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Localization by in situ hybridization of a low copy chimaeric resistance gene introduced into plants by direct gene transfer

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Summary. An in situ hybridization method was developed for detecting single or low copy number genes in metaphase chromosomes of plants. Using as a probe ^{3}H-labelled plasmid pABDI, which confers kanamycin resistance (Km^r) to transformed cells, DNA introduced into the plant genome by direct gene transfer was detected with a high efficiency: about 60% to 80% of interphase and metaphase plates showed a strong signal. The insertion site of the Km^r gene in two independent transformants was localised on different homologous chromosome pairs. This result independently confirmed previous genetic data which had indicated that transformed DNA was integrated into plant chromosomes in single blocks.

Key words: In situ hybridization – Transformed plants – Low copy gene

Introduction

An understanding of the mechanism of expression and regulation of genes in eukaryotic cells will ultimately require a determination of their location within the genome. In situ hybridization is potentially a very powerful technique for this purpose and has been used successfully in animal cell systems (e.g., Brahic et al. 1978; Gerhard et al. 1981), but until recently the localisation of single genes in plants has not been reported. Ambros et al. (1986a, b) have recently reported the localisation of a complete copy of *Agrobacterium rhizogenes* T-DNA in the genome of a transformed *Crepis capillaris* root culture, using biotin or ^{3}H-labelled probes. However the detection of the biotin-labelled probe required the use of reflection-contrast microscopy and the distribution of label in both cases showed a fair degree of background hybridization. In addition there have been reports of the use of in situ hybridization for highly repetitive sequences of DNA in order to identify the chromosomes of each parent in hybrid plants (Hutchinson et al. 1981), or to locate genes with high copy number, such as zein genes (Viotti et al. 1982). We report here a method which has allowed the unequivocal identification of the presence of a small foreign DNA segment in transformed plants with virtually no background hybridization.

Tobacco plants transformed by direct gene transfer (Paszkowski et al. 1984) and containing a single or a few copies of the introduced plasmid (3–5 copies) provide ideal material with which to attempt localisation of low copy number genes by in situ hybridization. The foreign DNA present in the genome of such transformants has been analysed at the molecular level (Paszkowski et al. 1984) and genetically (Potrykus et al. 1985) and a probe is available which does not cross-hybridize to any other sites in the genome. Furthermore the foreign DNA is known to be integrated into different homologous chromosomes in independent transformants, thus allowing correlation between in situ signals and genetic data.

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Materials and methods

Plant material. Wild-type *Nicotiana tabacum* Petit Havana-SR1 (Maliga et al. 1973) was used both as karyotype reference and as control for in situ hybridization.

Homozygous transformed plants, produced by selfing the independent hemizygous primary transformants T_{2-1} and T_{2-2} were used for the in situ hybridization experiments (see clone 40/16, Table 5 and clone 11/05, Table 9, Potrykus et al. 1985). These plants have been shown to contain, at one Mendelian locus, the foreign gene for kanamycin resistance amongst 3–5 rearranged copies of the plasmid pABD1 (Paszkowski et al. 1984; Potrykus et al. 1985). It has also been shown that crosses between the two transformants yield 15:1 segregating populations indicating that the foreign gene is probably localized on different chromosomes in the different transformants.

Cytological preparation. Cuttings from wild-type SR1 tobacco plants and from the clones T_{2-1} (40/16) and T_{2-2} (11/05) were induced to root at 25° C in an aerated water bath containing Hoagland solution, under photoperiodic illumination (approx. 2000 lx, 14/10 h). Root tips were excised and immersed in a solution of α-chloronaphthalene (0.2% w/v in water) for 1.5 h (wild type) or 1 h (transformed plants) to arrest cells in metaphase. After washing, the root tips were subjected to enzymatic treatment for protoplast release (Mouras et al. 1978). The protoplast suspension was transferred to fixative (3 vol. absolute ethanol + 1 vol. acetic acid) and stored overnight at −18° C.

