Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in *Arabidopsis*

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UDP-glucose pyrophosphorylase (UGPase) is a key enzyme producing UDP-glucose, which is involved in an array of metabolic pathways concerned with, among other functions, the synthesis of sucrose and cellulose. An *Arabidopsis thaliana* UGPase-encoding gene, *Ugp*, was profoundly up-regulated by feeding sucrose to the excised leaves and by an exposure of plants to low temperature (5 °C). The UGPase activity and its protein content also increased under conditions of sucrose feeding and exposure to cold. The sucrose effect on *Ugp* was apparently specific and was mimicked by exposure of dark-adapted leaves to light. Drought and O_2 deficiency had some down-regulating effects on expression of *Ugp*. The sugar-signalling pathway for *Ugp* regulation was independent of hexokinase, as was found by using transgenic plants with increased and decreased expression of the corresponding gene. Subjecting mutants deficient in abscisic acid (ABA) to cold stress conditions had no effect on *Ugp* expression profiles. Okadaic acid was a powerful inhibitor of *Ugp* expression, whereas it up-regulated the gene encoding sucrose synthase (*Sus1*), indicating distinct transduction pathways in transmitting the sugar signal for the two genes in *A. thaliana*. We suggest that *Ugp* gene expression is mediated via a hexokinase-independent and ABA-insensitive pathway that involves an okadaic acid-responsive protein phosphatase. The data point towards *Ugp* as a possible regulatory entity that is closely involved in the homoeostatic readjustment of plant responses to environmental signals.

Key words: *aba* mutants, low-temperature stress, okadaic acid, sucrose signalling.

INTRODUCTION

UDP-glucose pyrophosphorylase (UGPase) (EC 2.7.7.9) is an important enzyme producing UDP-glucose for sucrose synthesis in leaves. The UDP-glucose can also be used, for example, in the synthesis of cell wall polysaccharides [1,2], in production of the carbohydrate moiety of glycolipids, glycoproteins and proteoglycans and as a precursor to UDP-glucuronic acid [3]. Furthermore, it has a crucial role for the 'quality control' of proteins transported to the endoplasmic reticulum, where glycosylation processes occur [4]. In non-photosynthetic tissues or developing leaves, UGPase is linked to sucrose degradation pathways by using UDP-glucose produced by sucrose synthase (Sus) and providing carbon skeletons for starch synthesis [2,5]. The activity of UGPase is fully reversible and it can produce or use UDP-glucose, depending on the metabolic status of a tissue [2].

Despite the central role of UGPase in carbohydrate synthesis and metabolism, very little is known about the regulatory events that control UGPase activity and the expression of the corresponding gene(s). The enzymic activity/protein content of UGPase increases with phosphate deficiency stress via regulation at the gene expression level [6]. In addition, expression of the gene for UGPase might be susceptible to regulation by high sucrose concentration and low temperature, as found for a UGPase gene from potato tubers [7,8], but the exact nature of the signal and its physiological significance are uncertain. Apart from that, no other significant regulatory mechanisms, either at the gene or the enzymic level, have been reported. The enzyme might exist in large excess over that required for carbon flow from or to sucrose, as found by 'anti-sense' DNA approaches in transgenic potato plants [8]. In contrast, Spychalla et al. [7] failed to engineer potato plants with an inhibition of enzyme activity greater than 30 %; however, this level of inhibition was sufficient to decrease sugar content in stored tubers. In other studies, storage of tubers from transgenic potato plants with a 50 % decreased UGPase activity resulted in decreased sucrose levels [9], which is consistent with the important role of UGPase in sugar synthesis.

In plants, changes in carbohydrate concentration have frequently been implicated in responses to a variety of stresses, including cold stress. Little is known of the exact mechanisms of these responses, but the up-regulation of several genes coding for certain enzymes of sucrose synthesis and metabolism has been proposed as a part of acclimation mechanisms [10]. For instance, genes for both sucrose phosphate synthetase and Sus have been found to be induced by sugars and/or osmotica and certain stresses [11–14]. Because of its strategic positioning at the crossroads of several pathways for carbohydrate synthesis, UGPase might also represent a suitable target for transcriptional regulation under changing conditions.

