Sugars and light/dark exposure trigger differential regulation of ADP-glucose pyrophosphorylase genes in *Arabidopsis thaliana* (thale cress)

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Expression of four *Arabidopsis* (thale cress) genes corresponding to the small (*ApS*) and large subunits (*ApL1*, *ApL2*, *ApL3*) of ADP-glucose pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis, was found to be profoundly and differentially regulated by sugar and light/dark exposures. Transcript levels of both *ApL2* and *ApL3*, and to a lesser extent *ApS*, increased severalfold upon feeding sucrose or glucose to the detached leaves in the dark, whereas the mRNA content for *ApL1* decreased under the same conditions. Glucose was, in general, less effective than sucrose in inducing regulation of AGPase genes, possibly due to observed limitations in its uptake when compared with sucrose uptake by detached leaves. Osmotic agents [sorbitol, poly(ethylene glycol)] had no effect on *ApS*, *ApL2* and *ApL3* transcript level, but they did mimic the effect of sucrose on *ApL1* gene, suggesting that the latter is regulated by osmotic pressure rather than any particular sugar. For all the genes the sugar effect was closely mimicked by an exposure of the dark-pre-adapted leaves to the light. Under both dark and light conditions, sucrose fed to the detached leaves was found to be rapidly metabolized to hexoses and, to some extent, starch. Starch production reflected most probably an increase in substrate availability for AGPase reaction rather than being due to changes in AGPase protein content, since both the sugar feeding and light exposure had little or no effect on the activity of AGPase or on the levels of its small and large subunit proteins in leaf extracts. The data suggest tight translational or post-translational control, but they may also reflect spatial control of AGPase gene expression within a leaf. The sugar/light-dependent regulation of AGPase gene expression may represent a part of a general cellular response to the availability/allocation of carbohydrates during photosynthesis.

INTRODUCTION

ADP-glucose pyrophosphorylase (AGPase) is the first committed step of starch biosynthesis in all plants [1–3]. The enzyme is encoded by two sets of genes corresponding to the small and large subunits. Both types of subunits are required for a functional AGPase protein [3–5]. Starch-less and starch-deficient mutants lacking either the small or large subunit of AGPase have been isolated and characterized in some plants [3,5], including *Arabidopsis* (thale cress) [4,6]. A starch-deficient background was also obtained through 'antisense' technology by introducing a specific DNA construct inhibiting expression of an AGPase gene in potato (*Solanum tuberosum*) tubers [1] and leaves [7]. In photosynthetic tissues, the AGPase enzyme is believed to be highly regulated, utilizing carbon skeletons that are derived from newly fixed photosynthetic intermediates, and requires 3-phosphoglycerate (PGA) for maximal activity [2,8]. In certain storage tissues, e.g. the seeds of barley (*Hordeum vulgare*) and legumes, the enzyme may be much less prone to metabolic regulation, reflecting the presence of isoenzyme(s) that are distinct from those in leaves [2,9–11].

Over the last few years, evidence has been obtained for the occurrence of multiple genes encoding AGPase. In barley and *Arabidopsis*, where cDNAs for AGPase were comprehensively amplified by using PCR [12,13], there is apparently only one gene for the small subunit and two or more for the large subunit. Restriction-fragment-length-polymorphism studies in barley have suggested that the large subunit is encoded by a family of genes, with up to five distinct loci mapped [14]. In maize (*Zea mays*), potato, legumes and some other plants, two genes for the small subunit and three or more for the large subunit have been documented [11,15–17].

Despite the progress in molecular cloning and characterisation of AGPase primary structure, relatively little attention has been paid to the regulation of AGPase gene expression in plants. This is surprising, considering the key role of the enzyme in starch biosynthesis, a major plant pathway. There have been indications that an AGPase gene is susceptible to the regulation by sucrose [18–21] and the light/dark regime [18,21], but no comprehensive attempt to analyse sucrose/light regulation for all AGPase genes in a given plant species has been documented. In view of the complex feedback regulation of starch and sucrose pathways in plants (see, e.g. [22–25]), sugar signalling may represent an important regulatory mechanism for AGPase gene expression [18,21]. Several other genes that are inducible/repressed by elevated levels of sugars have already been identified in various plant species (e.g. [23,26,27]), opening exciting new avenues of research that focus on molecular dissection of sugar-signalling pathways [25,28]. In some cases homologous genes encoding proteins of similar function were found to be differentially regulated by sugars, for example as shown for genes corresponding to a number of isoenzymes involved in sugar conversions that are located in plastids and cytosol of *Chenopodium* (red goosefoot) [20], or for genes for sucrose synthase in maize [27].

An obvious obstacle in studies on regulation of the expression of related homologous genes (e.g. those encoding AGPase isoenzymes) is the necessity to use gene-specific probes in order to monitor individual responses, but not a sum of responses of homologous genes. Fortunately, such cDNA clones are available

Abbreviations used: AGPase, ADP-glucose pyrophosphorylase; ApS, small subunit of AGPase; ApL, large subunit of AGPase; PEG, poly(ethylene glycol); PGA, 3-phosphoglycerate.

