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ORIGINAL ARTICLE

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Regulation of vacuolar invertase by abscisic acid or glucose in leaves and roots from maize plantlets

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Abstract Recent studies have demonstrated in leaves of maize (*Zea mays* L.) plants submitted to a moderate water stress an early enhancement of vacuolar invertase activity that paralleled the expression of the vacuolar invertase *Ivr2* gene and the accumulation of hexoses. In this paper, the direct role of abscisic acid (ABA) was checked by providing this hormone to the root medium of hydroponically grown maize plantlets. ABA supplied to 10-day-old seedlings appeared to enhance the vacuolar invertase activity within 1 h in roots and 2 h in leaves, the maximum being reached at 4 and 8 h, respectively. The *Ivr2* gene expression varied accordingly, except that the maximum values were earlier. During the first 8 h of activity enhancement, hexose and sucrose concentrations were not significantly affected by ABA. The changes in activity were correlated to leaf and root ABA concentrations and they were concentration dependent in roots and leaves. In contrast, the addition of 1% glucose or polyethylene glycol, at the same osmotic potential, was ineffective on invertase activity, but glucose supply enhanced *Ivr2* transcript levels, after 18 h, in a concentration-dependent manner in the leaf, whereas they were repressed at higher concentrations in intact roots. The latter result appeared specific to intact roots since similar treatments performed using excised leaf or root pieces confirmed a previous report on the enhancement of *Ivr2* and *Ivr1* transcript levels by glucose in roots [J. Xu et al. (1996) Plant Cell 8:1209–1220]. Therefore, ABA appears to be a strong inducer of *Ivr2*-invertase expression in roots and leaves.

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Abbreviations ABA: Abscisic acid · PEG: Polyethylene glycol

Introduction

In most plants, sucrose is the major end product of photosynthesis and the major form of carbohydrate transported to non-photosynthetic organs. It can be involved in numerous metabolic pathways but before being used it has to be cleaved into glucosyl and fructosyl moieties, either by an invertase or a sucrose synthase. Invertase (β-fructosidase, EC 3.2.1.26), can be considered as a key enzyme in carbohydrate metabolism since it catalyses the irreversible reaction that converts sucrose into glucose and fructose. Invertase belongs to a multi-enzyme family with various forms classified in accordance with their cellular location, optimum pH and solubility properties: two soluble forms (acidic in the vacuole and neutral in the cytoplasm) and a particulate form (acidic in the cell wall) (Doehlert and Felker 1987). Little is known about neutral invertases, whereas acid invertases have been well characterized. Acid invertase genes have been isolated from various plant species and they appear to be well conserved (for a review, see Tymowska-Lalanne and Kreis 1998).

In maize, six acid invertase genes are presently known: *Ivr1* and *Ivr2* (Xu et al. 1996) encoding vacuolar forms and *Incw1*, *2*, *3*, *4* encoding cell wall enzymes (Taliercio et al. 1999; Kim et al. 2000b). Although the predominant role of acid invertases is probably to provide glucose for cell energy production (Karuppiah et al. 1989), they have several other physiological functions depending, for example, on their cellular location. Invertases are likely to play a major role in sucrose partitioning and long-distance transport by modulating the sucrose gradient between phloem and unloading tissues,

and in the control of the relative sink strength of plant tissues (Roitsch et al. 1995; Godt and Roitsch 1997; Roitsch 1999). This latter function could be essential for grain filling, as suggested from the maize *miniature-1* mutant (Cheng et al. 1996). Invertases could also be involved in cell elongation, through maintenance of cell turgor (Pfeiffer and Kutschera 1995) via osmotic pressure control, as suggested by Gibeaut et al. (1990). However, osmotic adjustment depends on species and organ age since this was observed in growing organs (Westgate and Boyer 1985) but not in adult maize leaves (Pelleschi et al. 1997). The regulation of gene expression could be another important function of invertases. Indeed, glucose and sucrose have been shown to be effective in repressing photosynthesis-related genes (Sheen 1990) and activating carbohydrate-related genes (Koch 1996). As an example, transgenic tobacco plants expressing yeast-derived invertase showed a reduction of photosynthetic-enzyme-encoding gene transcripts related to hexose accumulation (Herbers et al. 1996).

Invertase activity has been shown to be regulated both temporally and spatially: organ-specific expression patterns have been reported in carrot, tomato, *Arabidopsis* and maize (Sturm et al. 1995; Godt and Roitsch 1997; Tymowska-Lalanne and Kreis 1998; Kim et al. 2000a). Invertase activity is also regulated by internal and environmental factors such as carbohydrates (Xu et al. 1996), auxins (Morris and Arthur 1984), cytokinins (Ehness and Roitsch 1997), gibberellins (Wu et al. 1993), gravity (Gibeaut et al. 1990), light (Huber et al. 1986), temperature (Zrenner et al. 1996), wounding and pathogen infection (see Sturm and Chrispeels 1990). Numerous studies have focused on the influence of water stress on invertases. Moderate water stress prevents the increase of both cell wall and vacuolar invertase activities in young maize kernels (Zinselmeier et al. 1995) while an increase of vacuolar invertase activity has been detected in leaves of bean and pigeon pea by Castrillo (1992) and Keller and Ludlow (1993), respectively. Conversely, severe water deprivation inhibited acid and cytosolic invertases in bean (Castrillo 1992). More recently, an early increase of acid vacuolar invertase activity was reported in mature maize leaves submitted to moderate water stress (Pelleschi et al. 1997). This was later related to the stimulation of *Ivr2* gene expression among all invertase genes (Pelleschi et al. 1999; Kim et al. 2000a).

Concerning this specific effect of water stress on acid vacuolar invertase activity and gene expression, one question still remains: what actually triggers this induction? A good candidate is the plant hormone abscisic acid (ABA). Indeed, its level increases in the entire plant as a result of water stress (Quarrie 1993), invertase activity is stimulated by ABA in soybean (Ackerson 1985) and finally, in carrot, two regions homologous to ABA-responsive boxes have been localised in the promoter region of a cell wall invertase gene (Ramloch-Lorenz et al. 1993). Nevertheless, although ABA is the major signal operating during drought stress, not all drought-induced genes are regulated by this hormone (for a review, see Shinozaki and Yamaguchi-Shinozaki 1997). Interestingly, both glucose and fructose accumulate, in parallel with invertase activity, in leaves of plants submitted to water stress (Pelleschi et al. 1997; Kim et al. 2000a), and expression of the *Ivr1* and *Ivr2* genes is enhanced by glucose incubation of root fragments (Xu et al. 1996). Thus, glucose also seems to be a potential inducer of vacuolar invertase, as well as ABA. Recent results obtained with excised maize leaves, fed either with 0.37 mmol m^{-3} ABA or 10 mol m^{-3} glucose, have shown a specific enhancement of vacuolar invertase activity by ABA (Trouverie et al. 2003) but as hexoses accumulated in the leaves, probably as a consequence of wounding, the partitioning between hormonal and carbohydrate effects was not clear cut.

In the present paper, regulation of acid vacuolar invertase was investigated using intact plantlets cultivated under hydroponic conditions and incubated with various concentrations of ABA or glucose. Media with the same osmotic potential were also tested by using polyethylene glycol (PEG), in order to determine a possible osmotic effect of glucose. The results were compared with those obtained on excised leaf and root pieces. Vacuolar invertase activity, various gene expressions, substrate and product concentrations were analysed. A strong concentration-dependent induction of invertase activity and *Ivr2* gene expression by ABA was observed both in leaves and roots. Glucose was rather ineffective on invertase activity of intact plantlets while it had a slight effect on excised leaf and root pieces.

