Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*

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Sucrose synthase (Sus) is a key enzyme of sucrose metabolism. Two Sus-encoding genes (*Sus1* and *Sus2*) from *Arabidopsis thaliana* were found to be profoundly and differentially regulated in leaves exposed to environmental stresses (cold stress, drought or O_2 deficiency). Transcript levels of *Sus1* increased on exposure to cold and drought, whereas *Sus2* mRNA was induced specifically by O_2 deficiency. Both cold and drought exposures induced the accumulation of soluble sugars and caused a decrease in leaf osmotic potential, whereas O_2 deficiency was characterized by a nearly complete depletion in sugars. Feeding abscisic acid (ABA) to detached leaves or subjecting *Arabidopsis* ABA-deficient mutants to cold stress conditions had no effect on the expression profiles of *Sus1* or *Sus2*, whereas feeding metabolizable sugars (sucrose or glucose) or non-metabolizable osmotica [poly(ethylene glycol), sorbitol or mannitol] mimicked the effects of osmotic stress on *Sus1* expression in detached leaves. By using various sucrose/mannitol solutions, we demonstrated that *Sus1* was up-regulated by a decrease in leaf osmotic potential rather than an increase in sucrose concentration itself. We suggest that *Sus1* expression is regulated via an ABA-independent signal transduction pathway that is related to the perception of a decrease in leaf osmotic potential during stresses. In contrast, the expression of *Sus2* was independent of sugar/osmoticum effects, suggesting the involvement of a signal transduction mechanism distinct from that regulating *Sus1* expression. The differential stress-responsive regulation of *Sus* genes in leaves might represent part of a general cellular response to the allocation of carbohydrates during acclimation processes.

Key words: ADP-glucose pyrophosphorylase, anoxia, cold stress, drought, hypoxia.

INTRODUCTION

Drought, low temperature, high salt concentration and oxygen deprivation (hypoxia and anoxia) caused by flooding are common adverse environmental factors encountered by land plants. To cope with these environmental stresses, plants execute a number of physiological and metabolic responses, including the expression of numerous stress-specific genes [1–3]. Drought, high salt concentration and cold stresses are all characterized by dehydration and can be classified as water/osmotic stress. It is not yet fully understood how plants can sense osmotic stress, but it is clear that abscisic acid (ABA) is involved in the expression of a number of stress-responsive genes [4]. However, studies conducted on ABA-deficient or ABA-insensitive mutants have indicated that some of these genes are expressed independently of ABA [5,6]. With regard to anoxia, it is known to quickly inhibit protein synthesis, except for a selected set of proteins called anaerobic proteins ('ANPs'); most of them are enzymes of glycolysis and fermentation [2]. The identity of one or more specific sensors and the nature of early events that lead to the perception of O_2 availability and the consequent reprogramming of gene expression have yet to be determined.

Sucrose synthase (Sus) is one of the key enzymes involved in sucrose synthesis/metabolism, especially in non-photosynthetic tissues. This enzyme catalyses the reversible conversion of sucrose and UDP into UDP-glucose and fructose. Under normal growth conditions, Sus activity has been linked to many important plant processes, e.g. phloem loading/unloading [7–9] and nodule function [10]. UDP-glucose, directly or after conversion into other activated sugars, can be used for biosynthetic pathways such as the synthesis of cell wall [11] and starch [12,13]. In young leaves (sink tissue), Sus has supposedly a role in breaking down sucrose imported from mature leaves (source tissue). In mature leaves, Sus serves no apparent metabolic function and there is usually a very low level of expression of *Sus* genes under normal physiological conditions. During stress conditions, however, the expression of *Sus* gene(s) is frequently stimulated in leaves and other organs. For instance, *Sus* has been reported to be induced by both osmotic stresses and O_2 deficiency. Oxygen deficiency induces *Sus* genes in maize and rice roots [14,15], and in sugar beet leaves [16]. Sus mRNA and protein levels increased also in *Aspen* water-stressed shoots [17]. Whereas its role in stress adaptation is not fully understood, Sus is thought to be involved in meeting the increased glycolytic demand during stress. *Sus* expression is also regulated by externally provided sugars [18]. The most detailed study has concerned maize Sus isoenzymes, in which the shrunken-1 (*Sh1*) gene was maximally expressed under conditions of limited carbohydrate supply, whereas *Sus1* was up-regulated when sugars were abundant [19]. It has been suggested [18] that a specific sugar molecule might serve as a signal to initiate a transduction pathway controlling *Sus* gene expression. A contribution from other sensing mechanisms, e.g. an osmoticum-sensing pathway, has not been entirely ruled out in most of the studies on regulation of *Sus* genes in plants.

Two *Sus* genes have been reported in *Arabidopsis*: *Sus1* was previously identified as being responsive to anoxia and cold treatment [7], whereas virtually no physiological information was available on *Sus2* [20]. In the present study we investigated the effects of both abiotic stresses and sugars/osmotica on the expression of the *Sus* genes in *Arabidopsis* leaves, by using both

Abbreviations used: ABA, abscisic acid; AGPase, ADP-glucose pyrophosphorylase; PEG, poly(ethylene glycol); Sus, sucrose synthase.

