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# ULTRASTRUCTURE AND PROTEIN PATTERN OF HORSE-RADISH (*ARMORACIA LAPATHIFOLIA* GILIB.) CROWN-GALL TISSUE*

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Two horse-radish (*Armoracia lapathifolia* Gilib.) crown-gall tissue lines, one unorganized and the other teratoma producing were compared with the control leaf in ultrastructures and electrophoretic protein patterns. Tumour cells were similar to unspecialized parenchyma cells. During tumourigenesis the chloroplasts transformed into amyloplasts or leukoplasts. Amylochloroplasts were present in teratoma cells. Large lobed nuclei were observed in both types of transformed cells. Leaf specific polypeptides of about 22–, 21–, 20– and 18 kDa disappeared in tumour extracts and the enzyme Rubisco quantitatively decreased. The polypeptide of about 85 kDa was present only in transformed tissues. In some other proteins only quantitative differences could be detected.

## Introduction

Crown-gall tumour is a neoplastic plant disease caused by *Agrobacterium*-mediated integration of oncogenes into plant genome (Hooykaas and Schilperoort 1992). The understanding of the molecular basis of *Agrobacteria*-plant interaction enables molecular biologists and biotechnologists to employ the system in plant genetic engineering (Zambryski et al. 1989, Walden et al. 1990). The ultimate aim of many research programmes concerning crown-gall is the production of transgenic plants. As the basic

* The authors dedicate this work to their teacher Prof. dr. Mercedes Wrischer on the occasion of her 65 th birthday.

molecular processes in cell replication are similar in plants and animals, the crown-gall tumour could also be useful better to understand neoplastic growth in general (Bednar and Linsmaier-Bednar 1989, Gaspar et al. 1991). A transcription of ras-related genes (rat sarcoma genes) has also been detected in plants (Hagege et al. 1992 a). By explaining transformation at the gene-level we still have not explained how a differentiated plant cell rearranges at the structural and biochemical level to become a tumour cell.

The aim of the present study was to compare the cell ultrastructure and protein pattern of horse-radish tumour and teratoma tissue with the leaf cells which were the origin of the tumour.

## Material and Methods

Horse-radish (*Amoracia lapathifolia* Gilib.) plants were multiplied *in vitro*. For bud induction, 1 cm long root fragments were placed on the MS (Murashige and Skoog 1962) medium containing 0.8 mg/l of indoleacetic acid (IAA) and 4.0 mg/l of kinetin and exposed to light. Complete plants developed on the medium without hormones. Crown-gall tumours were induced on the leaf fragments with the wild octopine strain B6S3 of *Agrobacterium tumefaciens* according to the method described by Horsch et al. (1985). Tumour tissue was subcultured on the MS medium without hormones (Krsnik-Rasol and Ham 1993).

Hand-made sections through fresh materials were examined in a Zeiss, Axiovert 35 light microscope.

Small pieces of leaves and crown-gall tumour tissue were fixed in 1% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) and postfixed in 1% $OsO_4$. Fixation was followed by dehydration in graded ethanol series and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss EM 10A.

The pigments were extracted in 80% acetone and measured spectrophotometrically on Specoll 10, Zeiss Jena. Chlorophylls were quantitatively determined according to Holden (1976), and carotenoids according to Davies (1976).

Soluble protein extracts were prepared by grinding 0.2 g of the leaf tissue or 1 g of tumour tissue in 1.5 ml of 0.1 M Tris/HCl buffer, pH 8.0 at 4°C. The buffer contained 0.5 M sucrose, 0.6 M ascorbic acid and 0.6 M cystein. Before grinding, insoluble polyvinylpirolidone (cca 50 mg) was added to the tissue. The homogenate was centrifuged at 42 000 g, for 1 hour at 4°C. The supernatant was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Protein concentration was detected according to Bradford (1976). Protein bands were visualised by silver staining (Blum et al. 1987).

