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Phenotypic and genetic variations in crown-gall tumour cells of tobacco

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Summary. Phenotypic and genetic variations of tumour cells were analysed both quantitatively and qualitatively in clones and subclones of a crown-gall strain. Thus, growth rates, grafting tests, octopine synthesis, estimations of the T-DNA contents, modifications in the numbers, and structures of chromosomes were examined. Phenotypic variations are closely associated with genetic changes, including variation in chromosome number (which is shown to be non-specific to the tumoral state) and, above all, variation in the copy-number of T-DNA sequences per cell, and structural rearrangements of chromosomes. Such rearrangements are characterized by specific marker chromosomes in the tumour cells and they correlate with the degree of oncogenicity of the cells.

Key words: Phenotypic/genetic variations – Tumour crown gall cells – Octopine – T-DNA – Chromosome markers

Introduction

Plant cells transformed by the Ti plasmid of virulent *Agrobacterium tumefaciens* become tumorous (crown-gall). Such cells are able to grow indefinitely on media devoid of growth hormones and form tumours when grafted on wild type plants (Braun et al. 1943; Gautheret 1975). The trait is transmitted through cell division, the transforming agent being a fragment of the Ti-plasmid (T-DNA) (Van Larebeke et al. 1974) which is integrated into the host genome (Chilton et al. 1977, 1980) to which it is covalently linked (Tomashow et al. 1980).

Crown-gall tissues may regenerate spontaneously. This is mainly the case with nopaline-type crown-gall tissues (Braun 1959; Braun and Wood 1976; Turgeon et al. 1976) but organized structures of the teratoma-type have been observed in octopine-type crown-gall tissues (Scott 1979; Wullems et al. 1981; Owens 1982). Regenerated plants may exhibit transformed traits (Braun and Wood 1976; Turgeon et al. 1976; Wullems et al. 1981). However, in normal looking fertile regenerants, the tumorous trait is no longer expressed (Turgeon et al. 1976; Yang et al. 1980; Wullems et al. 1981): sequences from the T-DNA responsible for opine genes were still present while genes involved in oncogenicity were absent (Wullems et al. 1981; Yang et al. 1981).

Most of the research on crown-gall carried out up till now has concentrated on the characterization of both the transforming agent and the phenotypic variation of cellular clones and regenerated plants. However, some reports have described the genetic variations of the transformed cells. For example, Sacristan et al. (1977) analysed the variation in the chromosome number of transformed cells.

In this paper, we report on the relationship between the phenotypic and genetic variations in cells from a crown-gall strain in which we have previously demonstrated the presence of marker chromosomes specific to tumorous cells (Mouras 1984). Such marker chromosomes are ideal tools for the study of changes occurring in tumorous cells. The use of single-cell clones allowed us to analyse the phenotypic and genetic variations at both intra- and inter-clonal levels.

Material and methods

Plant material

We used the tumoral strain (CG) isolated by Morel (1948) and described for many years as being a habituated line. In 1980, Yang et al. demonstrated that it was a crown-gall strain as it contained the T-DNA from the plasmid pTi-A6.

The clones M_{13}, A_{14}, S_{17}, M_4, and C_{17} are single-cell clones derived from the CG strain (Lutz et al. 1974). They were 3 to 5

years old when taken for investigation. Among them, some (C_{17}, A_{14-5}) exhibited a permanent organogenic capacity, while others (A_{14}, S_{17}) showed a temporary one. Those retained for observation were coded A_{14p} and S_{17p}. $S_{17/K}$ is an organogenic derivative of S_{17p}. The subclones A_{14-1} to A_{14-7} were derived from the clone A_{14} by mechanical isolation of single cells and were analysed 5 to 6 months after cloning. T_{222} was kindly provided by Dr. Syono and, being a habituated clone to both auxins and cytokinins isolated from *N. tabaccum* var. 'Bright-yellow', served as an additional control in all growth tests. In vitro grown seedlings obtained from *N. tabaccum* seeds were used as general controls.

Grafting tests

The expression of the tumoral growth potential was estimated by means of grafting tests carried out according to the method of Limasset and Gautheret (1950). Twelve to 24 grafts were performed for each callus clone, as well as for the tissue fragments (stem-leaf) taken from regenerated plants. The outgrowth that developed in the region of the graft was considered as the expression of the tumour growth potential. The latter was estimated 4 months after the grafting operation by measuring the diameter of the tumour. This measurement obligatorily includes the diameter of the stem on which the graft was performed (average 10 mm). Thus, a small-sized tumour engendering an outgrowth of 1 to 2 mm has a diameter of 11 to 12 mm, while a large tumour can reach 40 to 50 mm.

