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Sugar-induced changes in cellular and extracellular protein and glycoprotein patterns of sugarbeet cell lines

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Plants sense carbohydrates and transduce a signal which changes gene expression and the activities of many enzymes. The relationship between changes in gene expression by carbohydrates and the developmental state of the cells is still poorly understood. To gain more knowledge about this relationship, we have analyzed three sugar beet (*Beta vulgaris* L.) in vitro cell lines residing on distinct differentiation states. Cell suspensions were initiated and cells were incubated for 72h in the medium with sucrose as a control, or treated during the same period with glucose or 3-O-methylglucose (3OMG). Cellular and extracellular proteins, separated electrophoretically, showed that sugar-induced protein expression was cell line-specific. More differences were visible in extracellular and in glycoprotein than on cellular protein patterns. The 3OMG downregulated while glucose upregulated cellular glycoproteins. In the case of extracellular proteins, glucose and 3OMG were equally effective in both downregulation and upregulation of protein expression. Sialic acid was indicated as a glycan conjugate in sugar beet. Carbohydrate-induced gene expression was related to the developmental state of cells.

Key words: cell differentiation, glycoproteins, SDS-PAGE, sugar beet, sugar sensing

Abbreviations: **3OMG** – 3-O-methylglucose, **Suc** – sucrose, **Glc** – glucose, **SDS-PAGE** – sodium dodecyl sulphate polyacrylamide gel electrophoresis, **GNA** – *Galanthus nivalis* agglutinin, **MAA** – *Maackia amurensis* agglutinin, **Con A** – Concanavalin A, **N –** normal cell line, **HO** – habituated organogenic cell line, **HNO** – habituated nonorganogenic cell line, **Tris** – tris(hydroxymethyl)aminomethane

Introduction

Plant development is characterized by extreme plasticity and an open way of growth. It is influenced by a complex interaction of internal and external stimuli. Carbohydrates, sucrose and glucose, as signal molecules, change gene expression and affect developmental programs (ROLLAND et al. 2002, ROLLAND et al. 2006). To sense these molecules, plants em-

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ploy several mechanisms: (a) hexokinase-independent – probably by a sugar transporter homologue, (b) hexokinase-dependent – in which phosphorylation of glucose initiates a signaling cascade, and (c) glycolysis-dependent – by an unknown sensor downstream of hexokinase. The generated signal is transduced by protein kinases and phosphatases, and numerous genes with sugar response nucleotide sequence in their promoter regions were identified (SMEEKENS 2000, ROLLAND et al. 2006). For example, glucose changed the expression of more than 1000 genes in Arabidopsis (PRICE et al. 2004). Moreover, carbohydrate content is related to the developmental stage of the cell. High intracellular sucrose concentration is related to cell growth and maturation while high glucose concentration is related to cell division and dedifferentiation (WEBER et al. 1996, BORISJUK et al. 1998, BORISJUK et al. 2002). Still, little is known of whether the induction of genes by carbohydrates is related to the developmental state of the cell. In our study three in vitro sugar beet cell lines were analyzed (DE GREEF and JACOBS 1979). The lines originate from the same mother plant but differ in their developmental state: N cell line is differentiated but not organogenic, HO is differentiated and organogenic and HNO is fully dedifferentiated (DE GREEF and JACOBS 1979, CAUSEVIC et al. 2005). The N preferentially accumulates sucrose, the HO sucrose and hexoses, while the HNO preferentially accumulates glucose and fructose (OMARZAD 1998). We wanted to test the influence of exogenously supplied carbohydrates on protein expression patterns and to establish whether this response depends on the developmental state of cells. After treatment with different carbohydrates, cellular and extracellular proteins were separated electrophoretically. The analysis was extended to glycoproteins and different types of glycans were revealed by lectin hybridization.

