tre6P, (B) trehalose, (C) sucrose, (D) glucose, (E) fructose, (F) starch, (G) maltose and (H) maltotriose. Data are mean ± S.D. (n=3). Significant difference between ethanol and water treated plants from the same genotype are indicated by asterisks (Student’s t-test) *P<0.05, **P<0.01, ***P<0.001.
Figure 3. Inhibition of starch degradation at night by induced high levels of Tre6P.
Ethanol-inducible TPS29.2 plants were grown in soil with a 12-h photoperiod. Four-week-old plants were sprayed with water (open symbols) or 2% (v/v) ethanol (closed symbols) at the end of the day (A-D) or in the middle of the day (E-H). Pools of five rosettes were harvested at the end of the day and at 2 or 4 h intervals through the night for determination of (A,E) Tre6P, (B,F) starch, (C,G) sucrose and (D,H) maltose. Data are mean ± S.D. (n=3). Significant differences between the water and ethanol treated plants at the same time point are indicated by asterisks (Student's t-test), * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 4. Phosphate content and phosphorylation of starch granules isolated from induced and non-induced TPS29.2 plants at night.
Inducible TPS29.2 plants were sprayed with water (open symbols) or 2% (v/v) ethanol (closed symbols) 6 h before the end of the day. Rosettes were harvested at the end of the day and at 2-12 h into the night for determination of Tre6P (A,B) and isolation of starch granules. The phosphate content of the starch granules was determined after enzymatic and acid hydrolysis. (C,D) Pi content (C₆ only). (E,F) total phosphate content (C₆ + C₃). (G,H) In vitro phosphorylation of the granules by recombinant potato GWD and Arabidopsis PWD was determined by incorporation of ³²P from [³²P]ATP. Tre6P data are mean ± S.D. (n = 3 or 4). Significant differences between ethanol and water-treated plants are indicated by asterisks (Student’s t-test): *P<0.05, **P<0.01, ***P<0.001. Starch granule data are single measurements from 10 and 12 h time points from Expt. 1 (A,C,E,G) and Expt. 2 (B,D,F,H).
Figure 5. Effect of an early dusk on metabolite levels in Arabidopsis leaves.
Wild type Arabidopsis Col-0 plants were grown in soil with a 12-h photoperiod for 3 weeks. Pools of five rosettes were harvested at various intervals through two sequential diurnal cycles: (i) 12 h light/12 h dark (control), and (ii) 8 h light/16 h dark (early dusk treatment) for measurement of: (A) starch, (B) maltose, (C) sucrose and (D) Tre6P. Data are mean ± S.D. (n=4).
Figure 6. Integrated model of the control of starch breakdown by Tre6P and the circadian clock.

The maximum permissible rate of starch degradation is set by the circadian clock to ensure that starch reserves are not exhausted before the expected dawn. If sucrose export is restricted by low demand from sink organs, sucrose accumulates in the leaves and Tre6P rises leading to inhibition of an early step in the pathway of starch degradation upstream of maltose production. As the core components of the clock operate in the nucleus, the pathway whereby it regulates starch breakdown presumably involves transport of an unidentified signal into the chloroplast. Tre6P probably also inhibits starch breakdown via an intermediary that is formed in the cytosol and transmitted to the chloroplast.