## Results

Starting from the same primary crown-gall tumour two tumour tissue lines were established. One unorganized and the other teratoma line. Teratomas were composed of adventitious buds and shoots with fleshy leaves which

never rooted.Light microscopy showed that teratoma leaves lost the typical dorsiventral leaf organisation.

Electron microscopy showed differences between the control and crown-gall tissue at the ultrastructural level. Tumour and teratoma cells had more cytoplasm and fewer plastids than normal cells. Tumour plastids were of amyloplast or leukoplast type (Fig. 2). Teratoma contained, predominantly amylochloroplasts (Fig. 3), while the control cells had well developed chloroplasts (Fig. 1). In tumour and teratoma cells mitochondria were abundant. They also had a more abundant rough ER (Fig. 2, 4, 5) and free ribosome aggregates (Fig. 2, 3). Dictyosomes and vesicles at the periphery of the cell (Fig. 2, 4, 5), indicating a transport activity across the plasma membrane, were more frequent in tumour than in the control cells. Another difference between the tumour and control cells concerned the nucleus. Nuclei of tumour cells were usually irregular with deep invaginations of the nuclear envelope, the same feature of the nuclei was also observed in teratomas (Fig. 6).

Compared to the control, the content of pigments was lower in both crown-gall tissue lines, being a little higher in teratomas than in the unorganized tumour (Table 1).

Table 1. Chlorophyll and carotenoid content (mg/g of fresh biomass) in horse-radish leaves and tumour tissues.

| Tissue | Chlorophyll a + b | Carotenoids |
|---|---|---|
| Leaves | 3.163 | 1.058 |
| Unorganized tumour | 0.015 | 0.006 |
| Teratomas | 0.251 | 0.092 |

To determine a tissue specific protein pattern, soluble proteins were analysed by SDS-PAGE (Fig. 7). Approximately 30 protein bands were separated in the control leaf, however this number was slightly reduced in the tumour and teratoma extracts. Although many proteins were common for all extracts, polypeptides of about 22–, 21–, 20– and 18 kDa present in the leaf disappeared in tumour. Tumour and teratoma tissue coincided in the majority of bands and showed one distinct polypeptide of about 85 kDa which was not detected in the control leaf. A band of low mobility (about 110 kDa), was characteristic of teratoma tissue only. The polypeptides of about 95–, 33– and 15– kDa were remarkable in tumour tissue. The large (56 kDa) and the small (14 kDa) subunits of ribulose–1,5-biphosphate carboxylase (Rubisco) were quantitatively dominant in the leaf.

## Discussion

Both unorganized and teratoma crown gall tissue lines originated from the same primary tumour which had been induced with a wild octopine (B6S3) Ti plasmid. Usually this type of plasmid induces unorganized tumours (Nester et al. 1984), however octopine teratomas were also described

(Amosino and Miller 1982). The horseradish teratoma line produced spontaneously undifferentiated cell masses (Krsnik-Rasol and Ham 1993). Because of such changes from an organized to an unorganized way of growth horse-radish teratoma tissue may be a useful system in studies of morphogenesis. We have not studied the molecular basis of teratoma phenotype which we suppose is due to an aberrant T-DNA inserted into plant genome as stated by Perbolte et al. (1987) for *Nicotiana plumbaginifolia.*

Only a small portion of crown-gall cells are actively dividing cells of meristematic character (Beiderbeck 1977). The majority of cells we observed were vacuolated undividing cells. They differed from the control leaf cells, from which they had derived, in the cytoplasm amount as well as in organelles feature and composition. Generally, they had the characteristics of relatively unspecialized parenchyma cells. The nuclei with envelope invaginations were frequently observed. Such irregular nuclei were observed in potato primary crown-gall cells as well (Lorković et al. 1993). Hagege et al. (1992 b) described large nuclei with deep invaginations in habituated sugar-beet cells. They discussed the connection of morphological nucleus abnormalities with cancer in animals.

