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The Expression of Tumour Markers in Intraspecific Somatic Hybrids of Normal and Crown Gall Cells from *Nicotiana tabacum*

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Summary. Following fusion of protoplasts from crown gall tumour calli, characterized by hormone independent growth, and protoplasts from normal tissues of a streptomycin-resistant mutant, SR1, we selected hormone independent streptomycin-resistant calli in *Nicotiana tabacum*. The tumour line, B6S3, lost the ability to form shoots. Some of the selected lines, similar to SR1, however, are morphogenic. Both calli and shoots contained the tumour specific enzyme lysopinedehydrogenase. The hybrid shoots are resistant to *Agrobacterium* infection and do not root. These tumorous properties are dominantly expressed in the somatic hybrids.

Key words: Somatic hybrids – Tumour markers – Crown gall – Protoplast fusion – T-DNA – Tobacco

1 Introduction

Fusion of plant protoplasts is readily achieved with the poly-ethyleneglycol/high pH/Ca^{++} methods developed by Keller and Melchers (1973) and by Kao and Michayluk (1974). In many studies, the aim of protoplast fusion experiments is to construct hybrid plants from plant species which are difficult or even impossible to cross sexually. Recently this has been accomplished successfully e.g. for *Nicotiana sylvestris* with *N. knightiana* (Maliga et al. 1977) *Lycopersicum esculentum* with *Solanum tuberosum* (Melchers et al. 1978). *Datura species* (Schieder 1978) and *Arabidopsis thaliana* with *Brassica campestris* (Gleba and Hoffmann 1978).

In our studies, we were interested to know the influence of a normal genome on the tumorous character of crown gall cells in intraspecific hybrids. Recently detailed reviews on plant tumours have been published (Beiderbeck 1977; Braun 1978; Schilperoort et al. 1979). The crown gall tumour disease is caused by bacteria of the species *Agrobacterium tumefaciens*. The crown gall cells used in our study are characterized by the synthesis of octopine, by phytohormone autotrophic growth and by the inability to form shoots. Octopine is a tumour-specific amino acid derivative, the synthesis of which is catalyzed by the enzyme lysopinedehydrogenase. It has been demonstrated that its synthesis is determined by a gene or genes on an octopine Ti plasmid in *Agrobacterium tumefaciens* (Bomhoff et al. 1976). Ti plasmids are essential for the crown gall inducing capacity of *A. tumefaciens* (Zaenen et al. 1974). A fragment of the Ti plasmid, which is called T-DNA, is present (Chilton et al. 1977) and is expressed into RNA in octopine producing crown gall cells (Drummond et al. 1977; Ledeboer 1978). It could be shown that the gene(s) determining lysopindehydrogenase are localised on the T-DNA (Koekman et al. 1979). It is not yet known, however, whether a T-DNA gene directly codes for the enzyme or cooperates with the plant genome to produce the enzyme.

2 Materials and Methods

2.1 Culture Conditions

B6S3 callus tissue was derived from leaf tumours on *N. tabacum* c.v. White Burley. The tumors were induced by the octopine strain B6S3 (LBA2) of *A. tumefaciens* (Bomhoff 1974). The B6S3 tissue was cloned by isolation of protoplasts and cultured on an agar solidified Linsmaier and Skoog medium without phytohormones (LS-H medium; Linsmaier and Skoog 1965). It was kept in culture for several years without greening and morphogenesis. *Nicotiana tabacum* SR1 shoots were the kind gift of Dr. Maliga (Maliga et al. 1973). The shoots, and also tissues derived from these shoots, are resistant to streptomycin (1 mg ml^{-1}). Sterile SR1 shoot cultures were maintained in Erlenmeyer flasks on T-medium (Nitsch and Nitsch 1969) under long day conditions (12 hours light, 12 hours dark) at 2000-4000 lux and a temperature of 28°C. Properties of the parental lines are summarized in Table 1.

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2.2 Protoplast Isolation

B6S3 crown gall protoplasts were isolated from 3 week-old callus cultures. The callus tissue was cut into small fragments and incubated in petri dishes containing the enzymes cellulase R-10 (2% w/v) and macerozyme R-10 (0.5% w/v) (Kinki Yakult MFG-Co., Ltd. Japan), dissolved in K3 culture medium (Nagy and Maliga 1976).

