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The Role of Carbohydrate-Metabolizing Enzymes in Sugar Sensing and Differentiation in Sugar Beet Cell Lines

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Summary

Plant development is influenced by changes in the levels and types of sugars produced metabolically. The normal (N), habituated organogenic (HO) and habituated nonorganogenic (HNO) sugar beet cell lines originate from the same mother plant but exhibit distinct levels of morphogenesis and differentiation, and contain different levels of simple carbohydrates. We aim to elucidate whether differences in the abundance and activity of enzymes involved in carbohydrate metabolism and sugar sensing/signalling help explain the different carbohydrate profiles and differentiation states of the cell lines. Using ^{13}C NMR spectroscopy to analyze cultures of the cell lines over 28 days, we found that N cells accumulated sucrose; HO cells sucrose, glucose and fructose; and HNO cells glucose and fructose. Of three invertase isoforms, the activity of cell wall invertase (CWI) was highest in all the cell lines, and CWI activity was greatest in HNO line. The specific accumulation of intracellular carbohydrates during subculture correlated strongly with CWI activity but less so with the vacuolar and cytoplasmic invertase isoforms, or with sucrose synthase activity. Cell lines showed differences in how sugars regulated invertase and sucrose synthase activity. The role of sugar sensing in the regulation of CWI activity was investigated in the cell lines using glucose and sucrose, as well as carbohydrate analogues such as mannitol, 2-*O*-deoxyglucose and 3-*O*-methylglucose. Differences in the regulation of CWI activity by carbohydrates across the three cell lines suggest that CWI can be fine-tuned according to the specific carbohydrate requirements of each line during growth. Differences in sugar signalling pathways across the cell lines were explored using glucose and sucrose in the presence of inhibitors of protein kinases or phosphatases. Taken together, our findings suggest that specific regulation of CWI activity plays an important role in determining the intracellular carbohydrate levels of sugar beet cell lines, and possibly their differentiation state as well.

Key words: carbohydrate metabolism, differentiation, invertases, NMR, sucrose synthase

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Abbreviations: ATP, adenosine triphosphate; 2-DOG, 2-deoxyglucose; DTT, dithiothreitol; 3-OMG, 3-*O*-methylglucose; CWI, cell wall invertase; CI, cytoplasmic invertase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Glc, glucose; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNO, habituated nonorganogenic; HO, habituated organogenic; HXK, hexokinase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance spectroscopy; N, normal; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Suc, sucrose; SuSy, sucrose synthase; UDP-Glc, uridine diphosphoglucose; VI, vacuolar invertase

Introduction

The role of the carbohydrates sucrose (Suc), glucose (Glc) and fructose (Fru) in plant development has been clearly established in recent years (*1,2*). High intracellular Suc levels are associated with morphogenesis, cell elongation and differentiation, while high intracellular hexose levels are associated with mitotic activity and dedifferentiation (*3,4*). Sensing of carbohydrates is a regulatory process in which interaction between a sugar molecule and a sensor protein generates a signal within the cell (*5*). Sugar sensing occurs by two main pathways: the disaccharide and monosaccharide sensing pathways. Sucrose is probably sensed by an atypical sucrose transporter SUT2/SUC3, analogous to SNF3 and RGT2 glucose sensors in yeast (*6*), while Glc is sensed at the cell surface by membrane-bound G-protein-coupled pathways, and intracellularly by hexokinase (HXK)-dependent, HXK-independent and glycolysis-dependent pathways (*7,8*). In all cases, the signalling triggers cascades involving protein phosphatases, kinases and calcium ions, which ultimately lead to cellular responses (*9,10*).

Enzymes of Suc metabolism, such as invertases (EC 3.2.1.26) and sucrose synthase (SuSy; EC 2.4.1.13), participate directly in sugar-regulated plant development by helping to control the levels and types of sugars produced metabolically (*11,12*). Invertases catalyze irreversible cleavage of Suc into Glc and Fru. They provide hexoses for metabolism and influence photosynthesis, carbohydrate partitioning between tissues, senescence and cell rejuvenation (*13,14*). Three invertase types have been identified based on their solubility, optimal pH and isoelectric point; these isoforms show different subcellular localizations. The isoforms are cell wall-bound invertase (CWI) and vacuolar invertase (VI), both of which have an acidic pH optimum; and cytoplasmic invertase (CI), which has a neutral pH optimum (*15*). In plants, invertase isoenzymes are encoded by a multigenic family (*14*). In sugar beet, two CWI and one VI gene have been identified (*16*). Sucrose synthase catalyzes the degradation and synthesis of sucrose by the following reaction:

$$\text{Suc+UDP} \leftrightarrow \text{Fru+UDP–Glc} \qquad /1/$$

Its activity is crucial in the synthesis of cell wall and storage compounds. Most plant species contain several isoforms of SuSy (*11*), and two have been purified and characterized from sugar beet (*17*).

Three sugar beet cell lines have been extensively used in the studies of cell differentiation (*18–22*). Normal (N) cells are photosynthetically active, with a diameter ranging from 25 to 600 μm, and they are growth regulator-dependent. These cells are differentiated but nonorganogenic. Habituated organogenic (HO) cells are photosynthetically active, similar in size to N cells, hormone-independent and organogenic. In contrast, habituated nonorganogenic (HNO) cells are nonphotosynthetic, ranging in diameter from 25 to 200 μm, hormone-independent, nonorganogenic and dedifferentiated. Their cell wall is less elaborated than that of N and HO cells, and they divide and respire more rapidly, which is associated with the accumulation of ATP. The lack of photosynthesis in HNO cells is related to a deficiency in tetrapyrrole-containing compounds (*23*). In addition, these cells contain extremely low amounts of cytochrome b5, P450, P420, total haem and chlorophyll. These findings suggest an alteration in the control of tetrapyrrole metabolism. Both HO and HNO cell lines are thought to carry out photosynthesis, whereas in HNO cells, CO_2 fixation is believed to occur only *via* a non-photosynthetic pathway involving phosphoenolpyruvate carboxylase (*24,25*). Omarzad (*26*) measured the photosynthetic activity in the three cell lines after 14 days in culture using an oxygraph and 50 mM $NaHCO_3$ as the carbon source. The activity in N cells (~9 μmol O_2/(mL·h)) was not significantly lower than in HO cells (~12 μmol O_2/(mL·h)). As expected, HNO photosynthetic activity was significantly lower (~2 μmol O_2/(mL·h)). Omarzad also studied the metabolism of $NaH^{13}CO_3$. By analyzing the isotopically labelled synthesized compounds, he found that $^{13}CO_2$ fixation occurred by an anaplerotic pathway involving phosphoenolpyruvate carboxylase in all three cell lines.