Growth tests

Habituated cells are autotrophic for growth regulators. Basal media MS (Murashige and Skoog 1962) and MB (which contains the macroelements of Knop, the microelements of Heller, vitamin B1 at 1 mg/l, sucrose at 35 g/l, and agar at 7 g/l) were used for growth tests. The cultures were exposed to continuous light (approximately 1,500 lux) and were maintained at 25 °C.

Growth rate was estimated at 4 weeks as being equal to $(w-w_o)/w_o$ where w_o represents the initial fresh weight. The measurements were performed after 3 consecutive transfers and each point represents the average of 10 samples, the average weight of the inoculum being 100 µg.

Opine tests

Approximately 200 µg of tissue were used per sample and were treated with 500 µl HCl 1%, for 5 min at 100 °C. Opines were extracted according to Petit et al. (1983) and Dahl et al. (1983).

Chromosome analyses

Chromosome analyses were carried out on cell colonies and young regenerated plants by both the squash method and the karyological method employing protoplasts described by Mouras et al. (1978). The latter method facilitates the morphological study of the chromosomes and has enabled the demonstration of the existence of specific marker chromosomes in tumour cells (Mouras 1981, 1984). These marker chromosomes are absent in normal cells cultivated in vitro and in cells of the root meristem, which served as controls. Twenty to 50 cells were used for the chromosomal analysis of each colony and plant.

DNA analysis

DNA isolation and Southern hybridization were carried out as described in Paszkowski et al. (1984). The probes corresponded to the 16 kb Bam HI fragment of the T_R and the 6 kb Eco R_I fragment of the T_L from the T-DNA (De Vos et al. 1981). The filters were exposed to X-ray films with intensifying screens for 4 to 6 days.

Results

Phenotypic variations in transformed cells

Grafting tests, growth measurements of callus tissues, and opine synthesis were studied.

1 Grafting tests: a measure of the intensity of the tumoral response. Two types of responses were observed:

– in the case of the callus clones (M_{13}, S_{17}, M_4, A_{14}, A_{14-1}, A_{14-2}, A_{14-3}, A_{14-4}, A_{14-6} and A_{14-7}) the tumoral response was always positive, with variations in the amplitude of the response (Fig. 1). In fact, the variation is systematic for each callus after grafting (Fig. 1 a–e): this seems to be determined by the cell composition of the graft. For the clone A_{14}, for example, the diameter of the tumour varies from 28 to 42 mm.

– in the case of the organogenic lines (C_{17} and A_{14-5}) and tissue fragments from regenerants (S_{17p} and A_{14p}) there was either a weakly positive (Fig. 1 g), or a negative (Fig. 1 f) response. In the latter case, the response was similar to that of the controls. These experiments were repeated and the results were confirmed.

Note the difference between the tumoral responses of the grafts initiated from callus and plants regenerated from these calli (Fig. 1 a, f, g). The quantitative results are shown in Table 1. The standard deviations illustrate the variations shown in Fig. 1.

2 Growth measurements. Callus clones and fragments from regenerants from such clones were seeded on basal MS and MB media. The aim of the growth tests were, on the one hand, to establish the level of autotrophy and, on the other hand, to assess the ability of the transformed cells to grow on media with high (MS) or low (MB) salt content.

The results are shown in Figs. 2 and 3. On basal MS, both the callus and the organogenic clones exhibited high growth rates which were quite similar from one clone to another. The explants from regenerated plants and from controls formed a weak callus; only calli derived from S_{17p} and A_{14p} strains continued to grow, at slow rates, after several subcultures. Calli produced by controls became necrotic and died after the second passage.

