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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Electrophoretic protein patterns and peroxidase activity related to morphogenesis in *Mammillaria gracillis* tissue culture

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The cactus *Mammillaria gracillis* Pfeiff. was propagated *in vitro* on Murashige and Skoog (MS) medium without any growth regulators. At the bases of some plants abundant callus masses developed. Lower agar and higher MS salt concentrations stimulated callus production. In the culture, morphologically normal and hyperhydric shoots were regenerated. The growth of crown-gall tumour was induced on disc-like explants of *in vitro* grown plants infected with *Agrobacterium tumefaciens,* B6S3 strain. Calli appeared on both infected and control explants. The tumorous tissue grew more intensively than the control. The transformed character of the callus was confirmed by PCR amplification of a gene *6a* of T-DNA. Gene expression in cactus shoots (grown *in vitro* or in pot), callus, hiperhydric and morphologically normal regenerated shoots and crown-gall tumour were analysed at the level of the electrophoretic pattern of soluble proteins. Some tumour-specific polypeptides were detected (76, 32–39, and 23 kDa). That of 42 kDa was highly expressed in callus and hyperhydric shoots. A faint 58 kDa band was found in all extracts except in the pot-grown shoot. Relatively high peroxidase activity was detected in callus and shoot regenerants and it was lower in tumour and the lowest in plant shoots.

Key words: *Cactaceae*, *Mammillaria gracillis*, callus, regeneration, hyperhydrycity, crown gall tumour, electrophoretic protein profile, peroxidase activity.

Introduction

Native cacti occur exclusively in the New World, which is characterized by long dry periods with short rains. As a result of living in these conditions cacti developed certain adaptations. Some of these adaptive characteristics are fleshy, columnar stems (which provide capacity for water storage), protective spines and reduced leaves. A special physiological adaptation of cacti is the CAM (crassulacean acid metabolism) manner of photosynthesis. CAM plants are largely dependent upon nocturnal accumulation of CO_2 for their photosynthesis because their stomata are closed during the day, retarding water loss.

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As plants with crassulacean acid metabolism, cacti are highly affected by artificial environmental conditions in tissue culture (MALDA et al. 1999). We studied changes in morphogenesis related to stress conditions and tumour transformation in the cactus *Mammillaria gracillis*. Shoots, unorganised callus, normal and hyperhydric regenerated shoots and tumorous tissue were compared with regard to their protein patterns and peroxidase activity. The aim of this work was to obtain information about changes in gene expression occurring in habituated callus, *in vitro* regenerants and tumorous tissue.

Materials and methods

Plant material

Cactus (*Mammilaria gracillis* Pfeiff) plants were propagated *in vitro*, at 24 °C, on a solid MS (MURASHIGE and SKOOG 1962) nutrient medium (0.9% agar, 3% sucrose) without any growth regulator at a 16/8-hour-light/night-photoperiod. Spontaneously formed calli were subcultivated every three weeks on the hormone-free MS medium.

Tumour induction

Crown-gall tumour was induced by *Agrobacterium tumefaciens*, strain B6S3 harbouring the wild type of tumour-inducing (Ti) plasmid. A bacterial culture grown overnight, in a liquid medium containing 0.8% beef extract, 0.1% yeast extract and 0.5% sucrose, was used for infection. To the surface of disc-like explants, cut from *Mammillaria* plants grown *in vitro*, 30–40 μL of bacterial suspension was applied. A sterile nutrient broth was applied to the control. Explants were put on solid 1:1 diluted MS medium and kept in the dark. After 8 weeks, both types of callus were treated with antibiotic during 5-day subcultivation in the liquid medium and then transferred to hormone-free MS medium, using carbenicillin (500 mg/L) to stop bacterial growth. The carbenicillin was also added to the medium during the second and third subculture (3–4 weeks each).