Nuclear magnetic resonance is a convenient method for studying the metabolomes of plant species during various developmental processes and for certain types of analyses where it offers higher resolution than enzymatic or HPLC determination of metabolites (*27,28*). For example, ^{13}C NMR spectroscopy has been used to estimate intracellular sugar amounts in sugar beet cell lines (*19, 29*). These studies revealed high intracellular levels of sucrose in the N cell line, and high levels of glucose in the HO and HNO lines. Additionally, the activity of the glycolytic enzymes hexose phosphate isomerase and phosphofructokinase is lower in HNO cells than in N cells, which is compensated by higher activities of enzymes of the hexose monophosphate pathway, *i.e.* glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (*19*).

Thus, the three sugar beet cell lines differ not only in differentiation state but also in carbohydrate metabolism. These differences make these lines a convenient experimental model for studying possible relationships among internal sugar content, invertase and SuSy activities and cell differentiation states. In the present study, we have observed that the cell lines accumulated different amounts of sucrose, glucose and fructose during the subculture period. Carbohydrate accumulation correlated strongly with CWI activity and less so with VI, CI, or SuSy activity. The cell lines also showed differences in how sugar sensing and transduction of sugar signals regulated CWI activity. These findings lead us to conclude that cell line-specific regulation of CWI has important implications for differentiation and physiology of sugar beet.

Materials and Methods

Plant growth and treatments

Three cell lines of sugar beet (*B. vulgaris* L. *altissima*) that originated from the same mother plant were cultivated *in vitro* (*30*). Cell lines were grown in a growth chamber at 22 °C on a 16-hour photoperiod (80 μmol/(m·s)).

For growth measurement, 2 g of each cell line were transferred to a Petri dish containing PG0 medium (*31*) supplemented with 88 mM Suc, Glc or Fru. Tissue sam-

ples were collected at 3, 7, 10, 14, 21 and 28 days and weighed. Samples were quickly frozen in liquid nitrogen and stored at –80 °C until use.

Cell suspensions were established from 14-day-old callus cultures by transferring 2 g of cells to flasks with PG0 liquid medium and agitated on an orbital shaker. After 7 days, cells were collected and washed three times with sugar-free medium and transferred to new flasks. Suc was replaced with mannitol, which served as an osmoticum. After 24 h in complete darkness, cells were collected and washed three times with fresh medium lacking sugar. To initiate treatments, cells were incubated in PG0 medium supplemented with various sugars, sugar analogues or signal transduction inhibitors. Cells were harvested after 72 h, frozen in liquid nitrogen and stored at –80 °C until use.

Carbohydrates were prepared as 10× stocks in PG0 medium and filter-sterilized before use. The following carbohydrates were added to the liquid medium at a final concentration of 88 mM: mannitol, 3-*O*-methylglucose (3-OMG), mannose, Glc and Suc. The carbohydrate 2-deoxyglucose (2-DOG) was supplied at a final concentration of 1 mM together with 87 mM mannitol as a supplementary osmoticum. Okadaic acid (OA; Sigma-Aldrich, St. Louis, MO, USA) was used as a protein phosphatase inhibitor. A stock solution was prepared in dimethyl sulfoxide (DMSO) and used at a final concentration of 0.1 μM. Staurosporine (SS; Sigma-Aldrich) was used to inhibit protein kinases. A stock solution was prepared in DMSO and used at a final concentration of 0.8 μM. Control cell suspensions were prepared in the same way as the treated ones, but they received only the corresponding volume of solvent vehicle.

^{13}C NMR spectroscopy

^{13}C NMR spectra of sugar beet cell lines were collected as previously described (*29*). On day 0 (immediately after inoculation) and on day 14 of the subculture period, cells were filtered through Whatman no. 1 paper, and the filtered culture medium was lyophilized and stored at –20 °C. For NMR measurements, the lyophilized material was dissolved in D_2O. For *in vivo* measurements, spectra were recorded on a 7.04 T Bruker AC 300 spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 10-mm multinuclear probehead at 75.47 MHz. Experiments were performed for 13 h at 3 °C using 30° pulses with a 2-second repetition time and a spectral width of 17 857 Hz, yielding approx. 16 000 scans for each spectrum. A capillary column containing a solution of tetradeutero-3-(trimethylsilyl)-propionate in D_2O was used for deuterium frequency locking and referencing the ^{13}C chemical shift (d=0.00 ppm). For *in vitro* measurements, 2 g of sugar beet cells were flash-frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle in 1 mL of 5 % (by volume) perchloric acid. After thawing, the suspension was clarified by centrifugation at 3000×*g* for 10 min. The pH of the supernatant was adjusted to 6.5 using 2 M KOH and then centrifuged at 3000×*g* for 10 min to remove the $KClO_4$. The resulting supernatant was lyophilized and stored at –20 °C. Samples were dissolved in D_2O before measurements. NMR experiments were carried out on a Bruker DRX 400 spectrometer operating at 100.61 MHz and using sodium tetradeutero-3-(trimethylsilyl)-propionate as an internal standard. Acquisition time for the experiments was approx. 12 h.

Protein extraction

Extraction was performed at 4 °C unless otherwise noted. Soluble proteins were extracted by grinding plant tissue in a Retsch MM 200 automatic bead-mill homogenizer (Retsch, Haan, Germany) for 1 min at 30 Hz in 50 mM HEPES/KOH (pH=7.4), 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1 mM EGTA and 10 % (by volume) glycerol (*32*). Insoluble 2 % (by mass) polyvinylpyrrolidone was added before the grinding. The homogenate was centrifuged for 10 min at 15 000×*g*. Supernatant was desalted on PD-10 columns (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM HEPES/KOH (pH=7.4), 5 mM $MgCl_2$ and 5 mM DTT. The eluate (fraction A) was used for subsequent analysis.

The pellet obtained after homogenate centrifugation was washed three times with equilibration buffer. After each wash, the homogenate was centrifuged at 15 000×*g* for 10 min. After the final wash, the supernatant was removed, and the pellet resuspended in 100 mM sodium acetate (pH=5.0), 5 mM Na_2SO_3, 5 mM DTT and 1 M NaCl. The resuspension was incubated for 4 h on an orbital shaker (*33*). The extract was clarified by centrifugation at 15 000×*g* for 10 min. The supernatant (fraction B), containing cell wall proteins, was used in subsequent analysis.

Protein concentration in extracts was estimated by absorbance at 595 nm using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). Bovine serum albumin was used as a standard reference.

Enzyme assays

Invertases were assayed according to Ross *et al*. (*33*), with slight modifications. Protein extract (50 μL) was added to 200 μL of assay buffer and incubated at 37 °C for 30 min. For assaying CWI and VI, 50 mM sodium acetate buffer (pH=4.5) and 125 mM Suc as substrate were used. For assaying CI, 50 mM MES (pH=7.5) and 125 mM Suc as substrate were used. Levels of reducing sugars were determined using the Somogy-Nelson method by measuring absorbance at 540 nm in a microplate reader (BioTek, Winooski, VT, USA). The quantity of the released reducing sugars was calculated from a standard curve generated using glucose. One unit of enzyme activity was defined as the production of 1 nmol of hexoses per min.

