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Protein and Glycoprotein Patterns Related to Morphogenesis in *Mammillaria gracillis* Pfeiff. Tissue Culture

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Summary

As plants with Crassulacean Acid Metabolism (CAM), cacti are highly affected by artificial environmental conditions in tissue culture. Plants of *Mammillaria gracillis* Pfeiff. (Cactaceae) propagated *in vitro* produced callus spontaneously. This habituated callus regenerated normal and hyperhydric shoots without the addition of growth regulators. In order to compare habituated callus with the tumorous one, cactus cells were transformed with two strains of *Agrobacterium tumefaciens:* the wild strain B6S3 (tumour line TW) and the rooty mutant GV3101 (tumour line TR). Gene expression in cactus plants, habituated callus, regenerated shoots and two tumour lines was analysed at the level of cellular and extracellular protein and glycoprotein profiles. Proteins were separated by SDS-polyacrylamide gel electrophoresis and 2-D PAGE electrophoresis and silver stained. Concavalin A-peroxidase staining detected glycoproteins with D-manose in their glycan component on protein blots. Developmentally specific protein patterns of *Mammillaria gracillis* tissue lines were detected. The 2-D PAGE electrophoresis revealed some tissue specific protein groups. The cellular glycoprotein of 42 kDa detected by ConA was highly expressed in undifferentiated tissues (habituated callus, TW and TR tumours) and in hyperhydric regenerants. Tumours produced extracellular proteins of 33, 23 and 22 kDa. The N-glycosylation of cellular and extracellular proteins was related to specific developmental stage of cactus tissue.

Key words: *Cactacaeae*, crown-gall tumour, glycoproteins, morphogenesis, protein pattern

Introduction

Analysis of protein complement of the genome includes the identification and quantification of proteins to find out which proteins are present in particular tissue, which post-translational modifications occur and how protein patterns can be related to morphogenetic status of tissues. The changes in protein N-glycosylation follow morphogenic events (*1*,*2*). Analysis of these changes can allow an insight in post-translational modifications specific for a certain developmental stage. Physiological and environmental factors have profound effects on glycosylation in mammals (*3*,*4*). Relatively little is known about the effects of environmental conditions and developmental stage on proteins produced by plant tissues cultured *in vitro*. Some publications describe the role of proteoglycans and glycoproteins in the processes of plant development (*5*,*6*). As plants with Crassulacean Acid Metabolism (CAM), cacti are highly affected by the artificial environmental conditions in tissue culture (*7*). Plants of *Mammillaria gracillis* Pfeiff. propagated *in vitro* develop calli without any exogenous

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growth regulators (*8*). This habituated (hormone-independent) callus spontaneously regenerates normal and hyperhydric shoots. The phenomenon of habituation bears a striking similarity to tumour transformation in crown gall disease where tumour tissue grows independently of exogenous hormones (*9,10*). In our study, crown gall tumours were induced to compare habituated and transformed calli. In most cases, habituation appears to be reversible; and habituated cells keep their totipotency, as do genetic tumours, and they can regenerate plants or somatic embryos (*11–13*). The plasticity of changes from an organised to an unorganised way of growth in the culture of *Mammillaria gracillis* makes this system suitable for studies on plant development (*8*). The aim of this work was to compare *in vitro* propagated plants, callus and *Agrobacterium* transformed tissues of *Mammillaria gracillis*, *Cactaceae*, with regard to their protein and glycoprotein patterns with the intention to detect some developmentally specific proteins.

Materials and Methods

Tissue lines

Mammillaria gracillis plants were propagated *in vitro*, at 24 °C, on a solid MS (*14*) nutrient medium without any growth regulator. Habituated callus was subcultivated every three weeks on the hormone-free MS medium. In the callus culture, regeneration of morphologically normal, as well as hyperhydric malformed shoots occured. Tumours were induced on disc-like explants of *in vitro* grown plants. Wounded tissue was infected with *Agrobacterium tumefaciens*, the wild strain B6S3 (tumour line TW) and the rooty mutant GV3101 (tumour line TR) (*8*).