DNA isolation and PCR

Total genomic DNA was isolated from the both types of callus according to DELLAPORTA et al. (1983). The transformed character of the callus was confirmed by PCR amplification of a 336 bp fragment of the gene *6a* from T-DNA. The primers were 5'TGCTTCAGATGGATTGCTTGCC3' and 5'GATAGCACCATCTAACTCCACG3'. For PCR, a PRIMEZYME KIT (Biometra) was used in 25 μL of reaction suspension containing 50 ng of plant DNA. The initial denaturation step, taking 2 min at 94 °C, was followed by 30 amplification cycles: 30 sec at 94 °C, 30 sec at 60 °C, 1.5 min at 72 °C. Final extension occurred for 7 min at 72 °C. To avoid false positives due to contaminating agrobacteria, an additional PCR was performed in the same conditions, using primers for the amplification of a 317 bp fragment of the bacterial *virB* gene located outside the T-DNA borders. The primers were 5'AGAGGCGGTGTTAGTTGC3' and 5'AACATCCAGCGAATGCCG3.

Protein analysis and electrophoresis

Total soluble proteins were extracted in 0.1M Tris/HCl buffer, pH 8.0 at 4 °C. Homogenates were centrifuged 25 min at 18 000 rpm and 4 °C. The supernatants were cen-

trifuged again 60 min at 22 000 rpm at 4 °C. A supernatant was used as a crude extract. Protein content was determined according to BRADFORD (1976). Samples were denatured using 0.125 M Tris buffer (pH 6.8), containing 5% (v/v) β-mercaptoethanol and 2% (w/v) SDS. For the SDS-PAGE approximately the same amount (6 μg) of protein per sample was loaded onto the gel.

For peroxidase activity, measurement extracts were prepared in 0.26 M H_3PO_4/Tris buffer, pH 6.9 at 4 °C. Homogenates were centrifuged 15 min at 14 000 rpm and 4 °C. The supernatants were centrifuged again 60 min at 14 000 rpm at 4 °C. The total guaiacol peroxidase activity of extracts was determined spectrophotometrically by measuring the increase in absorbency at 470 nm. The test solution was prepared after SIEGEL and GALSTON (1967), and contained 5 mM guaiacol, 0.2 mM KH_2PO_4, 0.2 mM Na_2HPO_4 and 5 mM H_2O_2. Peroxidase activity was expressed in dA/min/mg of protein.

The proteins were analysed by SDS electrophoresis in 8–18% gradient polyacrylamide gels with the buffer system of LAEMMLI (1970). The protein bands were visualized by silver staining (BLUM et al. 1987).

Results

At the basis of *in vitro* propagated cactus plants, abundant callus masses were formed spontaneously without any exogenous growth regulator. The callus was detached from the plants and subcultivated, on the basic MS medium, as a hormone independent habituated tissue. The callus was snowy with yellowish to light green parts (Fig. 1a), and, occasionally, anthocyanin coloured spots appeared in the callus.

To test the culture conditions that would be favourable for callus induction, cactus shoots were cultivated on the media with different agar and MS salt concentrations (Tab. 1). The lower agar and higher MS salt concentrations stimulated callus production. Roots development was more intensive in the media with higher agar concentration. Roots, but not callus, growth was observed in the water agar without any MS salts.

In the callus culture, regeneration of cactus plants as well as of hyperhydric, malformed shoots occurred (Fig. 1b). Regenerated cactus plants were green, covered with spines and had an unaltered cactus morphology. Hyperhydric shoots were translucent, light green, bowl-shaped and partially covered with disarranged spines.

Crown-gall tumours were induced on disc-like explants. Macroscopically visible outgrowths appeared on both infected and control explants in six weeks (Figs. 1c, d). Occasionally, some roots developed on the infected discs. After eight weeks, primary tumours and the control callus were excised and transferred, for five days, to liquid MS medium containing the antibiotic carbenicillin (500 mg L^{-1}). After transfer to solid medium, tumorous calli grew intensively never expressing any organogenic potential. The majority of the control wound-induced callus explants died.