SuSy activity was assayed in the sucrose synthesis direction as described by Hubbard *et al*. (*34*). Briefly, 45 μL of fraction A were incubated with 70 μL of 50 mM MOPS-NaOH (pH=7.5), 15 mM $MgCl_2$, 10 mM Fru and 10 mM UDP-Glc. After 20 min of incubation, the reaction was terminated by the addition of 70 μL of 30 % (by mass per volume) KOH. Blanks were terminated without incubation. Unreacted hexoses and hexose-6-phosphates were degraded by boiling the tubes for 10 min. After the samples had cooled to room temperature, 1.0 mL of 0.14 % (by mass per volume) anthrone in 6.9 M H_2SO_4 was added and the tubes were incubated at 40 °C

for 20 min. The amount of released sucrose was measured spectrophotometrically at 620 nm. One unit of enzyme activity was defined as the production of 1 pmol of sucrose per min.

SDS-PAGE and protein gel electroblotting

Fraction A was mixed with SDS sample buffer and fractionated by SDS-PAGE in a 12 % polyacrylamide gel, using the vertical buffer system of Laemmli (*35*). Equal amounts of protein were loaded in each lane, *i.e.* 10 μg when anti-VI antibody was used and 5 μg when SuSy antibody was used. Samples moved through the stacking and separating gels at 100 and 200 V, respectively. Proteins were transferred to a nitrocellulose membrane as previously described (*21*). The quality of the transfer was verified by staining the membrane with Ponceau Rouge S. Membranes were treated with anti-VI and anti-SuSy antibodies diluted 1:1000 and 1:4000, respectively, in blocking buffer. Anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) was used as a secondary antibody according to the manufacturer's recommendations.

The levels of proteins detected on Western blots were estimated using densitometric analysis (ImageTool v. 3 Final, The University of Texas Health Science Center at San Antonio, TX, USA).

Statistical analysis

Data management and statistical analyses were performed with the SPSS statistical software package (v. 11.0.1 PC; SPSS, Chicago, IL, USA) and Sigmaplot (Systat Software, Chicago, IL, USA). Means are expressed with their standard error and compared by ANOVA using the General Linear Model (GLM) procedure.

Results

Cell growth, metabolite content and metabolism

Previous results have shown that sugar beet cell lines contain distinct amounts of primary metabolites, notably sugars, which could be related to their developmental state (*29*). To gain more insight into the differential accumulation of intracellular carbohydrates, growth of the cell lines was compared during 28 days of subculturing on solid medium supplemented with Suc (Fig. 1). The N and HO lines gained similar amounts of fresh mass (FM) during this period. HNO cells increased in FM until day 14, after which growth slowed down and eventually stopped. Further differences in growth were observed when cells were fed with monosaccharides (Fig. 1). On Glc, FM increased most quickly in HNO cells, slightly less quickly in HO cells, and least quickly in N cells. Growth was similar for N and HO cells on Fru during 14 days, but thereafter the HO line gained more fresh mass than N did. Among the studied lines, HNO grew slowest on fructose.

We then compared the residual concentrations of carbohydrates and organic compounds after 14 days of subculture in nutrient medium supplemented with Suc (Table 1). Sucrose was present at higher concentrations in N culture medium than in HO or HNO media. Based on residual levels of monosaccharides, it seemed that N cells imported similar amounts of Glc and Fru. HO preferentially imported Glc over Fru, but at a slower rate than N cells. Very low concentrations of Suc, Glc and Fru in HNO medium suggest that these cells took up all available carbohydrates very fast. Organic acids and aromatic compounds were observed only in HNO medium (Table 1).

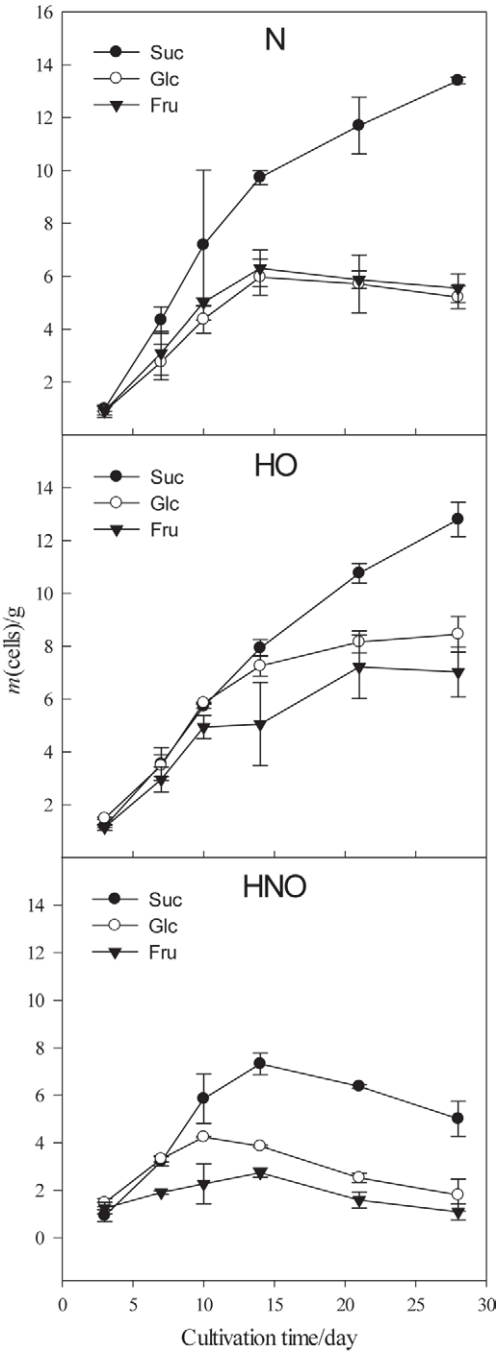

Fig. 1. Growth of sugar beet cell lines during 28 days of subculture. Normal (N), habituated organogenic (HO) and habituated nonorganogenic (HNO) cell lines were grown on solid PG medium supplemented with 88 mM of sucrose (Suc), glucose (Glc) or fructose (Fru). Values are the means of three replicates±standard error of the mean (SEM). Where no error bars are evident, the error was less than the area covered by the data symbol

Table 1. Concentration of carbohydrates, organic acids and aromatic compounds in sugar beet cell culture medium on days 0 and 14 of subculture, as measured by ^{13}C-NMR

		Sucrose	Glucose	Fructose	Organic acids	Aromatic compounds
Composition of medium on the initial day/(mmol/L)	Cell lines	90.00±0.01	0.00±0.01	0.00±0.01	0.00±0.01	0.00±0.01
Composition of medium after 14 days of culture/(mmol/L)	N	11.3±0.1	5.8±0.1	5.5±0.2	0.00±0.01	0.00±0.01
	HO	5.6±0.2	10.6±0.1	31.50±0.04	0.00±0.01	0.00±0.01
	HNO	1.1±0.2	1.5±0.3	1.3±0.2	3.2±0.1	1.8±0.2

Cell lines are as follows: N=normal, HO=habituated organogenic, HNO=habituated nonorganogenic. Values are the means of five replicates±SEM

