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Plant tumor reversal associated with the loss of marker chromosomes in tobacco cells

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Summary. In single cell clones from a tumorous tobacco strain and in plants regenerated from them, decrease or loss of tumor virulence is associated with the decrease or elimination of specific marker chromosomes. Cells without marker chromosomes called "normal cells", are obtained. Plants regenerated from strongly tumorous clones are obtained either from a mixed population of normal and weakly-tumorous cells or from normal cells only. In the first case, plants are weakly tumorous and in the second they are non-tumorous, like the check sample. Plant tumor reversal could be explained by such a mechanism.

Key words: Plant tumor – Recovery – Regenerate – Regression – Tumor virulence – Marker chromosome – Tobacco

Introduction

When recovery from the tumoral shape occurs, transformed cells revert to a normal state. Examples of this phenomenon are uncommon in animals and unusual in plants. Braun (1969) observed the loss of tumor virulence in plants regenerated from Crown-gall tissues but the existence in these tissues of normal cells from which plants can be obtained, cannot be excluded (Gautheret 1975). From anthers of teratoma plants regenerated from Crown-gall, Turgeon and al. (1976) obtained haploid plants devoid of any virulence. Recovery occurs during meiosis. Another proof of recovery is given by Lutz (1969): from strongly tumorous single cell clones issued from Morel's so-called habituated cultures, this author obtained non-tumorous plants; similar results were obtained by Sacristan et al. (1969, 1977).

Nevertheless, we observed that some plants regenerated from strong or weak tumorous clones produce, with grafting either weak tumors or none at all.

Since the tumoral state is inherited from cell to cell, genetic analyses are a means of studying the regression or recovery from the tumoral state. The specific marker chromosomes of tumorous cells in tobacco strain could supply the tool for this analysis. Tumor virulence and marker chromosomes are in fact linked: when the number of marker chromosomes increases the tumor virulence of tissues is shown by the size of the tumors, a decreased number of marker chromosomes being indicated by weak tumors (Mouras 1981). This fact suggests that regression or loss of tumor virulence could be associated with the decrease or loss of these marker chromosomes. Moreover marker chromosome elimination could lead to "normal cells" and perhaps to plant regeneration from "normal cells".

The presumption that the strain 'Tabac anergié', so-called habituated culture, is not habituated but an erroneously classified Crown-gall strain, does not modify in any way the results reported herein.

The pattern of tumor virulence regression and recovery from the tumoral state has been analysed from caryological investigations and from experiments which clearly demonstrate the tumorous or non-tumorous character of the tissues.

Material and methods

Material

Experiments are performed with two types of material:
(i) strongly tumorous clones of tobacco (A14 and S17) and plants issued from them.
(ii) weakly tumorous and highly organogenetic clones (C17 and A14-5) or a strain (S17/K).

Clones A14, S17 and C17 (Lutz and Belin 1974) are issued from single cells isolated from a tobacco strain 'Tabac anergié' (Morel 1948). The clones A14 and S17 have in the past produced shoots but this property is now either lost (A14) or sporadic (S17). The C17 clone is still producing numerous shoots. A14-5 is a highly organogenetic sub-clone derived from a single cell of A14 clone.

The S17/K strain results from regenerated S17 plant fragments cultivated on basal medium with added growth factors.

Culture mediums

Some clones are cultivated on Murashige and Skoog's medium with auxin and cytokinin (S17 and C17) and others with only cytokinin (A14 and A14-5) for shoot induction. The plants regenerated from A14 and S17 clones grow on mineral Murashige and Skoog medium.

The S17/K strain is cultivated on the basal medium (Knop macro-nutrients, Heller micronutrients vitamin B_1 (1 mg/l), sucrose (35 g/l)) supplemented with 2,4-D (0.1 mg/l) and kinetin (1 mg/l). This strain subcultured on basal medium has been giving plentiful shoots for years.

Methods

The tumorous character of cells and tissues can be determined by only two methods (Gautheret 1975):
(i) grafting of cells on normal plants (tobacco)
(ii) cultivation of cells (or tissues) on a medium which does not allow the development of normal cells (basal medium)

Growth test. Normal cells require auxin and cytokinin for their growth but tumorous cells do not. Thus the basal medium free of growth factors is a test medium.