Here we demonstrate that a UGPase gene (*Ugp*) in *Arabidopsis thaliana* is strongly and specifically induced on the provision of exogenous sucrose to excised leaves; we provide evidence for up-regulation of the gene after exposure of the leaves to light and under conditions of cold stress. Details of sucrose and cold regulation were analysed in transgenic plants with a modified

Abbreviations used: ABA, abscisic acid; HXK, hexokinase; OKA, okadaic acid; PP, protein phosphatase; Sus, sucrose synthase; UGPase, UDP-glucose pyrophosphorylase.

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expression level of *AtHXK1*, a gene encoding hexokinase (HXK), and mutants that were deficient in abscisic acid (ABA). In addition, evidence is provided for the involvement of a protein phosphatase sensitive to okadaic acid (OKA) in the sucrose-regulated signal transduction pathway for *Ugp*.

MATERIALS AND METHODS

Plant material and treatments

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, wild-type plants and ABA-deficient mutants (*aba-1* and *aba-2*) (seed stock nos CS155 and CS156 respectively, obtained from *Arabidopsis* Biological Resource Center) were grown for 6–7 weeks in pots with a mixture of soil, peat moss and perlite (2:1:1, w/w). All plants were cultured in a growth chamber under an 8 h light/16 h dark photoperiod, a photon flux density of 120 μmol/s per m^2, day and night temperatures of 23 and 19 °C, and a humidity of 80 %. Transgenic *A. thaliana*, ecotype Bensheim, plants with an increased (lines 9A3-2 and 9A14-2) or inhibited (lines A9Af2-6 and A9A2-7) expression of *AtHXK1* [15], as well as corresponding wild-type plants, were grown under the same conditions.

For the feeding experiments, leaves were detached from intact plants (plants were preadapted to the dark for 5–6 h) and placed into tubes with feeding solutions for 8–12 h (details are given in Figure legends). The submerged parts of the leaf petioles were removed from the samples before leaves were frozen with liquid nitrogen and stored at −80 °C. For stress treatments, wild-type and *aba* mutant plants were transferred for 1–7 days to a growth chamber with a 5 °C temperature regime (cold stress), under the same day/night regime and light intensity as in the controls. Wild-type plants were also subjected to water stress (drought) for 4–6 days or submerged into degassed water (flooding) for up to 2 days (other conditions as in controls) [14]. Leaves were sampled at the times indicated in Figure legends and compared with control leaves.

Activity assays and Western blot analyses

The protein extraction and UGPase activity assays were performed essentially as in [6]. The enzyme was assayed spectrophotometrically at 21 °C by measuring the reduction of NAD^+ at 340 nm with a Beckman DU 530 spectrophotometer.

SDS/PAGE and conditions of transfer of the resolved proteins to nitrocellulose membrane were as in [6]. UGPase protein was detected with rabbit antibodies (5 mg of serum protein in 10 ml of antibody buffer) raised against purified heterologously expressed (in *Escherichia coli*) barley UGPase ([19], and F. Martz and L. A. Kleczkowski, unpublished work) followed by goat anti-rabbit IgG coupled to peroxidase (Amersham). Specific labelling was detected with the ECL® kit (Amersham).

Northern blot analyses

Total RNA was isolated from *A. thaliana* tissues with a modification of the guanidine thiocyanate-based method, as described by Sokolov et al. [16]. Aliquots (20 μg) of total RNA were separated electrophoretically on 1.2 % (w/v) agarose/formaldehyde gel (equal loading was monitored by ethidium bromide staining of the gel), blotted on Hybond-N^+ (Amersham) membranes and hybridized with ^{32}P-labelled cDNA probes. The probes used were: *Ugp* (*Arabidopsis* expressed sequence tag no. T41638) corresponding to UGPase, *Sus1* [14], *AtHXK1* (*Arabidopsis* expressed sequence tag no. T20488) and *rab*18 [17]. The *Ugp* clone has at least 78 % and 86 % similarity, at the nucleotide and derived amino acid levels respectively, to UGPases from potato [18] and barley [19]. Hybridization and subsequent washes were performed at 65 °C, with standard procedures [20].