To compare more closely the levels of carbohydrates and their metabolism in the cell lines, ^{13}C NMR measurements of intracellular metabolites were performed over the course of the 28-day subculture. Peaks from data accumulation were assigned to sucrose (1), α-glucose (2), β-glucose (3), α-fructose (4), β-fructose (5) and glutamine (6) (Fig. 2a). At the beginning of subculture, a higher concentration of Suc was found in N and HO, while a much lower concentration of Suc was found in HNO cells (Fig. 2b). Over the course of the subculture, a sharper decrease in intracellular Suc was observed in N than in HO cells. In HNO cell line, low levels of Suc were slowly metabolized. Higher concentrations of Glc and Fru were recorded in HO and HNO lines than in N, indicating that the N line metabolized Glc and Fru very slowly. In the HO culture, Glc and Fru concentrations decreased quickly during the first 7 days, after which the Glc concentration remained constant while the Fru concentration decreased slightly towards the end of subculture. In the HNO line, monosaccharides were consumed faster than in N and HO lines. Glutamine was detected only in HO and HNO cells (Fig. 2b).

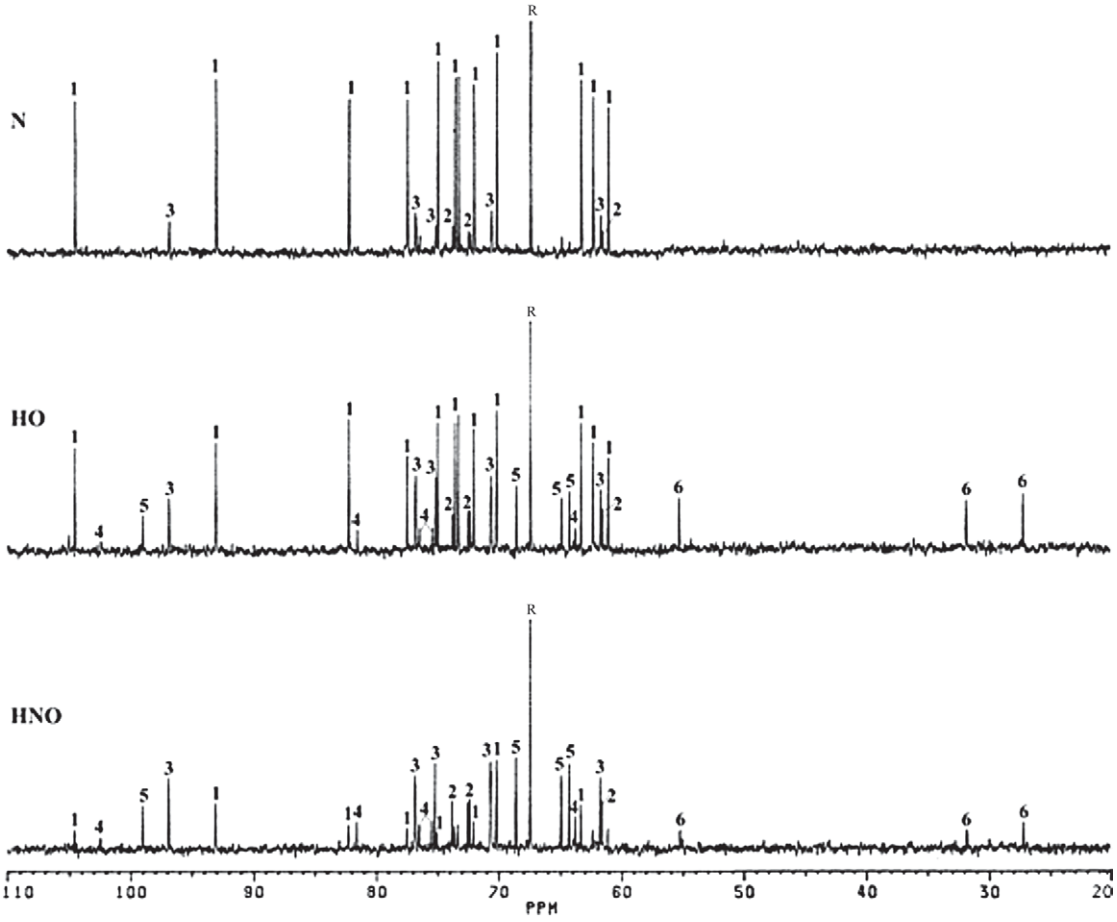

Fig. 2. a) Natural abundance proton-decoupled ^{13}C NMR (75.47 MHz) spectra of intact sugar beet cells (*in vivo*) obtained from normal (N), habituated organogenic (HO), and habituated nonorganogenic (HNO) cell lines. Each spectrum represents approx. 16 000 scans collected overnight. Peak assignments: 1 sucrose, 2 α-glucose, 3 β-glucose, 4 α-fructose, 5 β-fructose, 6 glutamine, R reference (dioxane)