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wild-type and ABA mutants, and studying *Sus* expression in both intact plants and excised leaves. The results, obtained with the use of gene-specific probes, suggest that *Sus1* and *Sus2* are differentially regulated by environmental stresses via distinct ABA-independent sensing/transduction pathways. An unexpected aspect of this work is that *Sus1* seems to be regulated entirely via stress-induced changes in internal osmotic pressure rather than via some sugar-specific signalling pathway. The nature of specific signal(s) initiating the transduction pathway is evaluated and discKussed.

MATERIALS AND METHODS

Plant material and treatments

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, plants and ABA-deficient mutants (*aba-1,* CS155; *aba-2,* CS156), in the same genetic background, were grown in a chamber with 8 h of white light (100 μE/s m^{-2} at 23 °C) and 16 h of darkness (19 °C) photoperiod regime for 6–7 weeks for all experiments. Plants were watered daily. For stress treatments, some plants were either transferred 5 h after the beginning of the day to a cold chamber (cold-stressed plants, 5 °C, same day/night regime and light intensity), deprived of water (water-stressed plants) or submerged into degassed water (O_2-deficient plants). Leaves were sampled at the time indicated in the figure legends and compared with control leaves collected before the treatments. For the feeding experiments, leaves were detached from intact plants, preadapted to the dark for 6 or 12 h as indicated in the figure legends, and placed into containers for 13 h with water, sugars/osmotica or ABA prepared in 10 mM Mes/KOH, pH 6.25, as indicated in the figure legends. The submerged parts of the leaf petioles were removed from the samples before leaves were frozen in liquid N_2 and stored at −80 °C.

Northern blot analyses

Total RNA was isolated from leaves and other tissues, by using a modification of the guanidinium thiocyanate method, as described in [21], and 10 μg aliquots of the RNA were used for Northern blot analyses. RNA was separated on 1.2% (w/v) agarose gels prepared with formaldehyde and stained with ethidium bromide. After alkaline Northern blot transfers, Hybond-N+ (Amersham) membranes with the blotted/immobilized RNA were hybridized with gene-specific cDNA probes. An 0.5 kb *Eco*RI–*Xho*I cDNA fragment from an *Arabidopsis* expressed sequence tag clone (no. ATTS0443) was used as the *Sus1* probe, whereas an 0.75 kb *Bam*HI–*Bgl*II fragment spanning the 5′ region of *Arabidopsis Sus2* [20] was used as the *Sus2* probe. A 0.54 kb cDNA encoding one of the large subunits of ADP-glucose pyrophosphorylase (AGPase) from *Arabidopsis* [22] corresponded to the *ApL3* probe. The *rab18* probe (0.56 kb) was kindly provided by Dr. E. T. Palva. The identities of the probes were checked by sequencing.

Western blot analyses

Frozen *Arabidopsis* leaves and pea seed coats were ground with a mortar and pestle in 50 mM Hepes/NaOH, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA and 2 mM dithiothreitol. The homogenates were centrifuged at 15000 ***g*** (4 °C) before being concentrated in Centricon 30 microconcentrators (Amicon). The conditions for protein electrophoresis, blot transfer and immunodetection were as described in [23], except that donkey anti-rabbit antibodies linked to horseradish peroxidase (Amersham) were used as secondary antibodies. The peroxidase activity was revealed with enhanced chemiluminescence reagents (Amersham).

Determination of sugars and leaf osmotic potential

Sugar concentrations were determined after ethanol extraction of 10–20 mg of freeze-dried leaf samples [80% (v/v) ethanol in water for 2 h at 4 °C] by an enzymic method [13]. Leaf osmotic potential was measured by a dew-point microvoltmeter (Wescor, Logan, UT, U.S.A.) on cell sap squeezed out of fresh leaves after rapid freezing in liquid N_2.

RESULTS

Differential regulation of *Sus1* and *Sus2* by environmental stress exposure

The response of *Arabidopsis Sus* genes to abiotic stresses was investigated. Intact 6–7-week-old plants were either transferred to 5 °C (cold stress), deprived of water (drought) or submerged into degassed water (O_2 deficiency) for the duration indicated in the legend to Figure 1. Figure 1(A) shows that *Sus1* and *Sus2* are

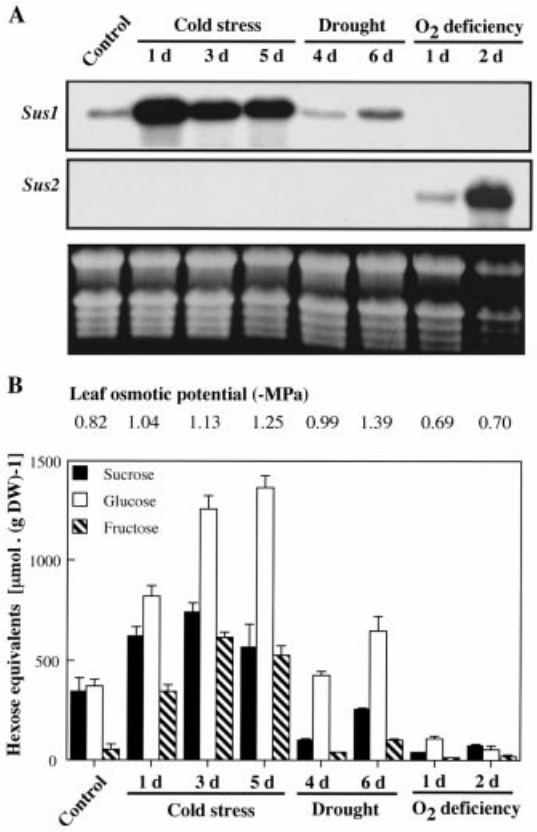

Figure 1 Effects of different environmental stresses on the expression of *Arabidopsis Sus1* and *Sus2* (A) as well as on leaf osmotic potential and sugar contents (B)

