Metabolizable and Non-Metabolizable Sugars Activate Different Signal Transduction Pathways in Tomato[1]

Alok K. Sinha, Markus G. Hofmann, Ulrike Römer, Walter Köckenberger, Lothar Elling, and Thomas Roitsch*

Lehrstuhl für Pharmazeutische Biologie, Julius-von-Sachs Institute, Julis-von-Sachs-Platz 2, Universität Würzburg, D–97082 Würzburg, Germany (A.K.S., M.G.H., T.R.); Heinrich-Heine-University Düsseldorf, Research Center Jülich, D–52426 Jülich, Germany (U.R., L.E.); and Magnetic Resonance Centre, School of Physics and Astronomy, University of Nottingham, Nottingham NG7 2RD, United Kingdom (W.K.)

To gain insight into the regulatory mechanisms of sugar signaling in plants, the effect of derivatives of the transport sugar sucrose (Suc), the Suc isomers palatinose and turanose, and the Suc analog fluoro-Suc were tested. Photo-autotrophic suspension culture cells of tomato (*Lycopersicon peruvianum*) were used to study their effect on the regulation of marker genes of source and sink metabolism, photosynthesis, and the activation of mitogen-activated protein kinases (MAPKs). Suc and glucose (Glc) resulted in reverse regulation of source and sink metabolism. Whereas the mRNA level of extracellular invertase (Lin6) was induced, the transcript level of small subunit of ribulose bisphosphate carboxylase (RbcS) was repressed. In contrast, turanose, palatinose, and fluoro-Suc only rapidly induced Lin6 mRNA level, whereas the transcript level of RbcS was not affected. The differential effect of the metabolizable and non-metabolizable sugars on RbcS mRNA regulation was reflected by the fact that only Suc and Glc inhibited photosynthesis and chlorophyll fluorescence. The activation of different signal transduction pathways by sugars was further supported by the analysis of the activation of MAPKs. MAPK activity was found to be strongly activated by turanose, palatinose, and fluoro-Suc, but not by Suc and Glc. To analyze the role of sugars in relation to pathogen perception, an elicitor preparation of *Fusarium oxysporum lycopersici* was used. The strong activation of MAPKs and the fast and transient induction of Lin6 expresssion by the fungal elicitor resembles the effect of turanose, palatinose, and fluoro-Suc and indicates that non-metabolizable sugars are sensed as stress-related stimuli.

In recent years, sugars have been recognized as important signal molecules that affect a variety of physiological responses and in particular regulate genes involved in photosynthesis, sink metabolism, and defense response (Koch, 1996; Smeekens, 1998; Roitsch, 1999; Sheen et al., 1999). Whereas the effect of sugars on gene regulation is well established, the nature of the sugar signal, and the molecular mechanisms involved in sugar perception and intracellular signal transmission, are largely unknown. Suc is the major form of translocated carbon in higher plants and was shown to regulate a number of carbohydrate-responsive genes. Whereas in many cases the effects of Suc could be mimicked by hexoses, such as Glc and Fru, a few studies demonstrated the existence of Suc-specific regulatory pathways (Chiou and Bush, 1998; Rook et al., 1998). In principle, a sugar signal could be generated by extracellular recognition via a soluble or membrane-bound receptor molecule or by intracellular sensing at different stages of sugar metabolism. For hexoses, a dual role of hexokinase in sugar sensing and glycolysis has been proposed (Jang and Sheen, 1997; Jang et al., 1997) that is a matter of a controversial debate (Halford et al., 1999). Additional membrane-based sensing systems have been implied both for hexoses and Suc. Primary lines of evidence are the finding that non-phosphorylatable Glc analogs can mimic the effect of Glc on the regulation of specific genes (Godt et al., 1995; Roitsch et al., 1995) and transgenic studies with specific subcellular targeting of a yeast (*Saccharomyces cerevisiae*) invertase (Herbers et al., 1996). Hexose transporters were shown to function as membrane sugar sensors in yeast (Özcan et al., 1998) and a dual function of plant sugar carriers in transport and sensing of sugars has been proposed (Lalonde et al., 1999). Additional sensing mechanisms involving metabolism of sugars such as glycolysis have also been suggested (Koch et al., 2000). In summary, results obtained with various experimental approaches and systems indicate the existence of different sensory mechanism and parallel sugar signaling pathways in higher plants (Jang et al., 1997; Smeekens and Rook, 1997; Lalonde et al., 1999).

To gain insight into disaccharide-specific signal transduction pathways, derivates of the transport

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant no. SFB380, Teilprojekt B26 to L.E. and grant no. Ro 4–1 to T.R.), by the Alexander von Humbold Foundation (to A.K.S.), and by the Studienstiftung des Deutschen Volkes (to M.G.H.).

* Corresponding author; e-mail roitsch@biozentrum.uni-wuerzburg.de; fax 49–931–888–6182.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010771.

sugar Suc were used in the present study. Turanose (3-O-α-d-glucopyranosyl-Fru) and palatinose (6-O-α-d-glucopyranosyl-Fru) are structural isomers of Suc composed of Glc and Fru with a different glycosidic linkage. They are not synthesized in higher plants and cannot be cleaved or transported by plant enzymes. Using these non-metabolizable Suc isomers, evidence for extracellular sugar sensing has been obtained in barley (*Hordeum vulgare*) and potato (*Solanum tuberosum*) tubers (Loreti et al., 2000; Fernie et al., 2001).

A Suc analog that is not cleaved by invertase is 1′deoxy-1′fluro-Suc (1-deoxy-1-fluorofructofuranosylα-D-glucopyranosid, fluoro-Suc). Because this sugar analog is not commercially available, it was synthesized by an optimized protocol. Here, we report a highly effective synthesis of fluoro-Suc with a recombinant Suc synthase 1 from potato to address the possible signaling function of this Suc analog.

Acitvation of mitogen-activated protein kinases (MAPKs) was shown to be involved in the stress-induced signal transduction pathways (Zhang and Klessig, 1998; Zhang et al., 1998). Thus, we also studied the activation of MAPK by different Suc analogs. Pathogen infection is known to affect source/sink relations and different lines of experimental evidence suggest a role of sugars in plant defense responses (Herbers et al., 1996; Ehness et al., 1997).

Photo-autotrophic cultures proved to be useful to study various aspects of sugar regulation (Krapp and Stitt, 1994; Roitsch et al., 1995; Godt and Roitsch, 1997). Sugar responses may be analyzed without the necessity of a sugar depletion period required for heterotrophic cultures and signal transduction events may be related to photosynthetic gene expression or photosynthetic activity as well as source/sink regulations. Using photo-autotrophic suspension culture cells of *Chenopodium rubrum*, it has been shown that source/sink relations and defense mechanisms are coordinately regulated both by sugars and stress-related stimuli (Ehness et al., 1997).