Protein samples

Total soluble proteins were extracted by grinding 0.5 g of fresh tissues in 1.5 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 and 4 °C for 60 min. Protein content was determined according to Bradford (*15*). Samples were denatured using 0.125 M Tris buffer (pH=6.8), containing 5 % (volume fractions) β-mercaptoethanol and 2 % (volume fractions) SDS (sodium dodecyl sulphate). For the SDS-PAGE, the same amount of protein (5–8 μg) per sample was loaded.

For two-dimensional (2-D PAGE) electrophoresis proteins were precipitated by the addition of 2 volumes of ice-cold acetone. The mixture was left for minimum 2 hours at –20 °C and then centrifugated at 10 000 × g for 15 min. After centrifugation the pellet was vacuum dried and solubilised in the sample solution (9.5 M urea, 1 % dithiotreitol, 1 % (volume fraction) pharmalyte 3–10 and 0.5 % Triton X-100).

Extracellular proteins were obtained from a liquid nutrient medium of 3-day old suspension cultures. The medium was decanted and passed through a mash filter to remove the cell debris. Extracellular proteins were concentrated by acetone precipitation and the pellet was resuspended in 100–150 μL of SDS sample buffer.

Electrophoresis and electroblotting

Both cellular and extracellular proteins were analysed by SDS electrophoresis in 12 % T (2.67 % C) polyacrylamide gels or in 8–18 % T (2.67 % C) gradient gels, with the buffer system of Laemmli (*16*).

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed according to Pearce and Svendsen (*17*). Carrier ampholytes pH=3–10 (Bio-Rad) were used in the first dimension (isoelectring focusing). The upper reservoir was loaded with 20 mM NaOH and the lower reservoir with 100 mM H_3PO_4. Pre-running conditions were 10 min at 200 V, 15 min at 300 V and 15 min at 400 V. First dimension was run at 310 V for 16 h and 450 V for 30 min. Second-dimensional analysis was performed according to Laemmli (*16*) on 8–18 % polyacrylamide gradient gels.

Protein bands and spots were visualised by silver staining (*18*).

Cellular and extracellular proteins were transferred to the nitrocellulose membrane in the vertical tank for electroblotting. Glycoproteins with D-manose in their glycan component were detected on nitrocellulose membrane by reaction with Concanavalin A. Bands were visualised by peroxidase reaction using 4-chloro-1-naphthol as a substrate (*19*).

Results

The morphology of *in vitro* plants, habituated callus (c), normal and hyperhydric regenerants (nr and hr), as well as two tumour lines (TW and TR) of *Mammillaria gracillis* Pfeiff. is shown in the Fig. 1.

SDS-PAGE of soluble cellular proteins showed a few tissue-specific bands. The 54 and 16 kDa polypeptides, most likely subunits of Rubisco, were missing from tumour extracts (Fig. 2, arrows). Band of 70 kDa (Fig. 2, lanes 4 and 5, arrows) and several bands in the range from 48 to 50 kDa and 56 to 60 kDa (Fig. 2, lanes 4 and 5, spots) are tumour-specific. The 26 kDa polypeptide was expressed more intensively in tumour TR than in other tissues (Fig. 2, lane 5, arrow). The 18 kDa polypeptide was specific for habituated callus and hyperhydric regenerants (Fig. 2, lanes 2 and 3, spots). The 17 kDa protein was present in all samples except in tumour TR (Fig. 2, arrows).

2-D PAGE electrophoresis revealed two groups of proteins common for all investigated tissues (Fig. 3, circles 1 and 2). In addition, in habituated callus, hyperhydric regenerants and tumour TW three common groups of proteins were found (Fig. 3, circles 3,4 and 5) Habituated callus and hyperhydric regenerants had two characteristic groups (Fig. 3, circles 6 and 7). Cactus plants and tumour TW showed some specific proteins (Fig. 3, circles 8,9,10 and 11,12,13,14, respectively).