Plants (6–7-week-old) were exposed to cold (5 °C), drought and anaerobic treatments for the periods indicated. (**A**) Total RNA was isolated from leaves and analysed on a Northern blot by using cDNA probes corresponding to *Sus1* and *Sus2*. The RNA was also isolated from leaves of plants before the treatments (control). The bottom panel shows an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane. (**B**) Sugars and leaf osmotic potential were measured as described in the Materials and methods section. Values are the means ± S.E.M. for three determinations on independent leaves.

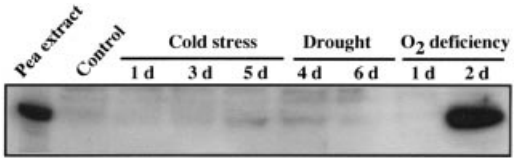

Figure 2 Effects of different environmental stresses on Sus protein contents in *Arabidopsis* leaves

Plants (6–7-week-old) were exposed to cold (5 °C), drought and anaerobic treatments for the periods indicated. Total protein fractions were isolated from leaves; 150 μg portions were analysed on a Western blot by using antibodies against native Sus from pea seed coats. Lane 1 contained 5 μg of protein fraction isolated from pea seed coats as positive control for the immunoreaction.

differentially regulated by stresses. *Sus1* responds very quickly to both cold stress and drought but we failed to detect any *Sus1* transcript with O_2 deficiency. Steady-state levels of *Sus1* mRNA increased profoundly after 1 day of exposure at 5 °C; the stress treatment as short as 8 h was sufficient for a significant increase in the transcript level (see Figure 4). Drought induced an increase in *Sus1* mRNA level after 6 days of treatment. In contrast with *Sus1*, *Sus2* expression seemed to be specifically triggered during O_2 deficiency but was not responsive to either cold or drought treatments (Figure 1A). It should be emphasized that the controls shown in Figure 1 (and Figure 4) for *Sus1* and *Sus2* did not change throughout the 1–6 days of stress exposure; for clarity we have therefore provided here only controls for the beginning of stress experiments. Changes in the expression patterns of *Sus* genes were studied in parallel with a detailed examination of leaf osmotic potential and sugar contents occurring during stress treatments (Figure 1B). Cold stress was most effective in inducing profound changes in both of these parameters. Soluble sugars, mainly glucose and fructose, increased markedly after only 1 day at 5 °C; after 5 days at 5 °C, glucose and fructose contents increased 3-fold and 8-fold respectively. Leaf water potential slowly decreased on cold treatment from -0.82 (control leaves) to -1.25 MPa. Drought induced a relatively small increase in the levels of glucose and fructose and resulted in a decrease in sucrose content and in large changes in leaf osmotic potential. In comparison with cold and drought stresses, O_2 deficiency induced completely different physiological disturbances: sugar contents were depleted after only 1 day of treatment and leaf water potential increased slightly in comparison with that of the control leaves.

In addition to studies on expression of *Sus* genes during environmental stresses, we also determined changes in Sus protein, by using polyclonal antibodies againstative Sus from pea seed coat [23]. On Western blots, Sus protein was easily detected after O_2 deficiency in *Arabidopsis* leaves, whereas only a faint band could be observed after long-term cold or drought exposures (Figure 2). Regardless of stress conditions, Sus appeared as a single protein band, having a molecular mass of approx. 95 kDa, which was analogous to that determined for pea seed coat enzyme [23].

Effects of sugar starvation on *Sus2* expression

Sus2 was specifically induced by O_2 deficiency (Figure 1B), which is characterized by a complete depletion of sugars within 1 day. To starve the leaves and to mimic the effect of O_2 deficiency *in vivo*, leaves were preadapted to the dark for 12 h, then detached and fed with 10 mM Mes/KOH, pH 6.25, in the dark for 24–48 h. This treatment induced a decrease in leaf sugar content

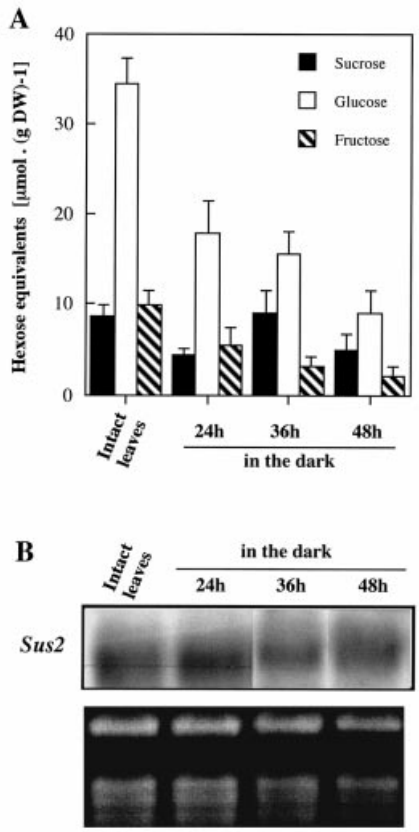

Figure 3 Effects of starvation on sugar contents (A) and *Sus2* expression (B) in *Arabidopsis*

Rosette leaves were detached from 6–7-week-old plants (preadapted to darkness for 12 h) and fed with 10 mM Mes/KOH, pH 6.25, for the durations indicated. (**A**) Sugars were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for five determinations on independent leaves. (**B**) Total RNA was isolated from leaves and analysed on a Northern blot by using cDNA probes corresponding to *Sus2*. The RNA was also isolated from leaves of plants before the treatments as a control. The bottom panel shows an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane. Note that the film was overexposed to detect possible changes in *Sus2* transcript content even if present in a very small amount.

