Theor Appl Genet (1989) 78: 715–720

Localization of the T-DNA on marker chromosomes in transformed tobacco cells by in situ hybridization

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Received March 2, 1989; Accepted June 27, 1989
Communicated by Yu. Gleba

Summary. Chromosome and molecular analyses were conducted on tobacco cells which had been transformed by the T-DNA of the Ti-plasmid. These analyses showed that there were specific chromosome rearrangements in the transformed cells (marker chromosomes). There was a positive correlation between the number of marker chromosomes per cell and the oncogenic potential of the transformed cells. However, we show, using the Southern hybridization method, that the T_L fragment of T-DNA, but not the T_R, clearly hybridizes with nuclear DNA. In situ hybridization was used to locate the insertion site of T-DNA: the hybridization signal was found on a small metacentric chromosome. This chromosome may occur single or translocated onto other chromosomes, to make marker chromosomes. Thus, by locating the T-DNA, we have confirmed the correlation between the marker chromosomes and the oncogenic potential.

Key words: Crown-gall cell – In situ hybridization – Marker chromosome – T-DNA

Introduction

During cell transformation by the bacteria *Agrobacterium tumefaciens*, part of the Ti-plasmid, the T-DNA, is transmitted to the cell and combines into the genome of the host cell in a stable fashion (Chilton et al. 1980; Tomashow et al. 1980). The majority of research on this transformation focused on the transforming element, e.g. it has been possible to determine the precise genetic structure of the T-DNA using molecular techniques (De Vos et al. 1981). Other works concentrated on the physiological changes of the transformed cells, i.e. on investigating to what extent the transformed trait is inherited or reverts to the wild phenotype. The reversion is usually associated with a virtually complete elimination of the T-DNA (Wullems et al. 1981a, b; Yang et al. 1981).

Nevertheless, the ways in which the transformation actually occurs, in particular the way in which the T-DNA is integrated, remain more or less unknown. Similarly, the location of the integration site has so far not been ascertained. It is only recently that it has been possible to envisage doing so with the perfecting of an in situ hybridization technique on metaphase chromosomes. This technique has made it possible to visualize DNA fragments approximately 20 kb long (Mouras et al. 1987a) and more recently 1–2 kb long (Mouras et al. 1989). We have attempted to locate the insertion site of the T-DNA into transformed cells, which we had previously examined cytogenetically. From these tests we have been able to show that there are multiple translocations of chromosomes, and that within these rearranged chromosomes, there are marker chromosomes whose presence and number correlate closely with the oncogenic potential (Mouras 1981).

We put forward the following hypothesis: supposing that the mathematical correlation is pertinent, then the oncogenic element (T-DNA) should be present on the marker chromosomes. To investigate this hypothesis we carried out the following tests: chromosome analyses, molecular hybridization with corresponding probes to the T-DNA fragments and in situ hybridization on metaphase chromosomes. The presence and the expression of the oncogenic element and its location on different chromosomes are discussed in light of the results obtained from the above experiments.

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Materials and methods

Material

In this study we used the tobacco crown-gall line isolated by Morel (1948) plus a series of its clones and sub-clones (Lutz and Belin 1974).

The in situ hybridization experiments were conducted on A_{14} clone. This clone seemed particularly suitable for the following reasons: it has good friability in culture in liquid medium; it has an adequate mitotic index in culture in the exponential growth phase (> 5%); it is easy to obtain protoplasts; finally, the genetic stock is relatively stable with time.

The pGV0153 probe was used for in situ hybridization and pGV0120 and pGV0153 for hybridizations using Southern's method: (i) pGV0120 contains the 16-kb, BamHI fragment 2 corresponding to the right fragment (T_R) of the T-DNA (De Vos et al. 1981). (ii) pGV0153 contains the 6-kb BamHI fragment 8 corresponding to the left fragment (T_L) of the T-DNA. The genes which code for the growth hormones and the octopine synthase are both found on this fragment (Akiyoshi et al. 1983).

Method

Cell cultures

The tobacco crown-gall line and 12 of its clones were cultivated in a solid medium which contains the Murashige and Skoog elements (1962) minus the growth factors. The cultures were placed in an air-conditioned room at 25 °C under continuous lighting and subcultured every 4–6 weeks. Cell suspensions were obtained by transferring the colonies into a liquid medium with the same composition as the solid medium.