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for AGPase from *Arabidopsis* [13], and they have already proved useful in characterizing starch-deficient AGPase mutants of this species [6]. In the present study we demonstrate that the multiple genes encoding AGPase in *Arabidopsis* are differentially regulated by both the exogenous provision of sugars and by light exposure, with the light effects mimicking closely those caused by the sugar feeding. In addition, metabolism of sugars fed to the leaves has been evaluated, and the nature of specific sugar signal(s) initiating the transduction pathway for AGPase genes is discussed.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, plants were grown in a chamber with 8 h white light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$, 23 °C) and 16 h darkness (19 °C) photoperiod regime. For the experiments, fully grown leaves of mature plants (6–7 weeks old) were used, after intact plants were kept in the dark for 6 h (unless stated otherwise). Petioles of the detached leaves were placed into containers with water or sugars/osmotica, and the leaves exposed to light or darkness, as indicated in the legends to Figures and Table. The submerged parts of the leaf petioles were removed from the samples before leaves were frozen in liquid N_2 and further stored at −80 °C.

Northern-blot analyses

Total RNA was isolated by guanidine thiocyanate-based method [29] with some modifications. Approx. 0.15 g of frozen tissue was ground in liquid N_2 and then homogenized in the presence of a denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 % sodium sarkosyl, 0.1 M 2-mercaptoethanol and 0.2 M sodium acetate, and extracted with 1 vol. of water-saturated phenol/chloroform (1:1, v/v). Total RNA from aqueous phase was precipitated with ammonium acetate and ethanol, and the pellet washed with 4 M LiCl to remove contaminating polysaccharides prior to a final double wash with 75 % ethanol and dissolving in deionized formamide. Aliquots of 15 μg of the isolated total RNA were used for Northern-blot analyses [30]. For RNA separation, 1.2 %-agarose gels with formaldehyde and ethidium bromide were used. Following Northern-blot transfer, Hybond-N+ (Amersham) membranes with the blotted/immobilized RNA were hybridized with gene-specific cDNA probes for AGPase (*ApS-1*, *ApL1-1*, *ApL2* and *ApL3*). Both *ApL2* and *ApL3* were isolated by PCR amplification of *Arabidopsis* transcripts [13], whereas *ApS-1* and *ApL1-1* represented near-full-length cDNAs (P. Villand, personal communication; [6]) isolated from an *Arabidopsis* cDNA library by probing with PCR-amplified *ApS* and *ApL1* cDNA clones [13]. Hybridizations with the probes and subsequent washes were performed at 65 °C, following standard procedures [30]). Multiple Northern blots were analysed in each experiment and a representative profile is presented.

Determination of sugars and starch

The frozen leaf material (approx. 100 mg) was ground to a fine powder in liquid N_2 and homogenized with a mortar and pestle in 0.8 ml of 0.2 M KOH/0.08 % Triton X-100. Soluble sugars were extracted on ice for 15 min. Glucose and fructose contents in the supernatant were determined by a spectrophotometric enzymic assay, recording the reduction of NAD^+ at 340 nm. The assay buffer contained 100 mM triethanolamine, pH 7.6, 5 mM $MgCl_2$, 10 mM ATP and 3 mM NAD^+, to which 2 units of hexokinase and 2 units of glucose-6-phosphate dehydrogenase were added to measure glucose. After completion of the reaction, 5 units of phosphoglucose isomerase was added to measure fructose. Sucrose was assayed in the same manner as glucose, after a 20 min hydrolysis step of an aliquot of the supernatant in a citrate buffer (200 mM, pH 4.6) containing 5 units of β-fructosidase. For starch determination the pellet was rinsed once in 0.5 ml of citrate buffer, then resuspended in 0.5 ml of citrate buffer and boiled for 10 min. Starch was digested at 55 °C for 24 h by adding 6 units of amyloglucosidase. The samples were then boiled for 10 min and the glucose released was assayed in the supernatant. All enzymes were supplied by Boehringer-Mannheim.

AGPase assay

The frozen leaf material (approx. 100 mg) was ground to a fine powder in liquid N_2 and homogenized with a mortar and pestle in the extraction buffer (buffer A) containing 40 mM Mops, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA and 10 mM dithiothreitol. AGPase was assayed in the supernatant in the direction of ADP-glucose synthesis [8]. The reaction medium contained 200 mM Tes, pH 8.0, 5 mM $MgCl_2$, 1 mM ATP, 0.25 mg/ml BSA, 0.5 mM [U-^{14}C]glucose 1-phosphate (sp. radioactivity 2500 d.p.m./nmol), 1 unit of inorganic pyrophosphatase (Sigma) and 1 mM PGA. The reaction was initiated by adding the sample and stopped after 10 min at 37 °C by boiling for 1 min. The sample mixture was then incubated with 2 units of alkaline phosphatase (Sigma) for 1 h at 37 °C. ADP-glucose was adsorbed on to DE81 ion-exchange chromatography paper (Whatman) and counted for radioactivity by liquid-scintillation spectroscopy.