RESULTS

Ugp is up-regulated by sucrose and light via a HXK-independent pathway

Possible effects of sugars and osmotica on *Ugp* expression were tested with excised leaves. The gene was found to be strongly up-regulated by sucrose and, to some extent, by a high concentration of PEG [poly(ethylene glycol)] 6000 (Figure 1A). The latter acts as a non-penetrating osmoticum and can induce several secondary effects, including sucrose accumulation [21]. Glucose, mannitol, sorbitol and KCl were ineffective in inducing any appreciable change in *Ugp* expression. In contrast with *Ugp*, all the feeding compounds used in the present study were found to stimulate the expression of *A. thaliana Sus1* (results not shown), an osmoticum-regulated gene [14]. When leaves were fed with different concentrations of sucrose (Figure 1B), the strong induction of *Ugp* was observed at as little as 50 mM sucrose. A further increase in sucrose concentration resulted in a relatively moderate increase in *Ugp* transcript content. A similar response was obtained when mixtures of different concentrations of sucrose and mannitol (the total concentration of both compounds was 300 mM) were fed to the leaves (results not shown). This helped to distinguish between the effects of sucrose itself from those exerted by osmotic pressure, and further supported data from Figure 1(A) that *Ugp* does not respond to changes in osmotic pressure. Feeding with sucrose also led to an increase in UGPase activity and protein content (Figure 2). The molecular mass of UGPase, as determined on immunoblots, corresponded to 50–51 kDa, which is identical with values previously obtained

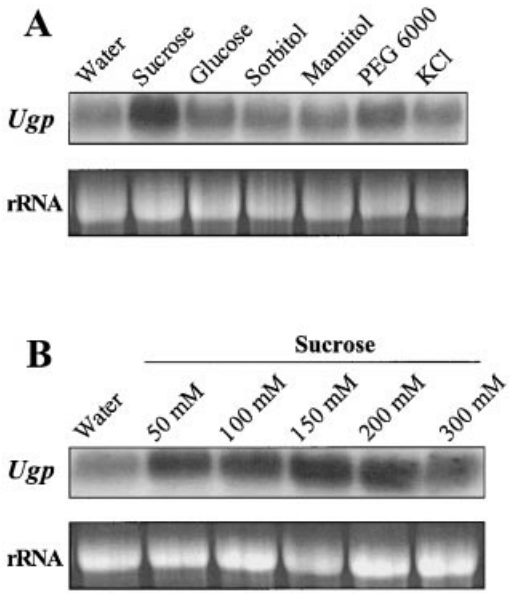

Figure 1 Effects of sucrose, glucose and osmotica on *Ugp* expression in *A. thaliana*

(**A**) Sucrose, glucose, sorbitol and KCl were applied at 100 mM each, and mannitol and PEG 6000 at 150 mM and 6% respectively. (**B**) Various concentrations of sucrose were applied (0–300 mM). In both panels sugars or osmotica were fed to the excised leaves in the dark for 10 h.

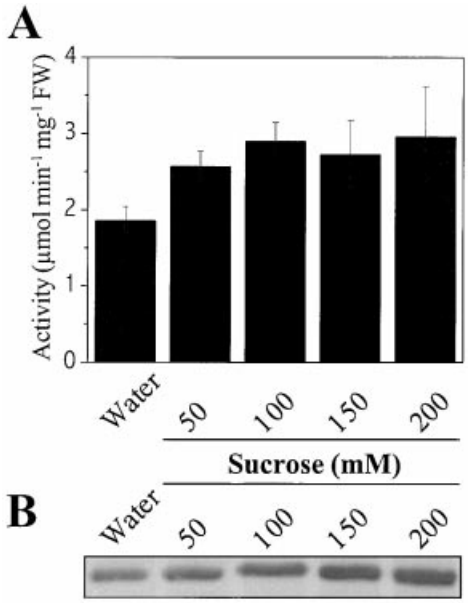

Figure 2 Effects of sucrose feeding on UGPase activity (A) and protein content (B) in *A. thaliana*

The conditions of the sucrose feeding were as described in the legend to Figure 1. Activity assays and immunoblots were done for samples from the same experiments. For activity assays, means ± S.D. ($n = 4$) are indicated. For immunoblots, protein aliquots corresponding to 2.25 mg of leaf sample were loaded in each lane.