The 42 kDa protein band was detected in all cactus tissues on the silver stained gels (Fig. 4A, arrows). The corresponding glycoprotein of 42 kDa, reacting with Con A, was highly expressed only in habituated callus, hyperhydric regenerants and both tumours (Fig. 4B, arrows). In both tumour extracts, TW and TR, four additional glycoproteins of 56, 62, 66 and 67 kDa were resolved (Fig. 4B, lanes 5 and 6, spots), and the first three

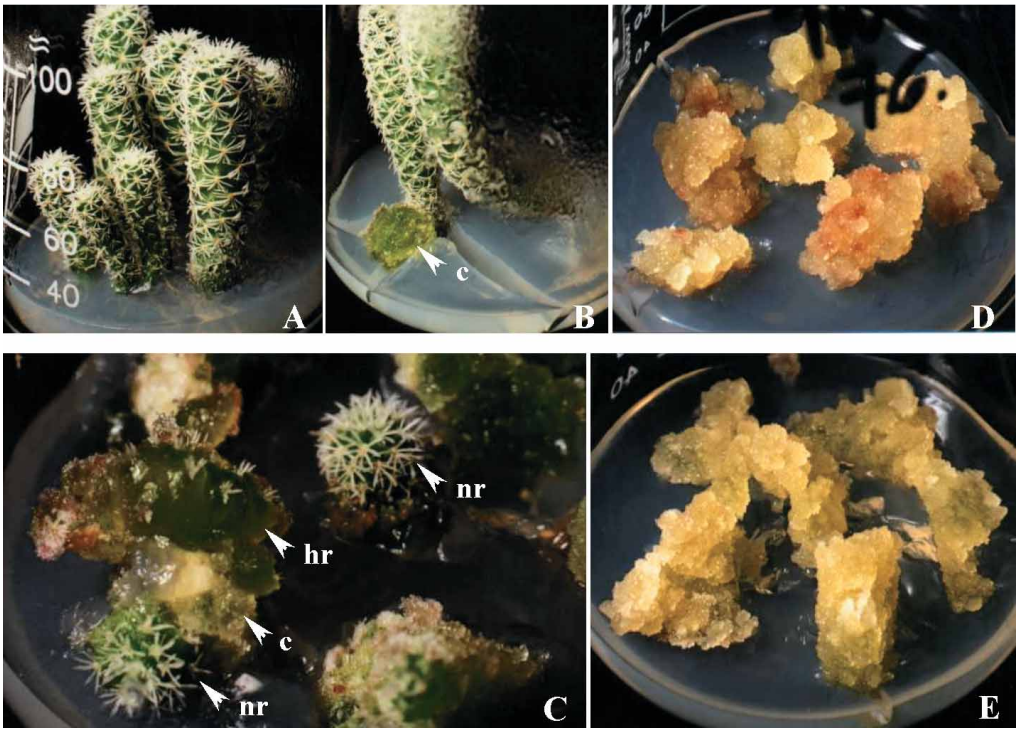

Fig. 1. ***Mammillaria gracillis in vitro*** **tissue culture.** A – cactus plants, B – spontaneously developed habituated callus (c), C – normal and hyperhydric regenerants (nr and hr) in the callus culture (c), D – tumour line TW, E – tumour line TR.

bands were found in habituated callus and in hyperhydric regenerants (Fig. 4B, lanes 2 and 3, spots). The proteins of 27 and 34 kDa were characteristic for tumour TR (Fig. 4B, lane 6, arrows).

Differences between *Mammillaria* tissues were more pronounced in the extracellular protein patterns (Fig. 5). Only clearly visible bands were considered. Almost no extracellular proteins could be detected in the medium of plants and normal regenerants (Fig. 5A, lanes 1 and 4). Very faint band of 34 kDa protein was characteristic for the habituated callus (Fig. 5A, lane 2, arrow). The 33 and 23 kDa proteins were highly expressed only in tumours (Fig. 5A, lanes 5 and 6, arrows). Both tumour lines contained protein of 22 kDa (Fig. 5A, lanes 2, 5 and 6, arrows).