Other methods

For the determination of AGPase protein content, leaves were ground with liquid N_2 using mortar and pestle, and the powder resuspended in buffer A. Conditions of protein electrophoresis were as described in [10]. The SDS/PAGE-resolved proteins were transferred on to nitrocellulose membrane (Bio-Rad) using a Bio-Rad protein-transfer system. Immunodetection of the small and large subunits of AGPase proteins was carried out using rabbit antibodies against purified AGPase from tomato (*Lycopersicon esculentum*) fruits [31]. In some experiments, rabbit antibodies raised against *Escherichia coli*-overproduced small subunit of AGPase from barley seed endosperm [32] were used. Binding of these primary antibodies to the AGPase protein(s) was detected with donkey anti-rabbit secondary antibodies linked to horseradish peroxidase (Amersham); the peroxidase activity was revealed using ECL fluorescence reagents (Amersham).

RESULTS

Sugar/light regulation and kinetics of AGPase gene responses

Prior to all experiments, *Arabidopsis* plants were kept in the dark for 6 h in order to lower the internal level of soluble sugars before the treatment. Total RNA fractions, isolated from darkened leaves of *Arabidopsis* that were fed for 12 h with various sugars/osmotica, were examined by Northern-blot analyses for the expression of four AGPase genes (Figure 1). Steady-state levels of transcripts for *ApS*, *ApL2* and *ApL3* increased profoundly upon feeding sucrose or glucose to the detached leaves in the dark, whereas transcript contents of *ApL1* decreased under the same conditions. Sucrose at 0.1 M was slightly more effective after 12 h in inducing changes in transcript levels when compared with its effects at 0.3 M (Figure 1). Substantial regulation was also observed at sucrose concentrations as low as 0.05 M (results

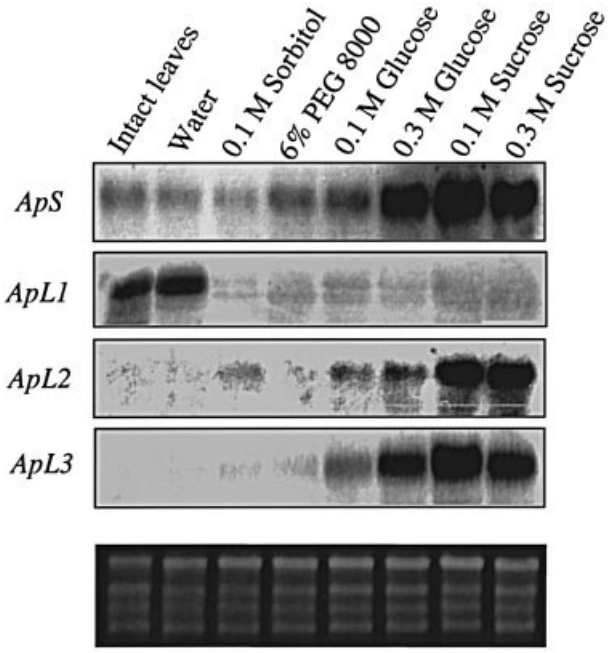

Figure 1 Sugar/osmoticum regulation of *Arabidopsis* AGPase gene expression

Rosette leaves detached from 6–7-week-old plants (pre-adapted to darkness for 6 h) were placed in glass tubes containing water or a given sugar/osmoticum and kept for 12 h in the dark. After feeding, total RNA was isolated from the leaves and analysed on Northern blots using cDNA probes corresponding to *ApS*, *ApL1*, *ApL2* and *ApL3*. The RNA was also isolated from leaves of intact plants that were pre-adapted in the dark. The bottom panel represents an ethidium bromide-stained gel demonstrating that equal amounts of RNA are present in each lane.

not shown). Glucose, with the exception of its effect on *ApL1*, appeared less effective than sucrose in regulating the levels of AGPase transcripts. With the exception of *ApL1*, responses to metabolizable sugars were independent of their osmotic properties, since osmotic agents such as PEG (non-penetrating osmoticum) or sorbitol (penetrating osmoticum) had little or no

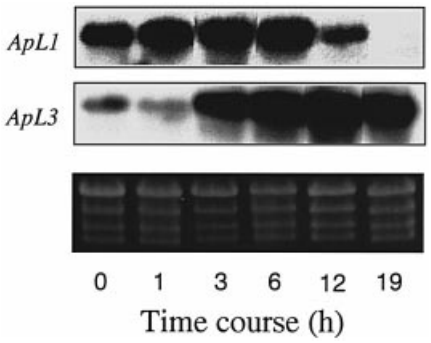

Figure 2 Time-course of sucrose regulation of *Arabidopsis ApL1* and *ApL3* genes

Rosette leaves detached from 6–7-week-old plants (pre-adapted to darkness for 6 h) were fed with 0.1 M sucrose in the dark. When indicated, total RNA was isolated from leaves and analysed on Northern blots for the expression of *ApL1* and *ApL3* genes. The bottom panel represents an ethidium bromide-stained gel demonstrating that equal amounts of RNA are present in each lane.