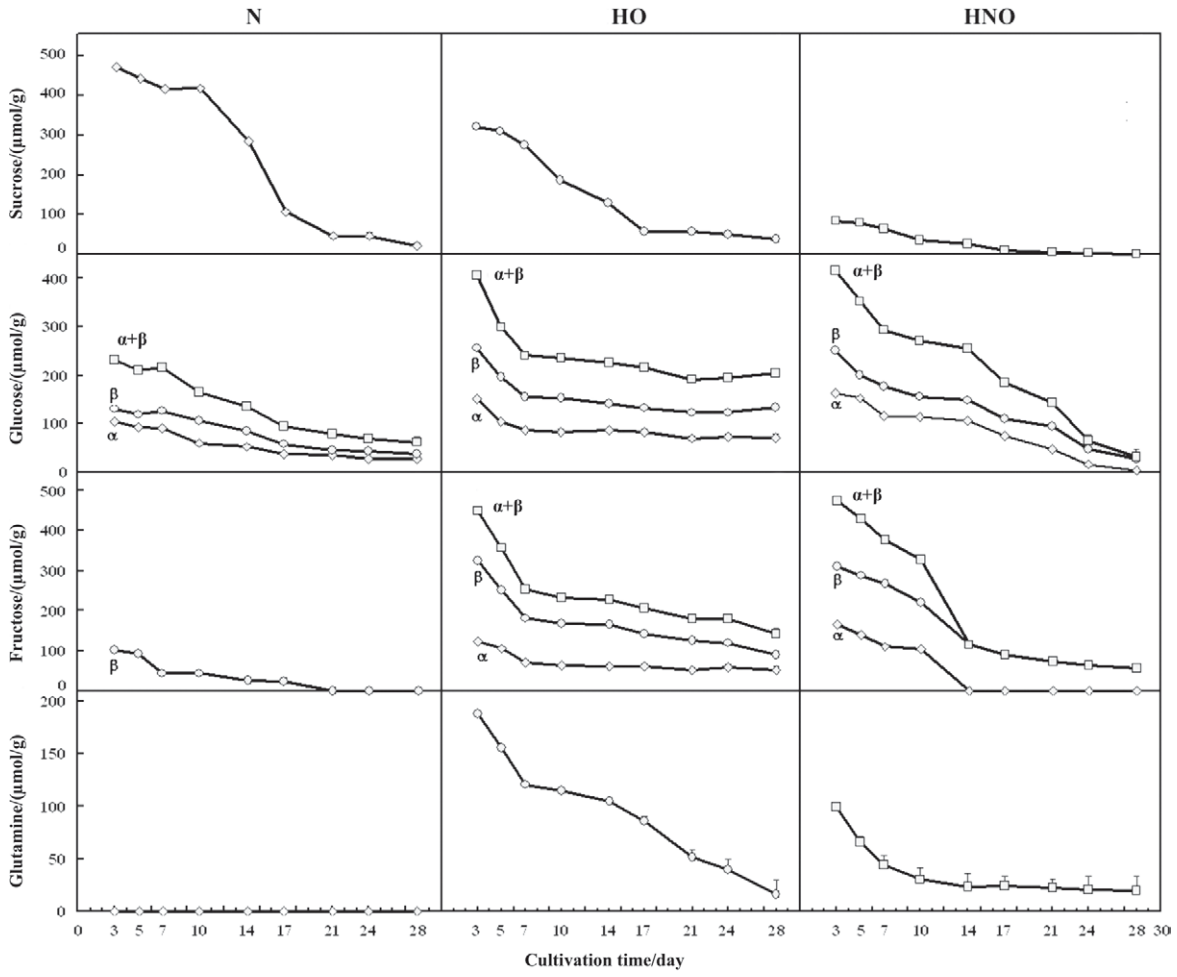

Fig. 2. b) Evolution in the levels of intracellular carbohydrates and glutamine (μmol per g of dry mass) during 28 days of subculture. At each time point, ^{13}C NMR (100 MHz) spectra of perchlorate extracts (*in vitro*) were recorded. Results for each time point are averages of 2–3 spectra recorded in independent experiments. Where no error bars are evident, the error was less than the area covered by the data symbol

Enzymes involved in carbohydrate metabolism

Differences in the types of imported carbohydrates and their intracellular levels depend on activities of enzymes such as invertases and SuSy (*11*,*36*). In sugar beet cell lines, these enzymes may help regulate levels of extra- and intracellular carbohydrates during subculture. We therefore measured the activities of three invertase isoforms and SuSy in the sucrose synthesis direction in the cell lines grown for 72 h on solid medium supplemented with Suc. Of the three invertase isoforms, the highest activity was recorded for CWI, followed by VI and lastly by CI (Fig. 3a). In HO and HNO cells, where higher concentrations of monosaccharides were recorded, CWI activity was higher than in the N line (Figs. 1b and 3a). SuSy activity was higher in N cells, which contain more intracellular sucrose, than in either HO or HNO cells, which contain less intracellular sucrose (Fig. 3b).

Differences in the regulation of enzymes involved in carbohydrate metabolism

Carbohydrates are powerful regulators of gene expression, in particular of genes that encode enzymes involved in their metabolism (*11*,*37*). To determine whether carbohydrates regulate invertase and SuSy activity in a similar manner in the three cell lines, cells were grown for 72 h on solid medium supplemented with Suc, Fru, or Glc. Enzyme activities were then compared across the different sugar treatments (Fig. 3a). Fru significantly increased total invertase activity in HO, but significantly decreased the activity in HNO cells. Glc inhibited the activity in N and HO but not in HNO cells. The influence of these carbohydrates on the levels of CWI and VI was assessed by Western blot (Figs. 3c and d). Anti-VI antibody from *Vicia faba* detected two main isoforms at 65 and 71 kDa (Fig. 3c), which are typical sizes for VI (*38*). The level of VI did not change in the presence of the sugars, except in HO cells treated with Fru, where additional isoforms appeared (Fig. 3e). Antibody against maize CWI did not yield clear results (data not shown), probably due to low homology between maize and sugar beet CWI.

SuSy activity did not differ significantly in the presence of the different sugars, although Glc elevated SuSy activity in the N line (Fig. 3b). Western blots showed two proteins of approx. 91 kDa (Fig. 3d). The SuSy2 isoform was more abundant than SuSy1, and only changes in the SuSy2 level corresponded roughly to the changes in SuSy activity (Fig. 3f).