Materials and methods

Plant materials

Maize seeds (*Zea mays* L., F-2 genotype, an early European flint line) were a gift from the Station de Génétique Végétale (91190 Gif-sur-Yvette, France). After water-logging in aerated water (48 h, 4°C), seeds were germinated in closed plastic boxes, on cellulose paper soaked with water, and maintained in the dark for 3 days at room temperature. Seedlings were transferred to a hydroponic system consisting of boxes containing a continuously aerated culture medium [basal medium (Hoarau et al. 1996), complemented with 13 mol m^{-3} KNO_3] that was changed every 48 h. Plantlets were grown for 7 days in a culture chamber with a circadian cycle of 16 h "day" (26°C, 70% relative humidity, 250 μmol quanta m^{-2} s^{-1} irradiance) and 8 h "night" (22°C, 90% relative humidity). All experiments were done with 10-day-old plantlets.

Exogenous product supply and material sampling

Experiments on intact plantlets Half an hour before the light period on day 10, the $t=0$ measurement was carried out for the control plantlets. At the start of the

light period, the culture medium of each batch was replaced by fresh culture medium, complemented with 0.037, 0.37 or 3.7 mmol m^{-3} (+)ABA, with 0.2, 1 or 2% (w/v) glucose, or with 2 or 7% (w/v) PEG 8000. PEG 8000 was used in order to obtain the same osmolarity potential as in 0.2 or 1% glucose medium. Osmolarity potential was determined with a Roebling cryo-osmometer (Messtechnik, Berlin, Germany). For each treatment, six plants were de-topped and the basal parts (root systems, seeds and hypocotyls) were maintained in the medium. Two 1-cm segments were taken from the middle part of four 2nd leaves, weighed (approx. 40 mg) and frozen in liquid nitrogen for determination of soluble carbohydrate content and invertase activity. The remaining parts of the four leaves were pooled with the two remaining leaves and frozen in liquid nitrogen for mRNA extraction. Xylem sap exuding from the de-topped part was collected for 20 min and frozen in liquid nitrogen for subsequent pH measurement using a micro-electrode (Hanna instruments) and ABA content determination. Then, roots of the six de-topped plants were excised and the excess medium carefully removed. Two pieces of the middle part of each root system, including primary and secondary roots, were sampled and weighed (approx. 40 mg). The remaining parts of these root systems were pooled and all root material was immediately frozen in liquid nitrogen and stored with the other samples at −80°C. This sequence of sampling was repeated at 1, 2, 4, 8, 12, 18 and 24 h after the light was switched on; light was maintained throughout the 24 h.

Experiments on excised roots and leaves The root system and the leaves of 20 plantlets were cut into approximately 1-cm pieces and independently incubated under light conditions for 24 h either in continuously aerated culture medium, or in culture medium with 10 mmol m^{-3} (+) ABA, 0.2% glucose or 2% PEG 8000. Plant material was sampled (excess medium being removed) and frozen in liquid nitrogen and stored at −80°C for biochemical determinations and mRNA extractions.

Biochemical measurements

Invertase activities and carbohydrate levels were determined using an enzyme coupling assay adapted from Bergmeyer and Bernt (1974) as described by Pelleschi et al. (1997). The protocol is based on the conversion of sucrose and hexose into glucose-6-phosphate and the final oxidation of glucose-6-phosphate into 6-phosphogluconate. The coupled NADH formation was measured spectrophotometrically at 340 nm. The 8 values on a fresh-weight basis (2 determinations, 4 independent samples) were averaged and standard errors calculated. Student's *t*-test comparisons were performed and a significant-difference hypothesis was accepted with $P \leq 0.05$.

ABA measurements

The ABA concentration was determined by a protocol adapted from Quarrie et al. (1988) by B. Sotta and A. Leonardi (Physiologie Cellulaire et Moléculaire des Plantes, Université Paris VI, Paris, France, and UMR de Génétique Végétale, INRA, Gif-sur-Yvette, France; personal communication), using a direct radio-immunoassay on aqueous root and leaf extracts or directly on an aliquot of xylem sap. The principle is based on MAC252 antibody competition between the endogenous ABA and exogenous [^{3}H]ABA: 100 µl [^{3}H]ABA solution [50% γ-globulin in 100% phosphate-buffered saline (PBS), 0.4 mg m^{-3} [^{3}H]ABA; Amersham], 100 µl antibody solution [1/8,000 MAC252, 0.5% (w/v) bovine serum albumin, 0.4% (w/v) polyvinylpyrrolidone in 100% PBS] and 200 µl 50% PBS were added to a sample aliquot (10 to 50 µl) and incubated for 45 min at 4°C. Then 500 µl saturated ammonium sulphate was added and precipitation proceeded for 30 min at room temperature. After a 4-min centrifugation at 4°C, 12,000 *g*, the pellet was dissolved in 1 ml 50% (w/v) 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 [^{3}H]ABA–AcMAC252 complex was counted twice with a 2-h interval between countings, using a Beckman counter (type LS 6000 IC).

RNA extraction and northern analysis

Samples were ground in a mortar in liquid N_2. Total RNA was extracted from a 300-mg aliquot of powder dissolved in 1.5 ml TriZol (Invitrogen Life Technologies) and incubated for 5 min at 30°C before addition of 300 µl chloroform and a further incubation for 3 min at 30°C. Samples were centrifuged for 15 min (10,000 *g*, 4°C) and 750 µl isopropanol was added to the aqueous phase. After 20 min precipitation at room temperature and centrifugation for 15 min (10,000 *g*, 4°C), the pellet was washed twice in 1.5 ml 70% (v/v) ethanol. After a 15-min centrifugation (6,000 *g*, 4°C) and drying, the pellet was finally dissolved in 20 or 40 µl distilled water at 55°C. RNA concentration was estimated spectrophotometrically at 260 nm (1 unit OD for 40 g m^{-3}). Denatured total RNA (10 µg/lane) was separated on a 1.2% (w/v) agarose gel according to Sambrook et al. (1989) and transferred to a positive membrane (Appligene) using 2×SSC as transfer buffer. Gel blots were hybridized to ^{32}P-labeled DNA probes generated with an oligo labelling kit (Pharmacia Biotech) as described by Kim (1998). The following probes were used: two vacuolar invertases (*Ivr1*: U16123 and *Ivr2*: U31451) and an ABA-responsive gene (*Rab17*: X15994). Relative

mRNA amounts were determined by densitometric scanning of the autoradiograms (Masterscan; Scanalytis, Billerica, MA, USA) or quantification by phosphoimager (Storm; Molecular Dynamics). The loading differences were standardised using an *18S* probe signal.