To test the transformed character of *Agrobacterium*-induced crown-gall tumour, a PCR amplification of a 336 bp fragment of the gene *6a* from T-DNA was performed. DNA electrophoretic analysis revealed the *6a* gene of the Ti plasmid in the tumour line only (Fig. 2 a), confirming the transformed character of the crown-gall tumour line. A bacterial contamination of tissues was excluded by amplifying a *virB* gene of the Ti-plasmid (Fig. 2 b), which was found only in the bacterial positive control.

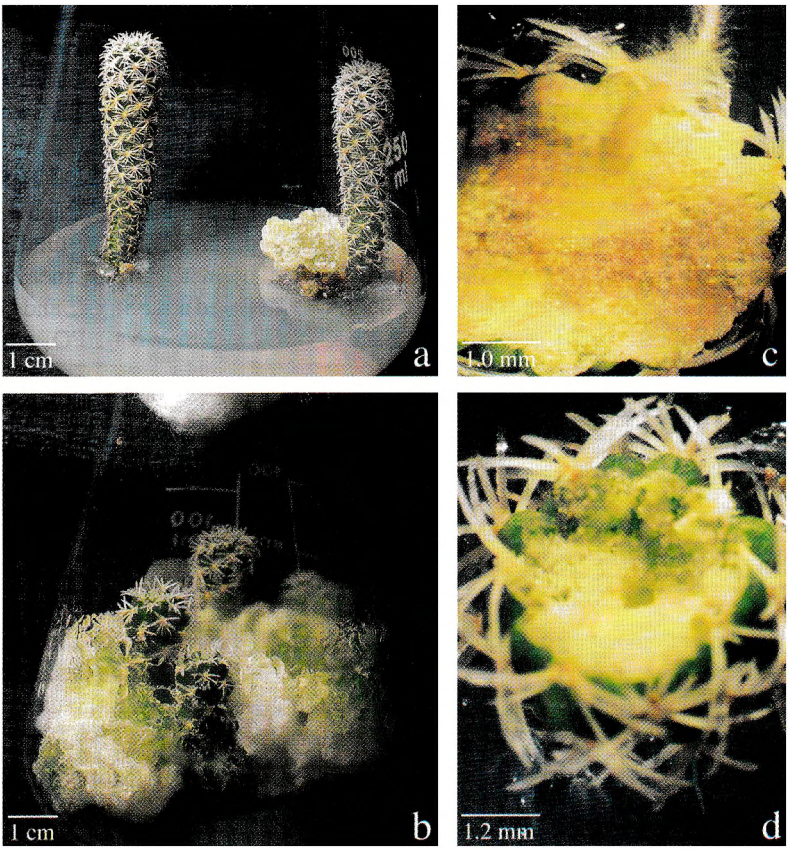

Fig. 1. **a – d** *Mammillaria gracillis* tissue culture. **a** Spontaneous callus formation at the basis of *in vitro* propagated cactus plant. **b** Regeneration of cactus plants and hyperhydric, malformed shoots in the callus culture. **c** Crown-gall tumour induced on disc-like explants of cacti plants. **d** Control wound callus induced on disc-like explants.

Gene expression in cactus shoots, unorganised callus, crown gall tumour tissue and in the regenerated shoots was analysed at the level of protein profiles. SDS-PAGE showed a few tissue-specific bands. Tumour-specific bands were those of approximately 76 and 23 kDa, as well as several faint bands in the range of 32 to 39 kDa (Fig. 3 line 6, spots). The 54 and 15.5 kDa polypeptides (asterisk), most likely subunits of Rubisco, were missing from the callus and tumour extracts. The polypeptide of 42 kDa (circle) was expressed more highly in the callus and hyperhydric shoots than in the other tissues. The 58 kDa protein (triangle) was missing only in the shoot of the pot-grown plant and might be connected with *in vitro* culture conditions.

Peroxidase is an enzyme widely distributed in plants and is involved in growth, differentiation, development processes as well as in stress response. In *Mammillaria* tissue culture, the highest total peroxidase activity was detected in callus and regenerated shoots (Fig. 4). The activity of tumorous tissue reached hardly a half of this value. Both cactus shoots cultivated in soil and *in vitro* had the low peroxidase activity.

