



RESEARCH PAPER

# The role of abscisic acid in the response of a specific vacuolar invertase to water stress in the adult maize leaf

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## Abstract

Among the numerous molecular and physiological modifications induced by water deficit, one of the earliest events observed in maize mature leaves subjected to water deprivation was a strong enhancement of acid vacuolar invertase activity, which occurred before the classical reduction in gas exchange due to stomatal closure. The increase in invertase activity coincided with the rapid accumulation of glucose and fructose that reached 8-fold the control leaf value. In addition, acid vacuolar invertase activity appeared to be highly correlated with xylem sap ABA concentration. In order to investigate the nature of the relationship between ABA and invertase activity, and to disconnect ABA from a likely sucrose side-effect, excised leaves were supplied with ABA or sucrose. As a consequence of ABA supply, a peak in leaf ABA appeared 4 h later which was followed by an enhancement of vacuolar invertase activity. ABA supply also produced a second maximum in leaf ABA. The transcript level of the *Ivr2* gene encoding one vacuolar invertase presented the same two peaks pattern as leaf ABA, with a 2 h lag. This response was specific since the other invertase genes were not responding. Thus, ABA appeared to be a powerful enhancer of the IVR2 vacuolar invertase activity and expression. In the present conditions, the addition of sucrose had no effect on the enzyme activity.

Key words: Abscisic acid, carbohydrates, vacuolar invertase, *Ivr2*, *Rab 17*, water stress, *Zea mays*.

## Introduction

Water deficit produces numerous molecular and physiological modifications that differ in vegetative and reproductive organs. In plants such as maize the main problem at an early grain-filling stage seems to originate from assimilate shortage (Boyle *et al.*, 1991) whereas, in young leaves, growth rate is affected from the onset of water stress (Bacon *et al.*, 1998). In adult stressed leaves, net photosynthesis rate decreases, mainly as a consequence of stomatal closure and not of photosystem dysfunction both in C<sub>3</sub> (Kaiser, 1987) and C<sub>4</sub> plants (Saccardy *et al.*, 1996).

In addition to photosynthetic modifications, water stress induces various early modifications at the level of carbon and carbohydrate metabolism. A reduction in Rubisco activity, not correlated to protein level, was reported in maize (Martinez-Barajas *et al.*, 1992), but most alterations dealt with carbohydrate partitioning. In *Phaseolus vulgaris*, sucrose-phosphate synthase (SPS) activity is affected by dehydration, partly through stomatal closure which slows down the Benson–Calvin cycle (Vassey and Sharkey, 1989; Vassey *et al.*, 1991). Carbon partitioning is modified in favour of sucrose and to the detriment of starch (Vassey and Sharkey, 1989). Accordingly, sucrose concentration increases in water-stressed plants during the day in sunflower (Fredeen *et al.*, 1991) and spinach (Zrenner and Stitt, 1991).

In adult maize leaves, where the level of sucrose cleaving enzymes, either sucrose synthase or invertase, is normally low (Nguyen-Quoc *et al.*, 1990), water deprivation was reported to induce a strong induction of vacuolar invertase activity associated with an equimolar increase in glucose and fructose without any enhancement of sucrose

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synthase activity (Pelleschi *et al.*, 1997). This increase in activity was shown to result from the expression of the *Ivr2* gene (Pelleschi *et al.*, 1999; Kim *et al.*, 2000) while none of the other invertase genes, either from vacuolar forms (*Ivr1*) or cell wall forms (*Incw1*, *Incw2*, *Incw3*, *Incw4*) were expressed or modulated (Kim *et al.*, 2000). Some induction of invertase activity by water stress has been reported on bean and pigeon pea by Castrillo (1992) and Keller and Ludlow (1993), respectively.

The accumulation of sucrose and hexose in a source leaf (Pelleschi *et al.*, 1997) may have several consequences such as a change in osmotic potential and an effect on the expression of some genes of carbohydrate metabolism. However, earlier studies have suggested that stress-induced accumulation of soluble carbohydrate did not change leaf osmolarity, which is consistent with the fact that maize belongs to the group of iso-osmotic plants (Pelleschi *et al.*, 1997) although osmotic adjustment has been reported in growing organs (Westgate and Boyer, 1985). Feedback inhibition of gene expression by carbohydrate may be suggested from the Sheen (1990) experiments that reported a specific co-ordinated repression of the transcriptional activity of photosynthetic gene promoters by soluble carbohydrate by transient expression in maize protoplasts. Conversely, added glucose was shown to enhance the expression of genes implied in carbohydrate metabolism such as  $\alpha$ -amylase in barley embryos (Loreti *et al.*, 2000) or acid invertase and sucrose synthase in maize root tips (Koch *et al.*, 1996). Sucrose and its cleavage products appear to be crucial components in some signal transduction pathways (Sturm and Tang, 1999). The increase in glucose and fructose by potato transgenic plants over-expressing a yeast invertase in leaf cell wall or vacuole was associated with an accumulation of proline and starch, an inhibition of photosynthesis and an increase in respiration. These responses mimic in some ways the symptoms of water stress (Heineke *et al.*, 1992; Scholes *et al.*, 1996).

ABA is also involved as a transduction signal in many stress responses. For example, stomatal closure is mainly mediated by an ABA signal and numerous genes are directly or indirectly ABA-dependent. Invertase activity was reported to be stimulated by ABA treatment in cotton (Ackerson, 1985) and two homologous regions to ABA responsive boxes have been mentioned in the promoter region of carrot invertase genes (Ramloch-Lorenz *et al.*, 1993). However, no direct comparative examination has been provided on the effect of ABA or carbohydrate supply on either photosynthesis and/or soluble invertase activity.