Materials and methods

Plant material, growth conditions and treatments

Three callus lines of sugarbeet (*Beta vulgaris* L. *altissima*) originated from the same mother plant were cultivated in vitro (DE GREEF and JACOBS 1979). Cell lines were grown in a growth chamber at 22 °C under a 16 h photoperiod (700 μmol m^{-2} s^{-1}). Cell suspensions were established from the 14 day old callus cultures by transferring five grams of tissue to flasks with PG0 liquid medium (NEGRUTIU et al. 1975) on an orbital shaker. The nutrient medium was supplemented with 88 mM of sucrose, glucose or 3-O-methylglucose (3OMG). Sucrose was applied as a control. The 3OMG was used as a sugar analogue to test hexokinase-independent sugar sensing (SMEEKENS 2000). After 72h the medium was collected, cells were harvested, and used for protein extraction.

Protein electrophoresis and electroblot

Total soluble proteins were extracted by grinding 0.5 g of fresh tissues in 1.0 mL of 0.1 M Tris-HCl buffer, pH 8.0 at 4 °C. Homogenates were centrifuged at 20 000 g and 4 °C for 15 min. Supernatants were centrifuged again at 20 000 g for 60 min. Protein content was determined according to Bradford using BSA as a standard (BRADFORD 1976). Prior to electrophoresis, samples were denatured for 5 min at 95 °C in a sample buffer (LAEMMLI 1970). Extracellular proteins were obtained from a liquid nutrient medium. The medium was passed through a mash filter to remove cell debris. Extracellular proteins were concen-

trated by an overnight acetone precipitation. The pellet was washed with acetone and resuspended in 100 – 150 μL of the sample buffer. For the SDS-PAGE, 5 μg of proteins per sample was loaded. Cellular and extracellular proteins were separated by vertical SDS-PAGE in polyacrylamide gel (12% T, 2.67% C), with the buffer system of LAEMMLI (1970). Proteins migrated through stacking and separating gels at 100 V and 220 V, respectively. Protein bands were visualized by silver staining (BLUM et al. 1987).

Separated proteins were electroblotted from gel to a nitrocellulose membrane in the vertical tank (Bio-Rad). The blotting was carried out for 1 h at 60 V. Glycoproteins were detected by lectin hybridization and subsequent visualization. Concanavalin A (Con A) was used to detect D-glucose and D-mannose in a glycan component of glycoproteins, GNA to detect terminal mannose, α(1–3), α(1–6) or α(1–2) linked to mannose and MAA to detect sialic acid linked α(2–3) to galactose. Glycoproteins that hybridized with Con A were visualized by peroxidase reaction using 4-chloro-1-naphthol as a substrate (HAWKES 1982). GNA and MAA lectins linked to glycans, were visualized using digoxygenin based kit and BCIP / NBT as a substrate to visualize secondary antibody hybridization (Roche Diagnostics GmbH, Germany).

Scanned gels and membranes were analyzed by Photo-Capt 3.1 application (Vilber Lourmat, Marne, France).

Tab. 1. Differences in the influence of carbohydrates on cellular protein and glycoprotein patterns of sugar beet cell lines. Proteins were extracted from fresh sugar beet tissue after 3 days subculturing in sucrose, glucose or 3-O-methylglucose, and separated in 12% SDS polyacrylamide gels. Sucrose served as a control. Lectins were used to label specific side glycans of the glycoproteins. **G** – glucose, **O** – 3-O-methylglucose; »**+**« – (glyco)protein upregulation, »**–**« – (glyco)protein downregulation.

	CELLULAR (GLYCO)PROTEINS											
	Silver stained			ConA			MAA			GNA		
kDa	N	HO	HNO	N	HO	HNO	N	HO	HNO	N	HO	HNO
73				O–								
65												O+
64				O–								
46			O–									O–
45												O–
33								O–				G+O–
32								O–				G+O–
30												G+O–
28								G+				G+O–
27			O–					G+	G–			G+O–
23			O–						G–			G+O–
22					O+							
21										O+		
18								G+O–				
16												
15												

Results

Cellular proteins of three day old sugar beet cell lines grown in cell suspensions with various sugars were separated electrophoretically and silver stained (Fig. 1A). Application of carbohydrates visibly changed the expression of few proteins: 3OMG downregulated proteins of 46, 22 and 21 kDa in the HNO line (Fig. 1A – arrows; Tab. 1). However, more differences were noticed when glycoproteins were detected by means of lectins (Fig. 1B; Tab. 1).