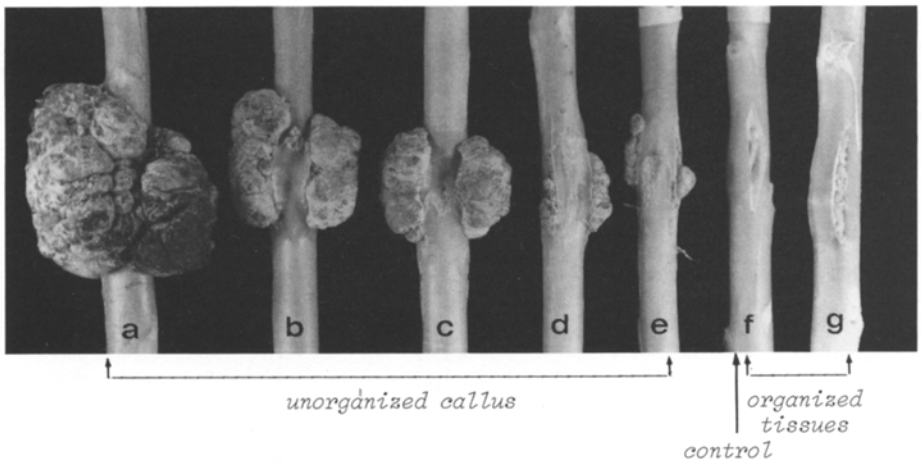

Fig. 1. Grafting tests showing the various degrees of oncogenicity of unorganized and organized cell clones derived from the crown-gall CG strain. Unorganized clones and sub-clones: $a = M_{13}$; $b = A_{14}$; $c = A_{14\text{-}2}$; $d = A_{14\text{-}4}$; $e = A_{14\text{-}3}$. Organized clones or regenerated plants: $A_{14\text{-}5}$ and C_{17}, A_{14p} and S_{17p}. In some cases, explants from A_{14p} and S_{17p} behaved as controls

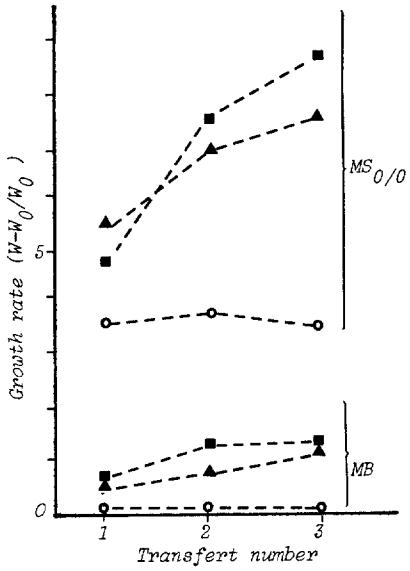

Fig. 2. Growth tests on MS and BM media of unorganized tumour cell colonies and a habituated cell-line (T_{222}). W_o: initial weight of the callus; W: final weight. ▲ clone M_{13} or A_{14}; ■ clone $A_{14\text{-}3}$; ○ clone T_{222}

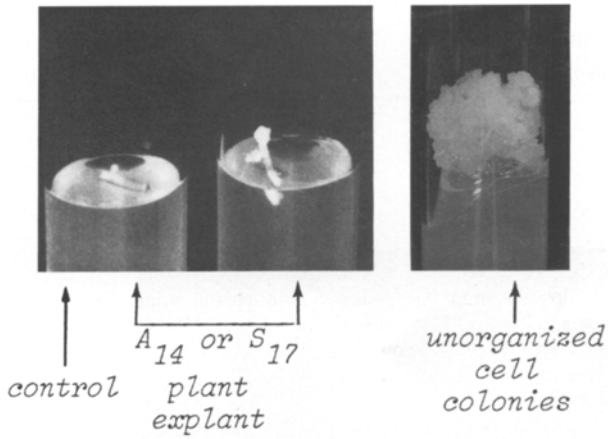

Fig. 3. Growth on BM medium of explants from plants regenerated from tumour cell clones and unorganized callus. Explants from certain regenerated plants behaved as controls (seedlings)

On MB, after a period of adjustment to the change of medium, the callus strains exhibited a slow but steady growth. In contrast, the organogenic strains become necrotic very rapidly, while the explants from regenerants produce a very poor callus, or, as with the controls, no callus at all (Fig. 3).

S_{17p} and A_{14p} show a slow, but otherwise steady growth, which appears to be due to the tumoral cells contained within these plants (Mouras et al. 1983).