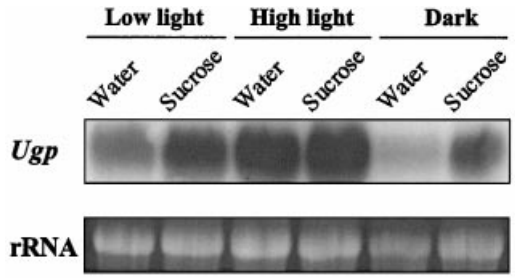

Figure 3 Effects of light intensity and sucrose on *Ugp* expression in *A. thaliana*

Excised leaves were fed with 100 mM sucrose either in the dark, low light (60 μmol/s per m^2) or 'high' light (150 μmol/s per m^2) for 8 h.

for UGPase from leaves of *A. thaliana* [6], barley [19] and potato tubers [18].

Exposure of the dark-preadapted leaves to light (Figure 3) resulted in expression patterns for *Ugp* that were largely analogous to those obtained for excised leaves fed with sucrose in the dark (Figure 1A). Light seemed to mimic the effect of sucrose, although in most cases the light plus sucrose conditions resulted in a more pronounced effect on *Ugp* transcript levels than individual treatments with sucrose or light. Light intensity as low as 60 μmol/s per m^2 was generally sufficient to affect the expression of *Ugp*, although clearly it was not saturating for maximal effect (Figure 3).

To analyse in more detail the mechanism of sucrose responsiveness of *Ugp*, we used transgenic plants with modified expression of the *AtHXK1* gene, which is thought to be involved in transmitting the sugar signal for the expression of a number of

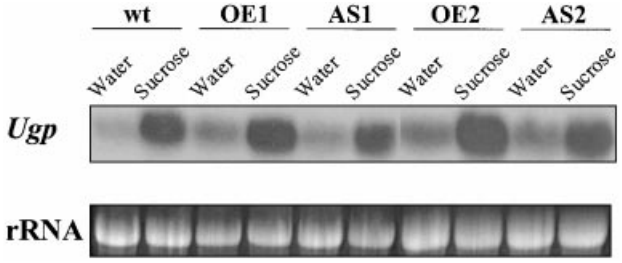

Figure 4 Effect of HXK status on sucrose-dependent expression of *Ugp* in *A. thaliana*

Excised leaves from wild-type and transgenic plants were fed with either water or 50 mM sucrose in the dark for 12 h. Abbreviations: OE, 'overexpressed' *AtHXK1* plants; AS, 'anti-sense' *AtHXK1* plants; OE1, 9A3-2 line; OE2, 9A14-2 line; AS1, A9Af2-6 line; AS2, A9A2-7 line [15]. The bottom panel shows the relative abundance of rRNA in each lane.

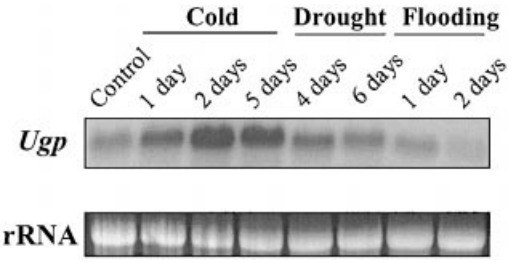

Figure 5 Stress responsiveness of *Ugp* expression in *A. thaliana*

Plants were treated under cold, drought and flooding conditions for the specified durations; leaves were harvested after a 4 h light period. The bottom panel shows the relative abundance of rRNA in each lane.