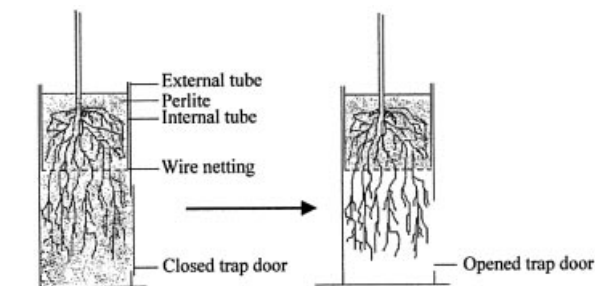
In this paper, the relationship between leaf carbohydrate, acid vacuolar invertase activity, stomatal conductance, and photosynthetic rate was analysed under moderate water stress. A parallel increase of vacuolar invertase activity and xylem sap ABA concentration was

observed, this response was earlier than for photosynthetic gas exchange. The same parameters have been measured in excised leaves supplied with ABA or sucrose. ABA xylem sap and leaf concentrations were measured in parallel. Exogenous ABA, but not sucrose, increased vacuolar activity with respect to the control. In addition, two maxima in leaf ABA concentration were observed which preceded a similar change in the *Ivr2* transcript levels.

## Materials and methods

### Plant material

Maize plants, F<sub>2</sub> genotype, an early European flint line (*Zea mays* L.), were grown in a greenhouse. After a 48 h imbibition in 4 °C aerated water, grains were planted in individual pots filled with perlite. Day/night temperatures were 24.5/18.5 °C, relative humidity was 60%, natural light was supplemented, when required (16 h d<sup>-1</sup>), with artificial light (NAV-T 400 W and HQI-T 400 W, 400  $\mu$ mol quanta m<sup>-2</sup> s<sup>-1</sup>). Nutrient solution [tap water 250 l, stock solution 1.0 l (made with a commercial solution 'Hydrocani C2' 1.0 l, a commercial powder 'Sequestrene solurapide Fe 100 SG' 15 g, NH<sub>4</sub>NO<sub>3</sub> 32 g, HNO<sub>3</sub> 34 g and MgSO<sub>4</sub>·7H<sub>2</sub>O 50 g)] was supplied nine times a day by an automatic system. For water stress experiments, bi-compartmented pots were used (Fig. 1): they were made of an external PVC tube (height: 250 mm; diameter: 100 mm) equipped with a sliding trap door in its basal part and an internal tube (height: 120 mm; diameter: 95 mm) in its upper part. The internal tube was terminated with a PVC wire netting (mesh 2×3 mm) allowing the lower roots to colonize the base of the external tube. A trap door opening allowed cleaning, with a gentle water jet, of the perlite from the roots which had colonized the lower part of the pots and so exposing these roots to air. The plants were divided in two equal groups, water-stressed plants and control, watered plants. In both groups, three plants were sampled daily for 6 d after water deprivation. In addition, five pots from each condition were weighed daily to compare the water content. The water loss was linear as a function of time, the final value was 15% of the initial weight (see insert to Fig. 3). At the end of the time-course, the difference in weight between stressed and control plants (8 g) was negligible compared with the total water loss (153 g). For experiments with excised leaves, simple pots made of a PVC tube (height: 250 mm; diameter: 100 mm) were used. The fourth leaves were excised in the morning, between 7–8 h after the beginning of the photoperiod. All studies (water deprivation, sampling and supplying) were started when 90% of the fourth leaf ligules were visible, about 15 d after sowing, according to Pelleschi *et al.* (1997).



**Fig. 1.** Schematic representation of the bi-compartmented pots allowing air exposure of the lower part of the root system. The onset of water stress was obtained by opening the trap door and removing the perlite in the lower part of the pot.

### Water-stress studies on intact plants

A portable open gas system (PP Systems; portable system CIRAS-1) allowed measurements of leaf net CO<sub>2</sub> uptake, transpiration, stomatal conductance, and internal CO<sub>2</sub> concentration (CO<sub>2i</sub>) of a 2.5 cm<sup>2</sup> medial part of the leaf at 400 μmol quanta m<sup>-2</sup>. Measurements were performed every morning. Three 0.5 cm<sup>2</sup> leaf discs (approximately 21 mg fresh weight in total) were punched out on both sides of the main vein from the median region of the fourth leaf used for photosynthetic measurements. All the results were expressed on a leaf area basis because this parameter is less influenced by water stress than fresh weight. This is also a common reference in photosynthesis-related studies. The samples were immediately frozen in liquid N<sub>2</sub> and stored at -80 °C until needed for the biochemical analyses. For osmotic measurements, the terminal part of the fourth leaves were ground on ice with de-ionized water and boiled immediately for 3 min in order to stop all enzymatic activity which could provide osmolytes. Measurements were made with an osmometer (Type 13 DR-Autocal, Roebing) after cooling at 4 °C and centrifugation at 14 500 g, for 3 min. Leaf water potential and xylem sap samples were obtained using the fifth mature leaf placed in a pressure chamber (PMS instrument company, Corvallis, Oregon, USA) (Turner, 1986). Water potential was measured when sap appeared at the cut end of the main vein, after a slow increase of chamber pressure; then xylem sap samples were collected by increasing the pressure further and immediately frozen in liquid nitrogen.

Experiments were repeated two or three times, and the results are means of all the experiments. In addition, all measurements in each experiment were carried out on three leaves from three plants.

### Excised leaves for supplying experiments

The fourth leaves of well-watered plants were excised, near the ligule, 7–8 h after the beginning of the photoperiod, left in distilled water for 30 min in the dark, and then transferred to various solutions where the leaf base was re-cut. At this time, light was switched on again and excised leaves were continuously lit for 24 h. The solutions were 10 mM sucrose or 0.37 μM (+)ABA in distilled water. The ABA stock solution was prepared by pre-dissolving 2 mg of (+/-)ABA powder (Sigma A7362) in 0.5 ml absolute ethanol and then finally making up to 1 ml with water. After dilution to 0.37 μM ABA the final ethanol concentration was only 0.005% (v/v). Every 6 h, three leaf discs were punched as previously described and the distal part of the leaves was used for measuring leaf water potential and for collecting xylem sap as described before. The leaf basal part was used for total RNA extraction and osmotic potential measurements. In two complementary experiments, ABA uptake was evaluated by providing 0.37 μmol <sup>3</sup>H-labelled ABA (2600 Bq ml<sup>-1</sup>) into the external solution. Radioactivity and ABA concentration in the xylem sap and leaf extract were measured after HPLC purification according to Julliard *et al.* (1992).