(Figure 3A) but *Sus2* mRNA level did not change even after 48 h in the dark (Figure 3B). Therefore starvation of *Arabidopsis* leaves could not mimic the effect of O_2 deficiency on *Sus2* expression.

Role of ABA in *Sus* expression

ABA signalling is known to be important in gene responses under osmotic stress [4]. We investigated the possible involvement of ABA in *Sus* gene expression by two different approaches: (1) by feeding ABA to detached leaves and (2) by using ABA-deficient *Arabidopsis* mutants in a cold stress experiment. ABA had little or no effect on steady-state levels of *Sus1* mRNA, whereas an ABA-responsive gene (*rab18*) [24] that was used as a control was strongly induced by the treatment (results not shown). This confirmed that ABA was indeed taken up efficiently by the leaves via the transpiration stream. A very low level of *Sus2* mRNA was detectable under both conditions (results not shown). When we subjected ABA-deficient mutants (*aba-1*, *aba-2*) to the cold stress conditions, *Sus1* was expressed in a similar way in both mutants and wild-type plants, whereas *rab18* mRNA

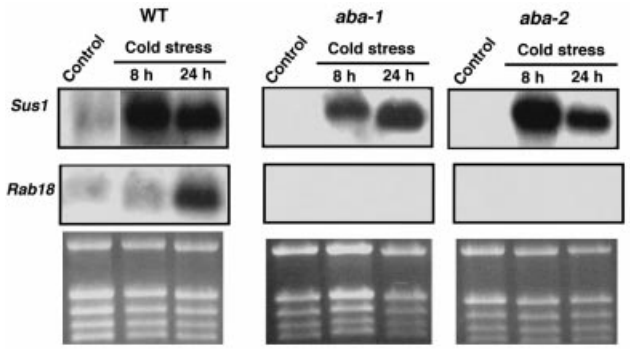

Figure 4 Effects of cold stress on *Sus1* expression in wild-type and ABA-deficient *Arabidopsis*

Plants (6–7-week-old) [wild-type (WT), *aba-1* and *aba-2*] were exposed to cold (5 °C) for the durations indicated. Total RNA was then isolated from leaves and analysed on a Northern blot by using cDNA probes corresponding to *Sus1* and *Rab18*, an ABA-responsive gene. The RNA was also isolated from leaves of plants before the treatments (control). The bottom panel shows an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane.

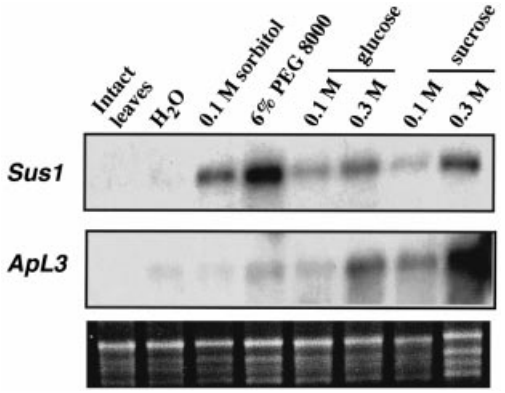

Figure 5 Effects of sugar/osmoticum on *Sus1* regulation in *Arabidopsis*

Rosette leaves were detached from 6–7-week-old plants (preadapted to darkness for 6 h) and fed for 13 h in the dark with water or a given sugar or osmoticum. After the feeding, total RNA was isolated from leaves and analysed on a Northern blot with cDNA probes corresponding to *Sus1* and *ApL3*, a sugar-responsive gene [21]. The bottom panel shows an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane.

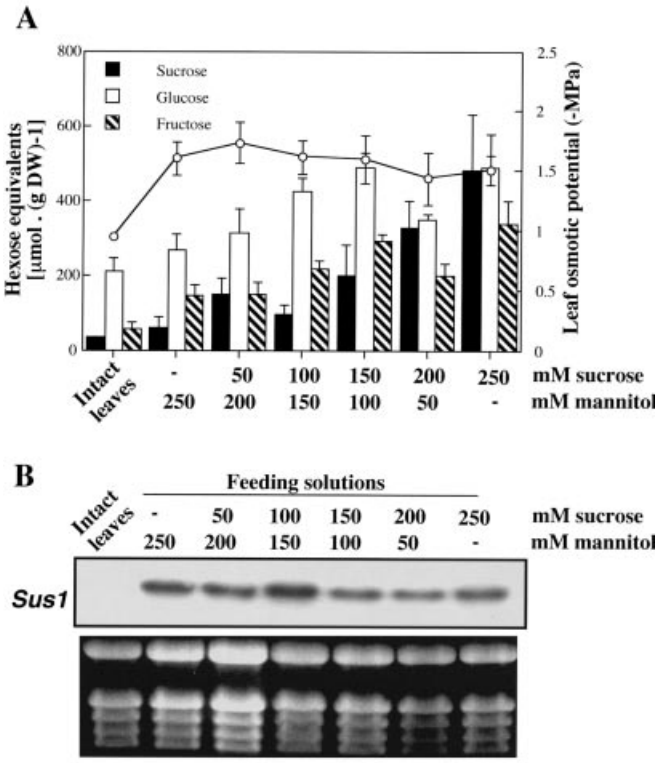

Figure 6 Specific effect of sucrose concentration in the feeding solutions on leaf osmotic potential and sugar contents (A) and on *Sus1* expression (B) in *Arabidopsis*