A photo-autotrophic culture of the model plant species tomato (*Lycopersicon peruvianum*; Beimen et al., 1992) was used in the present study to test the effect of Suc, Glc, and the non-metabolizable Suc derivatives turanose, palatinose, and fluoro-Suc on different cellular responses. The results were compared with the effect of an elicitor preparation of the tomato pathogen *Fusarium oxysporum lycopersici*. Extracellular invertase is the key enzyme for phloem unloading via an apoplastic pathway and was used as a representative marker gene for sink metabolism (Sturm, 1999; Roitsch et al., 2000). The analysis of the regulation of the mRNA for the small subunit of the Calvin cycle enzyme ribulose bisphosphate carboxylase (RbcS) was complemented by the measurement of the rate of photosynthetic oxygen evolution and chlorophyll fluorescence. The present study demonstrates that metabolizable and non-metabolizable sugars activate different signal transduction pathways. They were shown to differentially affect photosynthesis as well as MAPK activation. In contrast to Suc and Glc, the Suc derivatives had no effect on RbcS expression and photosynthesis but resulted in strong MAPK activation. The fact that the non-metabolizable sugars, like the fungal elicitor, activate MAPKs and results in a fast induction of Lin6 expression indicates that they are sensed as stress-related stimuli rather than extracellular carbohydrate signals.

RESULTS

Inverse Regulation of mRNAs for Extracellular Invertases Lin6 and RbcS by Suc and Glc

The time course of the regulation of mRNAs for the sink-specific extracellular invertase Lin6 and the photosynthetic marker gene RbcS by metabolizable sugars was analyzed by the addition of 50 mM Glc or Suc to autotrophically growing tomato cell cultures. Samples were taken before the addition of the sugars, and after 1, 4, 9, 24, and 48 h, mRNA levels were determined by RNA gel-blot analysis.

The low level of mRNA for extracellular invertase Lin6 was already elevated after 1 h in response to both Glc and Suc, further increased up to 24 h, and then declined (Fig. 1A). In contrast, the mRNA level of the photosynthetic protein was inversely regulated. The high steady state of RbcS mRNA was repressed already after 4 h by both sugars and further declined up to 48 h. Addition of 50 mM mannitol to the cultures as an osmotic control did not resulted either in the induction of LIN6 or repression of RbcS transcript level (data not shown).

Differential Uptake of Hexoses, Suc, and the Suc Isomers Turanose and Palatinose by Tomato Suspension Culture Cells

It has been shown previously that the Suc isomers turanose and palatinose are neither recognized nor transported by Suc transporters of soybean (*Glycine max*) cotyledons and broad bean (*Vicia faba*) leaves (M′Batchi and Delrot, 1988; Li et al., 1994). To rule out a possible uptake of these two Suc derivatives by the tomato suspension cultures used for the experiments, 50 mM of these sugars was added to the suspension culture cells and the concentration of the two Suc isomers in the culture supernatant were determined during a 48-h incubation period. Figure 2A demonstrates that the extracellular concentration of palatinose and turanose did not change even after a prolonged incubation time of 48 h compared with the initial concentration determined. No Glc could be detected during the course of the experiment. These results demonstrate that the two Suc isomers are neither cleaved nor taken up by the tomato suspension culture cells. In comparison, control incubations

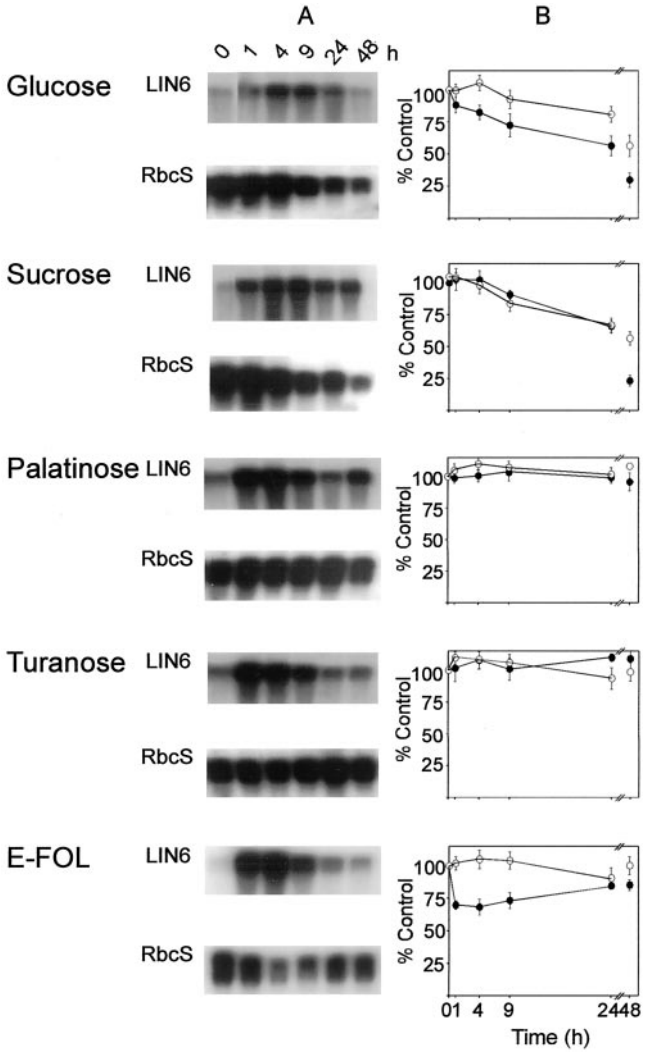

Figure 1. Differential effect of metabolizable sugars, Suc isomers, and an elicitor preparation of *F. oxysporum lycopersici* (E-FOL) on mRNA regulation and photosynthetic parameters. A, Regulation of mRNAs for extracellular invertase Lin6 and RbcS. Thirty micrograms of total RNA was separated on formaldehyde agarose gels, blotted onto nitrocellulose, and probed with random primer labeled cDNA fragments. Equal loading of RNA was confirmed by ethidium bromide staining of the rRNA (data not shown). The data presented are representative of five independent sets of experiments. B, Regulation of the rate of oxygen evolution (●) and effective photochemical yield Y (○). The data represent the mean values of five independent experiments.

with 50 mM Glc or Suc demonstrate a fast decrease of the concentrations of these sugars in the culture supernatant. Figure 2A shows that the Glc concentration starts to decrease after 1 h and further declines to 11 mM after 48 h. The concentration of Suc gradually declines to 0.3 mM at 48 h (Fig. 2B). Determination of the Suc cleavage products revealed a differential accumulation of Glc and Fru. Glc can be detected only after 24 h and the peak concentration of 12.7 mM at 24 h decreases to 9.4 mM at 48 h. In contrast, Fru starts to accumulate after 1 h, which further increases up to 29 mM at 48 h.