It is interesting to note that, contrary to the response in grafting tests (Table 1), there was no significant difference between the growth rates of the highly (A_{14}, M_{13}) and weakly tumoral ($A_{14\text{-}3}$) clones, on either MS or MB media (Fig. 2). Figure 2 also shows that the growth rates on the low ionic strength medium MB were strongly reduced; on this medium the habituated non-tumoral line, T_{222}, stopped growing after the first passage.

In conclusion, the growth measurements, unlike the grafting tests, appeared inappropriate for clearly assessing the degree of tumorigenicity of the various cell-lines tested.

3 Opine synthesis. Opine detection was attempted with the strain CG and with several derivative clones (S_{17}, A_{14}, $A_{14\text{-}3}$, C_{17}, A_{14p} and S_{17p}). Octopine was found in

Table 1. Results of karyological analyses and grafting tests from unorganized and organogenic colonies, and plants derived from the crown-gall CG strain. The standard error of the mean number of marker chromosomes and the mean tumour diameter were calculated respectively from 50 metaphase plates for each clone and from 12 grafts for highly tumourous colonies and 24 grafts for weak tumourous colonies. Normal cells: cells without marker chromosomes

		% of normal cells	Chromosome no.	Mean no. of marker chromosomes per cell	Difference	Observed mean tumour diameter
Highly tumorous cell colonies	M13	6.3	62–97	2.85 ± 1.36		45 ± 7
	S17	0	48–58	2.80 ± 0.93		40 ± 14.3
	M4	5.8	30–54	2.09 ± 1.16		35 ± 11.6
	A14	3.7	38–54	1.98 ± 1.03		35 ± 6.6
	A14-1	7.8	37–46	1.98 ± 1.12		30.5 ± 5.2
	A14-2	6.0	36–45	1.84 ± 1.06		30.5 ± 4.9
	A14-6	2.2	34–44	1.67 ± 0.84	HS	25.5 ± 3.3
	A14-4	13.5	32–44	1.51 ± 1.12	HS	28 ± 4.6
	A14-7	7.7	57–78	1.39 ± 0.69		23 ± 5.4
	A14-3	33.3	63–75	1.04 ± 0.97	NS NS	16 ± 1.4
Weakly tumorous colonies and tissues	A14 plant	20	39–47	1.10 ± 0.79		12 ± 1.6
	S17/K	23.1	45–55	1.07 ± 0.80	S	12 ± 1.5
	S17 plant	46.1	51–54	0.60 ± 0.63		11 ± 1.3
	A14-5	55.3	48–57	0.58 ± 0.75		11 ± 0.9
	C17	24.0	50–55	0.36 ± 0.57		11 ± 0.9
Seedlings (control)		100	48	0		*

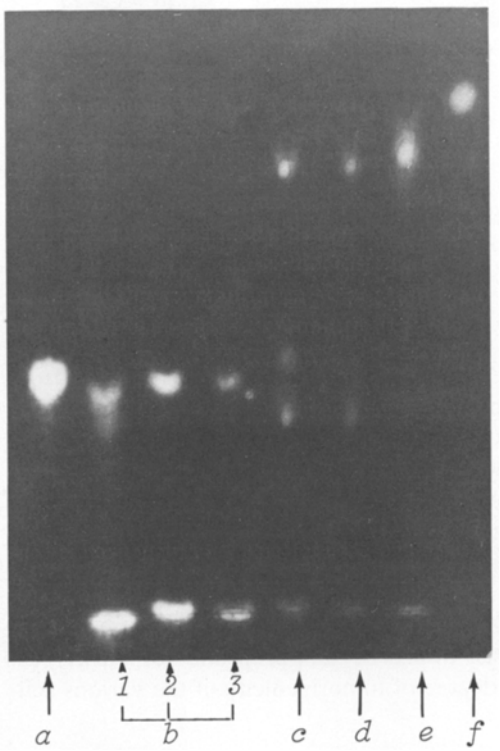

Fig. 4. Octopine synthesis test in the CG strain and clones or subclones derived from it. *a* = octopine (control); *b* = CG strain (b_1: callus 1-week old; b_2: 4-weeks old; b_3: 8-weeks old); *c* = A_{14} clone; *d* = $A_{14\text{-}3}$ clone; *e* = A_{14p}; *f* = arginine (control)

CG only; the other strains contained high levels of arginine. These results were confirmed when repeated with CG, A_{14}, $A_{14\text{-}3}$, and A_{14p} (Fig. 4). Furthermore, the analysis showed that neither agropine nor mannopine were present in these tissues.