On the blot, extracellular glycoproteins reacting with Con A were detected (Fig. 5B). The glycoprotein of 35 kDa was expressed in all cactus tissues, except in plants and normal regenerants (Fig. 5B, arrows). The polypeptide of 23 kDa was specific for tumour lines (Fig. 5B, lanes 5 and 6, arrows).

Discussion

Although, *Mammillaria gracillis* is not a commercially interesting plant species in Europe, a switch from an organised to a disorganised manner of growth in the culture makes the presented system suitable for studies of plant morphogenesis.

To our knowledge, no *Agrobacteria*-mediated transformation of *Cactaceae* has been reported hitherto. Crown gall tumours were induced to compare habituated and transformed calli, both hormone-independent.

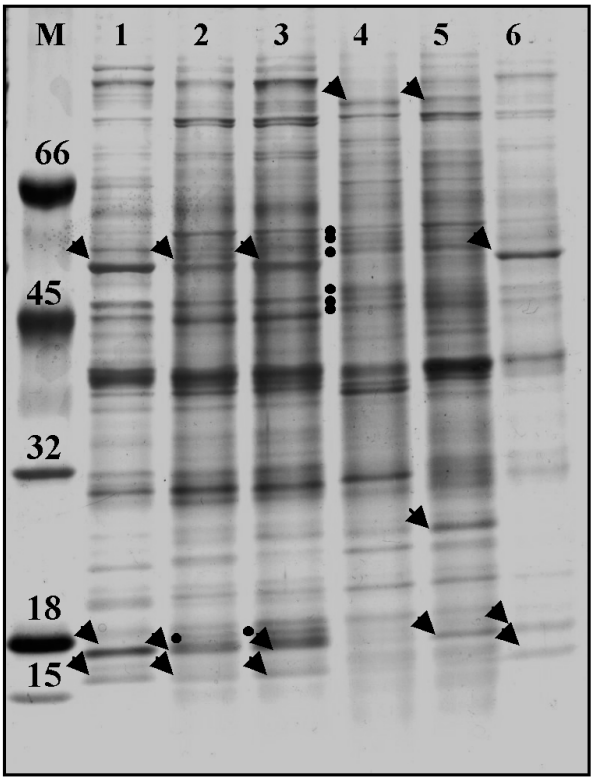

Fig 2. Electrophoretic pattern of soluble cellular proteins of ***Mammillaria gracillis*** **tissues separated by SDS-PAGE in 8–18 % gradient gel.** Lane: 1, cactus plant; 2, habituated callus; 3, hyperhydric regenerant; 4, tumour TW; 5, tumour TR; 6, normal regenerant; M, molecular mass markers. Arrows and spots indicate bands mentioned in the text.