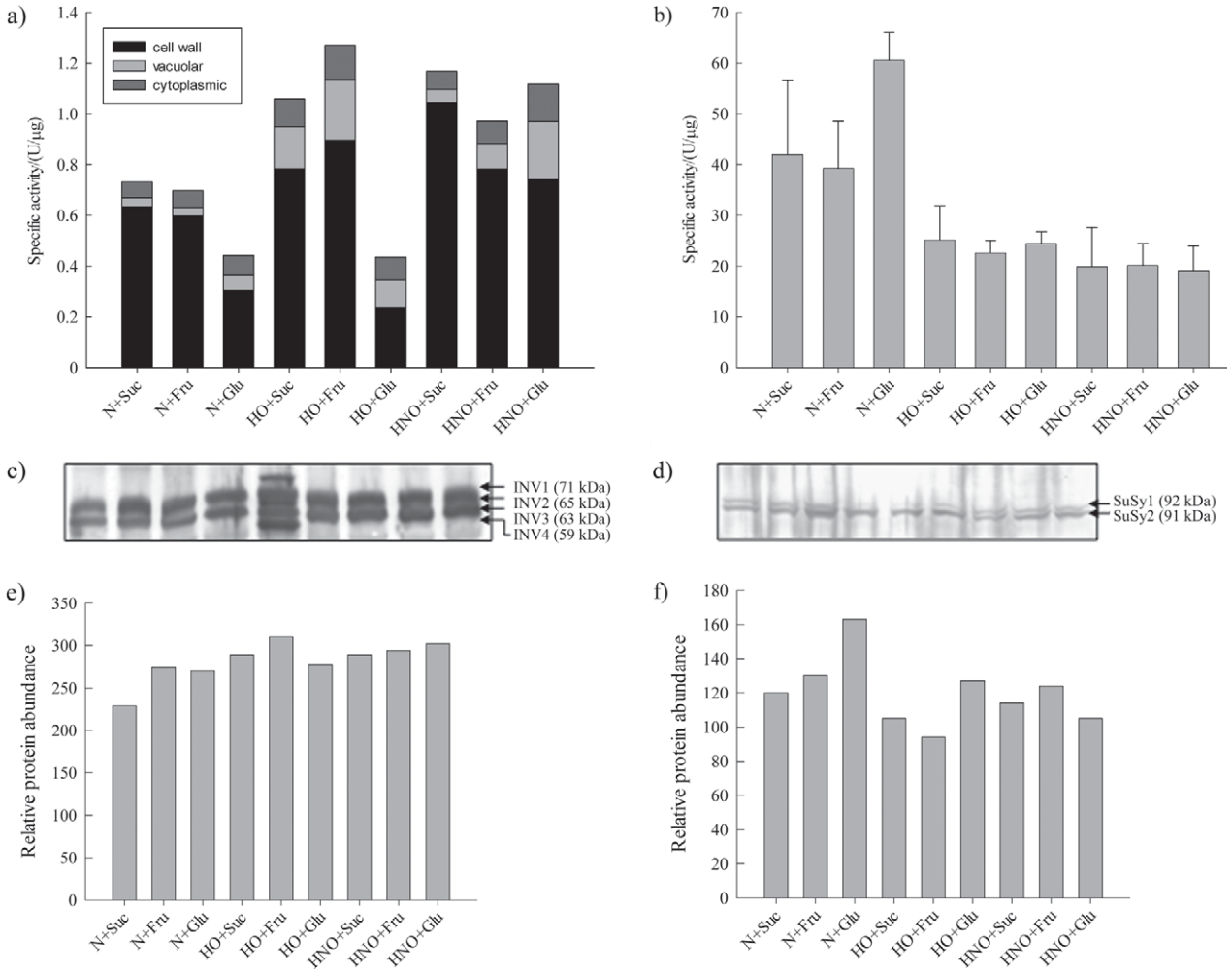

Fig. 3. Invertase and sucrose synthase activities and protein levels in sugar beet cell lines after 72 h of growth on solid medium supplemented with 88 mM sucrose (Suc), glucose (Glc) or fructose (Fru): a) invertase activity, b) sucrose synthase activity, c) vacuolar invertase Western blot, d) sucrose synthase Western blot, e) densitometric analysis of the abundance of two detected proteins of vacuolar invertase, f) densitometric analysis of the abundance of two detected proteins of sucrose synthase. Protein sizes are indicated in kDa. Values are the means of three replicates±SEM. Where no error bars are evident, the error was less than the area covered by the data symbol. Different letters indicate significant differences ($p \leq 0.05$) between sucrose on the one hand and glucose or fructose on the other

To further elucidate the influence of the enzymes on intracellular levels of carbohydrates, the activity of invertases and SuSy during the 28 days of subculture was correlated with the concentration of selected metabolites analyzed by ^{13}C-NMR, as well as with FM (Table 2 and Fig. 4). A significant negative linear correlation was found

Table 2. Pearson's coefficients (r) describing linear correlation between metabolites measured by ^{13}C-NMR and enzyme activities in sugar beet lines ($N \geq 36$)

Variable / Variable	Internal glucose content	Internal fructose content	Internal glutamine content	Cell wall invertase activity	Cytoplasmic invertase activity	Vacuolar invertase activity	Sucrose synthase activity	Fresh mass
Internal sucrose content	NS	NS	NS	–0.62***	NS	NS	NS	–0.47***
Internal glucose content		0.87***	0.71***	–0.62***	NS	NS	–0.31*	–0.64***
Internal fructose content			0.80***	–0.41*	NS	NS	NS	–0.59***
Internal glutamine content				NS	NS	NS	NS	–0.45***
Cell wall invertase activity					–0.35***	NS	0.35***	0.59***
Cytoplasmic invertase activity						0.61***	NS	NS
Vacuolar invertase activity							NS	NS
Sucrose synthase activity								NS

Significance levels are indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, NS=not significant

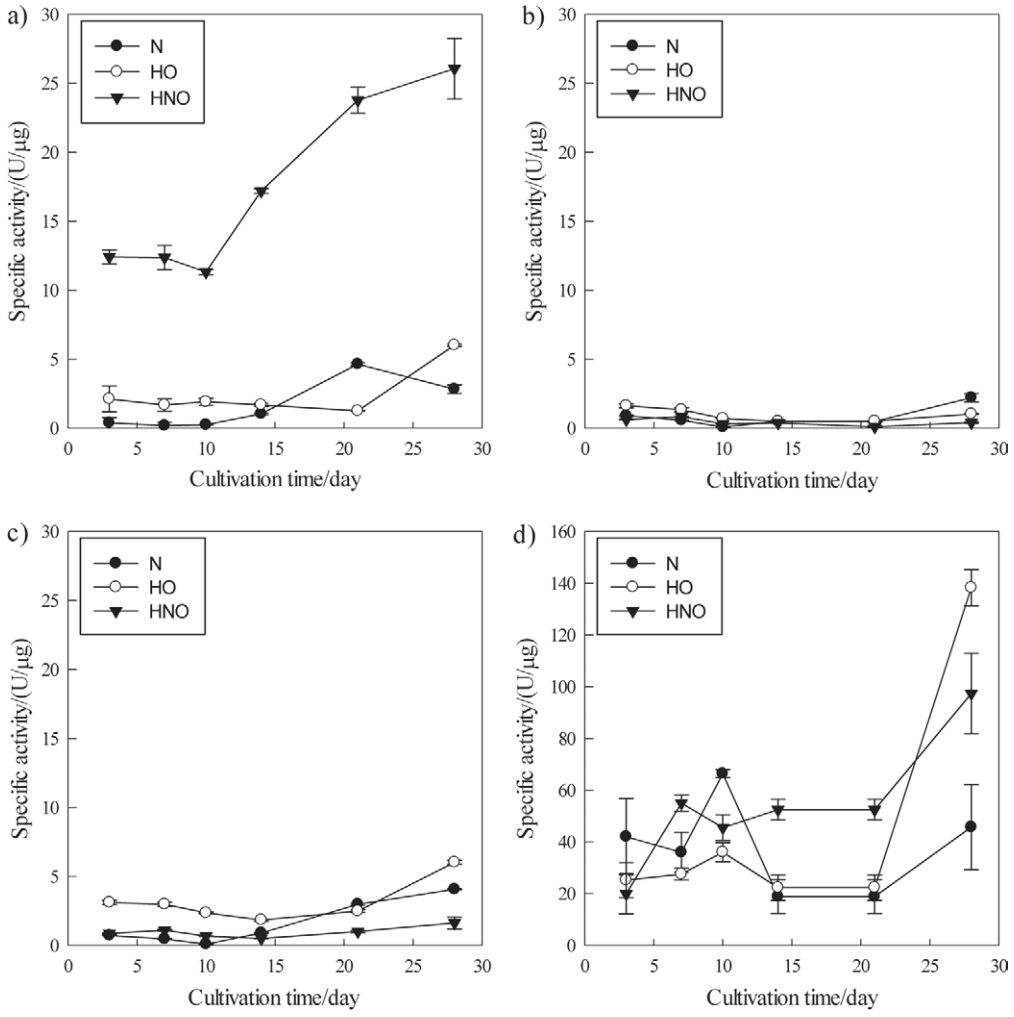

Fig. 4. Evolution of invertase and sucrose synthase activities in three sugar beet cell lines grown for 28 days on medium supplemented with 88 mM sucrose: a) cell wall invertase, b) vacuolar invertase, c) cytoplasmic invertase, d) sucrose synthase. Values are the means of three replicates±SEM. Where no error bars are evident, the error was less than the area covered by the data symbol

between CWI activity on the one hand and the concentrations of Suc, Glc or Fru on the other. In contrast, CWI activity was strongly positively correlated with FM. SuSy activity was slightly negatively correlated only with Glc concentration. A negative correlation was also observed between FM on the one hand and the levels of Suc, Glc or Fru on the other. Finally, significant linear relationships were also detected when enzyme activities were compared among themselves: CWI activity was negatively correlated with CI activity but positively with SuSy activity, while CI activity was positively related with VI activity. It seems that CWI, and to a lesser extent SuSy, may be involved in regulating intracellular carbohydrate content.