Grafting test. Tumor virulence is shown by using grafting tests according to the method of Limasset and Gautheret (1950). 12 grafts are performed for each test and another 12 are made when results are doubtful. The results recorded 4 months later are expressed by the mean diameter of tumors in millimetres. The diameter of tobacco stems on which grafts are performed is generally 10 mm and for this reason the diameter of tumors produced are equal or greater than 10 mm respectively for negative or positive responses. For example, a weak tumor shows an outgrowth of 1 or 2 mm from the scar corresponding to the inoculation point of the grafted tissue. In a strong tumor the outgrowth can reach 50 to 60 millimetres (Fig. 3).

Chromosome analyses. Caryological analyses are carried out with a new method using protoplasts (Mouras et al. 1978). Callus and shoots of regenerated plants are analysed by this new technique which make the morphological analyses of chromosomes easier. Thus, 3 dicentric chromosomes specific of tumorous cells (marker chromosomes) have been identified (Mouras and Lutz 1980) but the *m* element cannot be precisely characterised (Fig. 1). Nevertheless statistical treatment of the results suggests a similar origin for the *m* element (Mouras 1981). The number of analysed cells and the results of marker chromosome counting are reported in Table 1.

Results

We recall the results obtained on a tobacco tumorous strain and the clones issued from it (Mouras 1981):
(i) only tumorous cells contain marker chromosomes represented particularly by 3 dicentric chromosomes called m-mx, T2-m and m-a (Fig. 1). These marker chromosomes result from translocation.
(ii) the number of marker chromosomes are unchanged over a period of years.
(iii) the shapes of marker chromosomes are stable.
(iiii) the tumor virulence of clones varies in the same way as the number of marker chromosomes (regression line).

Results of experiments concerning marker chromosome counting propensity to grow on the basal medium and responses to grafting tests are reported in Table 1, Figs. 2 and 3.

Clone A14: callus and plants

On the basal medium the propensity to grow is greater with callus than with stem explants. In this latter case the tissues formed on stem explants grow slowly

Table 1. Results of caryological analyses and grafting experiments. (abnormal cells: cells with marker chromosomes)

		S_{17} clone		A_{14} clone		Organogenetic callus		
		Callus	Plant	Callus	Plant	$S_{17/K}$ (strain)	C_{17} (clone)	A_{14-5} (clone)
Cell number		26	15	54	20	39	25	47
Abnormal cells		26	8	52	16	30	19	21
%		100	53.3	96.3	80	76.9	76	44.7
Average number of dicentric chromosomes per cell	m – mx	1.80±0.27	0.47±0.35	1.35±0.20	0.65±0.32	0.72±0.19	0.20±0.17	0.34±0.16
	T_2 – m	0.50±0.15	0	0.39±0.13	0.05±0.10	0.33±0.18	0.08±0.11	0.24±0.12
	m – a	0.50±0.15	0.13±0.20	0.26±0.12	0.40±0.28	0.02±0.05	0.08±0.11	0.02±0.04
Average number of chromosome markers per cell		2.80±0.35	0.60±0.28	2.00±0.13	1.10±0.27	1.07±0.15	0.36±0.18	0.60±0.12
Mean tumor diameter		45 ±6.80	11 ±1.31	35 ±6.60	12 ±1.64	12 ±1.54	11 ±0.94	11 ±0.94

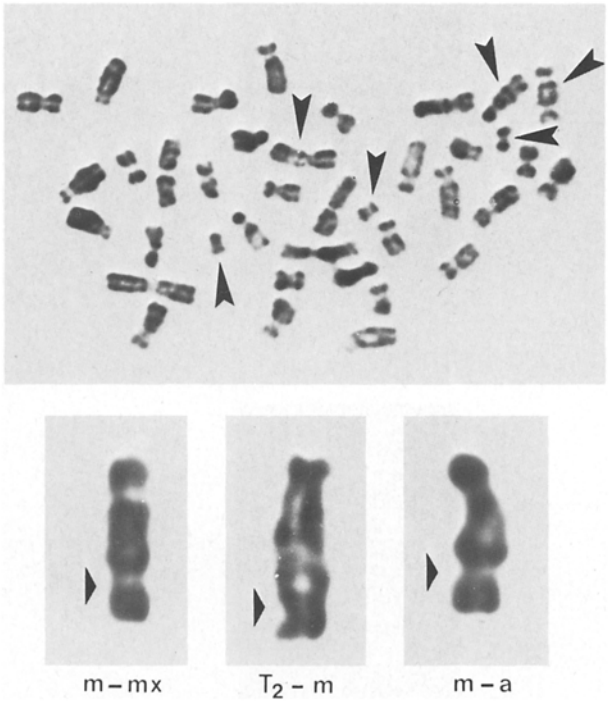

Fig. 1. Example of a metaphase plate in a cell of A_{14} clone: broken and translocated chromosomes are indicated by an arrow; the 3 marker chromosomes ($m - mx$, $T_2 - m$ and $m - a$), specific of tumorous cells, show the m common element

