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Developmentally specific soluble and membrane proteins and glycoproteins in *Mammillaria gracillis* Pfeiff. (Cactaceae) tissue culture

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Artificial environmental conditions in the tissue culture influence growth and induce aberration from the characteristic organization pattern. As CAM plants, cacti are particularly susceptible to altered growth environment. *In vitro* propagated *Mammillaria* plants spontaneously produce callus which regenerates normal and hyperhydric shoots without the addition of any growth regulator. In order to compare habituated callus with tumour, cactus cells were transformed with *A. tumefaciens*. Cactus tumour has never expressed any organogenic potential. The aim of this work was to detect changes in protein and glycoprotein profiles in different developmental stages of *Mammillaria* tissues. Proteins were separated by SDS-PAGE and silver stained. Glycoproteins were detected according to their affinity to Con A, while the glycan components were further characterized with lectins GNA, DSA, MAA, PNA and SNA. Only few morphogenesis-specific polypeptides were observed on the gels. Differences were more pronounced in the glycoprotein pattern. The greatest number of glycosylated proteins in all cactus tissues was detected by Con A, according to which all untransformed tissues were characterized by the presence of 40 kDa glycoprotein, while the tumor exhibited some specific protein bands. No signal was observed with DSA, while GNA-, PNA-, MAA- and SNA-profiles partially correlated with those detected with Con A. The results demonstrated changes in protein glycosylation related to disarrangement or loss of characteristic tissue organization pattern in *Mammillaria gracillis* tissue culture.

Keywords: Cactaceae, development, glycoproteins, lectins, *Mammillaria gracillis*, plant tissue culture

Abbreviations: **CAM** – Crasulacean acid metabolism, **Tris** – tris(hydroxymethyl)aminomethane, **DTT** – dithiothreitol, **SDS-PAGE** – sodium dodecyl sulphate polyacrylamide gel electrophoresis, **TBS** – Tris buffered saline, **Con A** – Concanavalin A, **GNA** – *Galanthus nivalis* agglutinin, **DSA** – *Datura stramonium* agglutinin, **PNA** – *Arachis hypogaea* agglutinin, **MAA** – *Maackia amurensis* agglutinin, **SNA** – *Sambucus nigra* agglutinin, **Man** – mannose, **2D electrophoresis** – two dimensional electrophoresis

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Introduction

Artificial environmental conditions in the tissue culture, such as elevated relative humidity and rich nutrient medium, can influence and modify tissue growth and induce spontaneous changes from the characteristic organization pattern to unorganized callus (ELIAS--ROCHA et al. 1998, KRSNIK-RASOL and BALEN 2001). As succulent plants with Crasulacean Acid Metabolism (CAM), cacti are particularly susceptible to this altered growth environment. *Mammillaria gracillis* plants propagated *in vitro*, develop callus in the absence of exogenous growth regulators (KRSNIK-RASOL and BALEN 2001). After being removed to the fresh medium, this callus grows as a habituated tissue and spontaneously reorganizes into morphologically normal or malformed hyperhydric shoots. Since the phenomenon of habituation bears a striking similarity to tumour transformation in crown gall disease where tumour tissue grows independently of exogenous hormones (GASPAR 1995), cactus cells were transformed with *Agrobacterium tumefaciens* (KRSNIK-RASOL and BALEN 2001).

Only few studies have been conducted on plants to reveal whether environmental conditions and developmental stage act upon protein glycosylation. It was found that *N*-glycosylation of extracellular proteins varies with organization level of plant tissues cultured *in vitro* (KRSNIK-RASOL et al. 2000, BALEN et al. 2002, PAVOKOVIĆ et al. 2007). The glycosylation profile of endogenous proteins can be altered by plant development and growth conditions (STEVENS et al. 2000). However, modest information about the glycoprotein patterns related to cell differentiation, dedifferentiation and transformation in tissue culture is available so far. The aim of this work was to reveal whether the adaptation of plant cells to artificial environment in tissue culture is reflected in glycosylation of water soluble and membrane proteins in different *Mammillaria gracillis* tissues.