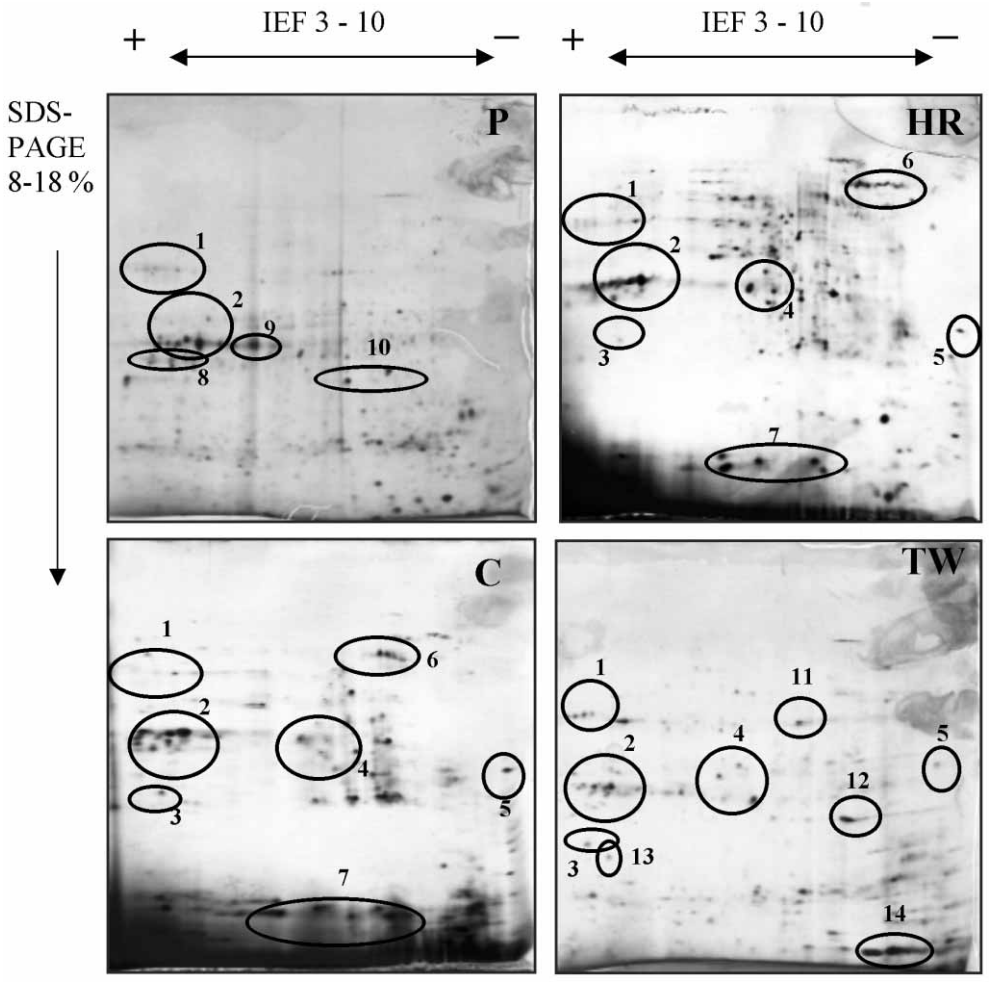

Fig. 3. 2-D PAGE electrophoresis of soluble cellular proteins of *Mammillaria gracillis* tissue lines. P, cactus plant; C, habituated callus; HR, hyperhydric regenerant; TW, tumour TW. Circles and numbers indicate groups of proteins mentioned in the text.

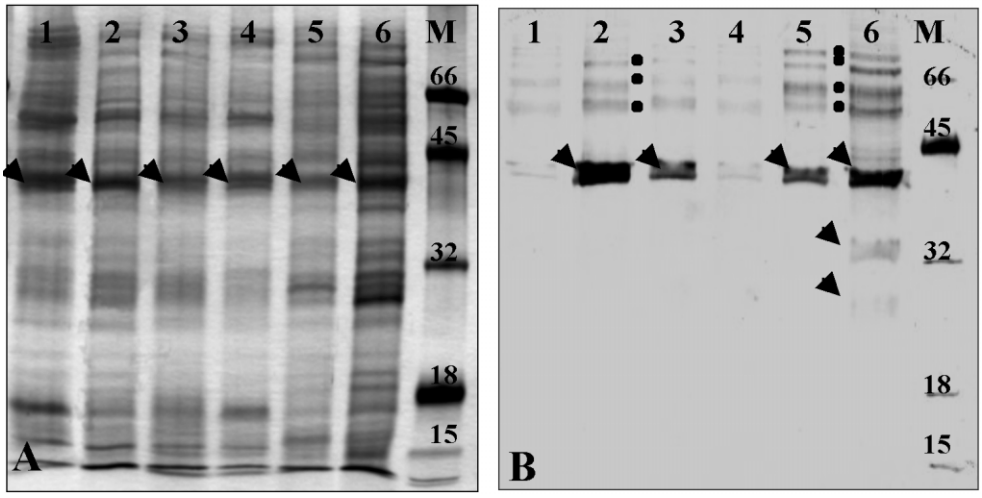

Fig. 4. A – Soluble cellular proteins of *Mammillaria* tissue lines separated by SDS-PAGE in 12 % gel. B – Electro-blot of the same gel. Lane: 1, cactus plant; 2, habituated callus; 3, hyperhydric regenerant; 4, normal regenerant; 5, tumour TW; 6, tumour TR; M, molecular mass markers. Arrows and spots indicate bands mentioned in the text.