Rosette leaves were detached from 6–7-week-old plants (preadapted to darkness for 6 h) and fed for 13 h in the dark with 10 mM Mes/KOH, pH 6.25, containing sucrose and mannitol. (**A**) Sugars and leaf osmotic potential were measured as described in the Materials and methods section. Values are means ± S.E.M. for three determinations on independent leaves. (**B**) Total RNA was isolated from leaves and analysed on a Northern blot by using cDNA probes corresponding to *Sus1*. The bottom panel is an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane.

was detected only in wild-type plants (Figure 4). Very low or no expression of *Sus2* was observed in both wild-type and mutants exposed to cold (results not shown).

Effects of sugar/osmoticum on *Sus1* expression

Because sugar contents in leaves were profoundly affected during stress exposure (Figure 1B), we investigated the putative involvement of sugars (glucose and sucrose) in *Sus* gene expression. Before all experiments, *Arabidopsis* plants were kept in the dark for 6 h to lower the internal level of soluble sugars before the treatment. Total RNA fractions, isolated from darkened leaves that were fed for 12 h with sugar/osmotica, were examined by Northern blot analyses for the expression of *Sus1* (Figure 5). *Sus1* mRNA did increase on feeding with sugar; however, osmotic agents such as poly(ethylene glycol) (PEG) (non-penetrating osmoticum) or sorbitol (penetrating osmoticum) had an even stronger effect, suggesting that the response to sugars actually reflects their osmotic properties rather than being any sugar-specific effect. In comparison, *ApL3*, a sugar-responsive gene encoding one of the large subunits of AGPase, a key enzyme of starch synthesis [21,25], showed a completely different expression pattern: it was specifically induced by glucose or sucrose feeding, but not general osmotica (Figure 5). The expression of *Sus2* was very low and unchanged under any of the above-described feeding conditions (results not shown), supporting the data in Figure 3, in which the depletion of the internal sugar concentration had no effect on the *Sus2* transcript level.

To evaluate further the specifics of *Sus1* regulation, two different sets of feeding solutions were used to distinguish between the effects of sucrose itself and those exerted by osmotic pressure. First, sucrose concentration was varied from 0 to 250 mM in the six solutions used, whereas the total solute concentration was kept constant at 250 mM by adding an adequate amount of mannitol, a non-metabolizable sugar. Leaf sucrose content increased with sucrose concentration in the feeding solutions (Figure 6A). At relatively low sucrose concentrations, glucose and fructose accumulated to high levels in the leaves, most probably reflecting a high rate of conversion of sucrose into these two sugars. In contrast, at a sucrose concentration of 150 mM or

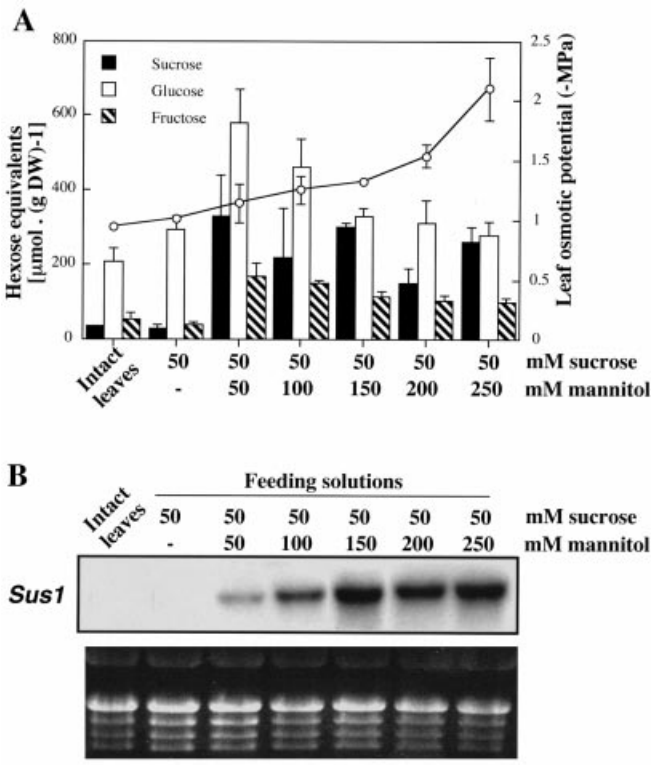

Figure 7 Specific effects of the osmolarity of the feeding solutions on leaf osmotic potential and sugar contents (A) and on *Sus1* expression (B) in *Arabidopsis*