To test whether the components of sugar sensing pathways are responsible for cell line-specific regulation of CWI activity, the cell lines were cultured in the presence of non-metabolizable and metabolizable carbohydrates (Fig. 5a and Table 3). Cell suspensions are routinely used in such experiments as they offer a fast and uniform cellular response. Preliminary measurements of CWI activity showed the greatest differences in activity after 72 h of incubation (data not shown). To address the possible osmotic effect of the added compounds, control experiments were carried out with identical concentrations of mannitol. The metabolic substrate Glc significantly increased CWI activity only in N and HNO cells, while Suc was the only carbohydrate that provoked the same response in all cell lines, namely, an increase in CWI activity (Fig. 5a). Involvement of the HXK-independent sugar sensing pathway was tested using the glucose analogue 3-*O*-methylglucose (3-OMG), which is transported into cells but not phosphorylated by HXK (*5*). In both the N and HNO lines, 3-OMG caused a significant increase in CWI activity. Conversely, it decreased CWI activity in HO cells (Fig. 5a). Involvement of the HXK-dependent sugar-sensing pathway was tested using 2-DOG or mannose, which are phosphorylated by HXK but poorly metabolized further (*37*). Neither compound affected CWI activity in the N line. In HO cells, mannose reduced CWI activity, while 2-DOG reduced it slightly but not significantly. In the HNO line, both compounds reduced CWI activity. These results show that a given carbohydrate, or its analogue, can provoke distinct cellular responses in different cell lines, leading to an increase or decrease of CWI activity. N and HNO cells

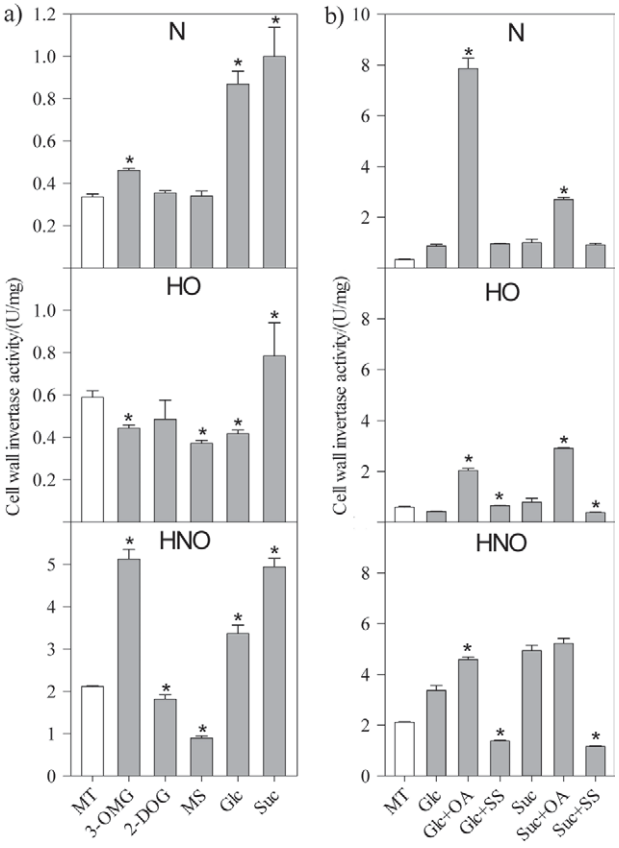

Fig. 5. Activity of cell wall invertase in normal (N), habituated organogenic (HO), and habituated nonorganogenic (HNO) cell lines after 72 h in liquid nutrient medium supplemented with: a) sugars or sugar analogues, and b) sugars and inhibitors of protein kinases or phosphatases. Values are the means of three replicates±SEM. Asterisks indicate significant differences ($p \leq 0.05$) between the treated and control cultures. Suc, sucrose; Glc, glucose; MT, mannitol (white bar, osmotic control); 3-OMG, 3-*O*-methylglucose; 2-DOG, 2-deoxyglucose; MS, mannose; Suc+OA, sucrose and okadaic acid; Glc+OA, glucose and okadaic acid; Suc+SS, sucrose and staurosporine; Glc+SS, glucose and staurosporine

Table 3. Names and concentrations of sugars added to the culture medium and the possible sensing mechanism in the sugar beet cells

Sugar	*c*/mM	Possible sensing mechanism
mannitol	88	osmotic control
3-*O*-methylglucose	88	prior to hexokinase (HXK) phosphorylation
2-deoxyglucose	1	triggered by HXK phosphorylation
mannose	88	triggered by HXK phosphorylation
glucose	88	glycolysis
sucrose	88	glycolysis

showed greater similarities in their responses to the different compounds, consistent with the fact that they are morphologically more similar to each other than to HO cells (*39*).

The possibility that protein phosphatases and kinases involved in signal transduction might be regulating the Glc- and Suc-induced increase in CWI activity was tested using the phosphatase inhibitor okadaic acid (OA) and the kinase inhibitor staurosporine (SS) (*37,40*). We compared CWI activity in cells fed Glc or Suc in the presence or absence of OA and SS. Culturing the cells for 72 h in the presence of Glc or Suc and 0.1 μM OA led to a significant increase in CWI activity in all cell lines, although this increase was not significant in HNO cells treated with OA and Suc (Fig. 5b). When cells were treated with 0.8 μM SS in the presence of Glc or Suc, the response of CWI activity was cell line-specific (Fig. 5b). N cells cultured in the presence of either Glc or Suc and SS showed similar CWI activity to the cells cultured in the absence of SS. In the HO line, CWI activity increased in the presence of Suc and SS, while it decreased in the presence of Glc and SS. In HNO cells, both carbohydrates inhibited CWI activity in the presence of SS.

Thus, inhibition of phosphatases by OA increased CWI activity in all three cell lines in the presence of Glc or Suc. In contrast, inhibition of kinases by SS affected CWI activity differently depending on the cell line and carbohydrate (Glc or Suc).

Discussion

In this study, we wanted to elucidate possible relationships among internal sugar content, invertase and SuSy activity, and cell differentiation state in organogenic and non-organogenic sugar beet cell lines. The N, HO and HNO cell lines showed differences in growth as well as in the uptake and metabolism of carbohydrates (Figs. 1 and 2). The differences in growth might be due to different sizes of the cells and due to metabolic differences.