Extraction of DNA

DNA isolation and Southern hybridization were carried out as described in Paszkowski et al. (1984).

Chromosome preparations

For chromosome analyses and in situ hybridization experiments on metaphase chromosomes, cell suspensions were subcultured every 3–4 days and the mitotic index was determined by sampling the cell suspensions at regular intervals. The mitotic index reached 5%–7% about 40 h after the third transfer. In order to block the chromosomes in metaphase, the cell suspension was transferred into a 0.2% solution of α-chloronaphthalene (mitoclasic agent) for about 45 min. The suspension was then washed in running tap water and submitted to an enzymatic cell wall digestion solution according to Mouras et al. (1978). The protoplasts then underwent a hypotonic shock in order to disperse the chromosomes within the cells, much the same as with animal cells. After fixation in ethanol-acetic acid (3:1) for at least 30 min, the protoplast suspension was distributed either onto clean dry slides for chromosome studies or onto specially precoated slides for in situ hybridization (5×10^4–10^5 protoplasts per slide). The latter may be used immediately or stored at −70 °C.

In situ hybridization

Treatment of histological slides. In order to avoid aspecific fixation of the probe at the surface of the slides, they were coated prior to application of the cellular suspension, according to the method developed by Gerhard et al. (1981).

Probe labelling. The radioactive probes for hybridization experiments using the Southern method were prepared from pGV0120 and pGV0153 plasmids. They were obtained by nick-translation (Rigby et al. 1977) using a single radioactive nucleotide (^{32}P-ATP). The specific activity of the probes used here was of the order of 10^8 cpm/μg of DNA.

The radioactive probes for in situ hybridization were prepared by nick-translation, this time using three radioactive nucleotides (10 μ*M* of ^{3}H-dATP at 26 mCi/mmol, ^{3}H-dCTP at 67 mCi/mmol and ^{3}H-dTTP at 105 mCi/mmol); the fourth nucleotide was non-radioactive and was added in excess (60 μ*M* of dGTP). Only the T_L fragment (6 kb, BamHI fragment from the pGV0153 plasmid) was used for this probe. The specific activity of the resulting probes was of the order of $4–5 \times 10^7$ cpm/μg of DNA.

Hybridization. The cytological preparations were pre-treated according to the method developed by Mouras et al. (1987a), i.e.: (i) prehybridization: the preparation was treated with RNA'seA (100 μg/ml) at 37 °C for 1 h, then washed in 2 × SSC, dehydrated in a series of ethanol baths from 70 °C and 95 °C, then air-dried. (ii) denaturation: the radioactive probe was added to a mixture of the following composition: DNA probe (1 μg/ml), sonicated carrier DNA (0.25 mg/ml), dextran sulphate (10%), formamide (50%) dissolved in a saline solution of 3 × SSC. This mixture was denatured at 70 °C for 15 min, then rapidly chilled in ice water or liquid nitrogen. Thirty to forty microliters of this mixture was added to each cytological preparation (10–20 ng of DNA probe per slide). The preparation was then covered with a siliconized coverslip and denatured again at the same time as the chromosomal DNA. The preparations were denatured by incubating them in water at 80 °C for 30 s. Finally, the preparations were allowed to incubate at 25 °C or at 40 °C for 15 or for 63 h in each case. (iii) posthybridization: the coverslips were removed and the preparations were carefully washed two to three times in a 2 × SSC solution at 37 °C and then three to four times at room temperature. This treatment effectively removed all excess probe. Probes that were weakly hybridized onto the DNA and any aspecific hybridizations were removed by a more stringent treatment which involved incubating the preparations in 1 × SSC at 60 °C for 15 min. Next the preparations were dehydrated in ethanol, dried in air and then coated with the Kodak emulsion NTB_2 for autoradiography.

Results

The working hypothesis is that there is a direct relationship between the existence in the analyzed lines of specific marker chromosomes on which T-DNA sequences are located and their oncogenic potential.