plant genes [15,22]. First, we analysed the transgenic plants for their expression of *AtHXK1*; the plants overexpressing *AtHXK1* had an at least 10-fold higher transcript content for *AtHXK1*; those with an 'anti-sense' construct had an approx. 50 % decrease in *AtHXK1* expression compared with wild-type plants (results not shown). These results are analogous to those obtained by Jang et al. [15]. When excised leaves of the transgenics were fed with sucrose to study the expression of *Ugp*, the patterns were analogous to those found for leaves of wild-type plants (Figure 4). Neither the excess of *AtHXK1* transcript nor its decrease in leaves had any marked effect on *Ugp* expression. There was a slightly lower content of *Ugp* transcript in sucrose-fed leaves of wild-type and 'anti-sense' plants in comparison with 'over-expressing' plants, but its significance is not entirely clear. The enzymic activity of HXK is concerned with hexose metabolism; an increase or decrease in HXK content might affect both the status of phosphorylated hexoses and that of the ATP pool [23]. This in turn might lead to a readjustment of metabolism in transgenic plants, with possible effects on gene expression in comparison with wild-type plants.

Ugp is up-regulated by cold stress via an ABA-unresponsive pathway

The responses of the *A. thaliana Ugp* gene to abiotic stresses were investigated. *Ugp* was strongly up-regulated by cold stress, but some decrease in transcript content was observed for drought (after an initial increase) and O_2-deficiency conditions (flooding) (Figure 5). Under cold stress, steady-state contents of the *Ugp*

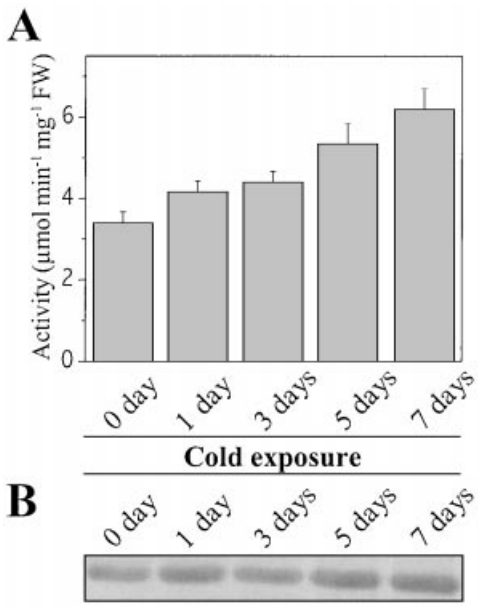

Figure 6 Effects of low-temperature stress on UGPase activity (A) and protein content (B)

Plants were exposed to a cold regime for up to 7 days; leaves were harvested after a 4 h light period. Activity assays and immunoblots were done for samples from the same experiments. For activity assays, means ± S.D. ($n = 4$) are indicated. For immunoblots, protein aliquots corresponding to 3.75 mg of leaf sample were loaded in each lane.

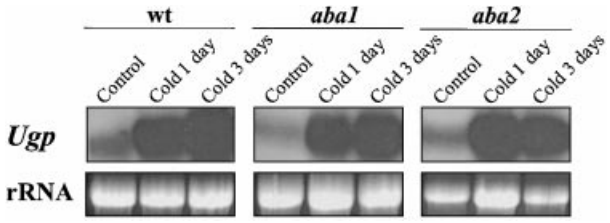

Figure 7 Effects of ABA status on cold-dependent expression of *Ugp* in *A. thaliana*

The wild-type plants and *aba-1* and *aba-2* mutants (deficient in ABA content) were exposed to cold (5 °C) for 1 and 3 days. Leaves were harvested after a 4 h light period. The bottom panel shows the relative abundance of rRNA in each lane.

transcript increased markedly after only 1 day of exposure at 5 °C; a stress treatment as short as 8 h was sufficient for a significant increase in the transcript level (results not shown). The expression increased further for plants kept in the cold for 3 days; however, a 5-day cold stress resulted in a decreased content of *Ugp* transcript (Figure 5). Up-regulation of the *Ugp* gene was accompanied by changes in UGPase activity and protein content (Figure 6), which increased steadily during the cold exposure. On the basis of activity assays, there was an approx. 80 % increase in UGPase activity during 7 days of stress treatment.