Suc Isomers Differentially Affect the mRNAs for Extracellular Invertases Lin6 and RbcS

The time course of the regulation of mRNAs for the sink-specific extracellular invertase Lin6 and the photosynthetic marker gene RbcS by metabolizable sugars was analyzed by the addition of 50 mM turanose and palatinose to autotrophically growing tomato cell cultures. Samples were taken before the addition of the sugars and after 1, 4, 9, 24, and 48 h, and mRNA levels were determined by RNA gel-blot analysis.

A fast and strong induction of the Lin6 gene could be observed in response to both Suc isomers. The low level of mRNA for Lin6 was highly induced already after 1 h and the elevated level declined after 24 h

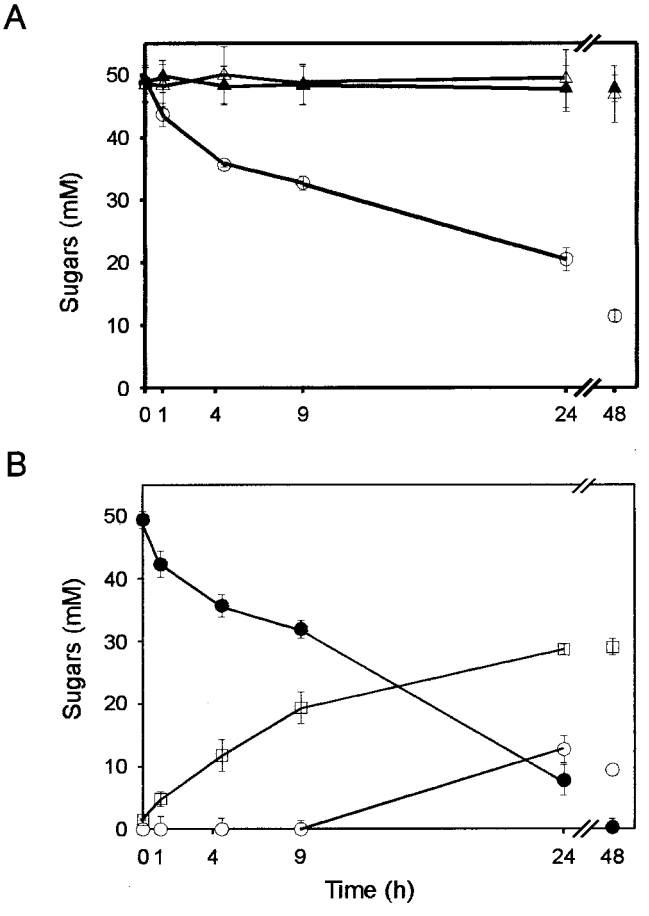

Figure 2. Time course of changes in external sugar concentrations. Suspension cultures cells were treated with 50 mM of the indicated sugars and the remaining concentration in the culture supernatant was determined at the given time points. A, Suspension cultures were treated with Glc (○), palatinose (▲), or turanose (△). B, Suspension cultures were treated with Suc and the concentration of Suc (●), Glc (○), and Fru (□) in the same supernatant were determined. The data represent the mean values of six independent measurements.

(Fig. 1A). In contrast, neither turanose nor palatinose had an effect on the high RbcS mRNA level throughout the 48-h experiment. Thus addition of the two non-metabolizable Suc isomers turanose and palatinose results in a differential effect on the source- and sink-specific marker enzymes tested.

The Differential Effect of Glc, Suc, and Suc Isomers on RbcS mRNA Is Reflected by the Rate of Photosynthetic Oxygen Evolution and Chlorophyll Fluorescence

In further experiments, it has been addressed whether the differential effect of the metabolizable and non-metabolizable sugars on the mRNA level of the photosynthetic gene RbcS is reflected by physiological parameters.

The rates of oxygen evolution were measured with the help of a liquid phase oxygen electrode. Glc treatment results in an immediate decrease of the rate of oxygen evolution, which further declines up to 48 h (Fig. 1B). Suc treatment also resulted in a pronounced reduction of the rate of oxygen evolution although with a different time course. An initial lag phase of 4 h was followed by a constant decline to result in a final reduction to values comparable with the Glc-treated cultures. In contrast, the non-metabolizable Suc analogs palatinose and turanose did not reduce the rate of oxygen evolution throughout the experiment.

To analyze whether the photosynthetic apparatus is also differentially affected by the metabolizable sugars and the Suc analogs, chlorophyll fluorescence measurements were carried out with a PAM 2000 portable fluorometer as described in "Materials and Methods." The F_v/F_m values, reflecting the maximal photochemical quantum efficiency of PSII reaction centers of dark-adapted samples, remained unchanged by all the treatments (data not shown). Photochemical yield Y, an indicator of effective photochemical quantum efficiency of illuminated sample, was differentially affected by the metabolizable and non-metabolizable sugars. Glc and Suc resulted in a constant decline of the photochemical yield Y with comparable low values after 48 h (Fig. 1B). In contrast, the photochemical yield remained unchanged in turanose- and palatinose-treated cultures.

Fast, Transient, and Inverse Regulation of mRNAs for Extracellular Invertase Lin6 and RbcS by an Elicitor Preparation of *F. oxysporum lycopersici*

Using photo-autotrophic cultures of *C. rubrum*, it has been shown that during a short incubation time of 6 h, the mRNA for an extracellular invertase and RbcS are coordinately regulated both by Glc and the fungal elicitor chitosan (Ehness et al., 1997). These findings were reevaluated and extended in the present study by comparing the effect of sugars with a fungal elicitor on the autotrophic tomato suspension culture over a 48-h period. *F. oxysporum lycopersici* is a wilt-inducing pathogenic fungus specific for tomato (Armstrong and Armstrong, 1981). An elicitor preparation of this fungus (E-FOL), shown to elicit secondary metabolite production in the photoautotrophic tomato suspension culture line used for the experiments (Beimen et al., 1992), was used to address the regulation of Lin6 and RbcS in response to this stress-related stimulus.

Treatment of the tomato suspension culture with 150 μg mL^{-1} E-FOL resulted in fast and transient effects on the levels of mRNAs for Lin6 and RbcS (Fig. 1A). After 1 and 4 h, the Lin6 mRNA was highly induced, then declined to a low level again. The transient effect on RbcS mRNA showed a similar time course. The high level of mRNA for RbcS was most strongly repressed at 4 h and then increased again to the normal level.