Higher levels of sucrose were measured in the differentiated N and HO lines than in the dedifferentiated HNO line, which instead contained higher levels of hexoses. Previous work on *Vicia faba* found that high levels of intracellular sucrose are related to differentiation and starch storage, while high levels of intracellular hexoses are related to cell division and delayed differentiation (*4*). Our results suggest that this relationship also holds in sugar beet cell lines. Differences in the rates at which the cell lines consumed carbohydrates during subculture may reflect differences in their metabolism. N and HO cells are capable of photosynthesis, whereas HNO cells are not. During growth, HO and HNO consume more oxygen than N, but HNO cells contain higher ATP levels, and their enzymes of the pentose phosphate pathway are more active, suggesting that this cell line shows substantial metabolic differences from the other two (*20*). To provide readily available fuel for such metabolism in the form of glucose, CWI is significantly higher in HNO cells. High CWI activity has been linked to faster depletion of sucrose, increased cell division, delayed cellular differentiation and organogenesis in plants (*1,3,4,41*).

We detected glutamine only in the habituated cell lines. While it remains unclear why glutamine was not detected in the HNO line, this could indicate a disturbance of carbon or nitrogen metabolism. These two metabolic pathways are interconnected and carbohydrates can

modify the expression of genes involved in nitrogen transport and metabolism, as well as the enzymatic activities of the encoded proteins (*42,43*).

Enzymes of carbohydrate metabolism and cell line-specific differences in their regulation

Our results confirm the previous suggestion that the HNO line differs from the other lines in sugar metabolism and in the enzymes involved (*19*). We found cell line-specific differences in the regulation of invertase and SuSy activity (Fig. 3), but the changes in the activity did not seem to correlate with protein levels. One explanation for this is that SuSy is phosphorylated, which alters its activity and cellular localization without changing its abundance (*44*). Carbohydrates have been reported to both repress and activate SuSy and invertases (*36,45*). In those studies, the regulatory effect of the carbohydrates depended on the type of tissue, leading to the suggestion that distinct carbohydrate sensing mechanisms in different tissues are responsible for the differences in regulation (*45*). Thus, the cell line-specific differences that we observed in the regulation of enzymes involved in carbohydrate metabolism may be due to differences in sugar sensing and signalling (Fig. 5). We observed cell line-specific regulation of CWI activity by carbohydrates and their analogues that target specific components of sugar-sensing pathways. The localization of CWI is crucial for providing cells with sucrose or hexoses; in this way, it determines the type of sugar signals perceived by cells (*13*).

Proper plant growth and development is believed to depend on cellular responses to carbohydrates detected in the environment (*10,45*). During early development of *Vicia faba* embryos and sugar beet roots, CWI-generated glucose serves as a signal for cell division (*46*). In rapidly developing sugar beet roots, CWI is the primary supplier of hexoses for maintaining sink tissue (*45*). Cell line-specific differences in the regulation of CWI activity observed in sugar beet cell lines may help fulfill specific metabolic requirements. N and HO cells, which are autotrophic, differentiated and slow to divide, have no need for hexoses to sustain the type of metabolism and heterotrophic growth of HNO cells. These cells show higher metabolic activity, higher respiration, and greater stocks of ATP; this metabolism demands high levels of readily available hexoses. Hexoses may be provided by high CWI activity. In addition, CWI is regulated post-translationally by a small protein inhibitor (*47*). Whether this inhibitor plays a role in the regulation of CWI activity observed here remains to be seen.

The addition of carbohydrates to the cell lines in this study initiated a signalling cascade involving protein phosphorylation and dephosphorylation, ultimately leading to activation of CWI (Fig. 5a). When cells were exposed to carbohydrates in the presence of OA, CWI activity in all three cell lines was even greater than in the presence of carbohydrates alone (Fig. 5b). This suggests that OA-sensitive protein phosphatases participate in the regulation of invertase activity. OA inhibits type 2A protein phosphatases (PP2A) at a concentration of 1 nM, and type 1A phosphatases (PP1A) at 100 nM (*48*). In contrast, protein phosphatase 2C is not sensitive to OA (*49*). Thus, it seems that PP2A and PP1A enzymes inhibit carbohydrate-induced activation of CWI. OA-induced upregulation of CWI transcription was observed in *Chenopodium rubrum* cell suspensions, and OA increased transcription of SuSy and dark-inducible genes in *Arabidopsis* (*50–52*). In addition, OA was found to inhibit the expression of genes encoding UDP-glucose pyrophosphorylase in *Arabidopsis*, barley fructosyltransferase, vacuolar invertase in wheat and phloem-specific proton-sucrose symporter in sugar beet (*48,51,53,54*).

While protein phosphatases can act directly on carbohydrate-sensing pathways, they also regulate many other signalling pathways. Therefore,we cannot rule out the possibility that another signalling pathway was affected in our experiments, and that this contributed to our results. However, we observed the same reaction in all three cell lines when phosphatases were inhibited, suggesting that the phosphorylation events regulating CWI activity are conserved in the lines.

It is also possible that protein kinases are involved in the regulation of CWI activity. However, when we inhibited kinases using SS, we did not observe uniform regulation of CWI across the cell lines, suggesting differences in the signal transduction pathway(s) involving protein phosphorylation. While at higher concentrations (>2 μM) SS inhibits AGC protein kinases, at the concentration of 0.8 μM used here it selectively inhibits protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase (*55,56*). The existence of PKC in plants has not been unambiguously demonstrated, and it is unlikely that mitogen-activated protein kinases are involved in the transduction of sugar signals (*49,57*).

Similarly, abscisic acid induced VI and CWI activity *via* signalling mechanism that involved reversible protein phosphorylation, and inhibition of PKs with staurosporine additionaly induced VI and CVI activity but not a number of gene transcripts (*58*). In contrast, exposing *Chenopodium rubrum* cell suspensions to Glc in the presence of SS increased the levels of CWI mRNA (*9*).

Causevic *et al.* (*21*) observed that sugar beet cell lines differ in their levels of DNA methylation, including differences in methylation of enzyme activators such as phenylalanine-ammonia lyase, which in turn leads to differences in enzyme activity. Further research should examine whether differences in the methylation of the promoters of genes encoding components of sugar-sensing pathways was responsible for the differences in CWI regulation observed here.

Conclusion

In conclusion, we have shown cell line-specific differences in the uptake and intracellular levels of soluble carbohydrates, as well as in their metabolism in sugar beet. Cell line-specific differences in the activity of CWI may play an important role in generating these different carbohydrate profiles. In addition, the ability of metabolizable and non-metabolizable carbohydrates to regulate CWI activity was cell line-specific. These differences may be due to differences in either sugar sensing or signalling *via* pathways involving several protein kinases. Thus we propose that sugar beet cell lines achieve a specific carbohydrate profile by regulating cell wall invertase ac-

tivity, and this profile helps to determine the specific morphology and differentiation state of each cell line. Our finding that sucrose-cleaving enzymes can help to regulate morphogenesis and differentiation is yet another example of the metabolic flexibility of higher plants.

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