Material and methods

Plant material and growth conditions

Mammillaria gracillis plants were propagated in vitro, under 16/8-hour-light/night--photoperiod (light intensity 90 $\mu E\ s^{-1}\ m^{-2}$) at 24 °C on solid, MS nutrient medium (0.9% agar, 3% sucrose) (MURASHIGE and SKOOG 1962) without any growth regulators. Spontaneously formed callus was detached from the plants and subcultivated on the same nutrient medium as a hormone-independent habituated tissue (KRSNIK-RASOL and BALEN 2001). In the callus culture, regeneration of morphologically normal as well as of malformed hyperhydric shoots appeared. Regenerated shoots were green, covered with spines and had a normal growth. Hyperhydrated shoots were translucent, light green, rounder in shape and partially covered with softer spines (POLJUHA et al. 2003). Tumour tissue culture was established from primary tumours induced on shoot explants by Agrobacterium tumefaciens, the wild strain B6S3 (KRSNIK-RASOL and BALEN 2001). Transformed tissue never expressed any morphogenetic capacity.

Protein extracts, SDS-PAGE and electroblotting

Total soluble and membrane proteins were extracted by grinding 0.5 g of fresh tissue in liquid nitrogen. Pre-heated (80 °C) extraction buffer (0.1M Tris/HCl, 25% glycerol, 20% SDS and 0.01M DTT, pH 8.0), was added to the ground tissue and subsequently incubated

10 min at 80 °C. The homogenates were centrifuged at 20,000 g and 4 °C for 10 min and supernatant was collected. Total soluble and membrane proteins were analysed by SDS--PAGE in 12% T (2.67% C) polyacrylamide gels (LAEMMLI 1970). Proteins migrated through stacking and separating gels at 100 V and 200 V, respectively. Protein bands were visualized by silver staining (BLUM et al. 1987) and gels were scanned as an 8 bit grey scale Tiff-images with an HP Scanjet 2400 scanner (Hewlett-Packard Company, USA).

The proteins, separated by SDS-PAGE, were electroblotted to the nitrocellulose membrane (Pure nitrocellulose membrane – 0.45 μm, Bio-Rad) in a mini trans blot cell (Bio-Rad) at 60V for 60 min. The transfer buffer was 20 mM Tris-HCl, 150 mM glycine and 10% (v/v) methanol. The membrane was stained with Ponceau-S stain to confirm the complete transfer of the proteins. The stain was washed off with distilled water. The unoccupied sites of the membrane were blocked by incubating the membrane with 0.1% Tween® 20 in TBS buffer, pH 7.5 at 4 °C overnight.

Glycoproteins with D-manose in their glycan component were detected on nitrocellulose membrane by reaction with Con A. Bands were visualized by peroxidase reaction using 4-chloro-1-naphthol as a substrate (HRUBÁ and TUPÝ 1999). The glycan part of proteins was further characterized according to binding of digoxigenin-labeled lectins (DIG Glycan Differentiation Kit, Roche Diagnostics) GNA, DSA, PNA, MAA and SNA (Tab. 1). The staining procedure was performed following the manufacturer's instructions.

Tab. 1. Major N- and O-linked carbohydrate binding specificities of the different plant lectins applied.

Lectins	Major specificity	References
Con A	α-D-mannose, α-D-glucose	HRUBÁ and TUPÝ 1999.
(Canavalia ensiformis)	(N-glycans)	
GNA	mannose α-linked mannose	HRUBÁ and TUPÝ 1999.
(Galanthus nivalis)	(N-glycans)	
DSA	galactose-β(1,4)-N-acetylglucosamine	HIRABAYASHI 2004.
(Datura stramonium)	(N-glycans)	
PNA	galactose-β(1,3)-N-acetylgalactosamine	MERANT et al. 2005.
(*Arachis hypogaea*)	(O-glycans)	
MAA	sialic acid-α(2,3)-galactose	SHAH et al. 2003.
(Maackia amurensis)	(N- or O-glycans)	
SNA	sialic acid-α(2,6)-galactose	SHAH et al. 2003.
(Sambucus nigra)	(N- or O-glycans)	

Results

Only few morphogenesis-specific polypeptides were observed in different *Mammillaria* tissues (Fig. 1A): protein bands of 30 and 35 kDa (black arrows) were shoot-specific while those of 39, 47, 50 and 55 kDa (white arrows) were characteristic of tumour tissue.