Slides were precoated to prevent or reduce binding of the probe DNA to the glass. To precoat, the slides were immersed for 5 min in 1 M HCl at 95° C, neutralized in 1 M Tris-HCl, pH 7, for 1 min, rinsed in water for 1 min, dehydrated in 95% ethanol for 1 min and air dried. They were then incubated for 4 to 5 h in 1 × Denhardt's solution in 3 × SSC at 65° C (Denhardt's solution is 0.02% each of bovine serum albumin, Ficoll 400 and polyvinyl pyrrolidone), rinsed in distilled water, fixed in ethanol-acetic acid (3:1) for 20 min and air dried. Slides prepared in this way can be stored for several months. The slides could be mounted just before in situ hybridization or mounted and stored at −70° C until use. They were prepared by placing 2 drops of protoplast suspension (about 10^5 cells) onto precoated slides and air drying.

Recombinant DNA probes. The recombinant plasmid H10 (Saul and Potrykus 1984) contains an approx. 2.7 kb insert containing a middle repetitive sequence from *N. tabacum* Havana 425 cloned in pUC8. This insert cross hybridizes with all *N. tabacum* varieties tested.

The recombinant plasmid pABDI (5.4 kb) contains a selectable marker gene (1.2 kb) constructed by placing the protein coding region of the bacterial gene for aminoglycosidephosphotransferase (APH(3')II from Tn*5*) under the control of 5'/3' expression signals from cauliflower mosaic virus gene VI cloned in pUC8 (Paszkowski et al. 1984).

Preparation of ^{3}-H-labelled hybridization probes. Recombinant plasmids were labelled with tritiated deoxynucleotide triphosphates by a modified nick translation protocol using *Escherichia coli* DNA polymerase I (Rigby et al. 1977). The labelled nucleotides, which were obtained from Amersham, possessed the following activities: dATP 26 Ci/mmol, dCTP 76 Ci/mmol and dTTP 97 Ci/mmol. Aliquots containing 10 to 15 μmol of each of the three nucleotides were dried under vacuum and resuspended in 50 μl of the following solution: 40 μl H_2O, 5 μl of 10 × NTB buffer (NTB is 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$ and 10 mM β-mercaptoethanol), 60 μM cold dGTP, 4 ng/ml DNAase I, 500 ng of recombinant plasmid DNA and 5 units of *E. coli* DNA polymerase 1. After incubating for 2.5 h at 14° C, the reaction was stopped by addition of 5 μl of 300 mM Na_2EDTA, pH 8, and the mixture was passed through a Sephadex G100 column to separate the nucleotides from the labelled DNA.

Hybridization of ^3H-labelled DNA to chromosomes and cells. Pretreatment prior to hybridization. Slides were incubated at 37° C for 1 h with 100 μg/ml RNAase (DNAase free) washed thoroughly in 2 × SSC at room temperature, dehydrated through 70% and 96% ethanol and then air dried.

Denaturation and hybridization. The hybridization mixture contained, in 3 × SSC solution: 50% deionized formamide, 10% potassium dextran sulphate, sonicated salmon sperm DNA (500- to 1000-fold excess over probe DNA) and the probe DNA (1 μg/ml). The mixture was either denatured for 15 min at 70° C and quickly cooled in liquid nitrogen or denatured together with the chromosome preparation as described below.

Chromosome denaturation and hybridization were carried out in two different ways:

G method (the method normally used with human or animal cells; Gerhard et al. 1981). Slides without probe mixture were immersed in 70% formamide/2 × SSC solution for 2 min at 70° C, then immersed in 70% and 95% ethanol at 0° C. Following air drying 5 to 25 ng of the denatured, labelled probe (25 to 30 μl of hybridization mixture) was placed onto the slides, covered with a siliconized coverslip and sealed with rubber cement to prevent dehydration.

E method. The same amount of probe, either with previous denaturation (E_2) or without (E_1), was placed onto non-denatured slides. The samples were covered with a coverslip, sealed with rubber cement and then sealed in a plastic bag. Denaturation was then carried out in a water bath at 80° C for 30 s followed by cooling at room temperature.

Hybridization in both cases was for 1 day for experiments with the H10 probe or for 3 days with the pABDI probe, either at 25° C, as described by Brahic et al. (1978), or at 40° C (Gerhard et al. 1981; Harper et al. 1981).

Post-hybridization. After hybridization the coverslip was carefully removed and the slides were washed 4 times in 2 × SSC at 40° C (30 min each). Poorly matched hybrid DNA was removed by incubating the slides either in 1 × SSC, 60° C for 15 min, or twice in 50% formamide/2 × SSC solution, 40° C for 10 min. The slides were again washed in 2 × SSC, three times at 40° C and twice at room temperature (30 min each) and finally in distilled water. Afterwards, the slides were dehydrated in 70% and 95% ethanol containing 0.3 M ammonium acetate (5 min each). The slides were air dried and then immersed in Kodak NTB-2 emulsion diluted 1:1 with 0.6 M ammonium acetate, at 42° C, placed on a sheet of glass preequilibrated at 0° C for 5 min, and dried in an oven for 1 h at 40° C.