Rosette leaves were detached from 6–7-week-old plants (preadapted to darkness for 6 h) and fed for 13 h in the dark with 10 mM Mes/KOH, pH 6.25, containing sucrose and mannitol. (**A**) Sugars and leaf osmotic potential were measured as described in the Materials and methods section. Values are means ± S.E.M. for three determinations on independent leaves. (**B**) Total RNA was isolated from leaves and analysed on a Northern blot by using cDNA probes corresponding to *Sus1*. The bottom panel shows an ethidium bromide-stained gel, demonstrating the approximately equal amount of RNA in each lane.

more, both glucose and fructose reached a plateau, whereas sucrose accumulated markedly. Leaf osmotic potential decreased on feeding, owing to the high osmolarity of the solutions but then remained constant at approx. −1.6 MPa (Figure 6A); at the same time the *Sus1* transcript level was not affected by changes in sucrose concentration (Figure 6B). The second approach concerned experiments in which sucrose concentration was kept constant at 50 mM while the total concentration of the solutions was increased from 50 to 300 mM by adding an appropriate amount of mannitol. This induced a decrease in leaf osmotic potential from −1.01 to −2.10 MPa (Figure 7A). Leaf sucrose content increased on feeding but then remained relatively constant, at least in five cases out of six. The *Sus1* transcript level increased markedly when the osmolarity of the solution increased (Figure 7B). The two experiments involving the manipulation of sucrose and mannitol concentrations on feeding showed clearly that *Sus1* expression was not induced by changes in sucrose concentration (either in the solution or in the leaf) but was linked to leaf osmotic potential. However, on Western blotting we failed to detect Sus protein under any of these feeding conditions even when up to 170 μg of protein from leaf extract was loaded on the gel (results not shown).

DISCUSSION

Abiotic stresses trigger the differential expression of *Sus* genes

Abiotic stresses profoundly and differentially affected the expression of the two *Arabidopsis Sus* genes (Figure 1A): the transcript contents of *Sus1* increased markedly after cold treatment, and to a smaller extent after drought, whereas the content of *Sus2* mRNA was not affected by these osmotic stresses. In contrast, only *Sus2* was induced by O_2 deficiency. Sus protein level increased profoundly on O_2 deficiency but no major changes in Sus protein were observed after osmotic stresses (Figure 2). The antibodies used in the present study were raised against purified Sus from pea seed coats (which contain both Sus1 and Sus2 forms [26]) and they can effectively recognize both Sus forms in pea (A. Déjardin, unpublished work). As both *Arabidopsis* and pea enzymes are closely related (81 and 77 % identity at the amino acid level for the corresponding Sus1 and Sus2 proteins), and because *Arabidopsis Sus1* and *Sus2* share considerable similarity (66 % identity [7]), it seems reasonable that the two Sus forms can be immunodetected in *Arabidopsis* with these antibodies. Previously, an activating effect of cold treatment (25 h at 6 °C) on *Sus1* at the mRNA level was reported [7] but no results were provided at the protein or activity levels. In those studies, *Sus1* was also induced by anoxia caused by flooding, and an increase in Sus activity in flooded plants was observed, analogously to an increase in Sus protein in our studies (Figure 2). The actual internal O_2 concentration in the flooding solution used in [7] is unknown. Fine differences in oxygen concentration might have strong effects on the expression of oxygen-responsive genes. For example, in maize roots, *Sus1* was rapidly up-regulated by hypoxia (3 % O_2), whereas anoxia (no O_2) had very little effect. In contrast, *Sh1* mRNA increased strongly under anoxia but responded very little to hypoxia [15]. In our experimental design, the O_2 dissolved in water at the end of the treatment was between 6 % and 10 %, which corresponds to a mild treatment. In other plant species, anoxia decreased Sus activity and protein content in soybean nodules [27], but increased *Sus4* mRNA in potato [28] and in soybean callus tissue [29]. Other abiotic stresses have also been reported to affect the level of Sus protein or activity; for example, water stress induced Sus protein in *Populus* leaves [17] and increased Sus activity in bean leaves [30].

Sus2 was not affected by either a high concentration of sugars/osmotica or carbon starvation (Figure 3). This indicates that the induction of *Sus2*, as observed during O_2 deficiency (Figure 1), is not mediated at the level of internal sugar concentration in the leaves. Also, feeding ABA was ineffective in inducing *Sus2* (results not shown), ruling out an ABA-responsive transduction mechanism. The expression of *Sus2* is perhaps related directly to the oxygen status of a tissue via some O_2-sensing mechanism; however, its exact identity remains obscure.

Sus1 is induced by changes in leaf osmotic potential via an ABA-independent mechanism