Chromosome analyses of the transformed lines

The chromosomal composition (number and structure) of each transformed line was previously described (Mouras and Lutz 1983). For the sake of clarity, a brief chromosome analysis of the transformed lines is presented below. In each case, rearranged chromosomal elements arising from translocations were observed (Fig. 1). The specific rearranged chromosomes, called marker chromosomes (Fig. 2), are present in cells of each clone, the actual number varying from cell to cell. For example: (i) one cell may contain three configurations at once, m-mx, m-a and m-T; (ii) another cell may contain two

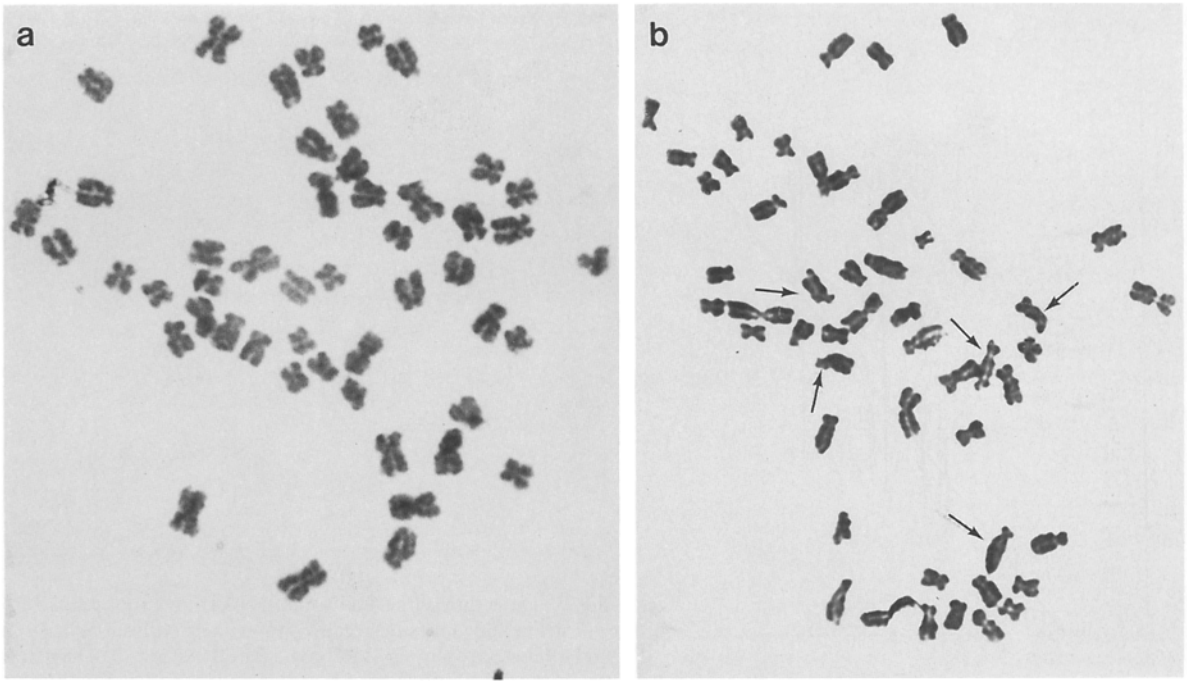

Fig. 1 a and b. Metaphase plate in **a** wild-type and **b** transformed cell. *Arrows* show rearranged chromosomes

configurations m-mx, with m-a and m-T absent; (iii) another cell may contain only one of the three dicentric configurations.

All of the above combinations have been reported and counted in a population of about 50 cells for each line. On the other hand, the oncogenic potential of the lines was estimated by grafting tests and was expressed in terms of the mean diameter of the tumors (measurements carried out on roughly 10–15 grafts for each line (Mouras 1981).

The results obtained from the marker chromosomes for each line and the mean diameter of the tumors were analyzed statistically. Because of the very high coefficient of correlation ($r=0.92$), it was possible to calculate the corresponding regression curve equation (Fig. 2). From this figure we were able to show that there is a relationship between the presence and the number of the marker chromosome and the extent of oncogenic potential (Mouras and Lutz 1983).