Results

Experiments on intact plants

Modulation of acid vacuolar invertase activity

Compared to control-plant invertase activity, which was relatively stable during the time course of the experiment, ABA treatment enhanced activity in a concentration-dependent way (Fig. 1a,b). In leaves, 0.037 and 0.37 mmol m^{-3} ABA produced no significant differences with respect to the control, except at 8 h where a 1.6-fold enhancement of invertase activity was observed. Plants fed with 3.7 mmol m^{-3} ABA showed a significant effect on invertase activity after 2 h treatment, reaching a peak value 2.8-fold greater than that of the control after 8 h (Fig. 1a) before decreasing to 1.2-fold the control value at 18 h, and rising to approximately 2-fold after 24 h. In roots, supplying ABA had a similar effect to that seen in leaves since invertase activity increased in a similar concentration-dependent manner; however, the effect was more immediate and more proportional to ABA supply (Fig. 1b). Roots incubated in ABA showed an increase of invertase activity between 2 and 8 h. The change was greater in roots treated with 0.37 mmol m^{-3} ABA than in those treated with 0.037 mmol m^{-3} ABA, except that a transient decrease was noted at 8 h before a regular decrease from 12 to 24 h. Again, the strongest effect was obtained with roots supplied with 3.7 mmol m^{-3} ABA where the response was detectable from 1 h, with a peak between 4 and 8 h that reached 2.3-fold the control-plant invertase activity. A progressive decrease of invertase activity occurred after 8 h, like for the other ABA treatments. In contrast to ABA, glucose or PEG treatments had no significant effect on either leaf or root invertase activities whatever the concentration (Fig. 1c,d; Table 1).

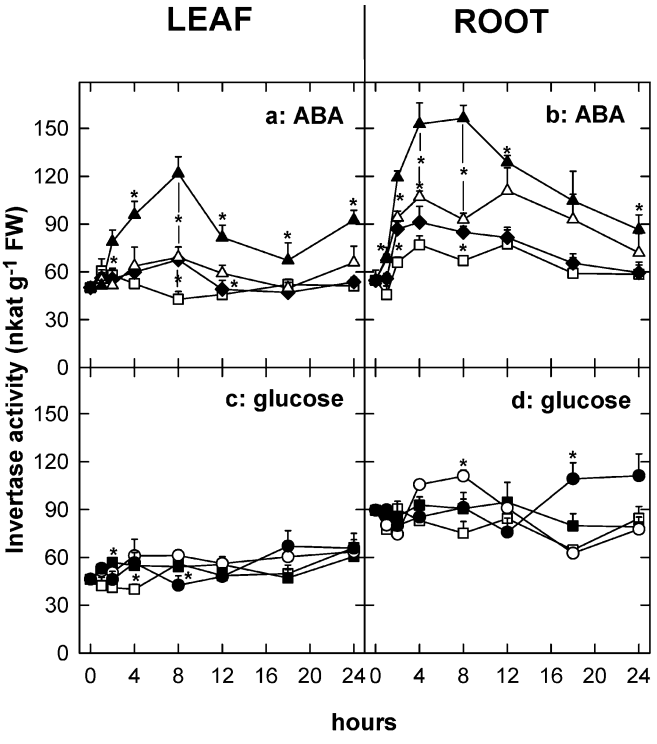

Fig. 1a–d Effect of ABA (**a, b**) or glucose (**c, d**) on vacuolar invertase activity in leaves (**a, c**) and roots (**b, d**) of intact 10-day-old maize (*Zea mays*) plantlets. Plants were supplied, for 24 h, with culture medium alone (□), or with culture medium complemented with 0.037 (◆), 0.37 (△) or 3.7 mmol m^{-3} (▲) ABA; or with 0.2% (■), 1% (○) or 2% (●) glucose. Means on a fresh-weight basis ± SE are from 4 independent determinations. A student's *t*-test was performed when the treatment effect was significant in a two-way ANOVA (time, treatment). *Asterisks* (*) denote significant differences: *t*-test at $P < 0.05$. An asterisk placed above the upper curve indicates a comparison between highest and lowest values, whereas an asterisk placed between values indicates a significant difference between these adjacent values

Changes in soluble carbohydrate levels

In control leaves and roots, hexose content remained rather constant during all experiments in contrast to sucrose content which increased by approximately 2- to 4-fold the initial value in leaves; this may be a consequence of the continuous light (Figs. 2, 3). The ABA supply tended to increase hexose and sucrose contents in leaves but the *t*-test showed that the effect was only significant for the highest concentration (3.7 mmol m^{-3}) from 8 h for hexoses and at 12 h for sucrose (Fig. 2a,c). In roots, ABA was much less effective, except for the slight increase in hexose levels that was detected at 8 h with the highest ABA concentration (Fig. 2b,d).

Supplying glucose had no significant effect on leaf hexose content until 12 h (Fig. 3a), after which, leaves of plants treated with 1% and 2% glucose showed a steady increase in glucose and fructose contents while 0.2% glucose remained ineffective. In contrast, in roots (Fig. 3b,d), incubation with glucose strongly and regularly enhanced hexose and sucrose contents. At each sampling time, a glucose-concentration-dependent effect on carbohydrate accumulation was observed, except for the sucrose content at 18 h in roots fed 1% glucose (Fig. 3d).

The leaf sucrose levels were also unaffected by glucose incubation during the first 8 h (Fig. 3c), after which sucrose was found to accumulate significantly, to a maximum of 3-fold the control value (18 h) when 2% glucose was supplied. PEG treatments had no significant effect either on hexoses or sucrose contents in leaves and roots (Table 1), despite a slightly higher level of glucose plus fructose in the roots of plantlets fed 7% PEG.

Table 1 The effect of PEG addition to culture medium on invertase activity (*Inv*; nkat g^{-1} FW), and hexose (*G* + *F*; mg g^{-1} FW) and sucrose (*Suc*; mg g^{-1} FW) contents during a 24-h time course, in leaves and roots from 10-day-old intact maize plantlets. Mean values and mean variation coefficients (*CV*) are from 3 independent determinations. Two-way ANOVAs (time, treatment) were not significant for leaf traits and root invertase, but were significant for hexose and sucrose in roots. In the latter case, significantly different values, at $P = 0.05$, in each column, are followed by different letters

Organ	Parameter	Time (h)								CV
		0	1	2	4	8	12	18	24	
Culture medium alone										
Leaf	Inv	44.87	55.71	54.19	49.18	60.14	50.13	52.41	58.09	0.11
	G + F	2.02	2.31	1.75	1.78	1.86	1.65	1.59	1.57	0.15
	Suc	1.15	1.92	1.33	1.54	2.31	2.82	2.26	2.00	0.13
Root	Inv	109.31	105.12	90.16	93.43	93.65	80.78	77.14	77.86	0.09
	G + F	2.73	2.55a	2.42a	2.35	2.67a	1.63a	2.38ab	2.43a	0.04
	Suc	1.34	1.47	1.50	1.59	2.28	1.11a	2.18a	2.44	0.05
Culture medium plus 2% PEG										
Leaf	Inv	44.87	53.23	54.58	45.45	50.60	46.40	47.67	65.37	0.12
	G + F	2.02	1.95	1.65	1.75	1.95	1.80	1.58	2.01	0.13
	Suc	1.15	1.09	1.35	1.10	2.13	2.81	2.58	3.39	0.15
Root	Inv	109.31	87.54	85.47	111.79	99.58	98.27	74.53	85.88	0.16
	G + F	2.73	2.13ab	1.99a	2.29	1.92b	2.34	2.11a	2.33a	0.10
	Suc	1.34	1.08	1.21	1.30	1.65	1.61	1.79b	2.12	0.11
Culture medium plus 7% PEG										
Leaf	Inv	44.87	53.43	65.61	54.47	54.93	51.43	45.79	54.51	0.10
	G + F	2.02	2.00	2.14	2.07	2.10	2.23	2.26	1.62	0.14
	Suc	1.15	1.48	1.52	1.89	2.44	3.42	3.96	3.27	0.14
Root	Inv	109.31	76.68	110.76	91.81	69.54	97.07	106.79	94.11	0.19
	G + F	2.73	2.12b	3.03a	2.94	3.24a	3.74b	3.65b	3.35b	0.07
	Suc	1.34	1.32	1.83	2.30	2.24	2.48b	3.13ab	2.76	0.10