Tab. 1. Effect of MS salt and agar concentration on rooting and callus formation of cactus plants.

Salts concentrations		Root development*	Production of callus	
			Shoots with callus (%)♦	Amount of callus*
0 MS	0.7% agar	+ +	0	0
	0.8% agar	+ +	0	0
	0.9% agar	+ + +	0	0
	1.2% agar	+ +	0	0
	1.4% agar	+ + +	0	0
¼ MS	0.7% agar	0	20	+
	0.8% agar	+	33	+
	0.9% agar	+ +	0	0
	1.2 %agar	+ +	0	0
	1.4% agar	+ +	0	0
½ MS	0.7% agar	0	33	+
	0.8% agar	+	66	+ +
	0.9% agar	+ +	66	+ +
	1.2% agar	+ +	0	0
	1.4% agar	+ +	0	0
MS	0.7% agar	0	100	+ + +
	0.8% agar	+	100	+ + +
	0.9% agar	+ +	100	+ + +
	1.2% agar	+ + +	0	0
	1.4% agar	+ + +	0	0

* 0 = No roots + = roots covering less than 1/3 of surface ++ = roots covering about 1/3 of surface +++ = roots covering whole surface
* 0 = No response + = < 0.5 g ++ = 0.5–4.0 g +++ = > 4.0g
♦ Number of replications = 6

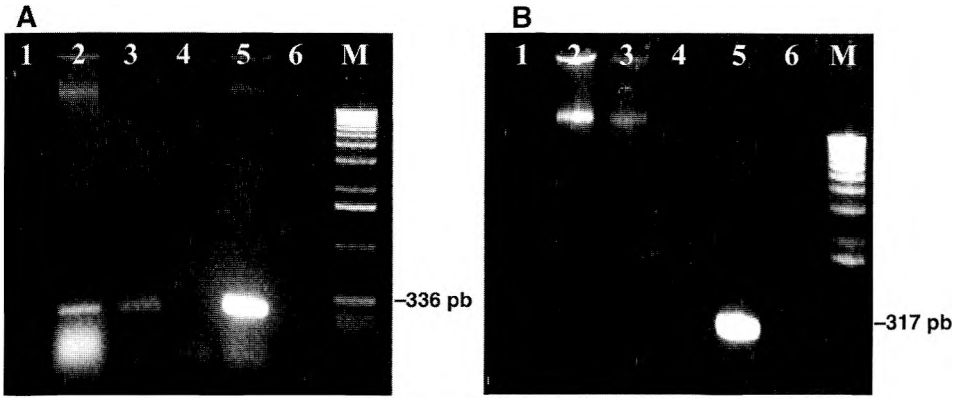

Fig. 2. **a** PCR analysis of the gene *6a* from T-DNA (336 bp) in *Mammillaria gracillis* callus lines. **b** PCR analysis of the bacterial gene *vir B* (317 bp) in *Mammillaria gracillis* callus lines. 1 – spontaneously formed callus, 2 – primary B6S3 tumour, 3 – crown-gall tumour, 4 – control callus, 5 – positive control (bacterial DNA), 6 – negative control (deH_2O)

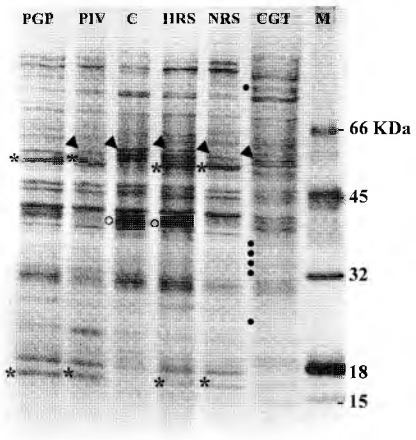

Fig. 3. Electrophoretic pattern of soluble proteins of *Mammillaria gracillis* tissues (8–18% gradient polyacrylamide gel). PGP – pot grown plant, PIV – plant *in vitro*, C – callus, HRS – hyperhydric regenerated shoot, NRS – normal regenerated shoot, CGT – crown-gall tumour