In our hands, *Sus1* behaved as a typical osmoticum-responsive, but not sugar-responsive, gene. Its up-regulation did not involve ABA signalling, as demonstrated by the feeding of ABA to detached leaves (results not shown) and the use of ABA-deficient mutants under cold treatment (Figure 4). *rab18*, an ABA-responsive gene [24] used in these studies as a control, was very strongly induced on feeding with ABA, whereas its expression was moderate in wild-type plants under cold stress. It has been shown [24] that low temperature results in only small changes in endogenous ABA level; in accord with that, only relatively small

changes in *rab18* mRNA level were observed. Cold exposure resulted in the accumulation of soluble sugars in the leaves and a decrease in leaf osmotic potential (Figure 1B); these processes are commonly observed during low-temperature stress [1,31]. The effect of an osmotic stress could be mimicked *in vitro* by feeding solutions of increasing osmolarity to detached *Arabidopsis* leaves (Figure 7). The induction of *Sus1* was not specific to a given type of osmoticum because sucrose, glucose, mannitol, sorbitol and PEG (molecular mass 8 kDa) could all induce this effect (Figures 5 and 6). It has been reported, with the use of *Arabidopsis* transformed with a reporter gene under the control of *Sus1* promoter, that *Sus1* is repressed (rather than induced) by sucrose in young plantlets [7]. This apparent discrepancy with our results might reflect differences in developmental stages; we were working on mature plants. The expression of *Sus* genes might require both 5′ and 3′ flanking regions and (as with, for example, potato *Sus4*) the leader intron to be sucrose-sensitive [32]. In addition, the leader intron of potato *Sus3* had positive and negative effects on the tissue specificity of *Sus3* [33], further underlining certain limitations imposed on gene activity studies that use only a promoter region coupled to a reporter gene.

To our knowledge, this is the first report on a *Sus* gene that is sensitive to osmotic pressure rather than specifically to sucrose or other sugars. The most detailed study on *Sus* regulation concerns maize genes, where *Sus1* transcript level in roots increased at high concentrations of glucose or other metabolizable sugars, whereas *Sh1* mRNA was maximally expressed under conditions of limited carbon supply [19]. In these earlier studies, L-glucose and mannitol were not effective in promoting changes in expression of maize *Sus1*/*Sh1*, suggesting that osmotic pressure is not a factor in the regulation. In potato, *Sus4* was also induced in detached leaves and petioles fed with sucrose [10], whereas mannitol had no effect on this induction [28]. A similar situation was reported for *Sus* from *Vicia faba* embryos [34] and rice embryos [35]. At present it is unclear whether these disparities reflect differences between species and/or organ physiological states, and/or the stage of development. In the present study we were careful to use different osmotica (PEG, sorbitol and mannitol) to avoid some possible artifacts. The results showed clearly that *Sus1* was sensitive to osmotic potential, whatever the osmoticum used in the experiment. *Arabidopsis* ***Sus1*** is one of the few plant genes encoding an enzyme of a major metabolic pathway that is regulated by changes in osmotic pressure. Previously, regulation by osmoticum was implied, for example for some α-amylase genes in rice seeds [36] and for *ApL1*, one of three genes for the large subunit of AGPase in *Arabidopsis* [21].

The regulation of *Sus1* probably involves sensing mechanisms distinct from those described for sugar signalling, where different pathways based on specific sensing of sugars via hexokinase-dependent and hexokinase-independent pathways and via a glycolysis-dependent pathway have been proposed [18,37]. Very little is known about osmoticum-mediated signal transduction pathway(s) in plants. Osmoticum-sensing pathway(s) have been most extensively studied in yeast; it seems that certain aspects of osmoticum regulation in this organism could be evolutionarily conserved in eukaryotes [3]. Yeast is able to sense changes in external osmolarity via membrane osmosensors (*Sln1p* and *Sho1p*), which activate a mitogen-activated protein kinase cascade, leading to the induction of genes involved in glycerol biosynthesis [38,39]. In *Arabidopsis*, three homologues of *Ypd1p*, which relays the phosphotransfer from *Sln1* in a two-component response mechanism, have recently been found [40] and several components of the mitogen-activated protein kinase pathway have been characterized (reviewed in [3]). Osmoticum regulation is probably the major factor in controlling *Sus1* expression during both cold and drought stresses (Figures 1 and 4). The stress-responsiveness of *Sus1* (at least during cold stress) proceeds via an ABA-independent pathway (Figure 4); whether this is simply a consequence of osmoticum-specific response is unclear.

Role of *Sus* genes in stress acclimation in leaves

Both *Sus* genes respond differentially at the mRNA level to the different stress treatments. Whereas transcript levels of *Sus1* (but not *Sus2*) could increase severalfold after osmotic stress exposures, it was unexpected to observe that Sus protein was almost undetectable on a Western blot after even long-term cold and drought treatments (Figure 2). It therefore seems that *Sus1* is under some tight post-transcriptional control during osmotic stresses. *Sus1* mRNA might perhaps be produced and stored for use during specific stages of cold-hardening or during plant recovery from the stress. In contrast, Sus protein was produced in large amounts during O_2 deficiency (Figure 2) after a substantial increase in *Sus2* transcript levels (Figure 1A). This might indicate that *Sus2* is important for O_2-shortage stress response in *Arabidopsis*. A role for Sus during anoxia has clearly been demonstrated in maize roots with the use of single or double Sus mutants, in which anoxic tolerance was correlated with the number of *Sus* genes [41]. Interestingly, *Arabidopsis Sus2* belongs to a small family of *Sus* genes that diverged very early from the main evolutionary branch for this gene; only sugar-beet *Sus* and pea *Sus2* belong to the same cluster [26]. Very little information is available about the role and regulation of enzymes corresponding to these genes. By analogy with *Arabidopsis Sus*2, the sugar-beet *Sus* mRNA level was not affected by cold treatment (4 °C, 48 h) or by sugar treatment (0–10 % sucrose) but it increased under anaerobic conditions [16]. The authors suggested that the onset of fermentative metabolism might lead to an increased demand for sugars. This hypothesis might still hold for a putative function for *Arabidopsis Sus*2 in leaves but the signal that mediates the activation of this gene is probably not related to the limited amount of sugars under anaerobiosis (Figure 3). A careful examination of the stress-induced expression patterns of *Sus* genes in tissues other than leaves needs to be performed for a complete picture of the role of Sus in *Arabidopsis*.