E-FOL treatment resulted in a fast and pronounced decrease of the rate of oxygen evolution after 1 h that recovered after 24 h, although values were still reduced compared with the control cultures (Fig. 1B). In contrast to the oxygen production, photochemical yield Y was not affected in the cultures treated with E-FOL (Fig. 1B).

MAPK Activity Is Induced Only by Non-Metabolizabable Sugars and an Elicitor Preparation of *F. oxysporum lycopersici*, But Not by Metabolizable Sugars

MAPKs play a key role in signal transduction cascades of animals and yeast. They are rapidly and transiently activated and characterized by phosphorylating the model substrate myelin basic protein (MBP) in an in-gel assay. There is also accumulating evidence for the importance of MAPKs in the transduction of various, in particular stress-related, stimuli in higher plants. Therefore, we have compared the effect of Suc, Glc, and the Suc derivatives with the effect of the fungal elicitor E-FOL on MAPK activation.

Tomato suspension culture cells were treated with the different stimuli for 5 min and crude extracts were analyzed for MAPK activity by in-gel kinase assays with the model substrate MBP. The dose response shown in Figure 3 demonstrates that concentrations of up to 100 mM of either Glc or Suc had no effect on MAPK. Only a concentration of 200 mM of the two metabolizable sugars resulted in a weak MAPK activity. Control incubations demonstrate that mannitol also resulted in weak MAPK activation at a concentration of 200 mM. This finding indicates that the weak MAPK activation by 200 mM Glc and Suc represents an osmotic effect rather than a specific effect of the two sugars applied. In contrast to the two metabolizable sugars, both palatinose and turanose resulted in strong MAPK activation. The dose response shown in Figure 3 demonstrates that

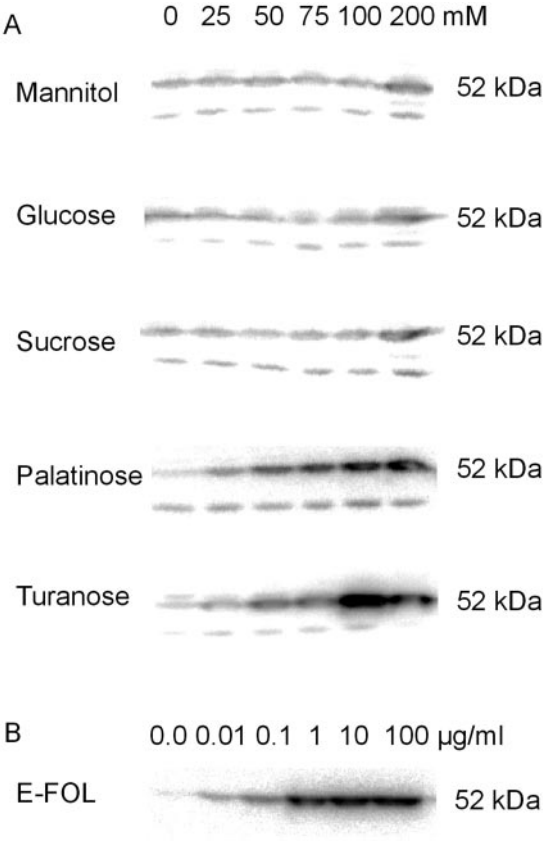

Figure 3. Differential effect of metabolizable sugars, Suc isomers, and an elicitor preparation of *F. oxysporum lycopersici* (E-FOL) on activation of MAPK. Cells were harvested exactly after 5 min after the addition of stimuli. A, Study of activation of MAPK by different sugars. Mannitol was taken as osmotic control for the different concentrations of sugars used. B, Activation of MAPK by different amount of E-FOL. The data presented in A and B are representative of five independent sets of experiments.

concentrations of 25 mM of both Suc isomers were sufficient to result in MAPK activation that further increases at concentration of up to 100 mM (Fig. 3A). The elicitor preparation E-FOL also strongly activated MAPK, even at the lowest concentration of 0.1 μg mL^{-1} tested (Fig. 3B). The observed differential effect of metabolizable and non-metabolizable sugars on MAPK activation further supports the activation of different signal transduction pathways.

Synthesis of the Suc Analog Fluoro-Suc

In further experiments, the question has been addressed whether the differential effects of the Suc isomers and Suc and Glc are related to the fact that turanose and palatinose are not transported. Fluoro-Suc was used as a Suc analog that is not subject to invertase hydrolysis, only slowly metabolized (Hitz et al., 1985) but efficiently transported into plant cells (Thom and Maretzki, 1992). We found that 80% to 85% of fluro-Suc was taken up by the cell culture within 6 h of incubation.

Because fluoro-Suc is not commercially available, it was synthesized by an optimized procedure. Card and Hitz (1984) previously synthesized fluoro-Suc by using a Suc synthase from barley seeds with an overall yield of 59% (507 mg). In the present paper, we could improve the synthesis with reference to the enzyme productivity by repetitive use of recombinant Suc synthase and alkaline phosphatase (Fig. 4). The synthesis yield after three batches was 100% with reference to the acceptor substrate. After purification, an overall yield of 85% (860.5 mg) for fluoro-Suc was obtained. The analysis by NMR confirmed the structural integrity of the product as described previously (Card and Hitz, 1984).

The Suc Analog Fluoro-Suc Differentially Affects the Regulation of mRNAs for Extracellular Invertases Lin6 and RbcS and Activates MAPKs

Because of the limited amount of fluoro-Suc available, the regulation of the mRNAs for the marker enzymes Lin6 and RbcS was analyzed only at one time point, 6 h after the addition of the Suc analog. Figure 5A shows that 20 mM fluoro-Suc strongly induces Lin6 mRNA, whereas the level of the RbcS mRNA was not affected. Thus, fluoro-Suc, like the Suc isomers, specifically affects only the expression of the sink-specfic extracellular invertase in contrast to the metabolizable sugars that also repress RbcS mRNA level.

To further substantiate the similar effect of fluoro-Suc and the Suc isomers, MAPK activation was tested. The in-gel assay shown in Figure 5B demonstrates that addition of 20 mM fluoro-Suc for 5 min also results in strong MAPK activation, whereas Suc was inactive. Thus, the ability of fluoro-Suc to activate MAPK activity also resembles the effect of turanose and palationse shown above.