During teratoma shoot morphogenesis some degree of cell differentiation towards leaf mesophyl cells was observed. Plastids developed more thylakoids and transformed into amylochloroplasts. Otherwise teratoma cells retained some characteristics of tumourous cells such as large irregular nuclei, abundant ER, dictyosomes and vesicles.

The low pigment content found in tumour tissue was in accordance with the plastid types observed.

A lower grade of tumour cells differentiation resulted in a decreased number of protein bands. According to plastid types, the low amount of pigments and Rubisco, the tumour should be completely glucose dependent. Four leaf specific polypeptides (22–, 21–, 20– and 18 kDa) are probably characteristic of photosynthetically active tissues, as they were also lacking in the root extracts (data not shown).

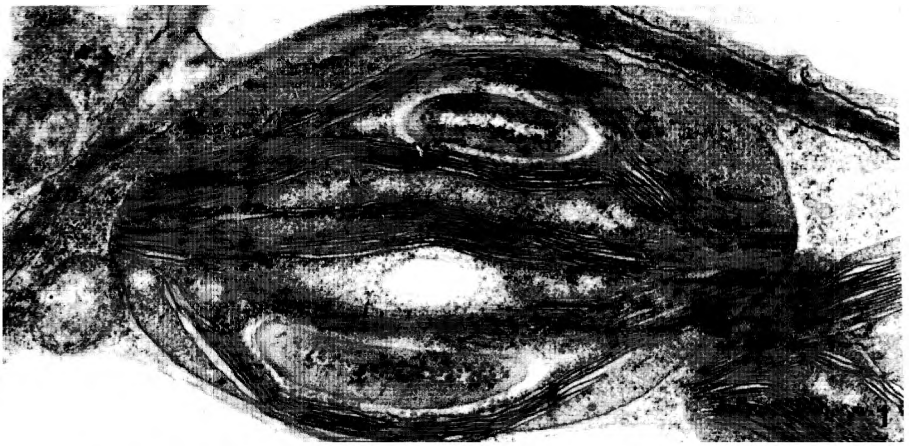
Differences in protein patterns between the control and tumour tissue are hardly a direct consequence of tumourigenesis, instead they could be caused by differentially specific gene expression. The same isoenzymes for tumour and control tissue were described, among others, by Curtis (1971) and Mader (1974).

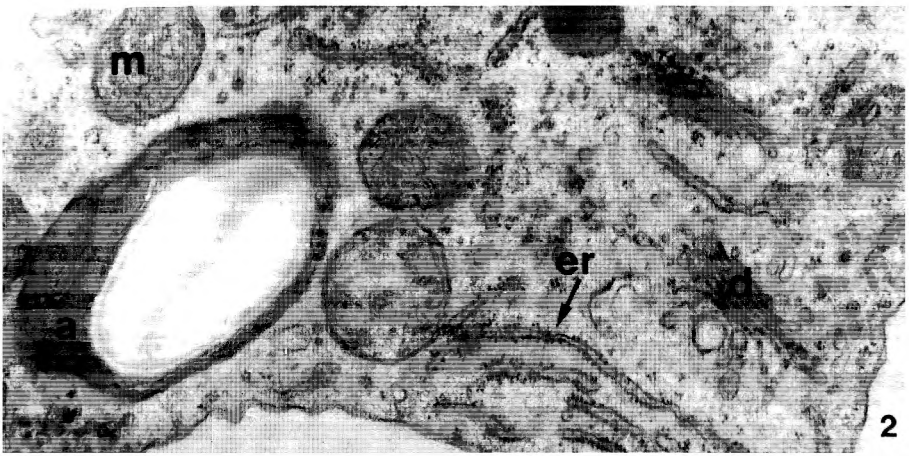
The results obtained show that the majority of horse-radish crown-gall cells grown in culture had the characteristics of relatively unspecialised parenchyma cells. Some degree of differentiation was observed in teratoma

Fig. 1. Chloroplast from the control horse-radish leaf. 22 000 : 1.