(Fig. 2 a1, a2). On the same medium plant, stem fragments taken on seedlings (check sample) do not produce any calli and brown rapidly (Fig. 2 d).

If the medium is supplemented with auxin and cytokinin, growth is better for tissues formed on stem fragments and unchanged for A14 callus.

These results show that callus and plant stem fragments do not require growth factors. However, stem explant does not produce enough growth factors to give a good growth.

Grafting tests results vary greatly according to the tissue used (Fig. 3):

(i) callus produces strong tumors.

(ii) plant fragments either produce a very small outgrowth or stay inert like the check sample.

The mean tumor diameter is about three times greater for callus than for plants. Thus the callus is always tumorous and plants weakly tumorous or not tumorous. The process involved in producing plants seems to attenuate or suppress tumor virulence.

From caryological analyses (Table 1) we observed that callus and plants both contain a high percentage of abnormal cells but the average number of marker chromosomes is higher in callus than in plants (twice).

Therefore, the plant organisation accompanied by a decrease in the number of marker chromosomes probably originates in tumorous cells.

Each of the three methods of analysis shows that in the process of organisation tumor virulence is attenuated or suppressed and at the same time the number of marker chromosomes is reduced.

Clone S17: callus and plants

On basal medium, S17 callus behaves similarly to A14 callus. On this medium S17 stem fragments produce a very small callus and some none at all. Most of the stem explants have the same behaviour as seedling stem explants (Fig. 2 d) and are unable to grow on medium free of growth factors.

Grafting test results are similar to those obtained with the A14 clone:

(i) callus is strongly tumorous

(ii) plants are weakly tumorous or not tumorous.

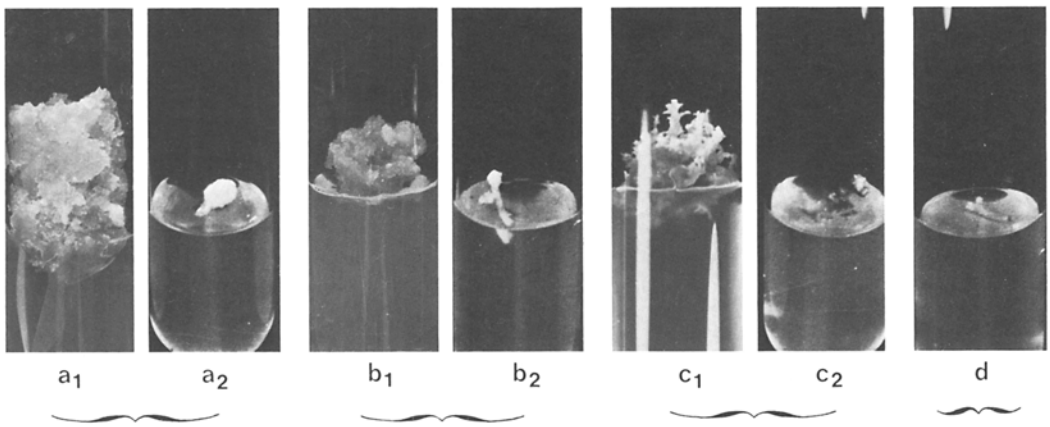

Fig. 2 Growth of callus and stem fragments of regenerated plants on basal medium (auxin and cytokinin free). Clone A_{14}: callus (a_1), stem plant fragment (a_2). Clone C_{17} or A_{14-5}: callus (b_1), stem plant fragment (b_2). Strain $S_{17/k}$: organogenetic callus (c_1), stem plant fragment (c_2). Check sample: stem fragment of seedling (d)