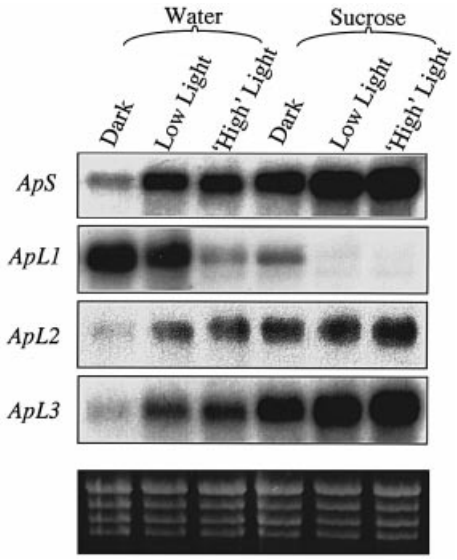

Figure 3 Effects of sucrose and light on AGPase gene expression in *Arabidopsis*

Rosette leaves detached from 6–7-week-old plants (pre-adapted to darkness for 6 h) were fed for 12 h with water or 0.1 M sucrose in the dark or under different light conditions [50 (low light) or 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ ('high' light)]. After feeding, total RNA was isolated from the leaves and analysed on Northern blots using cDNA probes corresponding to *ApS, ApL1, ApL2* and *ApL3*. The bottom panel represents an ethidium bromide-stained gel demonstrating that equal amounts of RNA are present in each lane.

apparent influence on AGPase gene expression (Figure 1). With respect to *ApL1*, the two osmotica were as effective as sucrose and glucose in down-regulation of this gene, suggesting that steady-state levels of *ApL1* mRNA are susceptible to changes in osmotic pressure.

We have chosen *ApL1* and *ApL3* as examples of AGPase genes that are down- and up-regulated respectively by sugars, and studied time-course of changes in the contents of the corresponding transcripts following feeding 0.1 M sucrose (Figure 2). For *ApL3*, a substantial induction occurred as early as 3 h after sucrose feeding, whereas the decrease in the *ApL1* transcript levels was observed only after 12 h treatment. Thus the two genes, besides being differentially regulated by sugars, differed also in kinetics of their responses. Interestingly, levels of *ApL1* transcript clearly increased during first 6 h of sugar treatment, preceding a subsequent decrease (Figure 2), suggesting a complex transcriptional/post-transcriptional regulation. The possibility cannot be excluded that the *ApL1* gene is in fact up-regulated, but its corresponding transcript is unstable in sucrose-fed leaves. Time courses of sucrose-dependent expression of *ApS* and *ApL2* genes followed that of *ApL3* (results not shown), i.e. their induction was almost complete after 6 h feeding.

Exposure of the dark-pre-adapted leaves to light (Figure 3) resulted in expression patterns for AGPase genes that were to a large extent analogous to those obtained for excised leaves fed with sugars in the dark. Light appeared to mimic the effect of sucrose, although in some cases the light-plus-sucrose conditions resulted in a more pronounced effect on AGPase transcript levels than individual sucrose or light treatments. Light intensity as low as 50 $\mu E \cdot s^{-1} \cdot m^{-2}$ was generally sufficient to affect the expression of AGPase genes, although clearly it was not saturating for maximal effect for the *ApL1* gene (Figure 3).

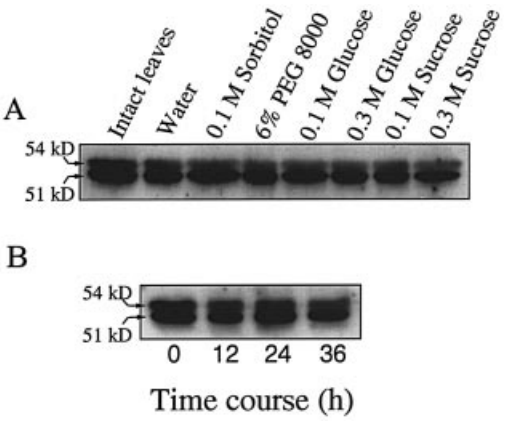

Figure 4 Effects of sugar/osmoticum treatments on AGPase protein contents in *Arabidopsis*

Rosette leaves detached from 6–7-week-old plants (pre-adapted to darkness for 6 h) were placed in containers with water or a given sugar/osmoticum and kept in the dark. After feeding, a total protein fraction was isolated from the leaves and analysed on Western blots using antibodies against native tomato-fruit AGPase [31]. (**A**) Qualitative studies. Feeding with a given sugar/osmoticum was carried out for 12 h. (**B**) Time-course kinetics with sucrose. Leaves were fed with 0.1 M sucrose for up to 36 h. At indicated time points, proteins were extracted from the leaves for Western-blot analysis. Abbreviation: kD, kDa.

AGPase protein and activity studies

Sugar-dependent patterns of expression of AGPase genes were examined further at a protein level (Western immunoblots), using antibodies raised against native AGPase from tomato fruits [31]. These antibodies recognize both small and large subunits of AGPase, which in *Arabidopsis* have relative molecular masses of 51 and 54 kDa respectively [4]. The immunoblots revealed no appreciable changes in the content of both subunit types, regardless of the sugar/osmoticum conditions tested (Figure 4). A similar constant pattern for the small subunit content was obtained when antibodies raised against the small subunit of barley endosperm AGPase [32] were used (results not shown). When we extended sucrose feeding from 12 to 36 h and studied the time course of the sugar effect (Figure 4B), we still could not discern any major changes in the amount of the small and large subunit proteins on Western blots.