SR1 protoplasts were obtained from leaves of sterile SR1 shoots which were cut into small fragments. The fragments were incubated in an enzyme solution of the same composition as used for B6S3 protoplasts. Digestion of the callus and leaf tissue was complete after 16 hours at 20°C in the dark. After digestion the protoplasts were freed from debris by subsequent filtration through 500 μ and 125 μ stainless steel filters. The filtrate was centrifuged at 50 xg for 5 minutes which caused flotation of the protoplasts to the top of the solution. The lower solution, containing fine debris and the pellet, was removed and the remaining band containing the protoplasts was washed three times with K3 medium.

2.3 Protoplast Fusion

Protoplast fusion was done as described by Power et al. (1976) with the following modifications. The protoplasts of both fusion partners were resuspended in a fusion medium containing 140 mM NaCl, 5 mM KCL, 0.75 mM Na_2HPO_4, 5 mM glucose and 125 mM $CaCl_2.2H_20$, pH 7.0. The B6S3 and SR1 protoplasts were mixed in a 1:1 ratio in 2 ml of the fusion medium (final concentration approximately 10^6 cells ml^{-1}) in a centrifuge tube. The cells were allowed to sediment for 5 minutes, and then 2 ml polyethyleneglycol (PEG, MW 6000) dissolved in fusion medium, adjusted to pH 7 with NaOH-glycine, was added giving a final concentration of 40% PEG. After 30 minutes incubation the PEG was gradually diluted by adding, at 5 min. intervals, fusion medium adjusted to pH 10.5, in volumes of 0.5 ml, 0.5 ml, 1 ml, 2 ml and 4 ml. The protoplasts were resuspended after each addition. Finally the protoplasts were collected by centrifugation at 50 X g for 5 minutes and were resuspended in K3 medium supplemented with streptomycin (1 mg ml^{-1}). As controls, B6S3 and SR1 protoplasts were treated separately in the same way, whereas a mixed population of cells was treated without addition of the fusion agents. The cells were cultured in the dark for 5 days at 28°C followed by a culture period of two to three weeks at 2000 lux.

2.4 Infection of Hybrid Shoots with A. tumefaciens

Normal SR1 shoots and hybrid shoots were infected with virulent strains of *A. tumefaciens*. This test used the LBA4013, an octopine strain derived from Ach5 (Klapwijk et al. 1978), and the LBA4058, a nopaline strain derived from a cross between LBA8374 (pT37 $Noc^c tra^c$) and LBA4011 (pAch5C3 rif^R) (Hooykaas, unpublished results). Suspensions of the bacteria were injected into the stems of the shoots. Tumour formation was scored after four weeks.

2.5 Lysopinedehydrogenase Assay

Samples of about 10 mg of callus or leaf tissue of the parental and hybrid cell lines were homogenised and the lysopinedehydrogenase (LpDH, E.C. 1.5.2.-) activity in the extracts was tested according to the method of Otten et al. (1978).

3 Results

3.1 Selection of Hybrid Cells

Protoplasts from SR1 leaf tissue and B6S3 callus tissue can be distinguished by the chloroplasts of the SR1 cells and cytoplasmic strands of the B6S3 cells. These visual markers allow the recognition of heterokaryons by microscopic observations (Fig. 1). Heterokaryons were observed 24 hours after fusion at a frequency of 1 to 2% of the cells present in the fusion mixture.

Following the fusion treatment, the mixture of SR1, B6S3 and fusion products was plated in liquid K3 medium with auxin and cytokinin and with streptomycin. This step permitted division of unfused SR1 or homokaryon SR1 protoplasts and of B6S3 + SR1 fusion products. During culture small colonies were formed which were plated for a second selection on solid, streptomycin-containing (1 mg ml^{-1}), K3-H (hormone free) medium. At this stage most of the cell clumps died within four weeks. A few

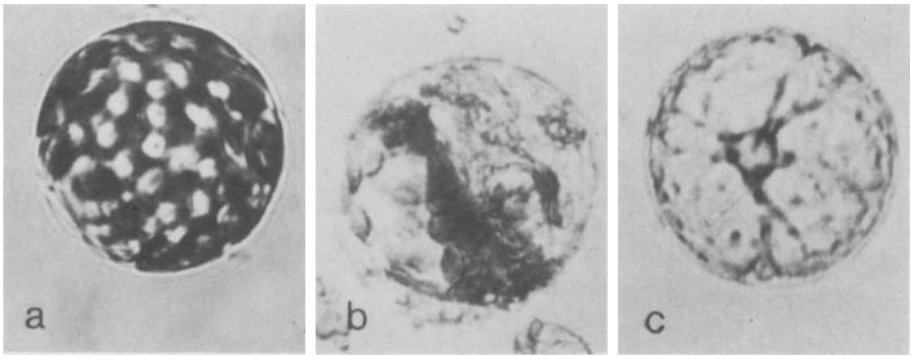

Fig. 1a-c. Fusion of isolated protoplasts from *Nicotiana tabacum* a, *Nicotiana tabacum* SR1 protoplast; b, SR1 + B6S3 heterokaryocyte; c, *Nicotiana tabacum* B6S3 protoplast