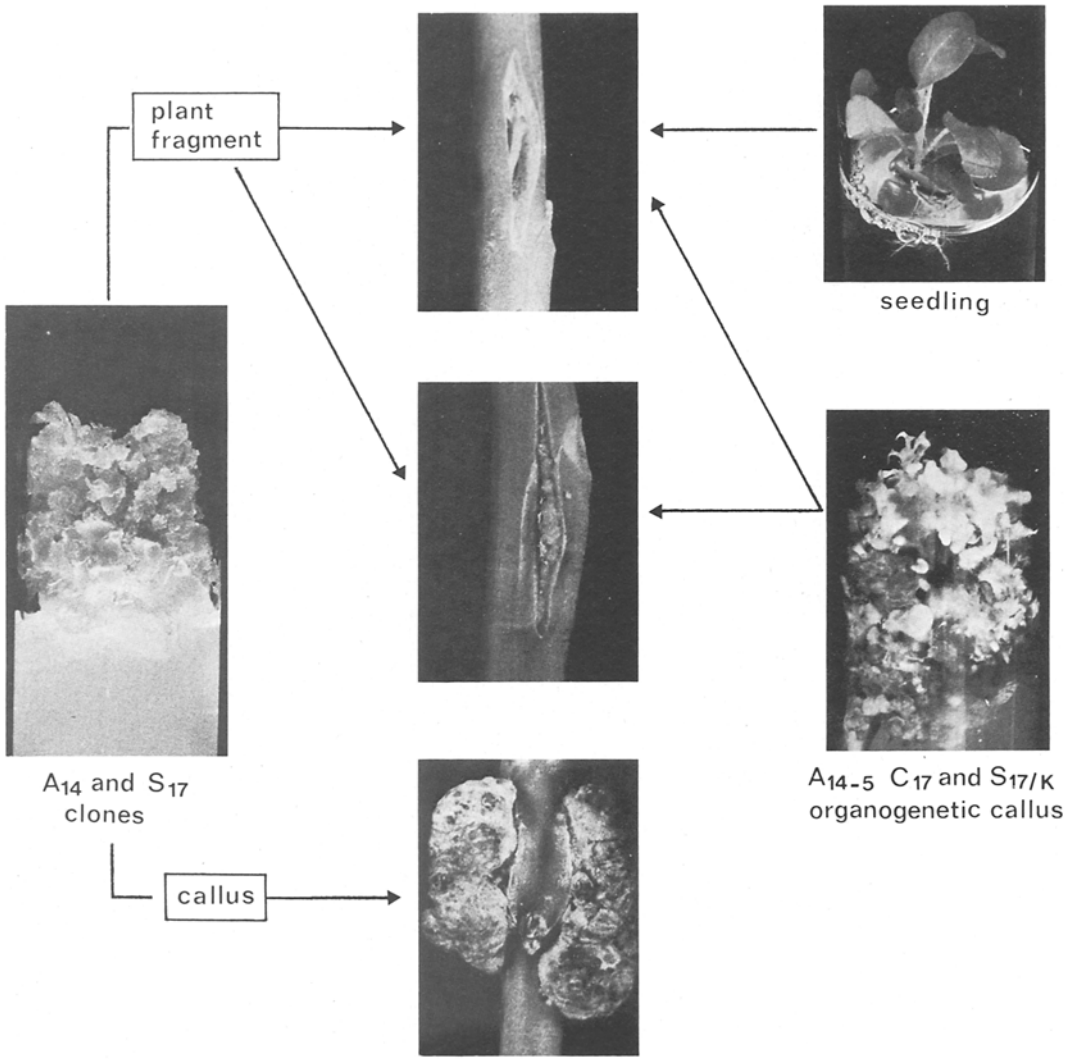

Fig. 3. Responses to grafting tests of callus (organogenetic or non-organogenetic) and tissues cutting off on plants issued from seeds or regenerated from callus

Caryological analyses reveal very great differences between callus and plants:

(i) the percentage of abnormal cells in plants is lower than in callus.

(ii) the number of marker chromosomes in plants is lower than in callus.

Cells devoid of marker chromosomes are called "normal cells" as opposed to abnormal cells. The high percentage of normal cells in S17 plants indicates that plant formation can arise from normal and abnormal cells.

The number of marker chromosomes (four times higher in callus than in plants) correlates with the tumor size (four times larger in callus than in plants).

These results agree with those observed with the A14 clone: plants formed from strongly tumorous callus are weakly tumorous. They contain normal and abnormal cells and few marker chromosomes. The decrease of tumor virulence and the number of these marker chromosomes vary in the same way.