Root, xylem sap and leaf ABA contents

Compared to control plants, where the ABA content was very low and constant in roots, xylem and leaves, supplying ABA over a 0.037 mmol m^{-3} threshold produced an increase that was earlier and higher in roots than in xylem sap and leaves (Fig. 4a,c,e). This increase was dependent on the supplied concentration since it was much higher at 3.7 than at 0.37 mmol m^{-3}. After

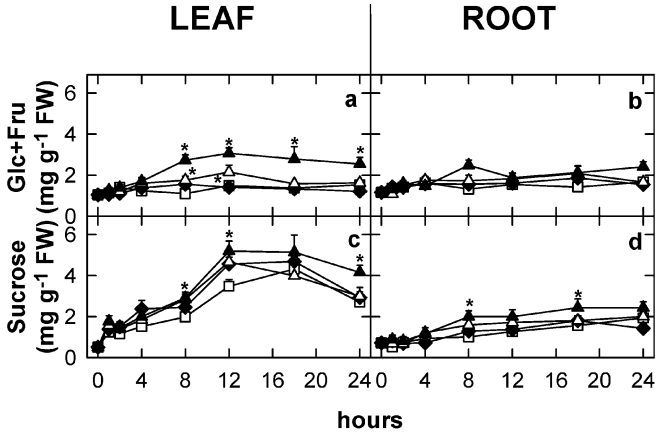

Fig. 2a–d Effect of ABA on hexose (*Glc* + *Fru*; **a, b**) and sucrose (**c, d**) contents in leaves (**a, c**) and roots (**b, d**) of intact 10-day-old maize plantlets supplied for 24 h with culture medium alone (□), or with culture medium complemented with 0.037 (◆), 0.37 (△) or 3.7 mmol m^{-3} (▲) ABA. Means on a fresh-weight basis ± SE are from 4 independent determinations. Statistical calculation and presentation, as in the legend of Fig. 1

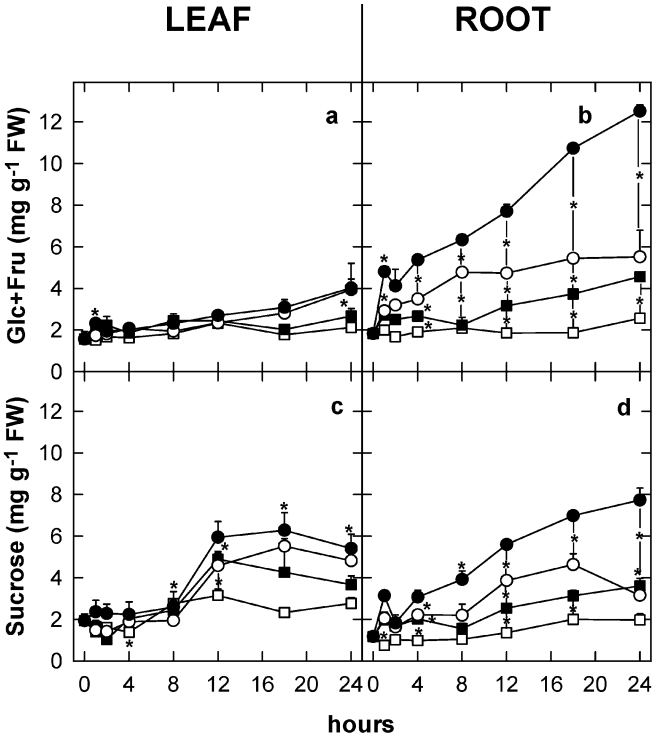

Fig. 3a–d Effect of glucose on hexose (*Glc* + *Fru*; **a, b**) and sucrose (**c, d**) contents in leaves (**a, c**) and roots (**b, d**) of intact 10-day-old maize plantlets supplied for 24 h with culture medium alone (□), or with culture medium complemented with 0.2% (■), 1% (○) or 2% (●) glucose. Means on a fresh-weight basis ± SE are from 4 independent determinations. Statistical calculation and presentation, as in the legend of Fig. 1