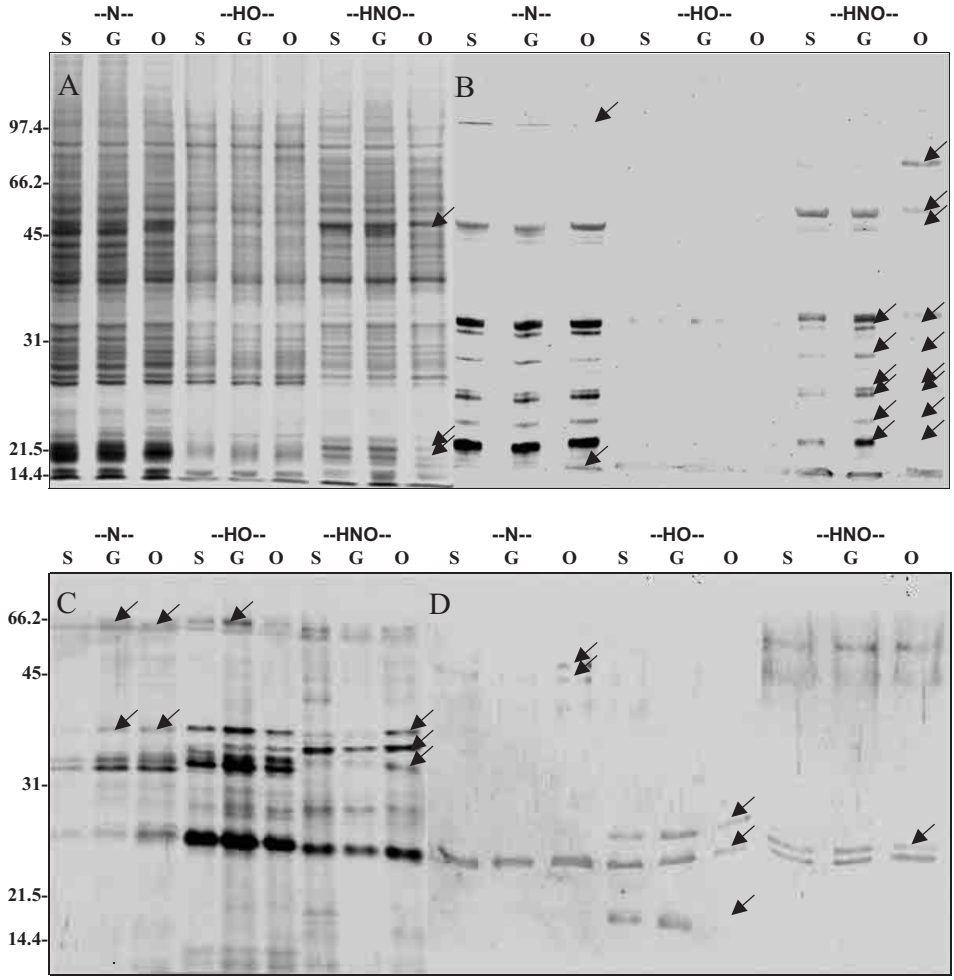

Fig. 1. The effect of carbohydrates on expression profiles of sugar beet proteins. Proteins were obtained after 3 days of subculturing and separated in 12% SDS polyacrylamide gels. (A) Silver stained cellular proteins, (B) Nitrocellulose membrane with mannose-containing glycoproteins detected with the GNA lectine, (C) Silver-stained extracellular proteins, (D) Nitrocellulose membrane with mannose-containing glycoproteins detected with the GNA lectine. N – normal, HO – habituated organogenic, HNO habituated nonorganogenic cell line. G – glucose, S – sucrose, O – 3-O-methylglucose. Arrows point to proteins whose expression was changed by carbohydrates.

Each cell line responded differently when treated with the same type of carbohydrate (Tab. 1). In general, glucose upregulated most of the glycoproteins, except the ones of 22 and 21 kDa in the HNO line. Application of 3OMG downregulated cellular glycoproteins in three cell lines, except those of 18 kDa in the HO and 65 kDa in the HNO lines. Particularly strong downregulation of glycoproteins was observed by 3OMG in the HNO line.