Control experiments were carried out to rule out intracellular cleavage of fluoro-Suc by Suc synthase. Crude extracts of fluoro-Suc-treated cells were analyzed by ^{19}F-NMR. The ^{19}F-NMR spectra revealed that fluoro-Suc was taken up by the tomato suspension culture cells, but that this Suc analog has not

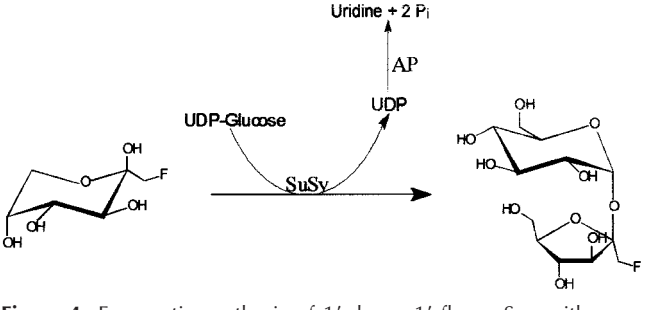

Figure 4. Enzymatic synthesis of 1′-deoxy-1′-fluoro-Suc with a recombinant Suc synthase (SuSy) from potato and alkaline phosphatase (AP).

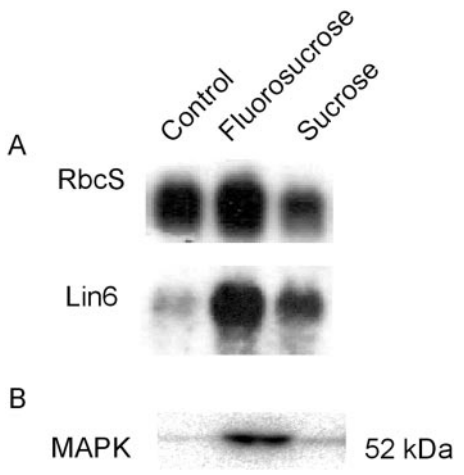

Figure 5. A, Regulation of mRNAs of extracellular invertase Lin6 and RbcS by fluoro-Suc and Suc. Suspension culture cells were treated with 20 mM fluoro-Suc and 50 mM Suc for 6 h. Thirty micrograms of total RNA was separated on formaldehyde agarose gels, blotted onto nitrocellulose, and probed with random primer-labeled cDNA fragments of extracellular invertase Lin6 or RbcS. Equal loading of RNA was confirmed by ethidium bromide staining of the rRNA (data not shown). B, Activation of MAPK by fluoro-Suc and Suc. The cell cultures were incubated with 20 mM fluoro-Suc and 50 mM Suc and harvested exactly after 5 min for the MAPK assay. The data presented in A and B are representative of three independent sets of experiments.

undergone any change in structure under the experimental conditions used (data not shown).

DISCUSSION

Although sugar-mediated signal transduction pathways have been recognized to be important to regulate a variety of physiological responses, the analysis in particular of the effect of the transport sugar Suc is complicated by the fact that it is readily cleaved by extracellular invertase. To circumvent this problem, the non-metabolizable Suc derivatives turanose and palatinose have been used to address disaccharide-specific signaling (Loreti et al., 2000; Fernie et al., 2001). The present study demonstrates that metabolizable sugars and non-metabolizable Suc derivatives activate distinctly different signal transduction pathways in photo-autotrophic tomato suspension culture cells. The data indicate that different disaccharide-specific pathways exist and that non-metabolizable Suc derivatives are sensed as stress-related stimuli.

Photo-autotrophic suspension culture cells of the model plant species tomato and Suc derivatives were used to get further insight in the mechanisms that mediate sugar recognition and signal transduction. Turanose and palatinose are isomers of Suc that differ in their glycosydic linkage between Glc and Fru. These two Suc isomers were shown to be neither cleaved nor taken up by the tomato suspension culture cells used. These data support previous findings that turanose and palatinose are not recognized or transported by Suc transporter (M'Batchi et al., 1984; M'Batchi and Delrot, 1988; Li et al., 1994). Fernie et al. (2001) also recently showed very poor absorption of palatinose by slices of potato tubers. The analysis was further complemented by the use of fluoro-Suc, a Suc derivative that is not subject to invertase hydrolysis but efficiently transported into plant cells (Thom and Maretzki, 1992). It was synthesized by optimizing the protocol of Kragl et al. (1993) using a recombinant Suc synthase. The results obtained with metabolizable sugars (Glc and Suc) and non-metabolizable sugars (turanose, palatinose, and fluro-Suc) were compared with the effect of an elicitor preparation of the tomato pathogenic fungus *F. oxysporum lycopersici* (E-FOL) as a specific and physiological stress-related stimulus.

Metabolizable Sugars and Non-Metabolizable Suc Derivatives Result in Differential Gene Regulation

In the present study, the regulation of mRNAs for extracellular invertase Lin6 and RbcS, chosen as representative marker enzymes for sink and source metabolism, have been analyzed over a 48-h incubation time. The metabolizable sugars Glc and Suc induce the expression of Lin6, whereas RbcS was repressed. This finding confirms results obtained with various experimental systems involving both monocotyledonous and dicotyledonous species showing that metabolizable sugars in general seem to repress photosynthetic genes, whereas sink-specific enzymes are induced (Ehness et al., 1997; Roitsch, 1999; Sheen et al., 1999; Pego et al., 2000). Whereas turanose and palatinose resulted in a strong, fast, and transient induction of extracellular invertase Lin6, the level of RbcS mRNA was not affected. Likewise, the Suc analog fluro-Suc, tested only with an incubation time of 6 h because of the limited amount available, also showed a fast induction of Lin6 transcript level, whereas RbcS transcript level was not affected. Palatinose was also shown to stimulate Suc degradation in discs of growing potato tubers (Fernie et al., 2001). The differential effect of the metabolizable sugars and the non-metabolizable Suc isomers on RbcS expression indicate that they activate distinctly different signal transduction pathways. The fungal elicitor E-FOL resulted in fast and transient repression of RbcS and induction of Lin6 mRNA. Induction of Lin6 pathway seems to be activated by all the stimuli tested, although at different time courses. The Suc derivatives and stress stimuli resulted in faster activation than the metabolizable sugars. In addition, the effect of the Suc isomers was transient like the elicitor, although it was not consumed like Suc.

The differential effect of the metabolizable sugars and the Suc isomers on RbcS expression is substantiated by the analysis of two physiological photosynthetic parameters. The correlation between the regulation of RbcS mRNA, the rate of oxygen evolution,

and the photochemical yield Y supports the use of RbcS mRNA as an appropriate marker for photosynthesis. The regulation of RbcS mRNA by E-FOL is also reflected by a transient decrease of the rate of oxygen evolution, whereas photochemical yield Y is not affected.