Autoradiography was performed at 4° C in light-proof boxes containing silica gel desiccant. The slides were developed in Kodak Dektol developer diluted 1:1 with water, for 3 min at room temperature followed by a brief wash in distilled water and fixation for 5 min in Kodak F-24 fixer. After washing in running tap water (10–15 min) the slides were stained for 30–45 min with 4% Giemsa stain in 0.15 M phosphate buffer pH 6.9, or in Wright's stain diluted 1:3 with 0.06 M phosphate buffer pH 6.8.

Controls were carried out for each experiment as follows:

For experiments with transformed material, protoplasts were treated with the hybridization mixture without probe DNA and wild-type SR1 cells were hybridized with the pABDI probe.

For repetitive sequences, wild-type SR1 protoplasts were treated with the hybridization mixture without probe and preparations made with *N. plumbaginifolia* protoplast suspensions were treated with the H10 probe.

Results

Success in localising a low or single copy gene in chromosomal DNA, by in situ hybridization depends on several factors including the accessibility of the target DNA for interaction with the probe DNA, the specific activity and the amount of probe DNA, the exposure time for autoradiography, the elimination of background hybridization and in particular the preservation of DNA throughout the entire procedure (especially during the storage of cell suspension and microscope slides and during denaturation of the chromosomes).

In order to optimize these factors for our work we carried out two series of experiments. In one we used as a

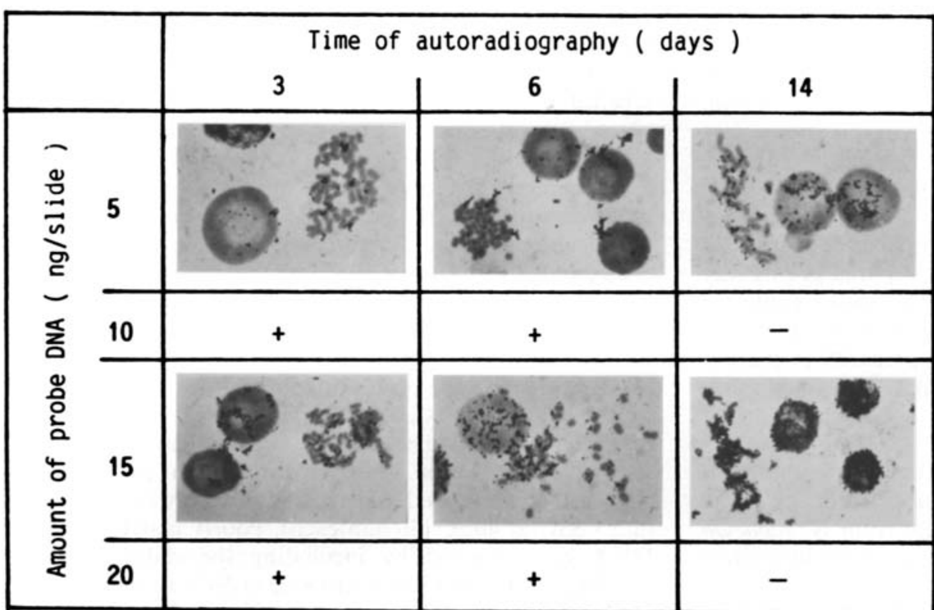

Fig. 1. In situ hybridization of a middle repetitive sequence of *Nicotiana tabacum* (probe H10) with *N. tabacum* Petit Havana SR1: effect of amount of probe DNA and time of autoradiography. (−) not tested, (+) tested but data not shown

probe the plasmid H10 described by Saul and Potrykus (1984), which consists of the plasmid pUC8 and a fragment of middle repetitive DNA isolated from *N. tabacum* which hybridizes to *N. tabacum* DNA with a copy number of approximately 3000 per genome. The high copy number meant that a significant signal could be obtained in a short time, thus allowing fast analysis of factors. In the other experiments the plasmid pABD1 (Paszkowski et al. 1984) was used to detect foreign DNA in plants which had previously been transformed with this plasmid.