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REFERENCES

1 Bray, E. A. (1997) Trends Plant Sci. **2**, 48–54
2 Drew, M. C. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**, 223–250
3 Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Plant Physiol. **115**, 327–334
4 Leung, J. and Giraudat, J. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**, 199–222
5 Nordin, K., Heino, P. and Palva, E. T. (1991) Plant Mol. Biol. **16**, 1061–1071
6 Gosti, F., Bertauche, N., Vartanian, N. and Giraudat, J. (1995) Mol. Gen. Genet. **246**, 10–18
7 Martin, T., Frommer, W., Salanoubat, M. and Willmitzer, L. (1993) Plant J. **4**, 367–377
8 Nolte, K. D. and Koch, K. E. (1993) Plant Physiol. **101**, 899–905
9 Fu, H. and Park, W. D. (1995) Plant Cell **7**, 1369–1385
10 Van Ghelue, M., Ribeiro, A., Solheim, B., Akkermans, A. D. L., Bisseling, T. and Pawlowski, K. (1996) Mol. Gen. Genet. **250**, 437–446
11 Amor, Y., Haigler, C. H., Johnson, S., Wainscott, M. and Delmer, D. P. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 9353–9357
12 Kleczkowski, L. A. (1994) Phytochemistry **37**, 1507–1515
13 Déjardin, A., Rochat, C., Wuillème, S. and Boutin, J.-P. (1997) Plant Cell Environ. **20**, 1421–1430

14 Ricard, B., Rivoal, J., Spiteri, A. and Pradet, A. (1991) Plant Physiol. **95**, 669–674
15 Zeng, Y., Wu, Y., Avigne, W. T. and Koch, K. E. (1998) Plant Physiol. **116**, 1573–1583
16 Hesse, H. and Willmitzer, L. (1996) Plant Mol. Biol. **30**, 863–872
17 Pelah, D., Wang, W., Altman, A., Shoseyov, O. and Bartels, D. (1997) Physiol. Plant. **99**, 153–159
18 Koch, K. E. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**, 509–540
19 Koch, K. E., Nolte, K. D., Duke, E. R., McCarty, D. R. and Avigne, W. T. (1992) Plant Cell **4**, 56–59
20 Chopra, S., Del-Favero, J., Dolferus, R. and Jacobs, M. (1992) Plant Mol. Biol. **18**, 131–134
21 Sokolov, L. N., Déjardin, A. and Kleczkowski, L. A. (1998) Biochem. J. **336**, 681–687
22 Villand, P., Olsen, O.-A. and Kleczkowski, L. A. (1993) Plant Mol. Biol. **23**, 1279–1284
23 Déjardin, A., Rochat, C., Maugenest, S. and Boutin, J.-P. (1997) Planta **201**, 128–137
24 Lång, V., Mäntylä, E., Welin, B., Sundberg, B. and Palva, E. T. (1994) Plant Physiol. **104**, 1341–1349
25 Kleczkowski, L. A. (1999) FEBS Lett. **448**, 153–156
26 Buchner, P., Poret, M. and Rochat, C. (1998) Plant Physiol. **117**, 719
27 Gonzalez, E. M., Gordon, A. J., James, C. L. and Arrese-Igor, C. (1995) J. Exp. Bot. **46**, 1515–1523
28 Salanoubat, M. and Belliard, G. (1989) Gene **84**, 181–185
29 Xue, Z., Larsen, K. and Jochimsen, B. U. (1991) Plant Mol. Biol. **16**, 899–906
30 Castrillo, M. (1992) J. Exp. Bot. **43**, 1557–1561
31 Strand, Å., Hurry, V., Gustafsson, P. and Gardeström, P. (1997) Plant J. **12**, 605–614
32 Fu, H., Kim, S. Y. and Park, W. D. (1995) Plant Cell **7**, 1387–1394
33 Fu, H., Kim, S. Y. and Park, W. D. (1995) Plant Cell **7**, 1395–1403
34 Heim, U., Weber, H., Bäumlein, H. and Wobus, U. (1993) Planta **191**, 394–401
35 Karrer, E. E. and Rodriguez, R. L. (1992) Plant J. **2**, 517–523
36 Yu, S.-M., Lee, Y.-C., Fang, S.-C., Chan, M.-T., Hwa, S.-F. and Liu, L. F. (1996) Plant Mol. Biol. **30**, 1277–1289
37 Jang, J.-C. and Sheen, J. (1997) Trends Plant Sci. **2**, 208–214
38 Madhani, H. D. and Fink, G. R. (1998) Trends Genet. **14**, 151–5
39 Gustin, M. C., Albertyn, J., Alexander, M. and Davenport, K. (1998) Microbiol. Mol. Biol. Rev. **62**, 1264–1300
40 Miyata, S., Urao, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) FEBS Lett. **437**, 11–14
41 Ricard, B., VanToai, T., Chourey, P. and Saglio, P. (1998) Plant Physiol. **116**, 1323–1331

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