Metabolizable Sugars and Non-Metabolizable Suc Derivatives Results in Differential MAPK Activation

The differential effects of sugar analogs and metabolizable sugars were further substantiated by the study of activation of MAPK, which is an important enzyme in a number of signal transduction cascades. MAPKs have been reported to be activated by several stresses in plants such as elicitors (Zhang et al., 1998), wounding (Stratmann and Ryan, 1997), cold and drought stress (Jonak et al., 1996), salinity (Munnik et al., 1999), and endogenous signals (Zhang and Klessig, 1997). In the present studies from the suspension cell cultures of tomato, MAPK was found to activated not only by the fungal elicitor E-FOL, but also by the sugar analogs, turanose, palatinose, and fluoro-Suc. In contrast, Suc and Glc did not result in MAPK activation at the corresponding concentrations. These results suggest that the perception of non-metabolizable Suc analogs and metabolizable sugars and transduction of the corresponding signals follow different pathways.

Implications for the Analysis of Sugar Signal Transduction Pathways

The finding that the metabolizable sugars and the different non-metabolizable Suc derivatives tested, turanose, palationose, and fluoro-Suc, activate distinctly different signal transduction pathways further supports the complexity and importance of carbohydrate-mediated signal transduction in higher plants.

The differential effect of the non-metabolizable Suc isomers and Suc on a very fast signal transduction event, the activation of MAPK, indicates the existence of distinctly different disaccharide specific pathways. Within the very short incubation time of 5 min, cleavage of Suc by extracellular invertase can be neglected. Thus, the observed difference between Suc and the non-metabolizable Suc may not be because of conversion of Suc into the hexose monomers. Using the Suc isomers turanose and palatinose, Loreti et al. (2000) also have demonstrated that both Glc and disaccharide-sensing mechanisms modulate the expression of α-amylase mRNA in barley embryos. Because effects on gene regulation have been analyzed, incubation times of at least several hours were required. The resulting Suc/Glc interconversion ruled out the comparison between the effect of Suc and the Suc isomers.

The finding that neither turanose nor palatinose is transported supports an extracellular recognition of these carbohydrate signals, which has been suggested before (Loreti et al., 2000; Fernie et al., 2001). Because the transportable fluoro-Suc elicited the same responses as turanose and palatinose, the corresponding effects are independent of the lack of a transport system. Both with respect to the time course of Lin6 mRNA induction and MAPK, the effect of the non-metabolizable Suc derivatives resemble the effect of the fungal elicitor E-FOL. These observations indicate that these Suc derivatives that are not naturally occurring in plants are sensed as stress signals rather than metabolic signals. The physiological significance of this assumption is supported by the fact that phytopathogens such as specific strains of *Erwinia* spp. are able to transform Suc into palatinose (Huang et al., 1998). By this mechanism, Suc is retrieved from the plants and converted to a form unavailable for the plant metabolism. Thus, the presence of unusual Suc derivatives may be signals for the presence of a pathogen. Therefore, these sugar analogs may not be appropriate tools to address extracelluar and disaccharide-specific sensing mechanism in plants per se.

Non-metabolizable Suc derivatives were shown to activate different signal transduction pathways than metabolizable sugars, thus demonstrating the complexity of carbohydrate-mediated regulatory mechanisms. Distinct sugar-sensing mechanisms and parallel signal transduction pathways may be a central part of a complex regulatory network of higher plants to integrate metabolism with development and defense responses.

MATERIALS AND METHODS

Growth of Suspension Culture Cells

Photo-autotrophic suspension cell culture cells of tomato (*Lycopersicon peruvianum*) were established by Beimen et al. (1992) These cell cultures are being subcultured every 2 weeks in Murashige and Skoog medium and are incubated shaking under continuous light conditions with an atmosphere containing 2% (w/v) CO_2.

Preparation of an Elicitor from *Fusarium oxysporum lycopersici*

The pathogenic fungus *F. oxysporum lycopersici* Schlecht. Fr. f. sp. *lycopersici* (Sacc.) was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). The fungus was cultured in a medium containing 50 g L^{-1}Glc, 8 g L^{-1}casamino acids, 0.5 g L^{-1}yeast (*Saccharomyces cerevisiae*) extract, 0.2 g $L^{-1}$$MgSO_4$and $FeSO_4$ each, 20 mg $L^{-1}$$CaCl_2$, and 1.5 mg $L^{-1}$$MnSO_4$ and Na_2MoO_4 each in 25 mM potassium phosphate at pH 7.5. After 4 d of shaking at 28°C, the culture was autoclaved, dialyzed against water, and lyophilized. For the induction of stress response,

150 mg $L^{-1}$of the dried hyphae was added to a tomato cell suspension culture.

Extraction of mRNA and RNA Gel-Blot Analysis

For the isolation of RNA, cells were harvested by centrifugation, snap frozen in liquid nitrogen, and ground in the presence of liquid nitrogen. Total RNA was isolated according to the methods of Chomczynski and Sacchi (1987). Northern-blot analysis was carried out as described previously (Godt and Roitsch, 1997).

Determination of Sugars

The concentrations of Glc were determined by a commercially available test system (GOD test, Roche, Mannheim, Germany). For the determination of Suc concentrations, the Suc present in the samples were hydrolyzed by 100 units of yeast invertase (Grade VII, Sigma, St. Louis) incubated at 30°C for 1 h. Glc concentration was determined before and after hydrolysis by invertase and the difference between these values was taken as the actual amount of Suc in the sample. The supernatant of Suc treated cells were used to estimate the build up of hexoses, Glc, and Fru. Glc was estimated by the GOD test, whereas Fru was estimated as described by Bernt and Bergmeyer (1970). Turanose and palatinose concentrations were determined according to Dubois et al. (1956).

In-Gel Kinase Assay for MAPK

The enzyme was extracted from the ground tissue in an extraction buffer consisting of 100 mM HEPES, pH 7.5; 5 mM EDTA; 5 mM EGTA; 10 mM dithiothreitol; 10 mM Na_3VO_4; 10 mM NaF; 50 mM glycerophosphate; 0.1 mM phenylmethylsulphonyl fluoride; 1 mM bezamidine; and 0.1 μg mL^{-1} antipain. The crude extract was centrifuged at 4°C at 20,000rpm for 10 min and an aliquot of supernatant equivalent to 40 μg of protein analyzed by Bradford assay (Bradford, 1976) was used for in-gel kinase assay. Polyacralymide gel (10% [w/v]) embedded with 0.3 mg mL^{-1} of MBP (Upstate Biotechnology, Lake Placid, NY) as substrate was used for the kinase assay. For control, MBP was substituted with histone or casein. After electrophoresis, proteins were renatured and assayed for kinase assay as described by Zhang and Klessig (1997). Activity were visualized by autoradiography and phosphor imager (Cyclone Phosphor Storage Systems, Perkin Elmer, Madison, WI).