Analysis of the T-DNA by Southern hybridization

With this information in mind, the oncogenicity of crown-gall lines and different clones can be estimated at the molecular level. Results of Southern hybridization are shown in Fig. 3.

(i) For the T_R fragment, the hybridization signal remains very weak throughout, irrespective of which cell line was studied.

(ii) In the case of the T_L fragment, signals were always positive but the intensity varied depending on which line was studied, with estimates ranging from two to five copies per diploid genome. A plant that has been regenerated from a line containing about five copies yielded an estimated one copy per genome.

Thus, the signal from the T_L fragment was much stronger than from the T_R fragment. Therefore, the T_L fragment was used in attempts to locate the T-DNA on metaphase chromosomes.

In situ hybridization

Each transformed line contained previously defined marker chromosomes (Fig. 1). Attempts to locate T-DNA were conducted on the A_{14} line for reasons already explained in 'Materials and methods'.

Hybridization efficiency. Significant hybridization signals were observed after exposure periods of 10–17 weeks. After 17 weeks, the signals on the interphase nuclei and on the metaphase chromosomes were of equal intensity and very much higher than the background signals. It should be noted that the controls to this experiment (conducted on suspensions of protoplasts from the roots of wild-type tobacco) were negative. The hybridization efficiency turned out to be between 60% and 70%, showing the accuracy of the method.

Results from hybridizations lasting 15 h were qualitatively much better than those from prolonged hybridizations, i.e. 63 h. The difference lies not in the intensity of the signal but in the morphology of the chromosomes: during hybridization, incubation in 50% formamide in

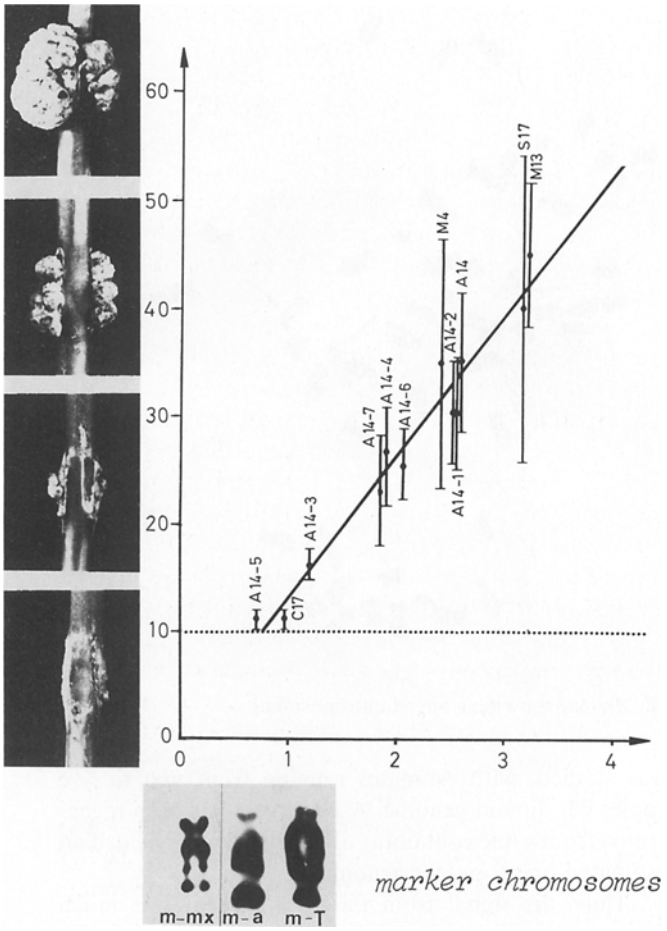

Fig. 2. Evidence for a relationship between the mean number of marker chromosomes per cell and the tumor size in 12 clones and sub-clones derived from the tobacco crown-gall strain isolated by Morel (regression curve equation: $y = 12.86x + 0.34$). Y-axis: tumor size (in mm); X-axis: mean number of marker chromosomes per cell. m: small metacentric chromosome common in each dicentric configuration. *Vertical bar:* tumor size in the 95% confidence limits. *Black points* correspond to the measured mean tumor size