Similarly to the protein patterns, we were unable to discern any major changes in AGPase activity promoted by the various sugar and light treatments (Table 1). The enzyme was assayed in the direction of nucleotide-sugar production, which is the most specific and sensitive assay for a pyrophosphorylase [10]. With this assay, we hoped to detect putative changes in AGPase activity caused by the possible (dis)appearance of enzyme forms encoded by *ApS*, *ApL1*, *ApL2* and *ApL3* genes which were differentially regulated by the sugar or light treatments (Figures 1–3). Only in the case of leaves kept in the dark on water or sucrose did we find about a 25 and 20% decrease in activity respectively when compared with other treatments (Table 1). Whether this decrease corresponds to the appearance of qualitatively new isoenzyme(s) of AGPase or reflects some post-translational modification is unknown at present. The data are similar to those obtained for two AGPase genes from potato, where sugar-dependent increases in mRNA contents were not transmitted to the level of subunit protein nor enzymic activity, which were relatively constant [21].

Table 1 Effects of sucrose feeding and light/dark regime on AGPase activity in *Arabidopsis* leaves

Rosette leaves were detached from 6–7-week-old plants kept for 6 h in the dark prior to the feeding experiment (control). Leaves were fed for 12 h with water or 0.1 M sucrose, in the dark or in the light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$). AGPase activity was assayed as described in the Materials and methods section. Values are the means ± S.E.M for three determinations on independent leaves.

Conditions	AGPase activity ($\mu mol \cdot min^{-1} \cdot g$ fresh $weight^{-1}$)
Control	0.213 ± 0.015
Dark	
+ Water	0.155 ± 0.014
+ 0.1 M Sucrose	0.167 ± 0.031
Light	
+ Water	0.212 ± 0.028
+ 0.1 M Sucrose	0.213 ± 0.028

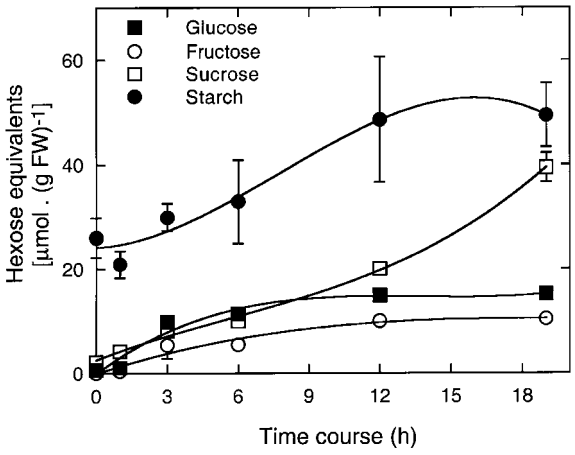

Figure 5 Time-course of changes in the carbohydrate contents in *Arabidopsis* leaves upon feeding sucrose in the dark

Rosette leaves were detached from 6–7-week-old *Arabidopsis* plants kept for 6 h in the dark prior to the feeding experiment (zero time). Leaves were fed with 0.1 M sucrose in the dark and then were collected at different times, as indicated. Soluble sugars and starch contents were measured as described in the Materials and methods section. Values are means ± S.E.M. for three determinations on independent leaves. Abbreviation: FW, fresh weight.

Carbohydrate metabolism during sugar feeding

Metabolism of sucrose fed to detached leaves was examined both for the dark and light conditions. For a time-course experiment (Figure 5), detached leaves were fed with 0.1 M sucrose in the dark, and samples were taken from 1 h to 19 h for sugar analyses. In the darkened leaves, sucrose was rapidly metabolized to hexoses and to starch. Synthesis of hexoses and starch reached a plateau after 12 h, whereas sucrose continued to accumulate steadily. After 19 h of feeding, the levels of sucrose, glucose, fructose and starch accumulated by a factor of 18-, 50-, 50- and 2-fold respectively when compared with the control. High levels of hexoses are probably the consequence of invertase activity hydrolysing sucrose, whereas starch synthesis must reflect AGPase involvement.

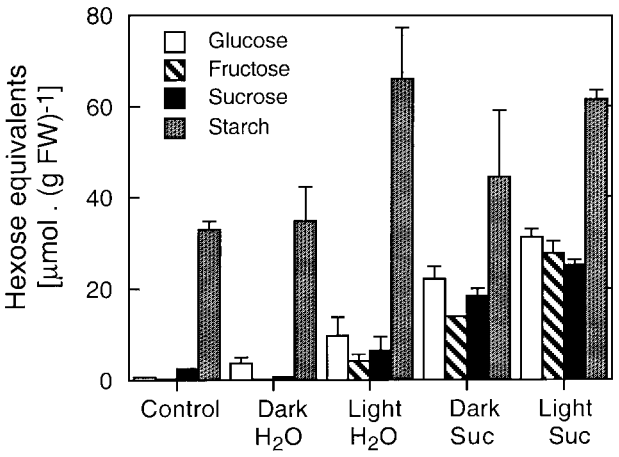

Figure 6 Dynamics of carbohydrate changes in *Arabidopsis* leaves exposed to sucrose treatment under dark and light conditions

Rosette leaves were detached from 6–7-week-old *Arabidopsis* plants kept for 6 h in the dark prior to the feeding experiment (control). Leaves were fed for 12 h with water or 0.1 M sucrose, in the dark or in the light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$). Soluble sugars and starch concentrations were measured as described in the Materials and methods section. Values are means ± S.E.M. for three determinations on independent leaves.