### Invertase activities

Acid vacuolar invertase activities were measured from extracts obtained by grinding leaf discs in an Eppendorf tube with a glass rod and some fine sand (<10 μm). 350 μl extraction buffer (50 mM HEPES-NaOH, pH 7, 10 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>EDTA, 2.6 mM dithiothreitol, 10% ethylene glycol, and 0.02% Triton) was added twice. The extract was centrifuged for 3 min at 12 000 g at 4 °C. An aliquot of each supernatant was desalted on G25 Sephadex columns equilibrated with 50 mM HEPES-NaOH (pH 7.5), 15 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>EDTA, 2.6 mM dithiothreitol, and 0.1% bovine serum albumin. Acid invertase activity of the desalted extract (25 μl) was determined after lowering the pH with 25 μl of 0.2 M Na acetate buffer (pH 4.8), adding 10 μl 600 mM sucrose at 30 °C and incubating for 15 min. Then, the pH was increased by adding 50 μl

0.5 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7) buffer, and samples were immediately boiled for 3 min in order to denature the enzymes. Glucose and fructose derived from sucrose hydrolysis were measured by adding 750 μl reaction buffer (50 mM HEPES-NaOH pH 7, 2 mM MgCl<sub>2</sub>, and 1 mM EDTA) containing 1 mM ATP, 0.4 mM NAD<sup>+</sup>, 3.5 units phospho-glucose isomerase (Roche; EC: 5.3.1.9), 2 units glucose 6-phosphate dehydrogenase (Roche; EC: 1.1.1.49), and 4.2 units hexokinase (Roche; EC: 2.7.1.1). After a 20 min incubation at 30 °C and a 12 000 g centrifugation for 2 min, NADH synthesis was measured spectrophotometrically at 340 nm (Bergmeyer and Bernt, 1974).

### Carbohydrate contents

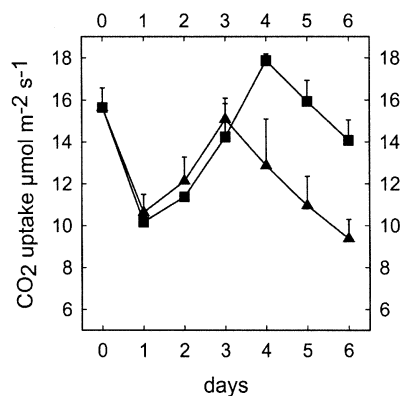
Samples were ground and centrifuged as described for the invertase activities. Carbohydrate contents were determined by enzyme assays (Bergmeyer and Bernt, 1974). The supernatant was used for the hexose content and the pellet for the starch content. The supernatant was boiled for 3 min and centrifuged at 12 000 g. The total soluble sugar content was determined from a 25 μl aliquot to which 15 units of β-fructofuranosidase (Roche; EC 3.2.1.26), in 320 mM sodium citrate (pH 4.6) buffer, was added; sucrose hydrolysis lasted 30 min at 30 °C. Then, the hexose content was determined in a 750 μl reaction mixture containing 235 mM triethanolamine-NaOH (pH 7.6), 3 mM MgSO<sub>4</sub>, 0.4 mM NAD<sup>+</sup>, 2.6 mM ATP, and an enzyme mixture (3.5 units phosphoglucose isomerase, 2 units glucose 6-phosphate dehydrogenase, and 4.2 units hexokinase) at 30 °C for 30 min. The NAD<sup>+</sup> reduction was measured at 340 nm. The free hexose content of the extract was measured similarly in another 25 μl aliquot, except that the β-fructofuranosidase treatment was omitted. The sucrose content was calculated from the difference between the total soluble sugar and the free hexose contents. Starch in the pellet was gelatinized by heating for 30 min at 100 °C with 0.1 ml 0.02 N NaOH. After the addition of 15 units amyloglucosidase dissolved in 320 mM citrate buffer (pH 4.2), starch hydrolysis was performed for 30 min at 50 °C. The extract was cleared by centrifugation at 12 000 g and any glucose starch derived was determined spectrophotometrically, as for the previous measurements, but without adding phosphoglucose isomerase.

### ABA concentration

The ABA concentration was determined by two protocols. The first one, adapted from Quarrie *et al.* (1988) by Sotta and Leonardi (unpublished data), used direct radio-immuno assay on an aqueous extract. The second one used methanol extracts, HPLC separation and an Elisa immuno assay on the purified ABA fraction as described in Julliard *et al.* (1992). The principle of the first method is based on MAC252 antibody competition between the endogenous ABA and exogenous ABA-<sup>3</sup>H: 100 μl ABA-<sup>3</sup>H solution (50% gamma-globulin in 100% PBS, and 0.4 pg μl<sup>-1</sup> ABA-<sup>3</sup>H), 100 μl antibody solution (1/8000 AcMAC252, 0.5% BSA, and 0.4% PVP in 100% PBS) and 200 μl 50% PBS were added to a sample aliquot (10–50 μl) and incubated for 45 min at 4 °C. Then 500 μl of saturated ammonium sulphate was added and precipitation proceeded for 30 min at room temperature. After a 4 min centrifugation at 4 °C and 12 000 g, the pellet was dissolved in 1 ml 50% ammonium sulphate. After another centrifugation, the pellet was dissolved in 100 μl distilled water and mixed with 1.4 ml scintillation medium. Radioactivity of the <sup>3</sup>H-ABA/AcMAC252 complex was counted twice during a 2 h time interval, using a 'Betamatic' counter (type Beta V, model No. 487).

### RNA extraction and northern analysis

Samples were ground in a mortar and stored in liquid N<sub>2</sub>. Total RNA was extracted from a 300 mg aliquot of the powder dissolved in 1.5 ml TriZol (Life Technologie) and incubated for 5 min at 30 °C.