Hybridization with a repetitive DNA sequence

Some of the factors listed above were tested by hybridization of plasmid H10 with chromosome spreads from *N. tabacum* SR1 (Maliga et al. 1973). A ^{3}H-labelled H10 probe was prepared with a specific activity of approximately 3.5×10^7 cpm/μg and hybridized under the conditions described in Materials and methods (methods G and E_2).

After 3 days of autoradiography a signal was detectable in about 90% of nuclei and metaphase plates and after 2 weeks the signal was so strong that chromosomes or nuclei were almost completely covered with silver grains (Fig. 1). The effect of the amount of probe DNA was analysed, using between 5 and 20 ng of probe per slide. As shown in Fig. 1, the signal increased as the amount of probe and the time of autoradiography were increased. Similar results were obtained with 15 ng of probe after 3 or 6 days as with 5 ng of probe after 6 or 14 days of autoradiography. Thus 5 ng of probe DNA proved to be enough for detection of such repetitive sequences of DNA in a relatively short time.

The variation of the post-hybridization procedure that included washing the slides in 1 × SSC at 60° C for 15 min most efficiently removed poorly matched hybrids. Thus, as shown in Fig. 1, it was possible to obtain a very low background signal using the H10 probe.

Little difference was observed in these experiments between hybridization methods G and E_2 (see Materials and methods). The results showed that the preparation of cells and slides and both hybridization methods were effective for this type of in situ hybridization.

Hybridization with a probe (pABDI) homologous to the foreign gene

Protoplasts isolated from root tips of two independent transformed plants – homozygous progeny plants derived from transformants T_{2-1} and T_{2-2} described in Paszkowski et al. (1984) and Potrykus et al. 1985, (Table 5, clone 40/16 and Table 9, clone 11/05) – were used to prepare slides with a high number of metaphase plates. The plants contain approximately 3–5 copy equivalents of the plasmid pABDI in a rearranged form, and all the copies are inherited as a single Mendelian factor. The copies were, therefore, assumed to be at one location in one chromosome and the target DNA in the chromosome which could hybridize with pABDI was thus estimated to be approximately 15–25 kb per chromosome.

Tritium labelled probes were prepared using pABDI DNA and hybridized using methods G, E_1 and E_2. With a probe of specific activity approximtely 2×10^7 cpm/μg a very weak signal was obtained, on only 5% of the metaphase plates, even after 8 weeks of autoradiography. However, with a probe of approximately 7.5×10^7 cpm/μg, using 12 or 20 ng per slide, a significant signal was obtained after 6 weeks of autoradiography. The signal of approximately two silver grains per metaphase plate on approximately 10% of plates did not allow precise localisation to a single pair of chromosomes. Preliminary localisation was, however, possible on the basis of 50 metaphase plates for each plant, where 40%–50% of grains were on sub-metacentric chromosomes for T_{2-1} and on sub-telocentric chromosomes for T_{2-2}. However, clear and unequivocal localisation of the foreign gene was possible after 12 weeks, when both homologous chromosomes carried a signal of 15–20 silver grains with virtually no background signal (Fig. 2 and see below). The strong signal at two positions was observed both in metaphase plates and in interphase nuclei.