Clones C17 and A14-5

On basal medium the organogenetic tissues of A14-5 and C17 clones go brown as early as the first transfer (Fig. 2 b1) but some plant stem fragments give a very small callus (Fig. 2b2) and some none. These tissues require auxin and cytokinin for their growth.

Grafting tests from plant fragments and organogenetic tissues give the same results (Fig. 3) as those obtained with A14 and S17 plants. Some plants or tissues produce very small outgrowth and some stay inert like the check sample (seedlings).

Caryological analyses of organogenetic tissues reveal a high percentage of normal cells (24 to 55%). This percentage is comparable with that (20 to 47%) of plants regenerated from strong tumorous callus (A14 and S17). Moreover, the number of marker chromosomes per cell is low but comparable to that of S17 plants and very different from that of disorganized tissues (A14 and S17 callus).

Increase in the percentage of normal cells, decrease in the number of marker chromosomes and weak tumor virulence are only observed in such organized tissues as organogenetic clones and plants. This has already been observed with the A14 and S17 clones.

S17/K strain

With the S17/K strain we observed the in vitro behaviour of weak tumorous organized tissues obtained from strongly tumorous clones. This strain obtained by cultivating fragment of S17 plants, has been growing for one year as non-organized tissue. It has become highly organogenetic on the basal medium. This shows that S17/K organogenetic tissues do not require growth factors. However, the growth is slow (Fig. 2 c1).

On the basal medium S17/K plant stem fragments produce calli from all parts of the explants (Fig. 2 c2) but, as with organogenetic tissues, the growth remains slow.

After grafting tests, plant fragments and organogenetic tissues both produce small outgrowths such as those obtained with other organized tissues and principally with the A14 plant. The tumor virulence of this strain is weak but higher than that of the S17 plant.

Caryological analyses (Table 1) reveal abnormal cells with marker chromosomes. The percentage of normal cells (23%) is lower than in S17 plants and the number of marker chromosomes higher than with S17 plants but similar to that observed with A14 plants.

The S17/K strain behaves similarly to the S17 plants but is a little more virulent than S17 plants. The percentage of abnormal cells in the S17/K strain is higher than in S17 plants and these abnormal cells contain more marker chromosomes than found in the S17 plant. Since this strain grows on basal medium and S17 plant on mineral Murashige and Skoog medium it would appear that the basal medium has a selective effect on tumorous cells. This could explain the growth of the S17/K strain on the basal medium while stem fragments of S17 plants produce only a very small callus.

Discussion

From the experimental data two separate states can be distinguished:

(i) non-organogenetic tissues, such as S17 and A14 clones, which do not require growth factors in culture medium, produce strong tumors and contain many marker chromosomes.

(ii) organogenetic tissues and organized cells in plants, such as the A14 and S17 plants, A14-5 and C17 clones and S17/K strain, which do not produce enough growth factors for good growth on the basal medium, produce very small tumors or none at all and contain few marker chromosomes.

It is clear that the organisation process arises from cells which undergo partial or complete loss of tumor virulence. This fact correlates with the decrease or loss of marker chromosomes.

These results require further remarks:

(i) each of the tissues analysed (except S17/K) is a single cell clone. For this reason, the S17 callus, for example, and plants regenerated from it, originate in theory from the same mother cell. Cells in callus and plants probably have the same potentiality. We showed that this is not true: the tissues of regenerated plants are weakly tumorous or not tumorous at all. The callus from which plants are obtained is strongly tumorous.

The single cell origin for each clone used proves the decrease or the loss of tumor virulence from callus to plant throughout cellular differenciation and plant organisation. It excludes any doubt about the recovery from the tumoral state.

(ii) some regenerated plants grow on basal medium (auxin and cytokinin free) although explants taken on seedlings do not.

If cellular multiplication and consequent callus formation is subordinated to the presence of auxin and cytokinin in the culture medium, then explants of regenerated plants synthesize growth factors. However, this synthesis is a property of tumorous cells. Consequently organized tissues contain tumorous cells i.e. the abnormal cells shown in our results. This could also explain why the S17/K strain grows on basal medium better than S17 plant fragments.

The presence of tumorous cells in plants clearly shows that plant organisation takes place from a population of both normal and tumorous cells. However, if tumorous cells are very virulent, they synthesize too many growth factors and this process leads to cell multiplication and prevents differentiation. Thus organisation in plants presupposes a weak virulence of tumorous cells, as observed with our material.