colonies, however, continued to grow and developed small callus tissues within 8 weeks (Fig. 2). Two independent fusion experiments yielded 4 and 12 colonies respectively after the second selection step. Each of these lines were obtained from about 10^5 cell aggregates that survived the first selection step. Hormone-independent, streptomycin-resistant colonies were not isolated from control plates in which SR1 and B6S3 protoplasts were cultured under selective conditions, either as separate cultures or as mixed cultures.

3.2 Shoot regeneration

All 16 independent lines of possible hybrid nature were maintained in culture on LS-H medium in the presence of streptomycin. Four green callus tissues started regeneration within two months after the first subculture on LS-H medium. They were cut and subcultured on LS-H medium. The morphology of the shoots is shown in fig. 3. The most remarkable difference between these shoots and the original SR1 shoots was the absence of rooting in the hybrids. In addition, the hybrid shoots remained shorter, had thicker stems and leaves than SR1 shoots and showed considerable spontaneous sprouting, suggesting decreased apical dominance. Regeneration was observed neither on the twelve yellowish lines, nor on the parental B6S3 tissue under the same conditions. Callus derived from SR1 protoplasts readily regenerates (Table 1). To study the hormone requirement of the individual cells, protoplasts were isolated from hybrid shoots and were compared with SR1 protoplasts. They were plated in liquid K3 medium and K3-H medium. It was found that protoplasts, both from hybrid and SR1 origin, proliferated only in the presence of hormones (naphthalene acetic acid 3 mg l^{-1}, 2.4-D 0.1 mg l^{-1} and kinetin, 0.04 mg l^{-1}). Taking into account a plating efficiency of 70%, all hybrid protoplasts developed callus tissues on K3 medium. After two passages approximately 200 individual callus tissues were transferred to LS-H medium where they all continued to grow. Callus tissues derived from SR1 protoplasts all became brown and died on LS-H medium. Through this treatment, a second generation of green, hormone independent calli was obtained from the hybrid protoplasts and all regenerated shoots on LS-H medium. Whenever these callus tissues were kept on LS medium supplemented with hormones, regeneration was not observed.

3.3 Determination of LpDH Activity

Callus tissues of B6S3, SR1 and B6S3 + SR1 hybrids, and leaf tissues of SR1 and B6S3 + SR1 hybrid shoots, have been screened for the presence of LpDH. Fig. 4 shows an example of one of these tests. LpDH activity was found in all 16 selected lines (Table 1). The enzyme was also present in the four different hybrid shoots that have been regenerated. LpDH was not found in extracts of SR1 shoots or in SR1 callus tissue. A number of (50) individual callus tissues from leaf protoplasts of hybrid B6S3

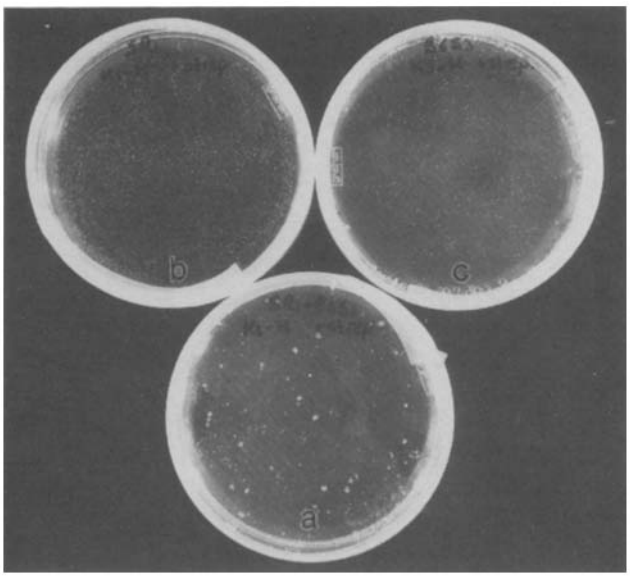

Fig. 2a-c. Selection of hormone independent, streptomycin resistant calli on K3-H medium supplemented with streptomycin (1 mg ml^{-1}); a, plate with aggregates from the fusion treatment; b, control plate with SR1 protoplasts; c, control plate with B6S3 protoplasts.