Glycosylated proteins were detected by Con A (Fig. 1B) according to which all untransformed cactus tissues were characterized by the presence of 40 kDa glycoprotein (arrows).

Cactus tumor exhibited specific bands of 33, 39, 45, 47 and 66 kDa (circles). Callus, hyperhydric regenerant and tumour had common bands of 55 and 56 kDa (stars), the expression of which was stronger in tumour tissue. The GNA-binding pattern revealed the glycoproteins of 31, 33, 40, 44 and 50 kDa in all cactus tissues (arrows) with stronger staining intensity in tumour tissue (Fig. 1C). The 47 kDa band (circle) was tumour-specific, while that of 26 and 27 kDa (stars) was missing in cactus shoot. PNA revealed the 50 kDa glycoprotein (arrows) in all tissues, while that of 44 and 51 kDa (stars) was present only in

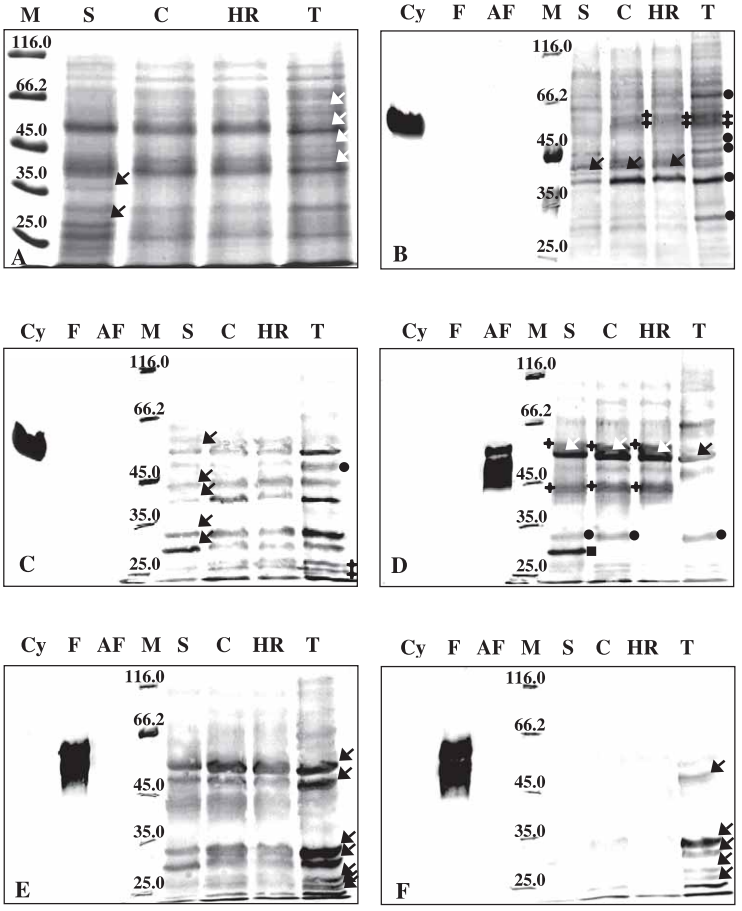

Fig. 1. A) Soluble and membrane proteins of *Mammillaria gracillis* tissues separated by SDS--PAGE in 12% gel and silver stained. M – protein molecular weight marker, S – shoot, C – callus, HR – hyperhydric regenerant and T – tumour. B) Con A-glycoprotein pattern. C) GNA – glycoprotein pattern. D) PNA-glycoprotein pattern. E) MAA-glycoprotein pattern. F) SNA-glycoprotein pattern. Cy – carboxypeptidase Y (positive control for Con A and GNA and negative control for DSA, PNA, MAA and SNA), F – fetuin (positive control for DSA, MAA and SNA and negative control for Con A, GNA and PNA), AF – asialofetuin (positive control for DSA and PNA and negative control for Con A, GNA, MAA and SNA), M – protein molecular weight marker, S – shoot, C – callus, HR – hyperhydric regenerant and T – tumour.