(iii) regenerated plants generally contain cells either with or without marker chromosomes. These cells have a low average number of marker chromosomes (0.6 for S17 plant) and arise from a cell population whose average number per cell is higher (2.8 for S17 callus). In this case, as in others, weakly tumorous cells and tissues can arise from strongly tumorous cells. Con-

sequently, the decrease or loss of marker chromosomes is accompanied by the decrease or loss of tumor virulence. This fact agrees with the relationship between tumor virulence and the number of marker chromosomes (Mouras 1981). On the other hand, if some regenerated plants proceed from strongly tumorous callus and callus from a single cell, then the single cell is tumorous. Since tumorous cells contain marker chromosomes, the loss of tumor virulence can lead to cells without marker chromosomes, considered as "normal cells". Some plants might only contain normal cells and not produce tumors after the grafting test. When plants contain normal and tumorous cells, weak tumor virulence seems to be due to these tumorous cells.

Plant tumor recovery could be explained by such a mechanism. Moreover, the elimination of marker chromosomes in tumorous cells of tobacco recall the loss of foreign T-DNA associated with the plant tumor reversal in Crown-gall tissues (Yang et al. 1980, 1981 Lemmers et al. 1981).

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References

Braun AC (1969) The cancer problem. A critical analysis and modern synthesis. Columbia University Press, New York London

Gautheret RJ (1975) Les phénomènes tumoraux. Masson Ed, Paris

Lemmers M, Holsters M, Engler G, Van Montagu Leemans J, De Greve H, Hernalsteens JP, Willmitzer L, Otten L, Schröder J, Schell J (1981) Hybridation interspécifique et amélioration des plantes. 5. – Le plasmide Ti, vecteur potentiel pour la modification génétique des plantes. CR Acad Agr France 67: 1052–1061

Limasset P, Gautheret RJ (1950) Sur le caractère tumoral des tissus de Tabac ayant subi le phénomène d'accoutumance aux hétéroauxines. CR Acad Sci Ser D 230: 2043–2045

Lutz A (1969) Etude des aptitudes morphogénétiques des cultures de tissus. Analyse par la méthode des clones d'origine unicellulaire. Rev Gén Bot 76: 309–359

Lutz A, Belin C, (1974) Analyse des aptitudes organogénétiques de clones d'origine unicellulaire issus d'une souche anergiée de Tabac, en fonction de concentrations variées en acide indolylacétique et en kinétine. CR Acad Sci Ser D 279: 1351–1353

Morel G (1948) Recherche sur la culture associée de parasites obligatoires et de tissus végétaux. Thèse, Paris, et Ann Epiphyt NS 14: 123–124

Mouras A, Salesses G, Lutz A (1978) Sur l'utilisation des protoplastes en cytologie: amélioration d'une méthode récente en vue de l'identification des chromosomes mitotiques des genres Nicotiana et Prunus. Caryologia 31: 117–127

Mouras A, Lutz A (1980) Mise en évidence d'une relation entre pouvoir tumoral et présence d'un élément chromosomique marqueur chez des clones issus d'une souche anergiée de Tabac. CR Acad Sci Ser D 290: 975–977

Mouras A (1981) Relation entre mutations chromosomiques et pouvoir tumoral chez des clones issus d'une souche tumorale de Tabac. Thèse Université Bordeaux II

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473–497

Sacristan MD, Melchers G (1969) The caryological analysis of plant regenerated from tumorous and other callus cultures of tobacco. Mol Gen Genet 105: 317–333

Sacristan MD, Melchers G (1977) Regeneration of plants from "habituated" and "agrobacterium transformed" single-cell clones of tobacco. Mol Gen Genet 152: 111–117

Turgeon R, Wood HN, Braun AC (1976) Studies on the recovery of Crown-gall tumor cells. Proc Natl Acad Sci USA 73: 3562–3564

Yang F, Montoya AL, Nester ED, Gordon MP (1980) Plant tumor reversal associated with the loss of foreign DNA. In Vitro 16: 87–92

Yang F, Montoya AL, Merlo DJ, Drummond MH, Chilton MD, Nester EW, Gordon MP (1980) Foreign DNA sequences in Crown-gall teratomas and their fate during the loss of the tumorous traits. Mol Gen Genet 177: 707–714