Thus, octopine, the biochemical marker characteristic of cells transformed by pT_i-A_6, appeared to be absent from all clonal lines analysed, despite the fact that these lines exhibited a similar, and sometimes stronger tumoral response as compared to the CG strain.

Genetic variation in transformed cells

Clonal and sub-clonal lines obtained from the original CG strain allowed us to correlate the genetic and phenotypic variations. The genetic drift within the lines was estimated by analysing the T-DNA content, and by karyological examination of several clones as well as the whole series of the A_{14}-sub-clones ($A_{14\text{-}1}$ to $A_{14\text{-}7}$). The regression of the tumoral trait was examined with A_{14p} and S_{17p}, regenerated from the corresponding tumoral clones.

1 Variation in the T-DNA copy number. DNA was extracted from the CG strain and from several of its clones and sub-clones. The results of hybridization with defined sequences from either the T_L or the T_R region

of the T-DNA were as follows. Weak signals were observed with the T_R sequences in all of the transformed strains analysed; the detection was at the limit of the resolution, suggesting the presence of T_R sequences, if any, in very low amounts.

On the other hand, probing with sequences from the T_L fragment (Fig. 5) showed that approximately 10, 5, and 2 to 3 copies of these sequences were present in the CG, A_{14} and $A_{14\text{-}3}$ clones respectively. In the case of S_{17p}, we estimated that about 1 copy of the T_L-DNA sequences was present (not shown). Thus, a relatively important variation in the copy number of T-DNA sequences occurred among the tested clones.

2 Genetic drift at the chromosome number level. Metaphase preparations were obtained by both the squash and the protoplast methods. The results were rather similar. The chromosome number distribution shown in Table 1 demonstrates an extensive degree of heterogeneity within each clonal strain, and this with both callus and organogenic structures. In regenerated plants, this heterogeneity was less important than that observed in corresponding callus clones. This aspect is illustrated in Fig. 6 (strains A_{14} and A_{14p}). The corresponding pictures of the tumoral response after grafting are also given. They show that different tumoral phenotypes may correspond to rather similar distribution patterns of chromosomes. In the case shown here, a very strong reduction in tumorigenicity takes place. We have shown previously (Mouras et al. 1983) that this was correlated with certain structural modifications of the chromosomes.

The fact that the analysed clones were 3 to 5 years old at the time of the analysis encouraged us to carry out two additional examinations in order to assess the eventual effects of the length of the in vitro passage on the observed heterogeneity. Thus, karyological analyses of subclones at 6 months and 1 to 3 years later were undertaken. Sub-clones $A_{14\text{-}1}$ to $A_{14\text{-}7}$ exhibited an important heterogeneity of the chromosome number (Table 1), which eventually became higher than that of the parental A_{14} strain. The results reflect the genetic variations arising in the progeny of the A_{14} clone; they also show that every cell line in culture is undergoing important and early variations within the original genetic background. Furthermore, analyses performed 1 to 3 years later showed that, under standardized culture conditions, the chromosomal distributions remained unchanged.

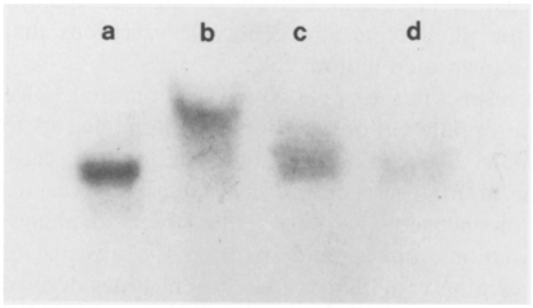

Fig. 5. Blot hybridization of DNA from the CG strain and clones with the probe corresponding to the 6 Kb Bam H_1 fragment of the T_L region within the T-DNA of the plasmid pTi-A_6. *Lanes a* and *b* = CG strain. The plant DNA was restricted with either Bam H_1 (*a*) or Eco R_1 (*b*) restriction enzymes. *Lane c* = Eco R_1 restriction fragment of the DNA from the A_{14} clone. *Lane d* = Eco R_1 restriction fragment of the DNA from the $A_{14\text{-}3}$ clone

3 Genetic drift at the chromosome structure level. The protoplast method of metaphase plate preparation enabled us to detect the presence of chromosomes exhibiting structural changes in the transformed cells (Fig. 7). Such rearrangements mainly concerned di- and polycentric chromosomes resulting from the breakage