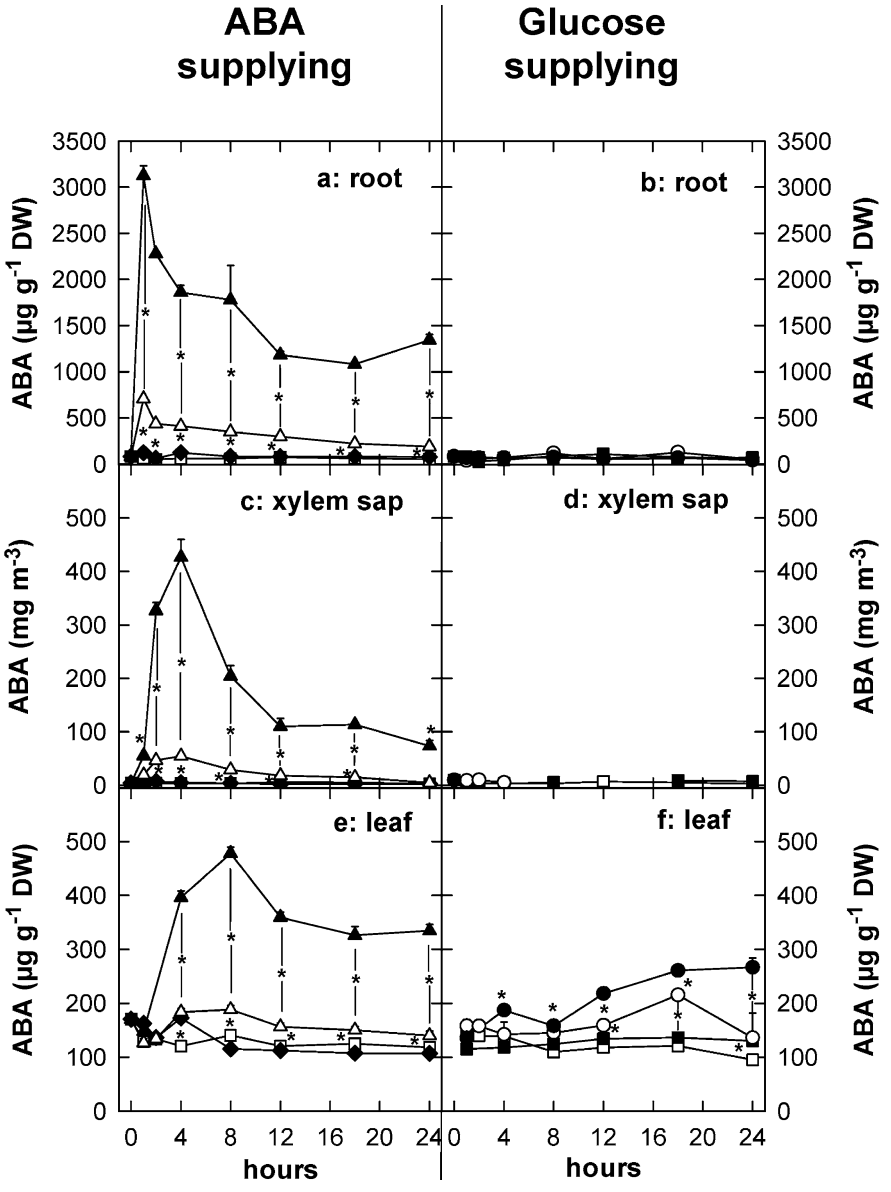

Fig. 4a–f Time course of root (**a, b**) xylem sap (**c, d**) and leaf (**e, f**) ABA accumulation in 10-day-old intact maize plantlets supplied for 24 h with culture medium alone (□), or with culture medium complemented with 0.037 (◆), 0.37 (△) or 3.7 mmol m^{-3} (▲) ABA; or with 0.2% (■), 1% (○) or 2% (●) glucose. For xylem sap and leaf ABA, means ± SE are from 3 or 4 determinations of 6 pooled plantlets. Statistical calculation and presentation, as in the legend of Fig. 1

the peaks reached at 1, 4 and 8 h, respectively, a similar pattern was observed: the tissue ABA concentration fell rapidly, then it stabilised from 12 to 18 h and slightly increased in roots and leaves at 24 h (Fig. 4a,c,e). The root-to-leaf concentration gradient and the peak delay are consistent with the root feeding ABA from the nutrient medium upward into the plant. By contrast, supplying glucose (Fig. 4b,d) and PEG (data not shown) at various concentrations had no significant effect on the root and xylem sap ABA contents. As xylem concentration may dependent on sap pH, this variable was checked in each extract; no significant differences were noted (not shown). Surprisingly, the leaf ABA content progressively increased after 8 h, in relation to glucose supply at 1 and 2% (Fig. 4f).

Invertase activity was plotted against tissue ABA in order to check for a possible relationship between the two variables. When ABA was supplied, the highest coefficient of correlation was observed between leaf vacuolar invertase activity and leaf ABA concentration ($R = 0.904$) but it was lower for roots ($R = 0.536$). There were no corresponding correlations when glucose was supplied ($R = 0.080$ and 0.033 for leaves and roots, respectively).

Modulation of gene expression

In control leaves, *Ivr2* gene expression was strongly down-regulated at 8 and 12 h (Fig. 5), which may be due to the memory of a circadian cycle as observed by Kim et al. (2000a). Comparatively, *Ivr2* transcript levels in control roots were stable. All ABA treatments up-regulated leaf *Ivr2* gene expression in a concentration-dependent way from 1 to 4 h; during this time period soluble carbohydrates were not yet affected (Figs. 5, 4e). The transcript level peak anticipated that of enzyme

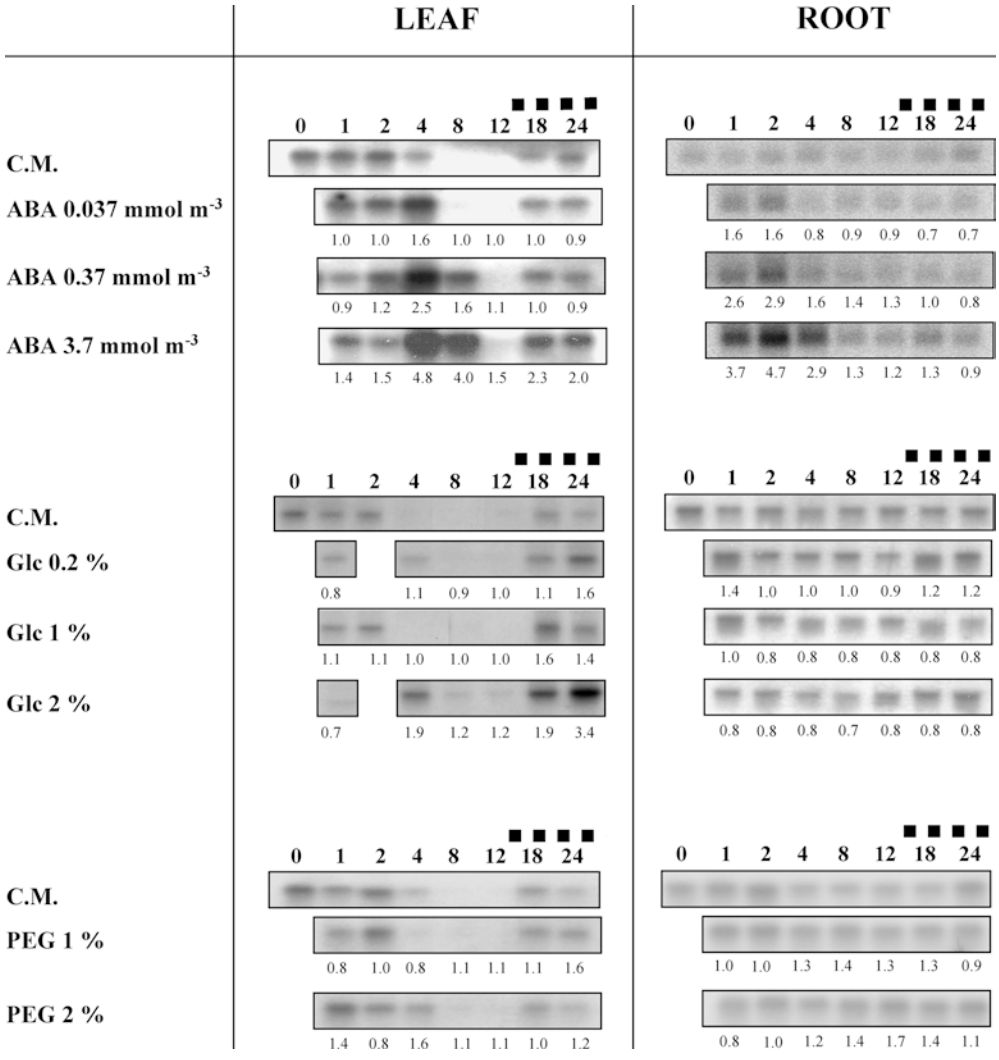

Fig. 5 Time course of *Ivr2* transcript levels in leaves and roots from 10-day-old intact maize plantlets supplied for 24 h, in continuous light, with culture medium alone (*C.M.*), or with culture medium complemented with 0.037, 0.37 or 3.7 mmol m^{-3} ABA; 0.2%, 1% or 2% glucose (*Glc*); or 1% or 2% polyethylene glycol (*PEG*). Total RNA samples (10 µg/lane), extracted from 3 pooled organs, were hybridized with an *Ivr2* cDNA probe. Hybridization signals were quantified with a Phosphoimager and the relative *Ivr2* mRNA expression was normalised using the *18S* signal intensity. The *numbers under the northern blots* are the treated/control ratios while the *dashed lines above the northern blots* indicate the position of the dark period before the treatments. The mean variation coefficient was about 3.3%

activity. When ABA was supplied at the two higher concentrations, a down-regulation of transcripts then occurred that was initially apparent at 8 h and particularly noticeable at 12 h. In roots of ABA-treated plants, a similar but earlier concentration-dependent pattern was observed, since the enhancement was visible from the first hour, the peak was reached at 2 h and the down-regulation occurred from 4 h. There was a remarkable parallelism between the time course of invertase activity and that of *Ivr2* transcript level both in leaves and roots (Figs. 1, 5). In contrast, using an *Ivr1* probe, no signal was detected in leaves of ABA-supplied plantlets whereas in roots a faint response was only observed at 2 h and 4 h for the highest ABA concentration (Fig. 6).