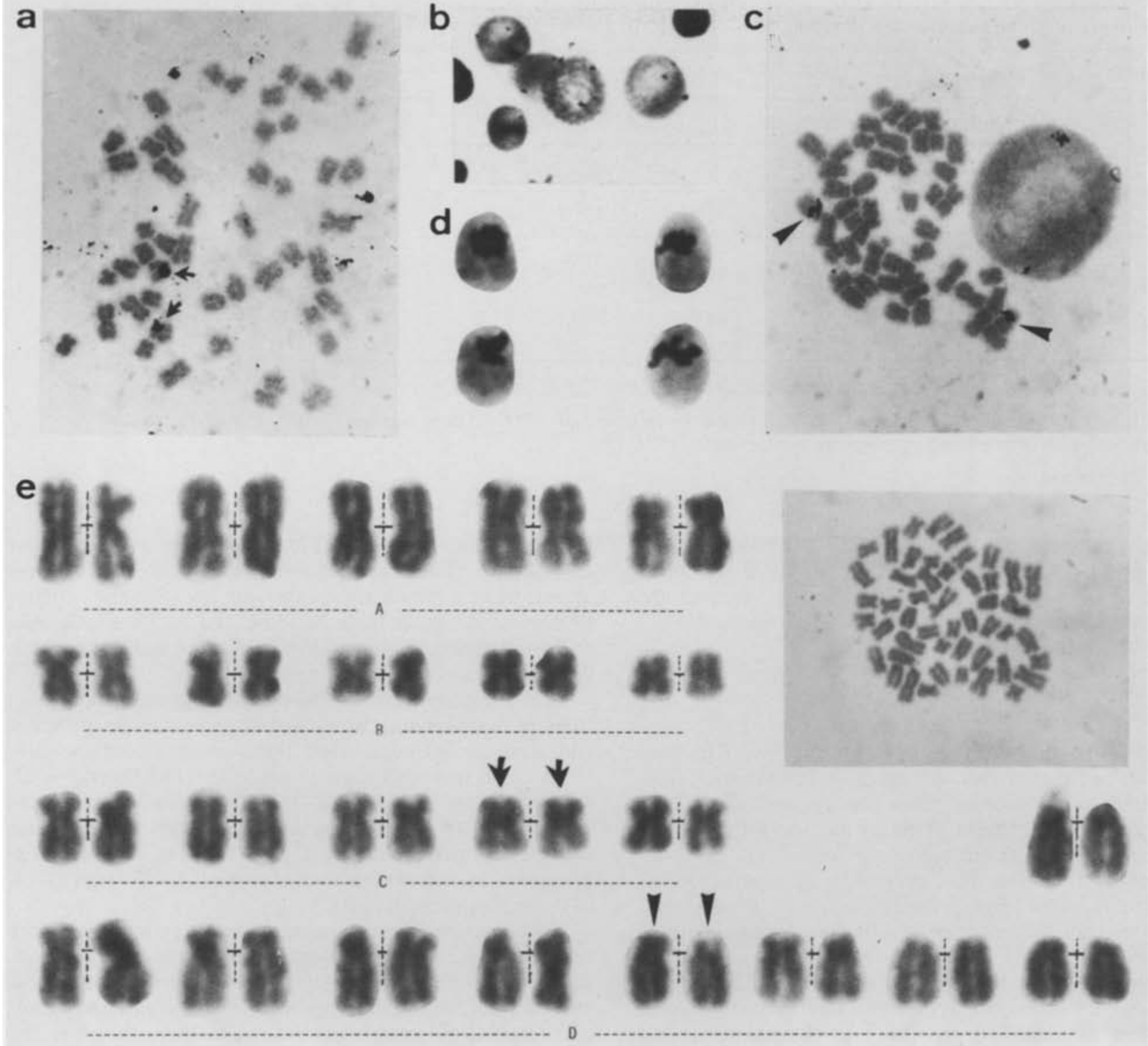

Fig. 2a–e. Localisation of the hybridizing signal in transformed plants. **a** Metaphase plate in transformed plant T_{2-1}. **b** Interphase nuclei with labelling (1 or 2 regions of labelling). **c** Metaphase plate in transformed plant T_{2-2}. **d** Individual chromosomes bearing a strong signal corresponding to the gene introduced by direct gene transfer into plants (T_{2-1}, left; T_{2-2}, right). **e** Wild-type SR1 metaphase plate and karyotype. The chromosomes bearing the foreign gene in transformed plants are indicated by *arrows* (T_{2-1}, group C) and by *arrowheads* (T_{2-2}, group D)

Hybridization to chromosomes or interphase nuclei from wild-type SR1 plants was never observed.

Factors affecting the sensitivity of the method

As outlined above, many factors can influence the success of the method. Several variations of the method were tried in order to assess their effect on the sensitivity. The results are presented in Table 1.

The effect of concentration of probe DNA (either 12 or 20 ng/slide) was not significant. In both cases the concentration was high enough to give a large excess of DNA for network formation.

The temperature of hybridization (25° C or 40° C) also appeared not to be very important in determining hybridization efficiency, although 40° C seemed to be slightly more effective.

The most significant difference in effect was that between different methods of denaturation of probe and chromosomal DNA in the various procedures (G, E_1, E_2). Method G, in which previously denatured probe was added to a denatured chromosome spread, gave significantly worse results than methods E_1 and E_2 in which probe and chromosomes were denatured together. Previous denaturation of the probe in solution followed by a second denaturation on the slide (E_2) also gave better results than denaturation only on the slide (E_1).