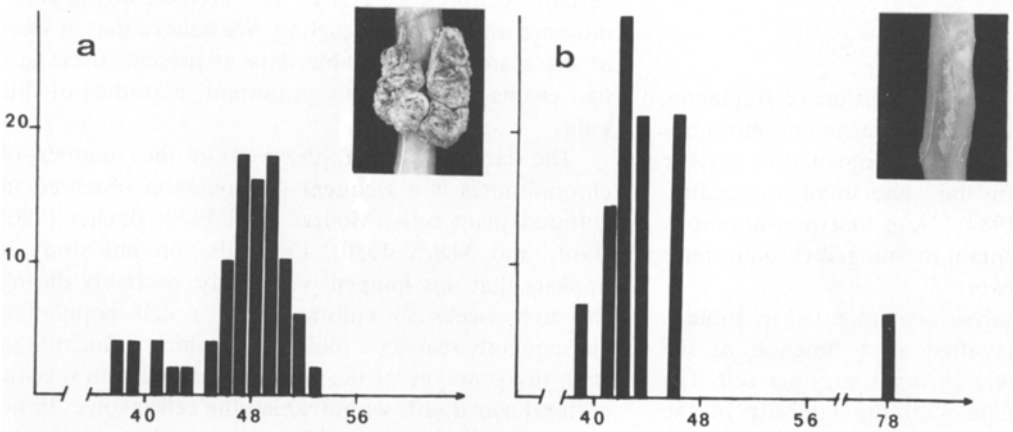

Fig. 6. Chromosome distribution in the A_{14} clone (**a**) and in A_{14p} (**b**). x-axis: chromosome number; y-axis: percentage. Photographs show the graft test results from the tumour callus (A_{14}) and regenerated plant (A_{14p}), respectively

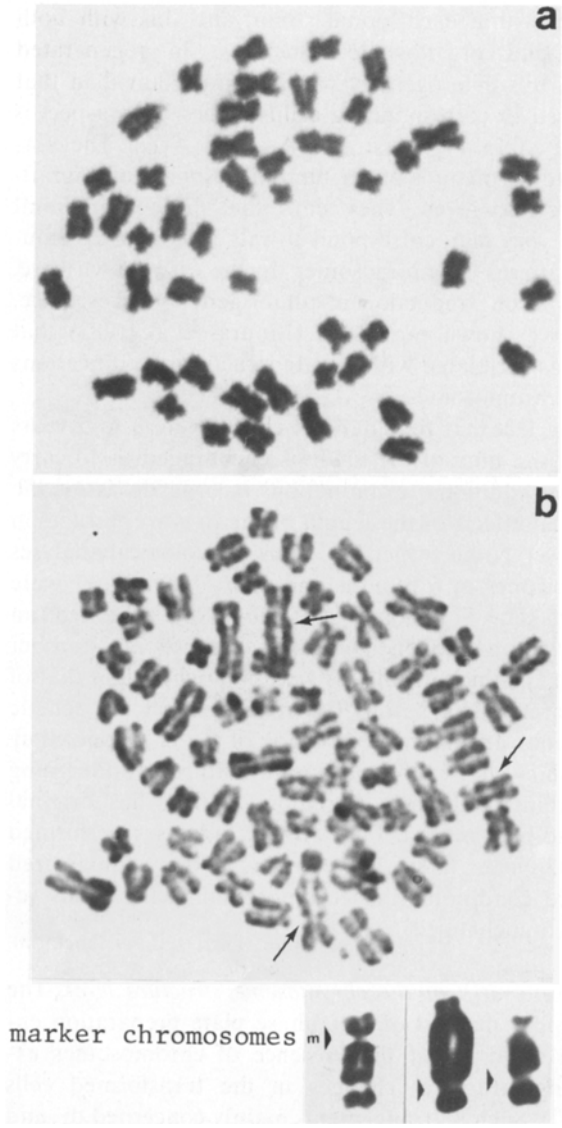

Fig. 7. Metaphase plates showing **a)** the wild type karyotype and **b)** the karyotype of a tumour cell with translocated chromosomes (*arrows*). Rearranged chromosomes specific to tumour cells are dicentric chromosomes designated as marker chromosomes

of chromosomes followed by the fusion of fragments with, or without, centromeres. Dicentric chromosomes specific to the transformed cells (designated as marker chromosomes) all contain the same small metacentric chromosome (Mouras 1984). Note that non-tumorous cells in culture do not contain rearranged chromosomes of the type described above.