Incubation with glucose produced an increase of *Ivr2* transcript levels in leaves at 18 and 24 h that was dependent on glucose concentration (Fig. 5). In contrast, in roots, the lowest glucose concentration had no effect while 1 and 2% glucose reduced *Ivr2* gene expression (Fig. 5). The PEG treatments had no appreciable effects on *Ivr2* transcript levels either in leaves or roots. In agreement with Kim (1998), *Rab17* transcripts were not observed in young leaves (Fig. 6 and data not shown) except for a slight signal at 12 h exclusively in plants incubated with 3.7 mmol m^{-3} ABA. However, a rapid ABA-induced *Rab17* response was seen in roots of plantlets treated with 0.37 mmol m^{-3} ABA and especially in 3.7 mmol m^{-3} ABA. In roots of control, glucose-, PEG- and 0.037 mmol m^{-3} ABA-treated plants no *Rab17* mRNA was detected.

Experiments on excised roots and leaves

Since the response of *Ivr2* expression to glucose was different in intact plants from that described in root pieces by Xu et al. (1996), experiments were performed on excised roots and leaves from similar plantlets.

Modulation of acid vacuolar invertase activity in excised organs

Whatever the conditions studied, the invertase activity of root pieces was approximately 4- to 8-fold lower than

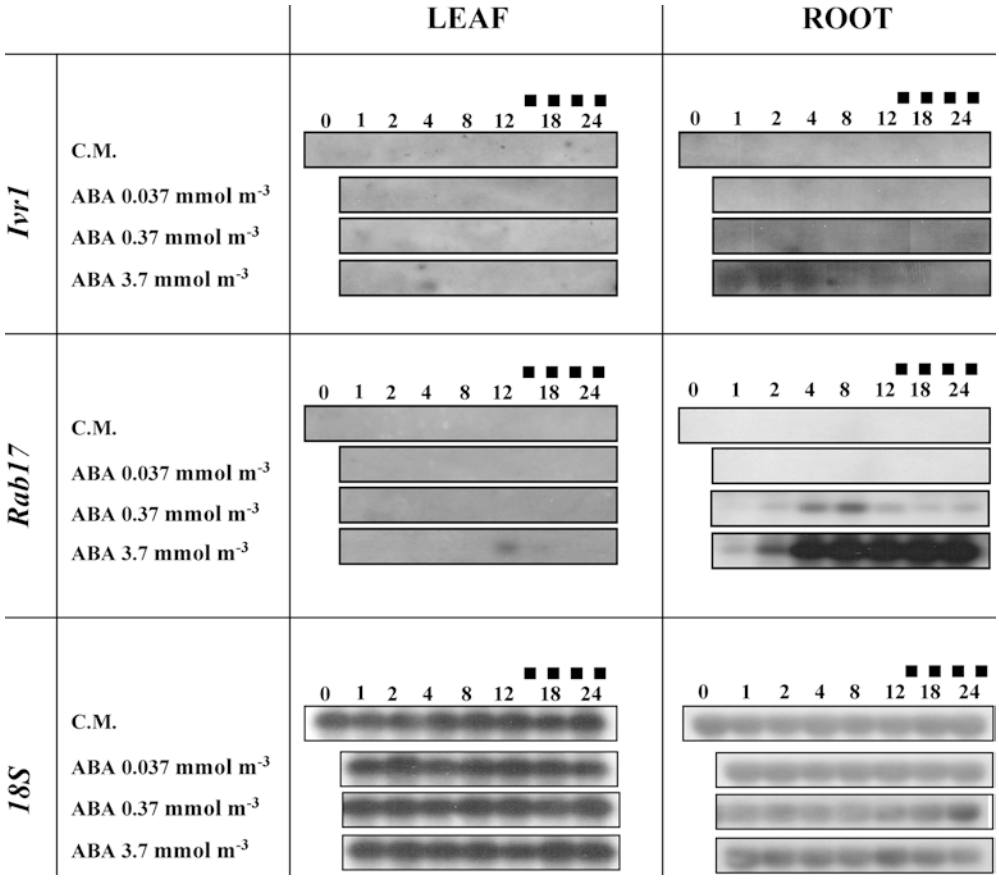

Fig. 6 Time course of *Ivr1* and *Rab17* transcript levels in leaves and roots from 10-day-old intact maize plantlets, supplied for 24 h, in continuous light, with culture medium alone (*C.M.*), or with culture medium complemented with 0.037, 0.37 or 3.7 mmol m^{-3} ABA. Total RNA samples (10 µg/lane), extracted from 3 pooled organs, were hybridized with *Ivr1* or *Rab17* cDNA probes. Hybridization signals were quantified with a phosphoimager and the relative mRNA expressions were normalised using the *18S* signal intensity. The *dashed lines above the northern blots* indicate the position of the dark period before the treatments. The mean variation coefficient was about 3.3%

in leaves (Fig. 7a,b). Invertase activities in leaf pieces, incubated for 24 h in 10 mmol m^{-3} ABA or 2% PEG, were respectively 1.4- and 1.2-fold higher when compared to control leaf pieces incubated in culture medium, while 0.2% glucose produced a non-significant decrease in invertase activity (Fig. 7a). In roots, invertase activity was increased in the pieces incubated in 10 mmol m^{-3} ABA (3.8-fold) and 2% PEG (2.7-fold) compared to culture medium (Fig. 7b). In contrast to leaves, glucose produced a slight increase of invertase activity in root pieces (1.5-fold).

Changes in soluble carbohydrate level in excised organs

The reducing-carbohydrate content was approximately 2-fold higher in leaf pieces than in root pieces, whatever the treatment (Fig. 7c,d). Leaf hexose content was related to invertase activity for the ABA and PEG treatments but not for the glucose treatment (Fig. 7c). The higher level of internal hexoses when compared to attached leaves (Figs. 2a, 3a) may have been due to exogenous glucose accumulation (3 mg g^{-1} FW is equivalent to 16 mol m^{-3} glucose). In contrast to leaves, the hexose content of root pieces was not related to invertase activity since the hexose concentrations were rather similar in roots incubated in 10 mmol m^{-3} ABA, 2% PEG and culture medium. As for leaves, the measured hexose content was strongly increased in 0.2% glucose-incubated root pieces where the activity was lower than in the control. In contrast to hexose levels, sucrose content remained close to the control value in 10 mmol m^{-3} ABA-treated leaves but it was higher in glucose- and PEG-treated leaf pieces (Fig. 7e, Table 1). In root pieces, the sucrose concentration remained very low, whatever the treatment, although it was enhanced by incubation in 10 mmol m^{-3} ABA or 0.2% glucose (Fig. 7f), but this effect was non-significant (ANOVA).