To minimize the background hybridization an additional washing step, in addition to the the general washes in $2 \times$ SSC at 40° C and at room temperature, was used. From

Table 1. Accessibility of target DNA (Km^r gene) in interphase nuclei and metaphase plates, shown by the efficiency of *in-situ* hybridization (labelling %)

Transformed plants		% of labelled cells						
		Denaturation method						
		G		E1		E2		
		25° C	40° C	25° C	40° C	25° C	40° C	Controls
T2-1	1	9.6	8.0	36.3	33.6	75.9	–	0-1
	2	6.9	8.6	–	–	–	81	0-1
T2-1	1	6.7	–	35.2	40.6	76.1	83.5	0-1
	2	4.2	4.6	–	–	–	61.5	0-1

G, E1, E2: denaturation method used, with hybridization temperature (25° C or 40° C)

1 and 2: Independent experiments carried out with preparations stored at −70° C (1) or prepared just before hybridization from protoplast suspensions stored at −18° C (2)

(−): not determined

the two variations tried (see Materials and methods), the inclusion of a 15 min wash in 1 × SSC at 60° C was more effective than two 10 min washes in 50% formamide, 2 × SSC (v/w) at 40° C.

Localisation of the Km^r gene on specific chromosomes

In order to locate the site of integration of the Km^r gene ten metaphase plates with well-spread chromosomes, from material from the homozygous transformed lines T_{2-1} (40/16) and T_{2-2} (11/05) treated by method E_2 using pre-denatured probe, were analysed.

Chromosomes were first ordered in groups according to type (metacentric large, metacentric small, sub-metacentric, sub-telocentric) and then within the groups according to the absolute length. Then the centromeric index (short arm length versus long arm length) was determined for each chromosome. Chromosomes carrying a hybridization signal could then be identified according to these criteria. For the transformed line T_{2-1} a sub-metacentric pair of homologous chromosomes (group C) with a centromeric index of approximately 0.62 was identified as carrying the foreign gene and with the line T_{2-2} a sub-telocentric pair (group D) with a centromeric index of approximately 0.48 (Fig. 2).

These data also confirmed that the partial and complete copies of pABDI are located within a small region of the chromosome, as suggested by the co-segregation of these as a single block into progeny (Potrykus et al. 1985). Furthermore, genetic data showing that, in these two transformants, the integration events had occurred on separate chromosomes were also confirmed.

Discussion

On the basis of published in situ hybridization methods, we have developed a procedure which allows the clear identification and localization of foreign genes, present in the chromosomes of transformed plants, with a target size of around 15–25 kb. Indeed the optimal conditions give us a hybridization efficiency greater than 80% with the pABD1 probe and 95% with the repetitive DNA probe H10. This compares favourably with the methods presented by Ambros et al. (1986a, b) for *A. rhizogenes* T-DNA and *C. capillaris.* The most important factors for this success appear to be a radioactive probe with high specific activity, protoplast isolation from dividing and meristematic cells and good denaturation and hybridization conditions, which retain the majority of the target DNA.

The presence of the hybridizing sequences at single positions in homologous chromosomes as well as on different chromosomes in independent transformants confirms previous molecular and genetic evidence (Paszkowski et al. 1984; Potrykus et al. 1985). This data and that of others (Hain et al. 1985; Czernilofsky et al. 1986) suggests that DNA integrated after direct gene transfer is most often integrated in one chromosome, with all the foreign DNA in close physical proximity.

Although the present method involves long exposure times (up to 12 weeks) the signal obtained, both on chromosomes and interphase nuclei, is very clear and unequivocal and does not require the counting of silver grains and subsequent statistical treatment of the results. Nevertheless recent improvements in probe technology (e.g. Landegent et al. 1985; Langer-safer et al. 1982; Ambros et al. 1986a, b) may improve this aspect and we are currently comparing the efficiency of in situ hybridization using [^{3}H]DNA and biotinylated DNA probes for a repetitive sequence and a single plant gene of *N. plumbaginifolia.* It can be envisaged that the method presented will allow the localization of original plant genes for which probes are available and thus the assignment of marker genes of linkage groups to chromosomes. In addition, the study of position effects on expression may be helped by this method. We hope that the results presented will stimulate further interest in in situ hybridization in plant research.

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