Fig. 2. Electron micrograph of tumour cell with an amyloplast (a), mitochondria (m), rough ER (er) and dictyosomes (d). 27 500 : 1.

Fig. 3. Electron micrograph of a teratoma cell with an amylochloroplast, mitochondria (m) and free ribosomes (r). 29 000 : 1.

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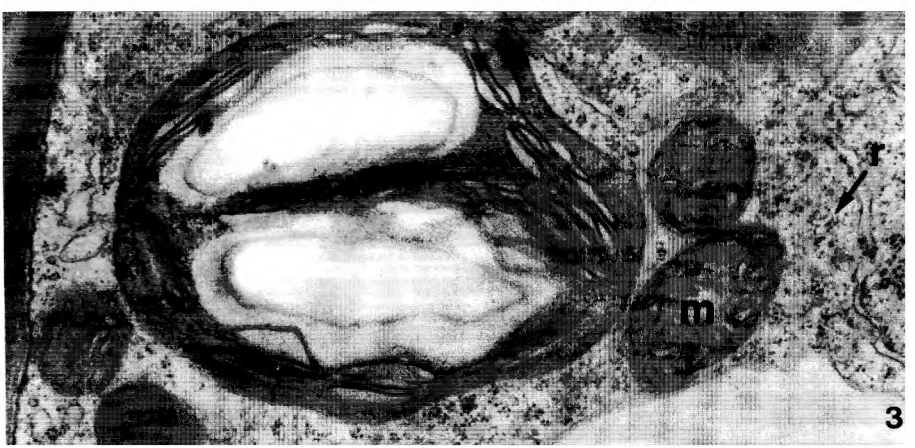
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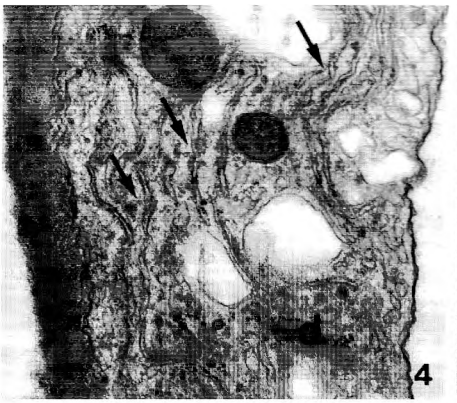

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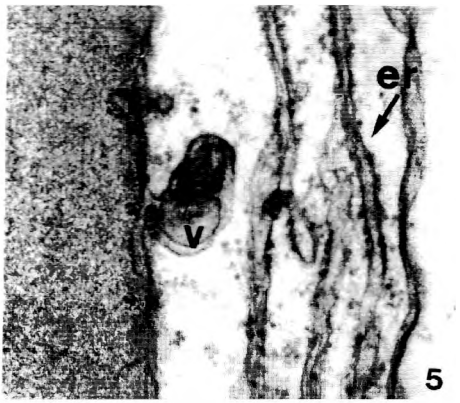


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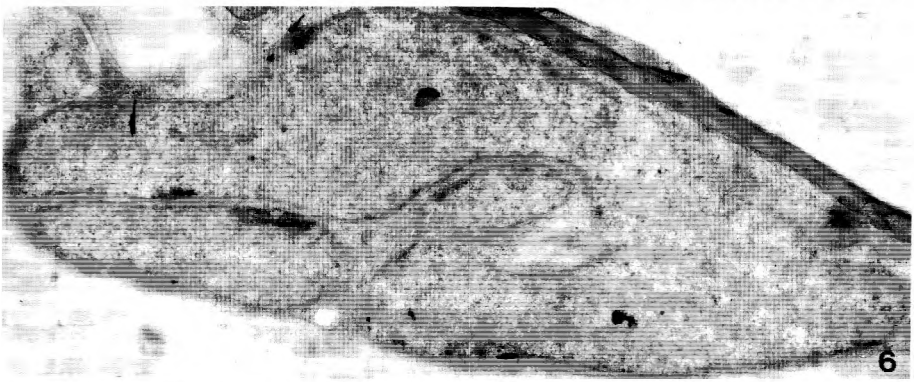