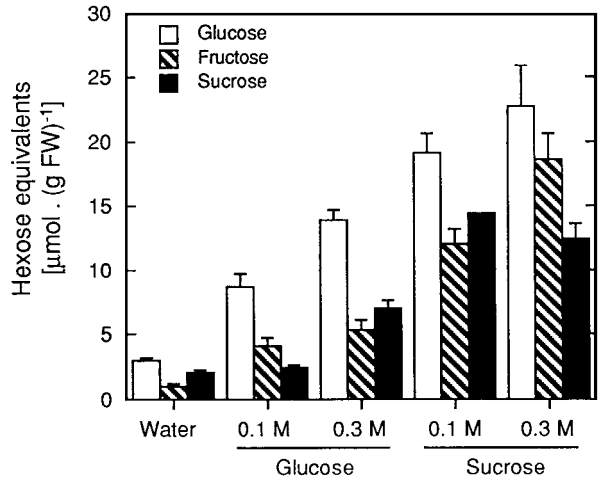

Figure 7 Comparative efficiency of uptake (and subsequent metabolism) of glucose and sucrose fed to *Arabidopsis* leaves in the light

Rosette leaves were detached from 6–7-week-old *Arabidopsis* plants kept for 6 h in the dark prior to the feeding experiment. Leaves were fed for 7.5 h with water, glucose (0.1 M or 0.3 M) or sucrose (0.1 M or 0.3 M) in the light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$). Soluble sugars were measured as described in the Materials and methods section. Values are means ± S.E.M. for three determinations on independent leaves.

For the light/dark experiment, detached leaves were fed for 12 h with sucrose (0.1 M) or water, in the presence or absence of light (Figure 6). The light treatment enhanced sugar and starch accumulation in leaves fed with sucrose or water in comparison with the control leaves or darkened leaves fed with water. The increase in starch, whether in sucrose-fed and/or illuminated leaves, although significant, was less than usually observed for illuminated intact *Arabidopsis* plants, where starch can accumulate over 10-fold at the end of the photoperiod [4]. However, Sicher and Kremer [33] reported no changes in starch concentration after 7 h of light. Also, starch increased only 6-fold in 48 h in detached *Arabidopsis* leaves fed with 6% sucrose under continuous light [34]. Starch accumulation may certainly depend on the growing conditions, age of the plants, and depending on whether intact plants or detached leaves are considered.

Feeding sucrose induced a higher increase in internal glucose content than did feeding equimolar amounts of glucose (Figure 7), suggesting that sucrose uptake by the leaves was far more efficient than glucose uptake. Even at a 0.3 M concentration, glucose appeared to be taken up less effectively than 0.1 M sucrose, as reflected by internal contents of glucose in the leaf. Glucose uptake was accompanied by significant increases in fructose and sucrose levels in the leaf, which is consistent with the involvement of a full set of cytosolic enzymes required for glucose-into-sucrose conversion in *Arabidopsis*.

DISCUSSION

Sugars/osmotica trigger differential expression of AGPase genes

Sugar feeding profoundly and differentially affected the expression of four *Arabidopsis* genes for AGPase (Figure 1): transcript contents for *ApS*, *ApL2* and *ApL3* dramatically increased upon sugar feeding, whereas the content of *ApL1* mRNA was down-regulated by sugar/osmoticum. The sucrose- or glucose-induced increase in mRNA levels of *ApS*, *ApL2* and *ApL3* appeared independent of changes in osmotic pressure, as PEG and sorbitol could not mimic the effect of these two metabolizable sugars (Figure 1). To what extent the changes in expression patterns were due to transcriptional regulation or to different stabilities of the mRNAs is unknown at present. Sugar-induced gene activation has previously been demonstrated for selected AGPase genes from other species, i.e. a large subunit gene was induced by sugars in potato [18,21] and after cold-girdling (preventing carbohydrate export) of source leaves in spinach (*Spinacia oleracea*) [35]. With respect to the small subunit gene(s), sugars only weakly affected a small subunit gene in potato [21], but caused a strong induction of a similar gene from sweet potato (*Ipomoea batatas*) [19] and from *Chenopodium* suspension cultures [20]. Sweet potato contains two genes for the small subunit [36] and, at present, it is unclear whether only one of them or both are sucrose-inducible. In *Chenopodium*, the induction of the small subunit gene was observed following a long-term (5 days) feeding of glucose [20].