In contrast to cellular proteins, extracellular protein pattern varied more between cell lines, and more differences in the pattern were observed after the sugar treatment (Fig. 1C, D – arrows; Tab. 2). Each sugar beet cell line excreted a specific set of proteins after the same carbohydrate had been applied. Glucose upregulated proteins of 66 kDa in N and HO. The 3OMG upregulated proteins of 37 kDa, both in the N and HNO lines and a 66 kDa glycoprotein was downregulated in both HO and HNO lines. The glucose and 3OMG were equally effective in both, upregulating and downregulating extracellular protein expression.

Tab. 2. Differences in the influence of carbohydrates on extracellular protein and glycoprotein patterns of sugar beet cell lines. Proteins were collected from cell suspension medium after 3 days subculture in sucrose, glucose or 3-O-methylglucose, and separated in 12% SDS polyacrylamide gels. Sucrose served as a control. Lectins were used to label specific side glycans of the glycoproteins. **G** – glucose, **O** – 3-O-methylglucose; »**+**« – (glyco)protein upregulation, »**–**« – (glyco)protein downregulation.

	EXTRACELLULAR (GLYCO)PROTEINS											
	Silver stained			ConA			MAA			GNA		
kDa	N	HO	HNO	N	HO	HNO	N	HO	HNO	N	HO	HNO
68				O+	O–							
66	G+O+	G+			O–	O–						
63												
54				O+								
45							O+			O+		
43						G+O–				O+		
42							O–	G+O+				
37	G+O+		O+		G+							
35			O+		O–	G+O+						
32			O+									
29	O+										O–	
25								G+O+				O–
23											O–	
22												
16								O–			O–	

Discussion

Protein expression profiles depended on the developmental state of the sugar beet cells. Upon addition of glucose or 3-O-methylglucose, each cell line responded by changing protein expression. Differences in response were also visible in glycoprotein patterns. Modification of gene expression by carbohydrates in cell suspension was already reported

(GAVISH et al. 1991, ROITSCH et al. 1995, EHNESS et al. 1997). Several isoforms of enzymes involved in sugar metabolism and sensing, such as hexokinase, invertase or sucrose synthase, have been identified. These enzymes differ in their subcellular and tissue distribution as well as in kinetic properties (DA-SILVA et al. 2001, ROITSCH et al. 2003). Different types of cells need to ensure proper production, transport and utilization of carbohydrates that suit their needs (ROITSCH and GONZALES 2004). This could lead to differential expression of proteins in various cell types, as demonstrated in the presented experiments, after the carbohydrate has been sensed and its signal transduced.

Proteins excreted in the nutrient medium were related to cell differentiation and were usually glycosylated (QUIROZ-FIGUEROA et al. 2006). Developmental stage influences glycoprotein pattern and glycan composition (ELBERS et al. 2001, BALEN et al. 2005). This offered the opportunity to monitor changes in glycoprotein expression with carbohydrate treatments. Several lectins were used to characterize types of glycoproteins affected by application of carbohydrates. Sialic acid was also found as a component of cellular and extracellular glycans using MAA lectins. Sialic acid is a common glycan found on animal glycoproteins but controversial in plants (TRAVING and SCHAUER 1998, SEVENO et al. 2004, REINHARD et al. 2006). However, recent papers reported that sialic acid could be a plant glycan conjugate (SHAH et al. 2003, BALEN et al. 2005). The indication of the existence of sialic acid in sugar beet tissue provides more evidence that plants can produce sialyated glycoproteins. The existence of sialyation could expand the use of in vitro plant systems for the production of more types of recombinant proteins (WALSH and JEFFERIS 2006, BALEN and KRSNIK-RASOL, 2007).

The results have shown that glucose and 3OMG-induced changes in gene expression depended on developmental state of sugar beet cell line. Differentially regulated proteins are interesting candidates for future experiments involving their identification by means of mass spectrometry. Our results underline the plant's remarkable plasticity in metabolic response where crucial molecules such as carbohydrates are involved.

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