Table 1. Properties of phytohormone independent calli obtained after fusion of B6S3 protoplasts and SR 1 protoplasts

Cell strain		Sm resistance	Hormone-independent growth	LpDH	Regeneration	Colour
B6S3		–	+	+	–	yellow
SR 1		+	–	–	+	green
B6S3 + SR 1	42-1	+	+	+	–	yellow
	42-2	+	+	+	+	green
	42-3	+	+	+	+	green
	42-4	+	+	+	+	green
B6S3 + SR 1	60-1	+	+	+	–	yellow
	60-2	+	+	+	+	green
	60-3	+	+	+	–	yellow
	60-4	+	+	+	–	yellow
	60-5	+	+	+	–	yellow
	60-6	+	+	+	–	yellow
	60-7	+	+	+	–	yellow
	60-8	+	+	+	–	yellow
	60-9	+	+	+	–	yellow
	60-10	+	+	+	–	yellow
	60-11	+	+	+	–	yellow
	60-12	+	+	+	–	yellow

Fig. 3. Somatic hybrid shoots regenerated from SR1 + B6S3-42-3 hybrid callus tissue (left); normal *Nicotiana tabacum* SR1 shoots (right)

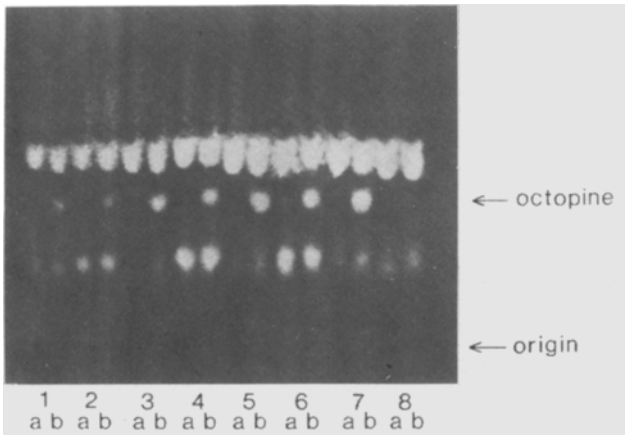

Fig. 4. Electropherogram of the products present in the reaction mixture to detect LpDH activity in extracts of different hybrid callus tissues and shoots. Channels a : reaction mixture at t = o; channels b : reaction mixture at t = 60 minutes. Channels 1 : B6S3 callus tissue; 2 : SR1 + B6S3-42-1 callus; 3 : SR1 + B6S3-42-1 shoots; 4 : SR1 + B6S3-42-3 callus; 5 : SR1 + B6S3-42-3 shoots; 6 : SR1 + B6S3-32-4 callus; 7 : SR1 + B6S3-42-4 shoots; 8 : SR1 shoots

+ SR1 42-3 and 50 callus tissues from protoplasts of the corresponding hybrid callus were also tested for the presence of LpDH. These tissues were taken at random from the 200 hormone independently growing tissues described before. All these independent, cloned hybrid tissues contained LpDH activity. These data indicate that the genetic information for LpDH activity is present in all individual cells of the hybrid shoots.

3.4 Infection of Hybrid Shoots with A. tumefaciens

When SR1 shoots were infected with the virulent *A. tumefaciens* strain LBA-4013, well developed tumours were present at the wound site within four weeks. No tumours arose, however, when the B6S3 + SR1 42-3 hybrid shoots were infected with this bacterial strain. Figure 5 shows SR1 shoots and a hybrid shoot infected with *A. tumefaciens*. The hybrid shoot (the middle one) did not undergo any remarkable change upon bacterial infection, whereas the SR1 shoot has developed tumours and is in a very bad condition. Infection of the hybrid shoot with a virulent nopaline bacterial strain (LBA4058) also did not result in tumour formation (result not shown).

4 Discussion

Parental protoplasts of both types gave rise to cell colonies in K3 medium with about 70% efficiency. The 16 hybrid colonies have been selected out of a total of 2×10^5 parental cells, so the frequency of hybrids obtained from the two experiments together may be estimated at approximately 1×10^{-4}. However, because of variation in batches of protoplasts, the aggregation of protoplasts during fusion and cell death, it is difficult to accurately assess the frequency of fusion.