Measurement of Rate of Oxygen Evolution

Rate of oxygen evolution of the cell cultures were measured using a liquid phase oxygen electrode (Frank Bros Ltd., Cambridge, UK) in the presence of saturating light provided by a halogen lamp projector. The cells in the sample cuvette were first allowed to respire for 1 min in the dark and then exposed to light for the measurement of oxygen evolution. Equal volumes of cells were used each time and immediately after the measurements, the cells were taken out to determine the fresh weight. The rate of oxygen evolution was calculated on the basis of fresh weight and represented as relative units.

Chlorophyll Fluorescence Measurements

Modulated chlorophyll fluorescences of the tomato cell suspension culture were measured using a PAM 2000 chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany). Maximum PSII quantum yield of a dark-adapted sample (F_v/F_m) and effective PSII quantum yield of illuminated sample (Y; for nomenclature, see van Kooten and Snel, 1990) were measured on cells dark adapted for 15 min as described by Schreiber et al. (1986). The preprogrammed protocol (Standard Run 3) was used for the determination of F_v/F_m and Y in a special cuvette designed for the purpose. The steady-state values obtained at the end of Run 3 were reported as the values of Y.

Synthesis of 1-Deoxy-1-Fluoro-Fru

1-Deoxy-1-fluoro-D-Fru was obtained by the reaction described by Card and Hitz (1984). In brief, the readily available 2,3:4,5-di-o-isopropylidene-D-fructopyranose was converted into the trifylate by the procedure described by Binkley et al. (1980). Trifylate was fluorinated by Tris (dimethylamino)sulfur (trimethylsilyl)difluoride (Sigma-Aldrich, Saint-Quentin-Fallavier, France) in refluxing tetrahydrofuran. After removal of the isopropylidene protection groups, 1-deoxy-1-fluoro-D-Fru was obtained as a syrup in 75% yield. The synthesis of 1′-deoxy-1′-fluoro-Suc was carried out by the repetitive batch technique (Kragl et al., 1993). The reaction mixture (100 mL) containing 0.96 mmol 1-deoxy-1-fluoro-Fru (176 mg) and 1 mmol UDP-α-D-Glc (Sigma, Deisenhofen, Germany) in 200 mM HEPES buffer, pH 8.0, was gently stirred at 30°C after the addition of 40 units of recombinant SuSy 1 from potato (*Solanum tuberosum*; Zervosen et al., 1998; Zervosen and Elling, 1999), and 200 units of alkaline phosphatase (Roche Diagnostics). The course of reaction was controlled by HPLC analysis of the product with an Aminex HPX-87C column (300 × 7, 8 mm, Bio-Rad, Munich) by elution with distilled water at 85°C. After 48 h, the enzymes were recovered by ultrafiltration and used in a second and third batch, respectively, by the addition of new substrates.

The yield of the combined product solutions was 2.9 mmol (100%) for 1′-deoxy-1′-fluoro-Suc. For isolation, the product solution was adjusted to pH 8.6 and loaded onto an anion exchanger column ($HCOO^-$ form) filled with AG 1-X8 resin (100–200 mesh, 122-mL bed volume, Bio-Rad), which was equilibrated with distilled water. Elution with distilled water (linear flow rate: 56.5 cm h^{-1}) gave a product pool, which was concentrated by in vacuoevaporation to a final volume of 5 mL. The disaccharide was further purified by chromatography on an AG 50W-X8 resin column (200–400 mesh, Ca^{2+} form, 1,532-mL bed volume, Bio-Rad). Elution with distilled water (linear flow rate: 3 cm h^{-1}) gave the fractions containing the disaccharide, which were pooled and lyophilized. The dry product was

dissolved in 10 mL of absolute methanol and crystallized at 25°C. 1′-deoxy-1′-fluoro-Suc was obtained in an overall yield of 85% corresponding to 2.5 mmol (860.5 mg) with an HPLC purity of 89%. NMR spectroscopy (11.7 Tesla) of 1′-deoxy-1′-fluoro-Suc revealed the typical couplings between ^{19}F and ^{1}H or ^{13}C: ^{1}H-NMR(D_2O): $H_{1'}$,δ′ 3a.39 ppm, m, $J_{1H\text{-}19F}$: 46.6 Hz, $J_{1H\text{-}1H}$: 10.4 Hz; ^{19}F-NMR(D_2O): δ_{CFCl3}: −229.4 ppm, m, $J_{19F\text{-}1H}$: 46.6Hz.; ^{13}C-NMR(D2O): $C_{1'}$,δ:80.7 ppm, d, $J_{13C\text{-}19F}$: 174.2Hz; $C_{2'}$,δ:101.8 ppm, d, $J_{13C\text{-}19F}$: 19.6Hz.

ACKNOWLEDGMENTS

W.K. is grateful for the support and advice of Drs. Claude Roby, Jacques Defaye, and André Gadelle (Commissariat à l'Energie Atomique, Grenoble, France) during the synthesis of fluoro-Fru. T.R. and A.K.S. would like to thank Werner Kremer (Institut für Biophysik und Physikalishe Biochemie, Regensburg, Germany) for the NMR analysis of tomato samples, and Dr. Ulrch Schreiber (Lehrstuhls Botanik I, Würzburg, Germany) for providing the PAM 2000 portable fluorometer. Authors are grateful to Dr. Ludwig Lehle for the help with the sugar determinations, Vinzenz Link and Mark Goetz for critically reading the manuscript, and Dr. Widmar Tanner for continuous interest and support (all from Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Regensburg, Germany).

Received August 22, 2001; returned for revision October 20, 2001; accepted December 18, 2001.

LITERATURE CITED

Armstrong GM, Armstrong JK (1981) Formae specialis and races of *Fusarium oxysporium* causing wilt diseases. *In* PE Nelson, TA Tousson, RJ Cook, *Fusarium*: Diseases, Biology, and Taxonomy. The Pennsylvania State University Press, University Park, PA, pp 391–399

Beimen A, Witte L, Barz W (1992) Growth characteristics and elicitor-induced reactions of photosynthetically active and heterotrophic cell suspension cultures of *Lycopersicon peruvianum*. Mill. Bot Acta **105:** 152–160

Bernt E, Bergmeyer HU (1970) D-Fructose. *In* HU Bergmeyer, ed, Methoden der Enzymatischen Analyze. 2 Auflage, Verlag Chemie, Weinheim Bergstrasse, Germany, pp 1349–1352

Binkley RW, Ambrose MG, Hehemann DG (1980) Synthesis of deoxyhalogeno sugars: displacement of the (trifluoromethanesulfonyl)oxy(triflyl) group by halide ion. J Org Chem **45:** 4387–4391