Modulation of gene expression in excised organs

Whatever the treatment, *Ivr2* gene expression was much higher in leaf than in root pieces (Fig. 7g,h) as noted for the activities (Fig. 7a). The pattern of *Ivr1* expression was the opposite, since a higher expression was observed in roots than in leaves. Leaf pieces incubated in 10 mmol m^{-3} ABA showed an enhanced *Ivr2* transcript level of approximately 2-fold, this being consistent with the measured invertase activity (Fig. 7g). By contrast, incubation with either 2% PEG or 0.2% glucose modulated *Ivr2* gene expression in an anti-parallel way with respect to invertase activity: an enhancement (1.5-fold) for glucose vs. a decrease (2-fold) for PEG. *Ivr1* mRNA was hardly detected in leaves, whatever the treatment. In root pieces, changes in *Ivr1* and *Ivr2* transcript levels were clear and consistent with vacuolar invertase activity, whatever the treatment: a very faint signal in

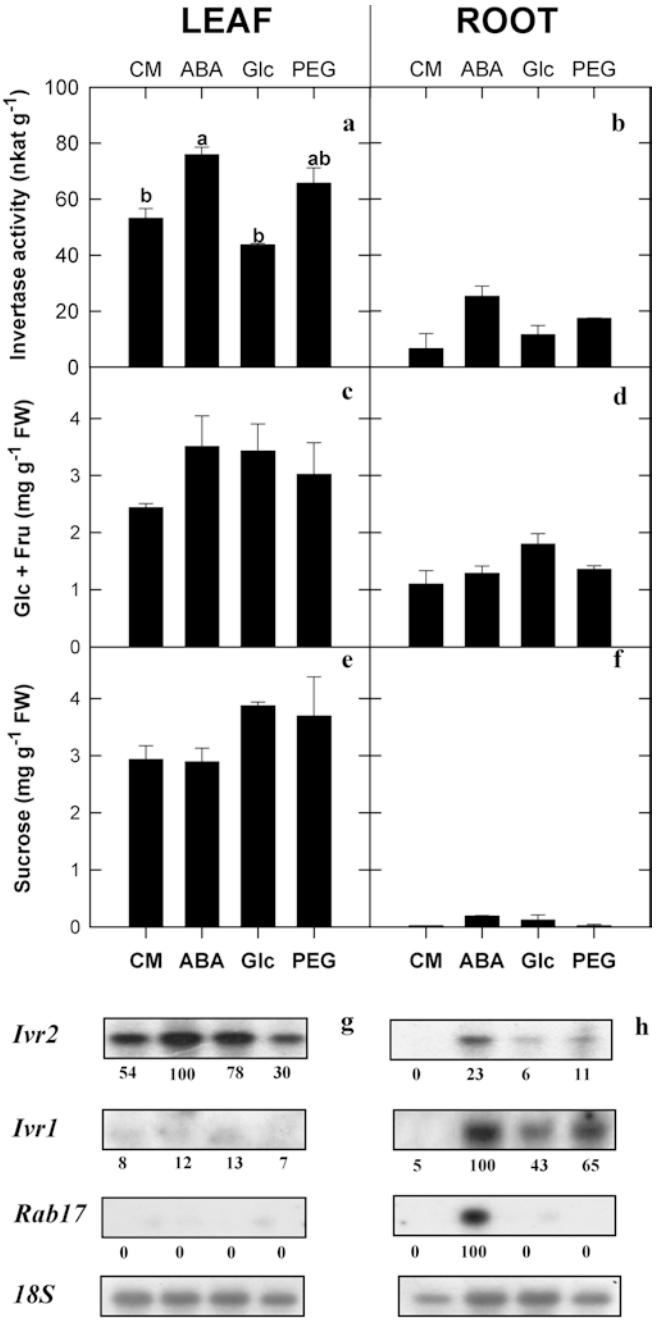

Fig. 7a–h Effect of a 24-h incubation of maize leaf (**a, c, e**) and root (**b, d, f**) pieces with culture medium alone(*CM*), or with culture medium complemented with 10 mmol m^{-3} ABA, 1% glucose (*Glc*) or 7% PEG on invertase activity (**a, b**), hexose (*Glc* + *Fru*; **c, d**) and sucrose (**e, f**) contents, and transcript levels of *Ivr2*, *Ivr1* and *Rab17* genes (**g, h**). The signal of each northern blot was quantified and the *numbers under the blots* show their relative intensities. For biochemical measurements, means ± SE are from 3 independent determinations. One-way ANOVAs were not significant for treatment effects in **b–f**; in **a**, *bars with the same letter* represent non-significantly different values ($P < 0.05$)

culture-medium-incubated roots, a strong enhancement in 10 mmol m^{-3} ABA, a less strong (half as much) enhancement in 2% PEG-treated roots, and finally a small increase with the 0.2% glucose treatment (Fig. 7h). Hybridization of RNA with the *Rab17* probe showed a very faint signal whatever the treatment and organ, except for root pieces incubated with 10 mmol m^{-3} ABA where *Rab17* transcripts were strongly enhanced (Fig. 7g,h).

Discussion

Hydroponic experimental model vs. excised organs

Hydroponic cultures of maize seedlings provide a good experimental model for analysis of the effects of exogenous effectors on invertase expression in both leaves and roots. In addition, enzyme activity, related gene expression and ABA contents in roots, xylem sap or leaves remain rather constant in control plants for the duration of the experiments, allowing the influence of possible effectors, like ABA or carbohydrates, to be detected against a stable background. This model is more adequate than excised leaves placed in an incubation medium because, in the latter case, side effects probably originating from wounding and/or phloem blockage lead to an accumulation of hexoses (Trouverie et al. 2003). Accordingly, wounding has been shown to affect invertase activity (Sturm and Chrispeels 1990). This is further supported, in our experiments, by the comparison of glucose effects between intact roots and root pieces since a slight decrease in *Ivr2* transcript levels was observed in intact roots of maize plantlets for a large range of internal glucose and sucrose levels whereas incubation of root pieces in a 1% glucose medium enhanced *Ivr2* and *Ivr1* mRNA levels. The latter observation confirms an earlier report by Xu et al. (1996) who described a similar 30% stimulation of *Ivr2* transcripts in excised roots. In addition, in the present excised-root experiment, a corresponding increase in vacuolar invertase activity was detected upon glucose supply, whereas in intact roots such a treatment had no effect on this activity, whatever the glucose level. Therefore, what is the origin of the difference in *Ivr2* gene response to glucose in intact vs. excised roots? It could be proposed as a working hypothesis that the glucose effect in excised roots reflects an interaction between glucose and wound-related signalling pathways. The question of the effector associated with organ excision should be addressed by further studies.

Glucose supply only affects *Ivr2* invertase transcript levels

In contrast to ABA, the effect of glucose supply on invertase activity and on *Ivr2* transcript levels both in roots and leaves from intact plants was not straightforward. During the 24-h time course, glucose, and to a lesser extent sucrose levels, increased as a function of time and of glucose supply. This led to a much higher final concentration internally than in the external medium, especially

in roots. This accumulation can be explained by both a significant glucose uptake by the roots and an increase in photosynthetic assimilation in the leaves under the continuous illumination conditions. In intact roots, glucose supply did not significantly affect invertase activity, even if some increase was noted at 2% glucose, while the *Ivr2* mRNA level was slightly depressed relative to the control for the two higher glucose concentrations. In leaves, invertase activity was insensitive to the carbohydrate accumulation whereas *Ivr2* transcript levels increased at 18 and 24 h. At the same time, leaf ABA content increased slightly while root and xylem sap ABA remained constant. Considering all of these observations, glucose seems to effect *Ivr2* gene expression but not invertase activity, and this depends on the organ. This glucose response is unlikely to be due to an osmotic effect since PEG treatment, at an equal osmotic potential, had no effect. However, a possible interaction with ABA can be assumed, as discussed below.