The results of the analysis are presented in Table 1. The cell lines were classified as a function of the average number of marker chromosomes per cell. The latter appears to correlate with the intensity of the tumoral response after grafting. There is an important amplitude of the standard deviation for the average number of marker chromosomes per cell; it is very likely that this is the consequence of the great number of cells having no such chromosomes, on the one hand, and, on the other hand, the presence of more than one marker chromosome in other cells (Table 1).

In conclusion, the cause of the observed variations seems to lie both in the variable number of T-DNA copies in the analysed clones and sub-clones, and in the structural modifications of certain chromosomes that are mitotically transmitted within the various clones that we isolated. Further analyses should attempt to correlate the presence of T-DNA sequences with that of marker chromosomes.

Discussion

Using a previously established technique of mitotic plate preparation and of successive series of clonal lines isolated from an original crown-gall strain, we were able to assess both qualitatively as well as quantitatively the phenotypic and genotypic variations that occur in transformed tumour cells.

With respect to the phenotypic variation, the choice of an appropriate culture medium (MB) enabled us to discriminate between tumour and habituated cells, while the grafting tests served to establish the various degrees of oncogenicity. Among the observed phenotypic variations, one should mention the absence of octopine synthesis in all cloned oncogenic lines derived from the tumoral strain isolated by Morel and which was shown to synthesize octopine. Thus, we confirm the observations reported by Yang et al. (1981), Wullems et al. (1981).

Our analysis of genetic variations in cultured plant cells, and particularly in transformed cells, included the identification of T-DNA signals, the reduction in the copy number of T-DNA sequences corresponding to diminished oncogenicity, and their absence in "revertant" cultures, as well as the variation in the chromosome number and structure. We believe that in view of the scarcity of available data at present, these last two events are extremely important in studies of this kind.

The large variation observed in the number of chromosomes is a frequent phenomenon observed in cultured plant cells (Mouras et al. 1979; Bayliss 1980; Binns and Meins 1980). From the present study it appears that this happens very early, probably during the first weeks in culture. Such a cell population subsequently reaches a ploidy level which is function of both the genotype of the parental cell and the specific cultural constraints within which the cells evolve. In no case were we able to establish any correlation between the chromosome number distribution and particular

phenotypic properties. These variations are most likely aspecific, as they can be observed in both tumoral and non-tumoral cells. On the contrary, structural modifications of chromosomes appeared to result from specific and non-specific rearrangements. As a matter of fact, we demonstrated (Mouras 1984) the presence of specific marker chromosomes in tumoral clones that were directly associated to the tumoral state: the loss of such chromosomes resulted in a change to a non-tumoral condition. The results resemble those reported with mammalian cells (Rowley 1977; Mitelman and Levan 1978), and bring experimental support to speculations made by Yang et al. (1981) and Binns et al. (1982) with respect to the heterogeneity of transformed cell populations and the existence of degrees of oncogenicity. In our case, this is better illustrated by the variation in the T-DNA copy number at the intra-clonal level, as described for the clones A_{14} and $A_{14\text{-}3}$. It should also be noted that, although oncogenic, these clones, unlike the original crown-gall strain isolated by Morel, do not synthesize octopine. As shown by Van Lijsebetens et al. (1986), this can result from the loss of the corresponding genes. This remains to be demonstrated in the case of our clones.

As already found by Wullems et al. (1981) and Yang et al. (1981), and in disagreement with results reported by Binns et al. (1982), clones derived from strains transformed by pTi-A_6 can simultaneously regenerate plants that may, or may not, exhibit the tumoral traits. As shown here, the first class of regenerants contain both T-DNA sequences and marker chromosomes.

In order to definitively demonstrate the specific relationship between the tumoral state, the presence of T_L-DNA sequences and that of marker chromosomes, such sequences must be localized within marker chromosomes by in situ hybridization. We have recently developed an in situ hybridization technique for a low-copy number foreign gene (the Tn-5 Kanamycin-resistance gene) on metaphase plant chromosomes (Mouras et al. 1987), and experiments are under way using the transformed cells reported here.

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