Agrobacteria mediated transformation might also be a way to introduce a foreign DNA into the *Mammillaria* genome with the aim of potential biotechnological application.

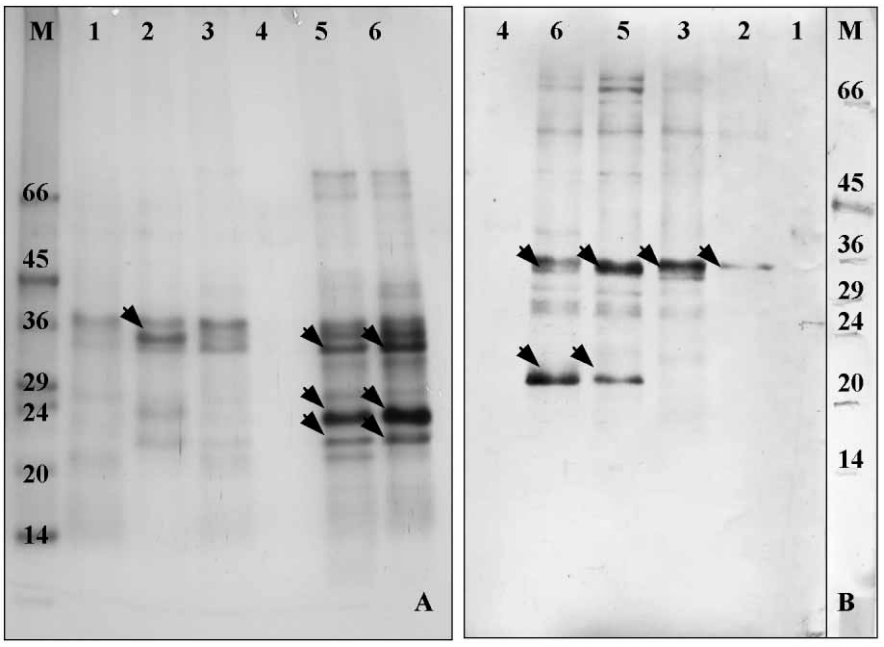

Fig. 5. A – Extracellular proteins of *Mammillaria* tissue lines separated by SDS-PAGE in 8–18 % gradient gel. B – Electro-blot of extracellular proteins of *Mammillaria* cell suspension culture. Lanes description as in Fig. 4. Arrows indicate bands mentioned in the text.

Despite obvious morphological differences between *Mammillaria* plants, habituated callus, regenerants (normal and hyperhydric) and tumours, only few morphogenesis-specific cell polypeptides were observed. In tumour tissues the Rubisco subunits were not detected, most likely as a consequence of chloroplasts dedifferentiation toward proplastids and low total chlorophyll content in tumours cells with reduced photosynthetic activity (Poljuha *et al.*, unpublished data).

In the search for a more reliable molecular marker of *in vitro* morphogenesis, 2-D PAGE electrophoresis, a more powerful protein separation technique, was applied. This method was already successfully used to study callus tissue proteins in *Cereus peruvianus* (*Cactaceae*) (*20*). Our results with 2-D PAGE electrophoresis revealed two groups of proteins common for all investigated tissues as well as spots characteristic of each tissue line. Further studies should identify some of the developmentally specific proteins and determine their localisation, modifications, interactions, activities, and ultimately their function.

Differences between *Mammillaria tissues* were more pronounced in the extracellular protein patterns. In the extracts of plant and normal regenerants almost no extracellular proteins were detected, most likely due to cell wall barrier and tight cell-to-cell adhesion (Poljuha *et al.*, unpublished data). The cells of habituated callus excreted only the 34 kDa protein. Such tissues with high content of water are characterised by reduced cellulose and lignin deposition in cell walls (*21,22*). The greatest number of extracellular proteins was revealed in tumours; three of them were tumour-specific. In sugar beet tumour tissue no tumour specific extracellular proteins could be detected (*1*).