ABA as a key hormone for vacuolar invertase regulation in leaves and roots

In our opinion, the most important results reported in this paper concern the enhanced invertase activity and *Ivr2* gene expression in both roots and leaves caused by the addition of ABA to the root medium. Root, xylem sap and leaf ABA levels were increased in proportion to the ABA concentration supplied to the root medium. ABA variation was found to be in phase with the changes in *Ivr2* transcript levels in roots and leaves, and this occurred before any significant changes in leaf and root carbohydrate contents (0 h to 8 h). During this first phase, the response began within 1 h in roots through a sharp increase of *Ivr2* transcripts and, later on, of vacuolar invertase activity. In leaves, the same sequence of events occurred (synchronised variation of leaf ABA and *Ivr2* transcripts and a delayed increase of activity) but approximately 1 h later than in the roots. The lag in leaf *Ivr2* expression can be simply explained by the time required to transport ABA from the roots to the leaves and to enter the mesophyll cells, as suggested by the delay between maximum root, xylem and finally leaf ABA contents.

During the second phase (8 h to 24 h), ABA content, *Ivr2* transcripts and invertase activity declined both in leaves and roots. In contrast, the hexose concentration increased. Thus, over the 24-h period, the magnitudes of the responses of invertase activity and *Ivr2* transcript levels were correlated in both roots and leaves. However, it should be noted that root and leaf ABA were maintained at a rather high level after 18 h and they slightly increased again at 24 h despite the very low *Ivr2* expression. The ABA-enhancing effect observed in attached organs was apparently not affected by organ excision since both IVR2 invertase activity and transcript levels were affected in root and leaf pieces incubated in 10 mmol m^{-3} ABA medium. Thus, in contrast to glucose, ABA appeared to be a powerful inducer of vacuolar invertase, both at the translational and enzymatic levels and under various experimental conditions.

Leaf hexose but not sucrose is related to invertase activity

In leaves from intact plantlets supplied with ABA, an increase of invertase activity and hexose content was observed while supplying glucose did not significantly modify either invertase activity or hexose levels. The delay between the increase of invertase activity and that of hexose concentration in ABA-treated plants suggested that the accumulation of hexose was likely to be a consequence of the enhanced invertase activity. Thus, in leaves, hexose content appears to be related to invertase activity. In contrast, there was no such relation in roots. The variations of sucrose, the invertase substrate, are more difficult to explain since sucrose content did not decrease in relation to invertase activity but rather increased both in control and ABA-treated plants. Thus, induction of vacuolar invertase activity is not reflected in changes in the hexose/sucrose ratio. However leaf sucrose concentration is not only dependent on invertase activity, since it is a balance between synthesis, export and hydrolysis processes. The observed accumulation might be better explained by a higher synthesis due to the continuous 24-h light period rather than a decrease in hydrolysis since invertase activity was enhanced. A lower export rate is unlikely since root soluble carbohydrate content, which is known to be dependent upon sucrose import from the shoots (Saglio et al. 1980), tended to increase. A higher sucrose synthesis rate could be provided by an indirect ABA effect on sucrose-phosphate synthase (SPS). Indeed, SPS is induced by glucose-6-phosphate (Doehlert and Huber 1983). The level of this effector might be increased by a higher rate of sucrose cleavage into glucose and fructose, followed by a rapid conversion by hexokinase and phosphoglucoisomerase.

Leaf vacuolar invertase may be dependent on a circadian rhythm

Under all tested conditions, a dramatic down-regulation of *Ivr2* gene expression occurred in leaves at 8 h and 12 h after illumination, and this was followed by a decrease in enzyme activity. Such a regulation did not occur in roots. This up and down variation is reminiscent of the day–night cycle effect described by Kim et al. (2000a) who reported a maximum vacuolar invertase activity and *Ivr2* transcript level at the beginning of the light period and their decline during the daytime in leaves but not in roots. This organ-specific response of vacuolar invertase could play a role in carbohydrate partitioning by increasing sucrose export from leaves and sucrose import from roots at the end of the day. Such a function has already been proposed for cell wall invertase (Godt and Roitsch 1997; Roitsch 1999).

Interaction between the ABA and carbohydrate signalling pathways

The glucose treatment results led us to question a possible interaction with ABA. Although these experiments were planned to consider only the monofactorial effects of ABA and glucose, it was clear that in some situations the effects of either factor were not independent with respect to the other. First, the leaf carbohydrate content increased after 8 h in ABA-treated plants. Second, glucose supply produced a concentration-dependent increase in leaf ABA concentration, which finally reached a maximum value intermediate between that obtained with the two higher ABA supplies. This observation can be compared to those of Cheng et al. (2002) who demonstrated that the expression of ABA biosynthesis genes was stimulated by 2% glucose but not mannitol. Surprisingly, in our glucose-supply experiment, ABA accumulation and its relationship with *Ivr2* expression was not associated with any effect on vacuolar invertase activity. In agreement with other reports, all these observations strongly suggest cross-talk between the ABA and glucose signalling pathways. Indeed, a number of studies using several *Arabidopsis* glucose-insensitive (*gin*) and ABA-deficient mutants (*abi*) showed evidence suggesting important connections between ABA and glucose signalling (Rook et al. 2001). However, further experiments will be required to explore the influence of interactions among ABA, glucose and water stress on invertase regulation in maize. Expression of *Ivr2* promoter–reporter gene constructs in transgenic systems might be a good approach to examine this question. To date, only part of the *Ivr2* genomic sequence is currently available; unfortunately, this does not include the promoter (Qin et al. 2004). Presently, the available expression data in maize shows decreased Ivr2 mRNA levels in water-stressed reproductive organs (kernel perianth) as opposed to leaves and roots (Andersen et al. 2002; Qin et al. 2004). Similarly, Dorion et al. (1996) mentioned a lower vacuolar invertase activity in pollen of water-stressed wheat plants. In maize leaves and perianth, a specific hybridization using the *Ivr2* 3′-UTR showed that the same gene is likely to be expressed in both organs, suggesting that the promoter is regulated by a different set of transcription factors in vegetative and reproductive organs. The opposite regulatory mechanisms may have a physiological significance when considering that an increase in invertase activity in source leaves would limit sucrose export because of sucrose-specific phloem loading. Conversely, the decrease in the IVR2 activity in the perianth would decrease osmotic pressure compared to that provided by glucose and fructose derived from sucrose hydrolysis. Thus, following the Münch model, postulating that source–sink flux is driven by osmotic gradients, a decrease in IVR2 activity would decrease phloem sucrose unloading in the seed.

Conclusions

Invertase regulation occurs at spatial, temporal, transcriptional and post-transcriptional levels. In this multifactorial regulation, ABA, in vegetative organs, seems to play a major role while this is less obvious for glucose. However, links between sugar response and ABA are likely to occur and should be considered. The fact that glucose addition led to a different *Ivr2* expression pattern in intact plants compared to tissues suggests that a wounding effect might give rise to an additional response.

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