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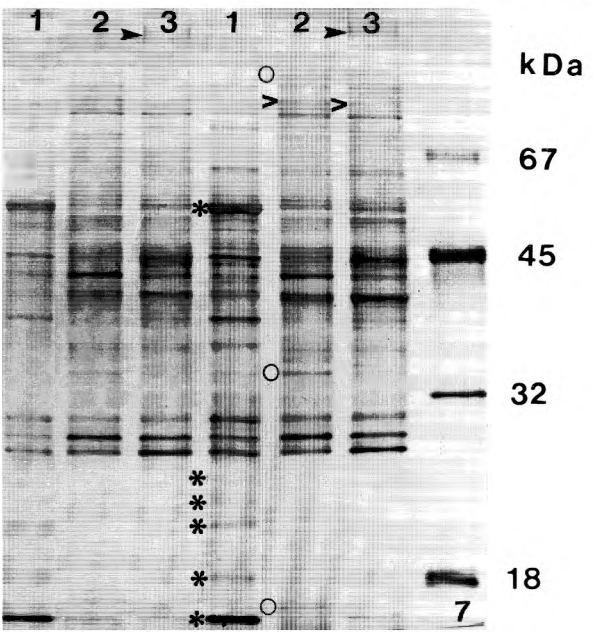


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cells which contained amylochloroplasts, but they did not reach a degree of control cell specialization. The number of protein bands was reduced in transformed cells, and some quantitative differences in protein patterns were also observed.

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Fig. 4. A part of a tumour cell showing rough endoplasmatic reticulum (arrows) and dictyosome (d). 15 100 : 1.

Fig. 5. Vesicle (v) at the periphery of tumour cell; rough ER (er). 52 000 : 1.

Fig. 6. Nucleus with deep invaginations from a teratoma cell. 9 500 : 1.

Fig. 7. Silver-stained SDS PAGE (12% gel) of soluble proteins extracted from the control leaf (1), tumour (2) and teratoma (3) tissues. Asterisks (*) indicate proteins of about 56–, 22–, 21–, 20–, 18– and 14 kDA present in the leaf extract.
Arrows (>) point the 85 kDa protein present in tumour and teratoma extracts.
Circles (o) indicate proteins of about 95–, 33– and 15 kDa remarcable in tumour.
Black arrows (▶) indicate teratoma specific band of about 110 kDa.

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## SAŽETAK

### ULTRASTRUKTURA I PROTEINSKI SASTAV TUMORA VRATA KORIJENA HRENA (*ARMORACIA LAPATHIFOLIA* GILIB.)

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Dvije linije tumorskog tkiva hrena (*Armoracia lapathifolia* Gilib.), neorganizirana i teratomska, uspoređene su s kontrolnim tkivom lista u ultrastrukturi i elektroforetskoj slici proteina. Tumorske stanice imaju osobine nespecijaliziranih parenhimskih stanica. Tijekom tumorske pretvorbe kloroplasti su se promijenili u amiloplaste ili leukoplaste. U teratomskim stanicama opaženi su amilokloroplasti. U tumorskim i teratomskim stanicama opažene su velike jezgre s uvratima jezgrine ovojnice. Polipeptidi svojstveni listu od oko 22–, 21–, 20– i 18 kDa nisu uočeni u tumorskom tkivu u kojem se također smanjuje sadržaj enzima ribuloza–1,5–bifosfat karboksilaza (Rubisco). Polipeptid od oko 85 kDa bio je prisutan samo u transformiranom tkivu. Što se tiče ostalih proteina pretežno su uočene kvantitativne razlike.

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