In contrast with *ApS*, *ApL2* and *ApL3*, the expression of *ApL1* gene was repressed by sugar/osmoticum treatments (Figure 1). To our knowledge it is the first time that such an observation has been reported for an AGPase gene. Kinetic studies showed that *ApL1* mRNA, after initial slight accumulation, decreased strongly after 12 h of sucrose feeding (Figure 2), which coincides with a major accumulation of soluble sugars (Figure 5). The similar effects of sugar and general osmotica, such as PEG and sorbitol, on the contents of the *ApL1* transcript suggest that the corresponding gene is sensitive to changes in osmotic pressure, as has been reported, for example, for some α-amylase genes [37].

Light mimics the effect of sugars on AGPase gene expression

Exposing the detached leaves to light has resulted in similar changes in the levels of AGPase transcripts as upon sugar treatments in the dark (Figures 1–3). Light did not appear essential in inducing changes in AGPase transcript contents as long as sufficient carbohydrate/osmoticum levels were available, and it stimulated changes in the mRNAs quantities in an intensity-dependent manner (Figure 3). It is possible that sugar regulation of AGPase transcripts reflects a mechanism where the

regulation occurs when the accumulation of sugars in cells exceeds some threshold value(s). For *ApS*, *ApL1* and *ApL3* transcripts, the cumulative effects of sucrose and light were larger than effects of sucrose or light alone (Figure 3), possibly due to higher sugar levels in leaves exposed to both sucrose and light (Figure 6). A possible effect of light through factors other than sugar(s) cannot be ruled out entirely, however, given close interactions between sugar and light signalling pathways in *Arabidopsis* [38]. While it is difficult to distinguish the causal effects of light and sugars on the quantities of AGPase transcripts, the observed light regulation of AGPase gene expression is likely to be mediated, at least in part, by carbohydrates produced by photosynthesis. Similar data were obtained, for instance, for a nitrate reductase gene [26], where its light-inducible expression in dark-adapted *Arabidopsis* plants could be mimicked by a supply of sucrose to plants in darkness. Feedback inhibition of photosynthetic genes by sugars [23,24] may represent another example of the interwoven nature of light/carbohydrate effect(s). The light regulation of expression of AGPase genes in *Arabidopsis* may be part of a general cellular response to an increased cell growth and metabolism promoted by the availability of sugars during photosynthesis.

Dynamic sugar/light-dependent changes in AGPase mRNA contents do not correlate with AGPase protein levels nor activity

Sugar/light-induced changes in the contents of AGPase mRNAs ranged, depending on experimental conditions, from a few fold for *ApS* to at least 10-fold for other genes considered (Figures 1–3). Despite these quite dramatic fluctuations at the mRNA levels, protein contents of both subunit types of AGPase (Figure 4) and its enzymic activity (Table 1) were relatively unaffected. A similar discrepancy was observed for potato leaf AGPase, where the light-dependent enhancement of transcriptional abundance was not accompanied by increases in protein content or enzymic activity [21]. This lack of correlation between mRNA levels and protein/activity was not due to a simple lag in protein accumulation, since the potato plants were exposed to a constant light for up to 7 days before final analyses. In our studies we did not observe any major changes in AGPase protein quantities after sucrose feeding for up to 36 h (Figure 4). There was some decrease (about 20 %) in the enzymic activity of AGPase upon sucrose feeding in the dark-kept plants (Table 1). These changes in activity, albeit relatively small, suggest that translation or post-translational events may contribute to regulation. A similar decrease in enzymic activity was reported for AGPase from *Chenopodium*, following a long-term (5 days) feeding of glucose [20].

Despite the apparent lack of sugar effect on AGPase protein content and enzymic activity, sucrose feeding in the dark resulted in an up-to-2-fold increase in starch levels (Figure 5). This observation supports the notion [21] that the increase in starch is not a direct consequence of an enhancement of AGPase gene expression, as initially suggested [18]. Rather, under these conditions, the net starch production may reflect an increase in substrate availability and/or, possibly, changes in the allosteric properties of AGPase. Similarly to that in potato leaves [21], AGPase in *Arabidopsis* is probably regulated via translational or post-translational events, possibly involving regulation at the level of protein turnover/proteolysis. As recently suggested [6,21], the two different subunit types, present in equimolar amounts in native AGPase enzyme [4,8,21], are likely to protect each other from proteolysis when complexed together. This may reconcile, depending on physiological conditions, differences in a steady-state level of corresponding mRNAs for both subunit types, providing a putative homoeostatic mechanism that balances changes in gene expression upon light/sugar regimes.