**Fig. 2.** Effect of drought stress on net CO<sub>2</sub> uptake on fourth leaves of well-watered (closed squares) and water-stressed (closed triangles) maize plants. Means  $\pm$ SD from two experiments. For each experiment, three leaves were measured.

Then 300  $\mu$ l of chloroform was added and the mixture was incubated for 3 min at 30 °C. Samples were centrifuged for 15 min, 10 000  $g$  at 4 °C, and 750  $\mu$ l of isopropanol was added to the aqueous phase. After 20 min precipitation at room temperature, centrifugation for 15 min, 10 000  $g$  at 4 °C, generated a pellet which was washed twice in 1.5 ml 70% ethanol. After a 15 min centrifugation at 6000  $g$  and at 4 °C and drying, the pellet was finally dissolved in 20 or 40  $\mu$ l distilled water at 55 °C. The RNA concentration was estimated spectrophotometrically at 260 nm (1 unit OD for 40  $\mu$ g ml<sup>-1</sup>). Denatured total RNA samples (10  $\mu$ g lane<sup>-1</sup>) were separated onto a 1.2% agarose gel according to Sambrook *et al.* (1989) and transferred to a positive membrane (Appligene) using SSC 2 $\times$  as transfer buffer. Gel blots were hybridized to <sup>32</sup>P-labelled DNA probes generated with the oligo-labelling kit (Pharmacia Biotech) as described by Kim (1998). The following probes were used: two vacuolar invertases (*Ivr1*: U16123 and *Ivr2*: U31451), two cell wall invertases (*Incw1*: U17695 and *Incw2*: AF050631), an ABA responsive gene (*Rab17*: X15994), the phosphoenolpyruvate carboxylase (*PEPc*: X17379). Relative mRNA amounts were determined by densitometric scanning of the autoradiograms (Masterscan, Scanalytis, Billerica, MA). The loading differences were standardized using a 18S probe signal.

## Results

### Experiments on intact plants

Water stress was obtained by water deprivation and exposure of the lower part of the root system to air by opening the trap door and removing perlite situated in the lower half of the pot.

**Changes in photosynthetic rate:** For the first 3 d after water deprivation there were no significant differences between control and stressed plants (Fig. 2). After 3 d, net CO<sub>2</sub> assimilation significantly decreased in the stressed plants. During this time, air-exposed roots appeared to be rapidly desiccated in the water-stressed plants.

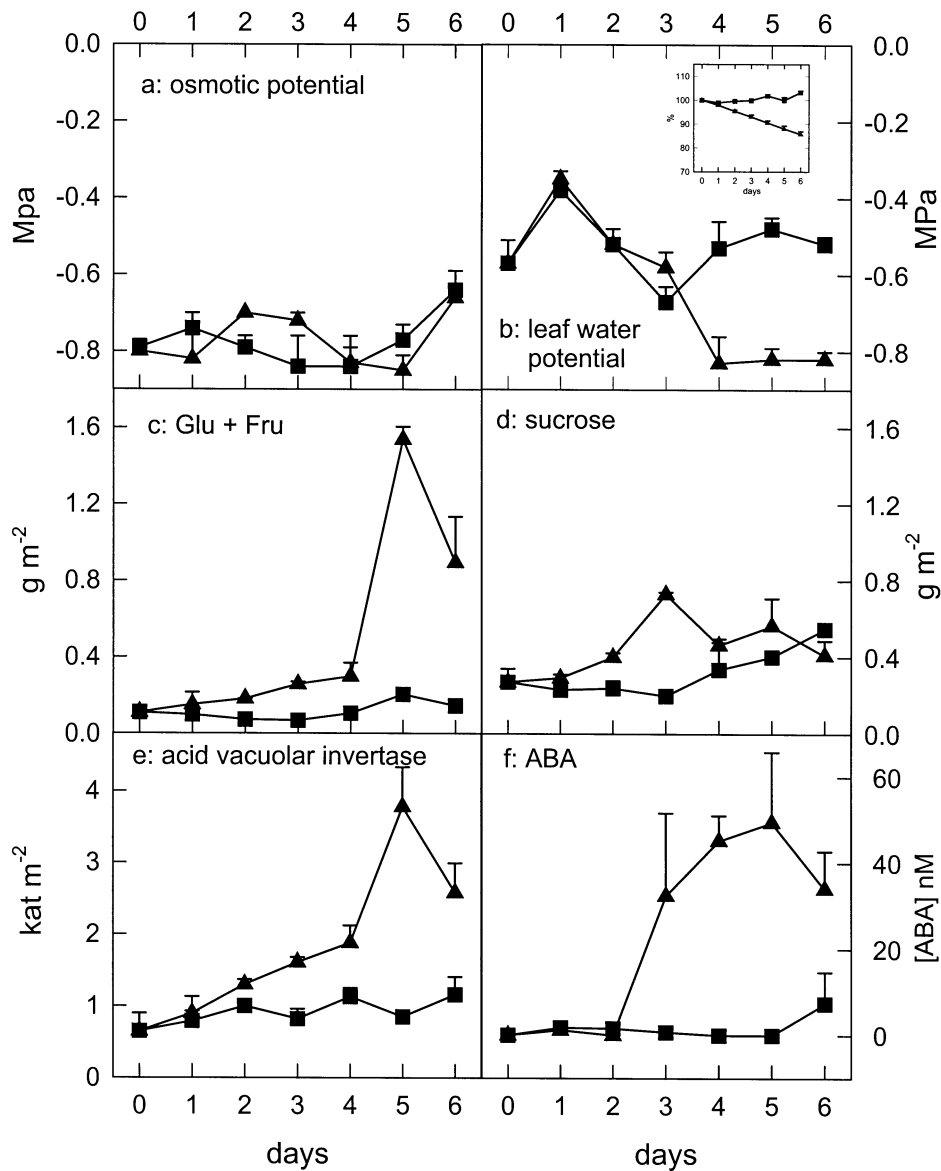
**Plant water status:** The osmotic potential did not change during the time-course of the experiment either in stressed

or in control plants (Fig. 3a). By contrast, the water potential of stressed plant leaves differed from the control plants after 4 d of water deprivation (Fig. 3b). The observed decrease did not change between day 4 and day 6, the stressed plant value being 1.7-fold less than the control plant water potential. Consequently, the decrease in water potential is likely to be due to a decrease in turgor potential if it is assumed that the volume of apoplastic water is low compared with total leaf water content. The wilting aspect of stressed plant leaves from the third day validates this approximation.