The selected lines can be divided into two classes. To

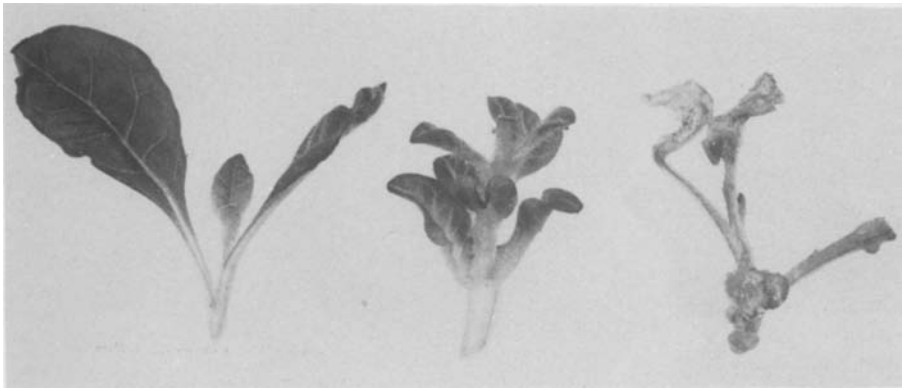

Fig. 5. Susceptibility to *Agrobacterium tumefaciens* strain LBA4013 of SR1 shoots and SR1 + B6S3 hybrid shoots. Left, SR1 shoot, not infected; Middle, SR1 + B6S3 hybrid shoot, infected; Right, SR1 shoot, infected

one class belong the 4 green calli that developed shoots. The combination of greening, regeneration and streptomycin-resistance on the one hand and the presence of LpDH activity and phytohormone independent growth on the other hand mark these callus tissues as hybrids. The second class concerns the calli that are yellowish, streptomycin-resistant, LpDH-positive and without regeneration. These lines can be distinguished only by their streptomycin resistance from the parental B6S3 tissue. Streptomycin resistance of the SR1 parental tissue is a cytoplasmic marker (Maliga et al. 1975), therefore the resistance observed in these lines need not to be due to nuclear fusion. It is important, however, that the spontaneous mutation rate for streptomycin resistance should be much lower than 10^{-4}, because in control experiments no streptomycin-resistant B6S3 lines were isolated. Unfortunately, the fusion of cells of the same species excludes the possibility of using other biochemical or cytological markers to prove the hybrid nature of the selected lines.

Development of shoots from the selected calli indicates that the lines are true hybrids. That is so because hybrid formation (nuclear fusion) between morphogenic and non-morphogenic cell types resulted in shoot-forming calli as described by Maliga et al. (1977) for *Nicotiana* and by Schieder (1978) for *Datura*. The ability to regenerate shoots apparently is also dominant when one of the parents is a tumour cell. The fact that all calli derived from the protoplasts have the property of synthesizing LpDH and growing hormone-independent rules out the possibility that these shoots developed from a mixture of parental cells having survived the selection procedure and shows that the genetic information for the tumour markers is present in all the cells.

Synthesis of LpDH could be shown in the shoots regenerated from the selected calli. The expression of this Ti-plasmid specified function is therefore not always coupled with the maintenance of neoplastic growth. The differential expression of these functions is also described by Wood et al. (1978). They found that leaf fragments from *nopaline* crown gall derived teratomas did not require hormones for callus formation, while pith explants required auxin, but no cytokinin, for only the first passage in tissue culture. These findings, however, are different from results we have obtained with *octopine* producing tissues of the B6S3 + SR1 hybrids. Leaf and pith sections taken from the hybrid shoots gave rise to shoots without callus formation on hormone free medium, whereas from the same explants fast-growing callus could be obtained only on medium containing both auxin and cytokinin (Wullems et al. 1979). Our findings, and those of Wood, demonstrate that the expression of the neoplastic condition of tumour tissue is influenced by the presence or absence of phytohormones in the culture medium.

The observed resistance of hybrid shoots to infection with *Agrobacterium tumefaciens* might be related to a changed cell wall composition, as has been postulated for crown gall cells by Lippincott et al. (1978) who studied the attachment of *Agrobacterium* on isolated cell walls from crown gall and normal cells. They found that *Agrobacterium* does not attach to cell walls from crown gall cells. This also may be the reason that tumours are not inducable on the B6S3 + SR1 hybrid shoots.

Concerning the LpDH activity, the morphogenic potential and the resistance to *A. tumefaciens*, the hybrid shoots have the same properties as shoots that developed from octopine-producing calli obtained after in vitro transformation of SR1 protoplasts by *A. tumefaciens* (Marton et al. 1979). Interestingly, root development has not been observed in that case either. This suggests that the suppression of root formation is a dominant tumour character in the hybrids.

The techniques of in vitro transformation of protoplasts and protoplast fusion, combined with genetic engineering of the Ti-plasmid using the recombinant DNA technology, may offer possibilities for genetic engineering in higher plants through the integration of desired genes into the plant genome. Fusion of undifferentiated tumour cells, carrying foreign genes, and of protoplasts prepared from normal plants may result in regenerants in which these foreign genes are expressed.

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