On the nature of signal(s) for AGPase gene regulation

It is unknown at present whether it is sucrose, hexoses or some of their metabolites that are responsible for the regulation of AGPase genes in *Arabidopsis*. Although sucrose feeding appeared to be more effective than glucose in regulating the levels of mRNAs (Figure 1), it also induced a larger increase in internal glucose concentration (Figure 7), suggesting that sucrose is taken up through the petioles more efficiently that glucose under our experimental conditions. A similar observation has been reported for sugar beet (*Beta vulgaris*) leaves, where sucrose (75 mM) feeding resulted in a 2-fold higher increase in glucose concentration than that caused by feeding an equimolar amount of glucose itself [39]. Also, a comparison of mRNA levels and internal sugar concentration showed a correlation between glucose concentration and the level of induction of the *ApL3* transcript (Figures 1, 2 and 5). The *ApL3* gene was rapidly induced (Figure 2) and its transcript levels increased in a manner parallel with that of glucose concentration (Figure 5): both reached their maximal values after 6 h of feeding. It is unclear whether this evidence directly points toward any of the sugars studied, since compartmentation of sugars may affect their availability as possible sensing molecules. An imbalance between the sugar concentration in different compartments can complicate the interpretation of the results obtained, and subcellular sugar determination might be useful to resolve some of the discrepancies. An alternative may involve the use of mutants or 'antisense' plants with altered internal levels of carbohydrates (see, e.g. [7,34,40]).

Generally, it is assumed that, in plants, the signal-transduction pathway for sugar regulation is mediated at the level of hexoses through hexokinase involvement, as in yeast [25,28]. However, other signal-transduction mechanism(s) are also plausible. For instance, expression of class I patatin promoter in transgenic *Arabidopsis* involves a sensor located upstream of hexokinase [41]. Studies with transgenic plants expressing invertase in different compartments of the cell argue for sensing events occurring at the endomembrane system [40]. Regulation by hexose derivatives, e.g. UDP-glucose [25] or glycolytic metabolites [23,35] also cannot be excluded. The AGPase genes themselves may be subject to different transduction mechanisms, especially with respect to *ApL1* versus other homologous genes. The *ApL1* gene is responsive to osmotic pressure rather than to any particular sugar (Figure 1); it is down-regulated [after initial up-regulation (Figure 2)] rather than induced (Figures 1–3), and it has a temporal expression pattern distinct from that of other AGPase genes (Figure 2 and results not shown). Whether these differences refer to entirely different sensing mechanisms or mechanisms sharing some common components of signalling pathways is unknown at present.

On the role of AGPase genes in *Arabidopsis*

In our experiments, the transcripts corresponding to the large subunit of AGPase were subject either to up- or down-regulation upon sugar/light exposure, whereas the amount of the small subunit mRNA increased considerably (Figures 1–3). As *Arabidopsis* contains only one gene for the small subunit [6,13], it may appear beneficial that changes in the amount of the ApS protein are compensated for by analogous changes in the net amount of the large subunit protein(s). In this way, a certain amount of the large subunit will always be available to form an active enzyme. There is apparently no fundamental objection to the possibility

that a given small subunit of AGPase may associate with different large subunits, resulting in a functional enzyme. Such a functional hybrid AGPase complex has already been reported by combining maize and potato small and large subunits of AGPase in a heterologous bacterial system [5]. Thus the lack of sugar effect on protein levels and activity of AGPase, as observed in the present study may actually reflect compensative processes where production of a given protein occurs in parallel with degradation of its functional homologues, without changes in an overall AGPase protein content nor changes in activity. With respect to *Arabidopsis*, however, such a presumption is challenged by the studies on a large-subunit-lacking mutant (*adg-2*), which was found to be starch-deficient [4]. Even though *Arabidopsis* contains at least three genes for the large subunit that are to various extents expressed in leaves, apparently only one ApL protein can functionally combine with ApS to form an active enzyme (but see further discussion below). Recent studies by Wang et al. [6] have indicated, through complementation analysis and mapping, that the missing AGPase protein in the *adg-2* mutant corresponds to *ApL1* gene. It is unknown at present whether complementation of the large subunit mutant with *ApL2* and *ApL3* gene products (e.g. by using constructs containing a sugar-unresponsive constitutive promotor) will also restore AGPase function *in vivo*. In this and other respects, it would certainly be beneficial if gene-product specific antibodies were available for AGPase homologues from *Arabidopsis* to study changes in the content of a given protein rather than monitoring a possible sum of all homologous proteins.

In the view of tissue- or cell-specific expression of AGPase genes in several plants [11,12,21,42,43], spatial and, possibly, developmental considerations should be taken into account with respect to AGPase gene regulation. Perhaps, within the leaf itself, different cell types may contain distinct isoenzymes of AGPase, as recently suggested for potato plants [21]. For instance, *ApL1* might be expressed in mesophyll cells (the major tissue of a leaf), with the expression of *ApL2* and *ApL3* confined to other chloroplast-containing leaf tissues, i.e. chlorenchyma, paravascular tissue, epidermal and guard cells or trichomes. An AGPase gene specifically expressed in the guard cells of potato leaves has already been described [43]. If the gene product of *ApL1* is indeed the major form of the large-subunit protein of AGPase in leaves [6], it will have to be present in mesophyll cells, which contribute the bulk of leaf volume and mass. Thus, assuming cell-specific expression of the large subunit transcripts in leaves, even if the *ApL2* and *ApL3* transcripts are translated and combine with ApS to form functional enzyme entities, their activities are rather unlikely to contribute significantly to an overall AGPase rate in leaf extracts.

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