**Changes in carbohydrate metabolism:** From the second day onwards, levels of glucose+fructose in the leaf were significantly higher in water-stressed plants than in control plants (Fig. 3c). This difference dramatically increased on the fifth day to give an 8-fold difference between the stressed and the control plants. Sucrose concentration in control plant leaves remained unchanged for the first 3 d while it increased significantly in stressed plants between the second and third day (Fig. 3d). Then, from 3–6 d sucrose concentration increased progressively from 0.2 g m<sup>-2</sup> to 0.55 g m<sup>-2</sup> in control plants, thus reaching the levels observed in the stressed plants between days 4–6. At the end of the 6 d experimental period, sucrose concentration was similar in stressed and control plants, while reducing sugar concentration remained 6-fold higher in stressed plants.

**Enhancement of acid vacuolar invertase activity:** Invertase activity (Fig. 3e) responded to water stress in a similar way to reducing sugar (Fig. 3c). While the activity remained unchanged in control plants, it increased slightly (2-fold) in stressed plants from the second day to the fourth day. On the fifth day, an abrupt 4-fold increase was observed, after which the activity declined to approximately 2-fold of the control activity. The correlated variations of invertase activity and reducing sugar concentration led to the assumption that glucose and fructose concentration were a result of the acid vacuolar invertase activity. On the other hand, sucrose concentration was less affected and not related to the measured invertase activity.

**Xylem sap and leaf ABA concentration:** ABA concentration in control plant xylem sap was constant, and its level was very low. By contrast, ABA concentration in the xylem sap of stressed plants increased between the second and the third day of water deprivation. At day 5, there was more than a 40-fold difference in ABA levels between the stressed and control plants (Fig. 3f). In water-stressed plants, a good correlation ( $r=0.84$ ) between ABA xylem sap concentration and leaf acid vacuolar invertase activity was obtained. In order to test the possible causal relationship between ABA and vacuolar invertase expression, exogenous ABA was provided to excised leaves.



**Fig. 3.** Effect of drought stress on osmotic potential (a), leaf water potential (b), (Glu+Fru) glucose and fructose concentration (c), sucrose concentration (d), acid vacuolar invertase activity (e), and xylem sap ABA concentration (f) in fourth leaves from well-watered (closed squares) and water-stressed (closed triangles) maize plants. Sampling and measurements were carried out 7 h after the beginning of the light period. Means  $\pm$ SE from three experiments. For each experiment, three leaves were measured. Insert: variation in soil (perlite) relative water content in control (closed squares) and water-stressed pots (closed triangles), means  $\pm$ SE from five pots. Error bars smaller than symbols are not visible.

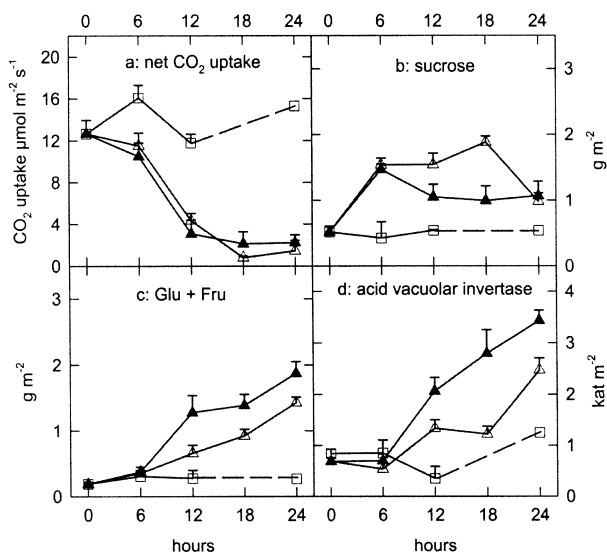
Furthermore, a close examination of Fig. 3 showing that sucrose, hexoses and vacuolar invertase start to increase before xylem ABA concentration indicates that xylem ABA is unlikely to be the causal factor of these responses. Thus, in further experiments, leaf ABA concentration was also measured which is probably an explanation of the response at cellular level.

#### Experiments on excised leaves

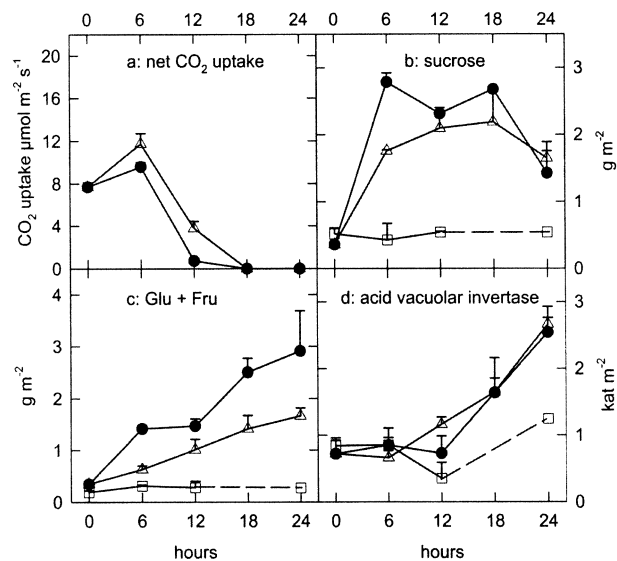
In these experiments ABA supply ( $0.37 \mu\text{M}$ ) was compared with  $10 \text{ mM}$  sucrose supply, since carbohydrates were previously reported to act on vacuolar invertase

expression (Xu *et al.*, 1996) and leaf carbohydrate content increased in parallel with invertase activity.