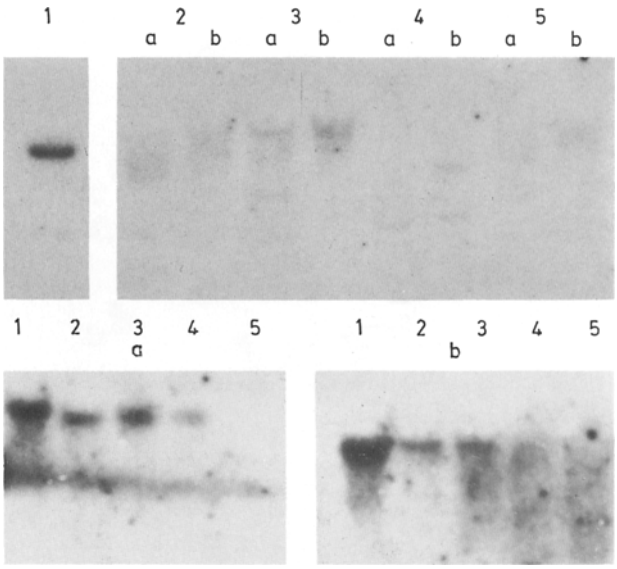

Fig. 3. Detection of the T-DNA in DNA from a tobacco crown-gall strain and clones derived from it with or without restriction and after electrophoresis of about 5 µg DNA/slot; the DNA was transferred onto nitrocellulose filter and hybridized with nick-translated T_R or T_L fragments of the T-DNA. *Top:* hybridization with T_R fragment (probe pGV0120). *a* = DNA restricted with EcoRI; *b* = DNA restricted with BamHI. *Lane 1:* T_R fragment of T-DNA (50 ng); *lanes 2 a–b:* clone A14-3; *lanes 3 a–b:* clone A14; *lanes 4 a–b:* untransformed tobacco DNA; *lanes 5 a–b:* crown-gall line. *Bottom:* hybridization with T_L fragment (probe pGV0153). *a* = unrestricted DNA; *b* = DNA restricted with BamHI. *Lane 1:* crown-gall line; *lane 2:* clone *A14-3; lane 3:* clone A14; *lane 4:* regenerated plant from clone S_{17}; *lane 5:* untransformed tobacco DNA

saline solution 3 × SSC leads to noticeable structural changes in the chromosomes, i.e. swelling and a reduction in the staining of the chromosomes.

Localization of the T-DNA. Figure 4 shows the signal obtained after hybridization of the T_L probe with metaphase chromosomes. The signal may occur on one or several small metacentric chromosomes (m) or on configurations of the type m-mx, m-a or m-T. However, it rarely occurs on other structures.

Table 1 shows the results of such an investigation. Consider, e.g. the configuration m: it can be seen that 17 cells out of the 25 metaphases analyzed contain 23 structures of the m-type carrying the signal. This example illustrates the fact that some cells may contain as many as three chromosome structures of the m type, with each

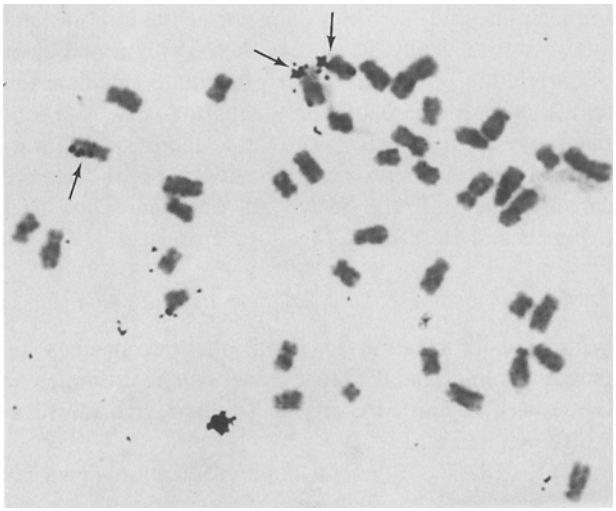

Fig. 4. Metaphase plate after in stiu hybridization (17 weeks of autoradiography). The label is clearly shown on marker chromosomes (*arrows*)

Table 1. Distribution of the labelling on metaphase chromosomes

	Chromosomic shape with hybridization signal (labelling)				
	m	m-mx	m-a	m-T	*
Analyzed cell number:for all categories: 25					
No. of chromosomes with labelling	23	11	8	3	11
Cell no. with a specific chromosome	17	11	6	3	10

* Means that the structure of the chromosome is different from dicentric configurations

one carrying a signal. On the other hand, the cells may contain several different structures (m-mx, m-a, m-T) as already observed during the chromosome analyses. The same explanation can be given concerning the other chromosome structures in Table 1.