untransformed tissues (Fig. 1D). The 33 kDa glycoprotein (circles) was missing in hyperhydric regenerant, while the 31 kDa one (rectangle) was shoot-specific. MAA gave the same glycoprotein pattern in all cactus tissues revealing the 25, 26, 27, 31, 33, 50 and 51 kDa bands (arrows) with stronger staining intensity in tumour tissue (Fig. 1E). SNA gave positive results only with tumour glycoproteins of 25, 26, 27, 31, 33 and 50 kDa (arrows) (Fig. 1F). No signal was observed after the treatment with DSA (data not shown).

Discussion

Many proteins destined for secretion or expression at the surface of plant cells are glycoproteins. N- as well as O-glycosylation are essential protein modifications required for many different aspects of their structure and function, including their targeting to the appropriate destinations, their stability, solubility and antigenicity, as well as their capacity to be recognized by receptors. Despite obvious morphological differences between *Mammillaria* tissues grown *in vitro*, only few morphogenesis-specific polypeptides were observed on the gels. Differences were more pronounced in the glycoprotein pattern. Glycoproteins were detected with Con A, which binds specifically to α-D-mannosyl residues, and with low affinity to α-D-glucosyl residues in N-glycosylated proteins (HRUBÁ and TUPÝ 1999). Some of the glycoproteins detected by Con A were also present on membranes treated with GNA, which recognizes terminal α-D-mannose groups, especially those with Man-α(1–3)--Man units present in high-mannose type N-glycans. This result suggests that these cactus glycoproteins have high-mannose type or hybrid type N-glycans (BALEN et al. 2007). The number of Con A- and GNA-reacting glycoprotein bands increased in tumour in comparison to untransformed cactus tissues. This is in line with an observation reported for different morphological stages of sugar beet tissue lines (KRSNIK-RASOL et al. 2000). Interestingly, the tumour-specific 39 kDa protein detected on silver-stained gels was also characteristic of tumour tissue on membranes treated with Con A. Moreover, the 47 kDa band, assigned as tumour-specific protein on gels, was exclusively present in tumour tissue on Con A- as well as on GNA-blots. The results obtained indicate that these proteins are tumour-specific N-glycoproteins with attached oligosaccharide(s) of the high-mannose type.

PNA, which specifically recognizes the Gal-β(1,3)-GalNAc sequence present in O-glycans, revealed several glycoproteins that also reacted with GNA. This result suggests that these proteins might posses both N- and O-glycosylation sites, although PNA and GNA may alternatively recognize different glycoproteins of similar molecular size, which are not resolved in SDS-PAGE (BALEN et al. 2007). MAA gave the same glycoprotein pattern in all cactus tissues while SNA gave positive results only with tumour glycoproteins indicating that tumour proteins have sialic acid α(2,3)- as well as α(2,6)- linked to galactose, respectively. Sialylated glycoconjugates have already been found in suspension-cultured cells of *Arabidopsis thaliana* (SHAH et al. 2003), in cellular and extracellular proteins of sugar beet tissue lines (PAVOKOVIĆ et al. 2007) as well as in soluble cellular glycoproteins of *Mammillaria gracillis* grown *in vitro* (BALEN et al. 2005) which suggests that a genetic and enzymatic basis for sialylation exits in plants.

Results obtained in this preliminary study by applied lectin-binding assay demonstrate changes in glycosylation of soluble and membrane proteins related to disarrangement or loss of characteristic tissue organization pattern in *Mammillaria gracillis* tissue culture. In

the search of more reliable markers of *in vitro* morphogenesis, 2D electrophoresis followed by mass spectrometry analysis of glycoproteins should be applied. Further efforts will be invested in this direction in the future.

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