*Changes in photosynthetic rate:* Leaf excision (Fig. 4a) caused a slight decrease of net  $\text{CO}_2$  assimilation during the first 6 h, then a dramatic decline was observed which was parallel to that of stomatal conductance (data not shown). However, leaf turgor was maintained for 24 h as confirmed by water and osmotic potential measurements (data not shown). The supply of  $0.37 \mu\text{M}$  ABA to the excised leaves from plants did not produce any significant differences in the time-course of net  $\text{CO}_2$  uptake when compared to the



**Fig. 4.** Effect of ABA supplying of excised leaves on net CO<sub>2</sub> uptake (a), sucrose concentration (b), (Glu+Fru) glucose and fructose concentration (c), and acid vacuolar invertase activity (d). (closed triangles) 0.37 μM ABA supplied leaves, (open triangles) water-supplied leaves and (open squares) leaves kept on intact plants. Means ±SE from two experiments. For each experiment, three leaves were measured.

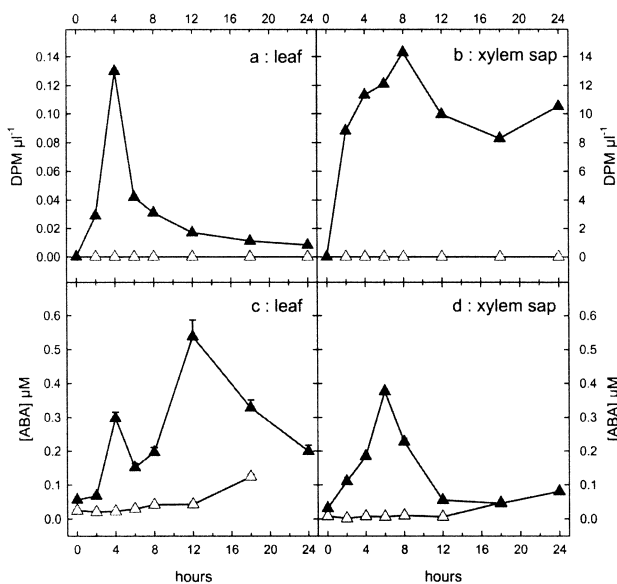


**Fig. 5.** Effect of sucrose supplying of excised leaves on net CO<sub>2</sub> uptake (a), sucrose concentration (b), (Glu+Fru) glucose and fructose concentration (c), and acid vacuolar invertase activity (d). (closed circles) 10 mM sucrose supplied leaves, (open triangles) water-supplied leaves and (open squares) leaves kept on intact plants. Means ±SE from two experiments. For each experiment, three leaves were measured.

water-treated controls (Fig. 4a); whereas 10 mM sucrose tended to have a slight but significant decreasing effect (Fig. 5a).

**Changes in carbohydrate metabolism:** Leaf excision produced a 3-fold increase in the sucrose level for the first 6 h of the treatment, without any significant difference between ABA-supplied or water-supplied leaves when attached leaves maintained a constant level (Fig. 4b). Over the same time, no changes were observed in the glucose and fructose concentrations (Fig. 4c). After 6 h, the ABA-treated leaves showed a larger increase in reducing sugar levels than the control leaves over the 24 h period. During the same 6–24 h period leaf sucrose levels decreased faster in the ABA-supplied leaves, but finally reached a level similar to that found in water-supplied leaves at 24 h (Fig. 4b). Starch content steadily increase from 0.29 g m<sup>-2</sup> to 1.40 g m<sup>-2</sup> in attached leaves, whereas the variation was from 0.29 g m<sup>-2</sup> to 1.90 g m<sup>-2</sup> in control and ABA-treated excised leaves.

When detached leaves were sucrose supplied, the sucrose concentration rapidly increased to a higher level than in the ABA experiments at 6 h and was higher than the corresponding water-supplied control leaves (Fig. 5b). Both treatments gave rise to a continuous increase in fructose and glucose levels during the supplying time-course, although the sucrose-supplied leaves contained higher reducing carbohydrate levels 3 g m<sup>-2</sup> versus 1.8 g m<sup>-2</sup> in control, at 24 h (Fig. 5c).



**Fig. 6.** Effect of supplying excised leaves with water compared to a solution containing 0.37 μM ABA labelled with <sup>3</sup>H-ABA (2500 Bq ml<sup>-1</sup>). <sup>3</sup>H-ABA radioactivity in leaf (a) and in xylem sap (b). Internal ABA concentration in leaf (c) and in xylem sap (d). (closed triangles) ABA-supplied leaves, (open triangles) water-supplied leaves. Means ±SE from three leaves.

**Invertase activity:** Compared with control attached leaves where invertase activity only increased slightly during the last 18–24 h period, leaf excision led, after 6 h, to an increase in invertase activity (Figs 4d, 5d). ABA-treated

leaves presented a 30–50% higher activity than excised control leaves from 6 h (Fig. 4d). By contrast, there was no significant difference between control and sucrose-supplied leaves, in the invertase activity time-course (Fig. 5d).

**Xylem sap and leaf ABA concentration:** Concentrations expressed on the basis of water content were in the same range for leaf and xylem sap. As a first step, ABA penetration was checked by providing  $^3\text{H}$ -ABA in the external medium and by measuring the radioactivity time-course in the ABA fraction separated either from xylem sap or the leaf. A sharp  $^3\text{H}$ -ABA peak was observed in the leaf at 4 h, followed by a more progressive decline up to 24 h (Fig. 6a). By contrast, xylem radioactive concentration increased progressively up to 8 h and partly declined for the last hours (Fig. 6b). Total ABA concentration in the leaf had a different time-course from  $^3\text{H}$ -ABA since it presented two peaks, at 4 h and 12 h (Fig. 6c). In the xylem sap, only one peak was observed at 6 h (Fig. 6d). In a separate experiment, using RAI assay for ABA detection in the xylem sap, no difference was observed between sucrose-supplied and water-supplied leaves, whereas concentration in attached leaves was too low to be detected (data not shown).