ABA signalling is known to be important in gene responses under cold stress [24]. We investigated the possible involvement of ABA in *Ugp* gene expression by either feeding ABA to detached leaves or using ABA-deficient *A. thaliana* mutants in a cold-stress experiment. Feeding with ABA had little or no effect on steady-state levels of *Ugp* mRNA, whereas an ABA-responsive

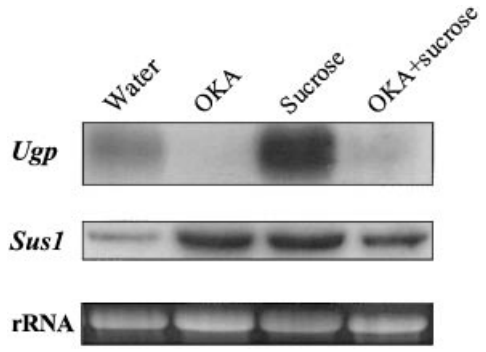

Figure 8 Effects of OKA on sucrose-dependent expression of *Ugp* and *Sus1* in *A. thaliana*

OKA (2 μM) and sucrose (150 mM) were fed to detached leaves for 10 h in the dark. The bottom panel shows the relative abundance of rRNA in each lane.

gene (*rab18*) [17] that was used as a control was strongly induced by the treatment (results not shown). When we subjected ABA-deficient mutants (*aba-1*, *aba-2*) to the cold stress conditions, the *Ugp* gene was up-regulated in a similar way for both mutants and wild-type plants (Figure 7). The *rab18* gene was expressed in wild-type plants but in *aba* mutants its transcript level was very low (results not shown) [14], which was consistent with the ABA deficiency in the mutants.

Sucrose induction of Ugp is sensitive to OKA

OKA, a potent inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A) [25], was found to inhibit the expression of *Ugp* completely when it was fed at 2 μM, regardless of the presence or absence of sucrose in the feeding solution (Figure 8). Expression of *Sus1* markedly increased after OKA application (Figure 8), indicating that *Sus1* is regulated via a distinct pathway. This is consistent with earlier results that the expression of *Sus1* responds to osmotic potential rather than to any specific sugar [14] and that components of the osmoticum-regulated pathways in plants are distinct from those comprising the sugar-signalling pathways [26]. An OKA/sucrose-dependent induction of a sweet-potato gene corresponding to Sus has already been reported [27].

DISCUSSION

The *Ugp* gene from *A. thaliana* was found to be up-regulated on feeding sucrose to excised leaves, after the illumination of darkened leaves, and on cold stress (Figures 1, 3–5 and 7). All these conditions result in a marked accumulation of sucrose and, to some extent, glucose and fructose in *A. thaliana* [14,16], possibly underlying a common transduction mechanism for *Ugp* regulation. Although, in our hands, sucrose seemed much more efficient than glucose (and other osmotica) in up-regulating *Ugp* (Figure 1), a possibility still exists that a product of sucrose hydrolysis and metabolism serves as the ultimate signal for *Ugp* up-regulation. In addition, sucrose might be more efficiently taken up through the petioles than glucose in excised *A. thaliana* leaves [14]. During both sucrose feeding and exposures to cold stress, an increase in transcript level for *Ugp* was subsequently accompanied by increased UGPase activity and protein content (Figures 2 and 6), suggesting that under these conditions UGPase is regulated at the transcriptional and/or post-transcriptional level. Earlier studies [6] demonstrated that phosphate deficiency

stress also up-regulated UGPase activity/protein via post-transcriptional control.

Similarly to the previously reported regulation of genes for ADP-glucose pyrophosphorylase (a key enzyme in starch synthesis) in *A. thaliana* [16], light did not seem to be essential in inducing changes in *Ugp* transcript contents as long as sufficient sucrose levels were available; it stimulated changes in the quantities of mRNA in an intensity-dependent manner (Figure 3). For *Ugp* transcript, the cumulative effects of sucrose and light were larger than the effects of sucrose or light alone (Figure 3), possibly owing to higher sugar levels in leaves exposed to both sucrose and light [16]. However, effects of light through factors other than sugar(s) cannot be ruled out entirely given the close interactions between the sugar and light signalling pathways in *A. thaliana* [28].