Discussion

In order to establish that there is a relationship between the marker chromosomes and the oncogenic potential of the transformed cells, it was necessary to locate the integration site of the T-DNA which was responsible for the tumor transformation of the cells. Only after the development of an in situ hybridization technique capable of showing up a single low copy number gene (Mouras et al. 1987a, 1989) was it possible to attempt locating the site. Prior to carrying out this technique, it was necessary to verify that T-DNA was in fact always present in the transformed lines, in order to choose a suitable probe to detect the integration site of the T-DNA. It was shown using Southern hybridization that the signal from the T_L fragment of T-DNA was satisfactory, whereas that of the T_R fragment was very weak or non-existent.

We only have indirect evidence concerning the expression of the genes on the T_L fragment: the transformed cells grew on hormone-free medium and developed tumors after grafting tests; for the octopine synthase gene we have observed that opine was absent from the cloned cell lines but present in the crown-gall mother line (Mouras et al. 1987b). Van Lijsebettens et al. (1986) sug gested that the non-expression could be due either to a methylation of the T-DNA or to the elimination of the octopine gene.

We have shown from in situ hybridization experiments carried out with the T_L fragment that the hybridization signals arose essentially from chromosome configurations which mainly correspond to the marker chromosomes. The presence of these marker chromosomes is, moreover, correlated to the oncogenic potential of the transformed cells. On the other hand, we have often observed signals on the small metacentric chromosomes which have the same morphology and size as the m element (small metacentric chromosome) in dicentric configurations, which also have hybridization signals. Differences in the chromosome structures carrying the transforming element (T-DNA) may be explained by the 'fusion – bridge – break' mechanism. A dicentric structure may undergo extensive rearrangements during cell division and, in particular, at the anaphase of the mitotic cycle: (i) dicentric chromosomes may, in certain cases, pass directly into a daughter cell without any modifications. The outcome of this disjunction leads to unequal daughter cells, one maintaining the dicentric configuration, the other not at all. This would explain the presence of cells which have recovered normal phenotype in 1%–20% of the cell population and the absence of marker chromosomes in plants regenerated from highly tumorous lines (Mouras and Lutz 1983). (ii) The dicentric structure may be broken when the centromeres move in opposite directions. The resulting elements may then develop in different ways. Either the element remains unchanged: this would explain the presence of a single m element bearing the T-DNA. Or the elements may translocate onto structures which are themselves broken. These translocations give rise to a diversity of dicentric chromosome configurations (m-mx, m-a, m-T) which make up the marker chromosomes (Mouras 1984). Among other things, this mechanism could well explain the presence of the T-DNA on different dicentric configurations.

The question as to how the specific dicentric chromosome forms in tobacco crown-gall cells is irrelevant and, without analyzing several independent tobacco crown-gall lines, any hypothesis remains largely speculative.

In conclusion, it has been confirmed that there is a relationship between the marker chromosomes and the oncogenic potential of transformed cells: we have shown that in the Morel strain of tobacco crown gall, the T-DNA integrates into the m element in marker chromosomes.

Acknowledgements. We are particularly indebted to I. Potrykus, (Friedrich Miescher Institut, Basel, Switzerland) who made it possible for us to conduct the experiments described in this paper in his laboratory. We would like to express our gratitude to Miss Z. Koukolikova-Nicola (F.M.I. Basel, Switzerland) and F. Salamon (U.L.B. Institute of Molecular Biology, Rhode-Saint-Génèse, Belgium) for kindly providing the probes pGV0120 and pGV0153, respectively. We are thankful to J. Mendes-France for helping us with the English version of this paper, and to J. Calcoen for the preparation of the manuscript.

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