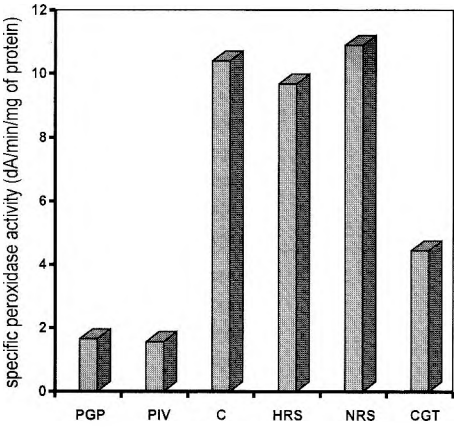

Fig. 4. Specific peroxidase activity in *Mammillaria gracillis* tissues. PGP – pot grown plant, PIV – plant *in vitro*, C – callus, HRS – hyperhydric regenerated shoot, NRS – normal regenerated shoot, CGT – crown-gall tumour

Discussion

Although, *Mammillaria gracillis* is not a commercially interesting plant species in Europe, the plasticity of the changes from an organised to an unorganised way of growth in culture makes the system presented here suitable for studies of plant development. *In vitro* propagated plants develop calli without any exogenous growth regulators. This fully habituated (hormone-independent) callus has regeneration potential. As xerophytes with crassulacean acid metabolism, cacti are highly affected by the artificial environmental conditions of *in vitro* culture. Hormone-independent callus growth could be caused by hyperhydric stress and relatively rich MS nutrient medium. ELIAS-ROCHA et al. (1998) propagated *Mammilllaria candida* plants using different combinations of growth regulators. The regenerated shoots were transferred to hormone-free media to induce rooting. Depending on the original medium, 4–49 % of shoots developed calluses. A switch from an organised to an unorganised manner of growth in the culture of *Mammillaria* species, indicates that culture conditions can cause a morphogenetic effect similar to that of growth regulators. At the other side, cytokinins as BA may induce hyperhydricity in the culture (PAQUES and BOXUS 1987, LESHEM et al. 1988). It remains to be elucidated why, phenotypically unaltered (normal) and malformed hyperhydric shoots develop in the same callus.

To our knowledge, no *Agrobacteria*-mediated transformation of *Cactaceae* has been reported hitherto. In the present work, crown-gall tumours were induced to compare habituated and transformed calli, both hormone-independent. *Agrobacteria* mediated transformation might also be a way to introduce a foreign DNA into the *Mammillaria* genome with the aim of potential biotechnological application.

Despite obvious morphological differences between *Mammillaria* shoots, unorganised callus, regenerants (normal or hyperhydric) and tumorous tissue, only a few morphogenesis-specific polypeptides were observed. In the search for more reliable molecular markers of *in vitro* morphogenesis, more powerful protein separation techniques, such as 2-D electrophoresis, should be applied. This method was performed in *Cereus peruvianus* (*Cactaceae*) to study callus tissue proteins (MANGOLIN et al. 1999). After impressive recent success in genome research the next step will include protein analyses that will include not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and ultimately their function (FIELDS 2001). The other way to identify differentially specific gene expression would be analyses of mRNA by means of a differential display method (LIANG and PARDE 1992)

Peroxidase is widely distributed in plants and catalyses the oxidation of many substrates using H_2O_2. An inverse relation between growth and peroxidase activity has been reported (JUPE and SCOTT 1992). Our results showed a relatively high activity of soluble peroxidase in callus and regenerants. Regenerated shoots with apparently normal phenotype had 7 x higher activity than the shoots of plants grown *in vitro* or in pot. Higher peroxidase activity in transformed tissue than in normal tissue was established in some other plant species (KRSNIK-RASOL 1991).

The present data show that *Mammillaria gracillis* affected by the artificial environment of *in vitro* culture may easily switch from an organised to an unorganised manner of growth, and that the morphogenetic status of the tissue is reflected in electrophoretic protein pattern and peroxidase activity.

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