Several studies have implied a possible role for HXK in sugar signalling, with HXK being proposed as a sugar sensor [15,22,29]. This was demonstrated both by using transgenic plants with a modified expression of the *AtHXK1* gene [15] and by using mannoheptulose, a specific inhibitor of HXK, and/or sugar analogues of D-glucose [29–31]. However, the role of HXK in sugar sensing and signalling is still a matter of debate; several HXK-independent pathways have been proposed [10,23,32,33], including those specifically sensing sucrose [34] and some product(s) of glucose oxidation [11,22,35]. The up-regulation of *Ugp* by sucrose is probably mediated via such a HXK-independent pathway, because the modulation of *AtHXK1* in transgenic plants had no apparent effect on *Ugp* expression (Figure 4).

Cold stress was shown to be an important factor in up-regulating *Ugp* expression in *A. thaliana* (Figures 5 and 7). It is unknown whether the cold signal effect is transmitted via the same pathway (or part of it) as the sucrose/light-mediated regulation, but sucrose content increases markedly during exposure of *A. thaliana* to cold [14]; sucrose signalling must therefore be seriously considered. Aside from general physiological considerations, this seems particularly important in the context of so-called 'cold sweetening', i.e. the accumulation of hexoses and sucrose observed when potato tubers are stored in the cold [9]. The effect of cold on *Ugp* expression was apparently transmitted independently of ABA, as found with ABA-deficient mutants (Figure 7). ABA signalling is not the only pathway for sensing cold; several genes have already been shown to be expressed independently of ABA [14,36,37]. *Ugp* was down-regulated by drought (after an initial increase) and, especially, flooding conditions (Figure 5). Flooding is characterized by a marked depletion of soluble carbohydrates in *A. thaliana* [14]; it is unknown at present whether the down-regulation of *Ugp* during flooding (Figure 5) is simply a consequence of low sucrose content in stressed leaves.

More details of the sucrose signalling pathway for *Ugp* have emerged from the use of OKA in the feeding solution. This compound is a potent and specific inhibitor of PP1 and PP2A [25] and its inhibitory effect on *Ugp* expression (Figure 8) indicates the involvement of one or both of the phosphatases in the sugar-mediated regulation of the gene. Similar external concentrations of OKA (0.5–5 μM) were effective in inhibiting PP1/PP2A activity in several plant response systems [38,39]. The activating effect of OKA on *Sus1* expression (Figure 8) indicates that a phosphoprotein ('X-P') that serves as a substrate for PP1 and/or PP2A is mediating the up-regulating signal for this gene, whereas up-regulation of *Ugp* requires the dephosphorylation of X-P or some other phosphoprotein that acts as a substrate for PP1 and/or PP2A. Both UGPase and Sus have previously been proposed to provide UDP-glucose for cellulose synthesis, the key process for cell growth in plants [1,2]. Because *Sus1* is believed to be the main gene for Sus under normal physiological conditions, and because *A. thaliana* probably contains only one *Ugp* gene (on the basis of genomic Southern blot analyses; results not shown), the presence of distinct signalling pathways for both genes might represent a mechanism ensuring the production of UDP-glucose even if one of the pathways is inactive or blocked.

In recent studies with mutants impaired in inorganic phosphate status [6], *Ugp* was found to be up-regulated by conditions of phosphate deficiency; this up-regulation was apparently not correlated with total soluble carbohydrate level in the mutants. Together with present results demonstrating the responsiveness of *Ugp* to sucrose regulation, to light/dark transition and to conditions of cold stress, it seems that the expression of the gene is susceptible to an array of signals and possibly to different transduction pathways. This is consistent with the emerging view that gene regulation in plants proceeds via the integration of several transduction pathways rather than a single one, responding to a plethora of signals. Whereas exact details of the regulation of *Ugp* are unknown, it is perhaps not surprising that this gene is subject to a complex transcriptional control, given the role of UDP-glucose, the product of UGPase, in anabolic pathways in plant cells. UGPase joins several other enzymes of sucrose synthesis and metabolism, such as sucrose phosphate synthetase, Sus and invertases [10–14,40], that are subject to a complex transcriptional regulation, underlying the importance of carbohydrate balance in growth and developmental processes.

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