**Modulation of gene expression:** To provide a better understanding of the regulation of carbohydrate metabolism under water stress, the expression of some genes was analysed with special emphasis on the soluble invertase transcripts, *Ivr1* and *Ivr2*. Among the examined genes (*Ivr1*, *Ivr2*, *Incw1*, *Incw2*, *Rab17*, and *PEPc*) a faint signal was detected with the *Incw1* probe and no signal using the *Incw2* and *Ivr1* probes (data not shown). By contrast, *Ivr2* and *Rab17* appeared to be up-regulated by ABA. However, this effect was superimposed on the variation observed in untreated control leaves where two peaks at 6 h and 18–24 h were separated by a drop at 12 h (Fig. 7). As the experiment took place in continuous light, the variation may be reminiscent of a circadian cycle. ABA supply increased the amplitude of the variations for both *Ivr2* and *Rab17* transcripts, especially at 6 h (Fig. 7). By contrast, the expression of phosphoenol-pyruvate carboxylase (*PEPc*) tended to be depressed by ABA. Sucrose supply had no significant effect on *PEPc*; *Rab17* expression was enhanced by 40–50% from 12 h whereas *Ivr2* expression was increased at 12 h (+68%) and 24 h (+20%) and decreased at 18 h (–45%).

## Discussion

### Early response of vacuolar invertase to water stress

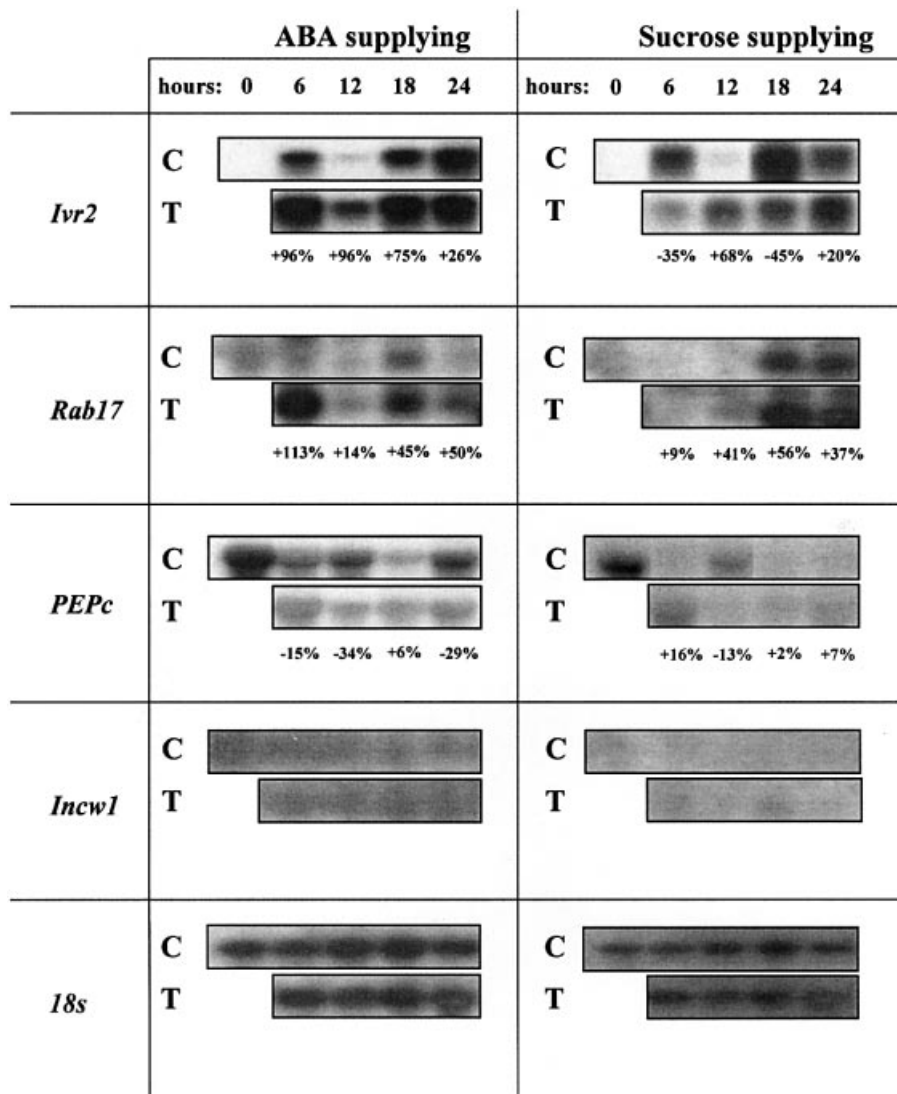
A progressive water stress induced by root tip exposure to air and the withholding of water produced dramatic changes on mature leaves. The earlier event was an

enhancement of acid vacuolar invertase activity related to an increase in glucose and fructose concentration confirming earlier reports obtained by simple water withholding (Pelleschi *et al.*, 1997; Kim *et al.*, 2000). Thus, the exposure of the distal part of the root system did not substantially modify the effect of water deprivation. Hexose concentration was strongly related to acid vacuolar activity, while sucrose level was not, although sucrose is the substrate of this enzyme. This is not surprising considering that sucrose concentration depends on synthesis, hydrolysis and export, and not only on invertase activity.

Water deprivation produced an increase of leaf acid vacuolar invertase activity before any significant modifications in other measured leaf parameters, like gas exchange, osmotic and turgor potential. Consequently, the regulation of leaf acid vacuolar invertase activity cannot be explained by the variation in photosynthetic or water status parameters. A more likely candidate is ABA since (i) its xylem concentration increase is correlated with vacuolar activity in stressed plants, and (ii) ABA is a signal coming from the roots when they perceive water stress (Davies and Zhang, 1991; Liang *et al.*, 1997). However, the effect of carbohydrate level either on vacuolar invertase activity (Kaufman *et al.*, 1973; Lopez *et al.*, 1988) or on the expression of *Ivr1* and *Ivr2* transcripts (Xu *et al.*, 1996) has been reported. As ABA mutants (*vp1* and *vp5*) are not easily used at the adult leaf stage because of pigment alterations, and glucose and fructose concentration cannot be manipulated independently from ABA in intact plants, it is difficult to establish causal relationships. ABA or sucrose supplying experiments on excised leaves provide some insights.