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Card PJ, Hitz WD (1984) Synthesis of 1′-deoxy-1′-fluorosucrose via sucrose synthase mediated coupling of 1-deoxy-1-fluorofructose with uridine diphospate glucose. J Am Chem Soc **106:** 5348–5350

Chiou TJ, Bush DR (1998) Sucrose is a signal molecule in assimilate partitioning. Proc Natl Acad Sci USA **95:** 4784–4788

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium thyocynate-phenol-chloroform extraction. Annal Biochem **162:** 156–159

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Ann Chem **28:** 350–356

Ehness R, Ecker M, Godt D, Roitsch T (1997) Glucose and stress independently regulate source/sink relations and defense mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell **9:** 1825–1841

Fernie AR, Rossner U, Geigenberger P (2001) The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers. Plant Physiol **125:** 1967–1977

Godt DE, Riegel A, Roitsch T (1995) Regulation of sucrose synthase expression in *Chenopodium rubrum*: characterization of sugar induced expression in photoautotrophic suspension cultures and sink tissue specific expression in plants. J Plant Physiol **16:** 231–238

Godt DE, Roitsch T (1997) Differential regulation of a tomato invertase gene family suggests an important function of extracellular isoenzmes in establishing and maintaining sink metabolism. Plant Physiol **115:** 273–282

Halford NG, Purcell PC, Hardie DG (1999) Is hexokinase really a sugar sensor in plants? Trends Plant Sci **4:** 117–120

Herbers K, Meuwly P, Frommer WB, Métraux JP, Sonnewald U (1996) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell **8:** 793–803

Hitz W, Schmitt MR, Card PJ, Giaquinta RT (1985) Transport and metabolism of 1′-fluorosucrose, a sucrose analog not subject to invertase hydrolysis. Plant Physiol **77:** 291–295

Huang JH, Hsu LH, Su YC (1998) Conversion of sucrose to isolmaltulose by *Klebsialla planticola*. J Micro Biotechnol **21:** 22–27

Jang JC, Leon P, Zhou L, Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell **9:** 5–19

Jang JC, Sheen J (1997) Sugar sensing in higher plants. Trends Plant Sci **2:** 169–174

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. Proc Natl Acad Sci USA **93:** 11274–11279

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Ann Rev Plant Physiol Plant Mol Biol **47:** 509–540

Koch KE, Ying Z, Wu Y, Avigne WT (2000) Multiple pathways of sugar-sensing and a sugar/oxygen overlap for genes of sucrose and ethanol metabolism. J Exp Bot **51:** 417–427

Kragl U, Gödde A, Wandrey C, Kinzy W, Cappon JJ, Lugtenburg J (1993) Repetitive batch as an efficient method for preparative scale enzymatic sythesis of 5-azido-neuraminic-acid and ^{15}N-L-glutamic acid. Tetrahedron **4:** 1193–1202

Krapp A, Stitt M (1994) Influence of high carbohydrate content on the activity of plastidic and cytosolic isoen-

zyme pairs in photosynthetic tissues. Plant Cell Environ **17:** 861–866

Lalonde S, Bolos E, Hellmann H, Barker L, Patrick JW, Frommer WB, Ward JM (1999) The dual function of sugar carriers: transport and sugar sensing. Plant Cell **11:** 707–728

Li ZS, Noubhani AM, Bourbouloux A, Delrot S (1994) Affinity purification of sucrose binding proteins from the plant plasma membrane. Biochim Biophys Acta **1219:** 389–397

Loreti E, Amedo A, Perata P (2000) Glucose and disaccharide mechanisms modulate the expression of a-amylase in barley embryos. Plant Physiol **123:** 939–948

M'Batchi B, Delrot S (1988) Stimulation of sugar exit from leaf tissues of *vicia faba* L. Planta **174:** 340–348

M'Batchi B, Pichelin D, Delrot S (1984) The effect of sugar on the binding of [^{203}Hg]-*p*-chloromercuribenzenesulfonic acid to leaf tissues. Plant Physiol **79:** 537–542

Munnik T, Ligterink W, Meskiene I, Calderini O, Beyerly J, Musgrave A, Hirt H (1999) Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. Plant J **20:** 381–388

Özcan S, Dover J, Jonston M (1998) Glucose sensing and signalling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J **17:** 2666–2673

Pego JV, Kortstee AJ, Huijser C, Smeekens SCM (2000) Photosynthesis, sugars and regulation of gene expression. J Exp Bot **51:** 407–416

Roitsch T (1999) Source-sink regulation by sugars and stress. Curr Opin Plant Biol **2:** 198–206

Roitsch T, Bittner M, Godt DE (1995) Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analogue and tissue-specific expression suggest a role in sink-source regulation. Plant Physiol **108:** 285–294

Roitsch T, Ehness, R Goetz M, Hause B, Hoffman M, Sinha AK (2000) Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. Aust J Plant Physiol, **27:** 815–825

Rook F, Gorris N, Kortstee A, van Kampen M, Borrias M, Weisbeck P, Smeekens S (1998) Sucrose-specific signalling represses translation of the Arabidopsis ATB2 bZIP transcription factor gene. Plant J **15:** 253–263

Schreiber U, Schliwa U, Bilger W (1986) Continous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res **10:** 51–62

Sheen J, Zhou L, Jang JC (1999) Sugars as signaling molecules. Curr Opin Plant Biol **2:** 410–418

Smeekens S (1998) Sugar regulation of gene expression in plants. Curr Opin Plant Biol **1:** 230–234

Smeekens S, Rook F (1997) Sugar sensing and sugar mediated signal transduction in plants. Plant Physiol **115:** 7–13

Stratmann JW, Ryan CA (1997) Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proc Natl Acad Sci USA **94:** 11085–11089

Sturm A (1999) Invertases: primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol **121:** 1–7

Thom M, Maretzki A (1992) Evidence of direct uptake of sucrose by sugarcane stalk tissue. J Plant Physiol **139:** 555–559

van Kooten O, Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth Res **25:** 147–150

Zervosen A, Elling L (1999) Application of sucrose synthase in the synthesis of nucleotide sugars and saccharides. *In* C Burke, ed, Methods in Biotechnology, Vol 10: Carbohydrate Biotechnology Protocols. Humana Press Inc., Totowa, NJ, pp 235–254

Zervosen A, Römer U, Elling L (1998) Application of recombinant sucrose synthase: large scale synthesis of ADP-glucose. J Mol Catalysis B Enzymatic **5:** 25–28

Zhang S, Du H, Klessig DF (1998) Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora spp*. Plant Cell **10:** 435–450

Zhang S, Klessig DF (1997) Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell **9:** 809–824

Zhang S, Klessig DF (1998) The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc Natl Acad Sci USA **95:** 7225–7230