The expression of arabinogalactan-proteins (AGPs) is highly regulated during plant development and correlates with cell differentiation (*23,24*). The analysis of N-linked glycans of soluble endogenous glycoproteins from leaves of tobacco plants of different age and under different conditions, demonstrated that developmental processes in plants could influence glycosylation (*2*). The glycosylation profile of endogenous proteins can be changed by plant development and growth conditions (*25*). The structure of extracellular N-linked glycans varies with different developmental stages and organisation level (*1*) but no specific role of N-linked glycans in biological processes in plants has been described as yet. In the present work it has been stated that the 42 kDa protein, although present in all cactus tissues, has different sugar composition or perhaps is not glycosylated at all in plant and normal regenerants.

Concavalin A staining of extracellular protein blots revealed high mannose oligosaccharide chains of glycoproteins. The characteristic patterns of extracellular glycoproteins could be related to developmental state of *Mammillaria* tissues. Further characterisation of 23 kDa highly expressed tumour-specific glycoprotein should contribute to better understanding of protein N-glycosylation in transformed and untransformed tissues.

Conclusions

A few morphogenesis-characteristic cell polypeptides were detected by SDS-PAGE. Two-dimensional electrophoresis separated cell proteins more powerfully and revealed some tissue specific protein groups. Differences between *Mammillaria* tissues were more pronounced in extracellular protein patterns separated by SDS-PAGE than in cellular protein patterns. No extracellular pro-

teins were detected in the medium of plants and normal regenerants. Tumour-specific extracellular proteins of 33, 23 and 22 kDa were found. The cellular glycoprotein of 42 kDa detected by ConA was highly expressed only in habituated callus, hyperhydric regenerants and in both tumours.

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Proteinski i glikoproteinski biljezi morfogeneze u kulturi tkiva kaktusa *Mammillaria gracillis* Pfeiff.

Sažetak

Kao kserofiti s CAM tipom fotosinteze kaktusi su vrlo osjetljivi na uvjete neprirodna okoliša u kulturi tkiva. Prilikom razmnožavanja biljaka kaktusa *Mammillaria gracillis* Pfeiff. (*Cactaceae*) spontano dolazi do rasta obilnog kalusa. Kalus može i bez dodatka regulatora rasta regenerirati izdanake s karakterističnim fenotipom kao i hiperhidrirane izdanake ove biljne vrste. Da bi se prilagođeni kalus usporedio s tumorom, provedena je transformacija tkiva kaktusa s dva soja bakterije *Agrobacterium tumefaciens*, divljim tipom B6S3 (tumorska linija TW) i »rooty« mutantom GV3101 (tumorska linija TR). U biljkama kaktusa, kalusu, regeneriranim izdancima i dvjema tumorskim linijama analizirana je ekspresija gena na razini staničnih i izvanstaničnih proteina te glikoproteina. Proteini su razdvojeni jednodimenzionalnom i dvodimenzionalnom poliakrilamidnom gel-elektroforezom, a elektroferogrami obojeni srebrom. Nakon elektroprijenosa proteina na nitrocelulozu membranu utvrđeni su u glikanskoj komponenti glikozilirani proteini s D-manozom pomoću ConA peroksidaze. Dvodimenzionalna elektroforeza pokazala je veći broj tkivno-specifičnih proteina. Stanični glikoprotein od 42 kDa bio je jako eksprimiran samo u nediferenciranim tkivima (kalus, tumori TW i TR) te u hiperhidriranim regenerantima. Tumori su izlučivali izvanstanične proteine od 33, 23 i 22 kDa u hranidbenu podlogu. Na osnovi dobivenih rezultata može se zaključiti da je stupanj organiziranosti tkiva kaktusa utjecao na N-glikozilaciju staničnih i izvanstaničnih proteina.