### Vacuolar invertase expression is enhanced by added ABA

$\text{CO}_2$  gas exchange of all detached leaves dramatically decreased after 6 h irrespective of what they were supplied. Thus, this phenomenon is unlikely to explain vacuolar invertase activity modulation. A similar drop was observed in heat-girdled maize leaves (Jeannette *et al.*, 2000) and was interpreted as a feedback inhibition due to carbohydrate accumulation. Accordingly, a large increase in sucrose was observed during the first 6 h period. After 6 h, the effect of ABA supply became apparent. Leaf vacuolar invertase activity increased in parallel with xylem sap ABA concentration and was preceded by a leaf ABA peak originating from added ABA. Meanwhile, sucrose, glucose and fructose also accumulated, so their role in the increase in invertase activity may be questioned. The sucrose supply experiments show that despite a further accumulation of glucose and fructose, no enhancement of invertase activity was observed. This rules out a direct carbohydrate effect on enzyme activity.



**Fig. 7.** Time-course of *Ivr2*, *Rab17*, *PEPc*, *Incw1*, and *18s* transcripts in excised leaves supplied with water (C) or treated (T) with a 0.37  $\mu$ M (+)ABA or 10 mM sucrose solution. The digits under northern blots are the treated/control ratio for spot intensity. For *Incw1* probe, the signal was to faint (4-fold exposure compared to other probes) for correct quantification. The mean variation coefficient between replicates is 0.26. For each extract, three leaves were pooled.

The observation of sucrose accumulation in all excised leaves, even in untreated controls, raises another question. It could be explained by an interruption of export through callose formation in phloem tissues or/and by a decrease of phloem loading capacity by ABA (Vreugdenhil, 1983). The callose hypothesis is not supported, since EDTA at 5 or 10 mM was ineffective on carbohydrate content at both concentration. Furthermore, 10 mM produced leaf wilting, a sign of toxicity (data not shown). Consequently, carbohydrate concentration appeared to be more dependent on invertase activity than invertase activity is dependent on carbohydrate level.

The accumulation of glucose and fructose in leaves may have other consequences, either on osmotic adjustment or

on gene expression: in the later case, it could interfere with the ABA effect. An osmotic adjustment has been reported in growing maize leaves by Westgate and Boyer (1985), but on mature leaves, these results confirm those of Pelleschi *et al.* (1997) showing an absence of osmotic adjustment. It is not surprising since maize is a well known iso-osmotic species. As a consequence, between days 3 and 4, turgor potential decreased with leaf water potential.

Recent results in *Arabidopsis* with carbohydrate-insensitive mutants (*isi*, *gin*) and ABA-deficient mutants (*aba*, *abi*) have demonstrated an interaction between ABA and the sucrose signalling pathways (Rook *et al.*, 2001, and references therein). In the present data, excision produced a continuous increase in leaf hexoses, thus it could be

hypothesized that the ABA effect on gene expression and invertase activity is favoured by hexose accumulation. The fact that the addition of 10 mM sucrose produced a further increase in leaf hexoses concentration, without any effect on invertase activity, rules out this hypothesis unless a threshold effect is assumed. Furthermore, in the case of ABA supply, the enhancement of the *Ivr2* transcript level corresponded with that of invertase activity, as in intact plants (Pelleschi *et al.*, 1999). It was not the same when 10 mM sucrose was added since, despite variations in the *Ivr2* transcript level, the invertase activity was not different from the control. The lower response of PEPc to sucrose, compared with previous reports, may be related to the difference in the carbohydrate concentration used: 30 mM sucrose solution for repressing the activity of PEPc-promoter-GUS constructs in maize protoplasts (Sheen, 1990). Interestingly, *Rab17* showed an enhanced expression both by ABA and sucrose supply. Such a response could suggest a feed-forward mechanism since ABA stimulation could be reinforced by the carbohydrate produced by the invertase activity. This hypothesis does not apply for IVR2 invertase because of the uncoupling between transcript and activity variations under sucrose supplying.

Among the four invertase genes studied (*Incw1*, 2, *Ivr1*, 2), only *Ivr2* gene expression was enhanced by ABA supply. It is interesting to note that both *Rab17*, defined as an ABA-responsive gene, and *Ivr2*, displayed, at transcript level, the same two peaks profile as the leaf ABA concentration, with a 2 h delay. The behaviour of *Ivr2* as an ABA-responsive gene was previously noted by Kim *et al.* (2000) who observed the existence of synchronous diurnal variations in both *Ivr2* and *Rab17* transcripts in leaves and sheaths, the maximum being at the beginning of the light period and the minimum at the end. The measurement of vacuolar invertase activity showed similar variation (Kim, 1998). As, in these results, the variation was observed under constant illumination, the existence of an endogenous rhythm may be suspected. By contrast with leaf ABA, xylem ABA had a different time-course and it is clearly not representative of intracellular ABA. Another question related to leaf ABA is the origin of the second peak. Radioactivity measurements clearly showed that it does not originate directly from external ABA, since its specific radioactivity is very low.

In the present study, ABA supply enhanced *Ivr2* gene expression in leaves, while sucrose supply did it less intensively and with a different time-course. Very recently, Andersen *et al.* (2002), applying water stress during early kernel development, did not observe a relationship between ovary ABA level and *Ivr2* expression. Thus, the response of the *Ivr2* vacuolar invertase to ABA seems to be organ-specific and may be due to different cross-talk in sucrose and ABA signalling pathways. Another hypothesis to explain the organ-specific expression could be the

existence of two *Ivr2* genes. Accordingly, *Ivr2* mapping revealed two loci, on chromosome 4L and 5S (Xu *et al.*, 1996). These points are currently under study.

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