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# Amplification of repetitive DNA from *Nicotiana plumbaginifolia* in asymmetric somatic hybrids between *Nicotiana sylvestris* and *Nicotiana plumbaginifolia*

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**Summary.** Asymmetric somatic hybrids were obtained between a chlorophyll-deficient mutant of *Nicotiana sylvestris* (V42) and a nitrate-reductase (NR)-deficient line of *N. plumbaginifolia* (cnx20 or Nia26), using each of the parents alternately as the irradiated donor. Irradiation doses applied ranged from 10 to 1,000 Gy of gamma-rays. Hybrid selection was based on complementation of NR deficiency with wild-type NR genes. To aid in the analysis of somatic hybrids, species-specific repetitive DNA sequences from *N. plumbaginifolia* (NPR9 and NPR18) were cloned. NPR18 is a dispersed repetitive sequence occupying about 0.4% of the *N. plumbaginifolia* genome. In turn, NPR9, which is part of a highly repetitive DNA sequence, occupies approximately 3% of the genome. The species-specific plant DNA repeats, together with cytological analysis data, were used to assess the relative amount of the *N. plumbaginifolia* genome in the somatic hybrids. In fusion experiments using irradiated *N. plumbaginifolia,* an increase in irradiation dose prior to fusion led to a decrease in *N. plumbaginifolia* nuclear DNA content per hybrid genome. For some hybrid lines, an increase in the quantity of repetitive sequences was detected. Thus, hybrid lines 1NV/21, 100NV/7, 100NV/9, and 100NV/10 (where *N. plumbaginifolia* was the irradiated donor) were characterized by amplification of NPR9. In the reverse combination (where *N. sylvestris* was the irradiated donor), an increase in the copy number of NPR18 was determined for hybrid clones 1VC/2, 1VC/3, 100VC/2 and oct100/7. Possible reasons for the amplification of the repeated sequences are discussed.

**Key words:** Asymmetric somatic hybrids – Species-specific repetitive DNA – DNA amplification – Gamma-irradiation – Tobacco

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## Introduction

The construction of asymmetric somatic hybrids, combining a partial donor genome with a complete genome of the recipient, has applications for plant breeding programmes. To direct the elimination of donor chromosomes in hybrid products, treatment of plant cells with X-rays and gamma-rays prior to fusion has been utilized (e.g., Gupta et al. 1984; Bates et al. 1987; Dudits et al. 1987; Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989; Wijbrandi et al. 1990a; Hinnisdaels et al. 1991). The relative contribution of DNA from each parent to hybrid genomes has been estimated by hybridization with total genomic DNA (Sala et al. 1985) or with gene probes showing RFLPs (Moore and Sink 1988; Wijbrandi et al. 1990b; Melzer and O'Connell 1992). However, both of these methods have a number of limitations for quantitative analysis. First, the parental species will share at least some DNA sequences, which may render hybridization with total DNA unsuitable for the analysis of hybrids among closely-related species. Second, RFLP analysis gives information about only a limited number of gene loci. Use of dispersed species-specific repetitive sequences as probes is, therefore, the method of choice for discriminating between the genomes of closely-related species, due to a combination of their ubiquitous distribution in the genome and their species-specificity. Examples include the analysis of somatic hybrids between *Nicotiana tabacum* and *N. paniculata* (Müller-Gensert and Schieder 1987), *Lycopersicon esculentum* and *Solanum acaule* (Schweizer et al 1988), *Solanum brevidens* and *S. tuberosum* (Pehu et al. 1990), and species of the genus *Brassica* (Itoh et al. 1991). Estimations of the relative contribution of parental genomes to hybrids between *N. tabacum* and *Hyoscyamus muticus* (Imamura et al. 1987), *N. tabacum* and *N. plumbaginifolia* (Piastuch

and Bates 1990), and *Lycopersicon esculentum* and *Solanum tuberosum* (Wolters et al. 1991) have been quantified using repetitive DNA sequences dispersed throughout the genome.

In this paper we have analyzed symmetric and asymmetric somatic hybrids between two phylogenetically close relatives (*Nicotiana sylvestris* and *N. plumbaginifolia*), both belonging to the section *Suaveolens* (Goodspeed 1954). The use of such close relatives allows the genetic incompatibility that often impedes growth and development of somatic hybrids between remote species (Harms 1983) to be avoided or minimized. Thus, genetically regular asymmetric hybrids between *N. plumbaginifolia* and *N. sylvestris* have already been obtained (Famelaer et al. 1989, 1990). In the present study, we compare the genomic status of hybrids obtained without irradiation and asymmetric hybrids obtained by irradiating the donor genome with different doses of gamma-rays prior to somatic cell fusion. In contrast to previous workers (Famelaer et al. 1989, 1990), we have included in our study somatic hybrids between *N. plumbaginifolia* and *N. sylvestris*, where each parent, alternately, was used as the irradiated donor. The range of irradiation doses was also significantly expanded (10, 100, 500 and 1,000 Gy) compared to that used in previous studies (Bates et al. 1987; Imamura et al. 1987; Wijbrandi et al. 1990a; Wolters et al. 1991). Quantitative estimation of the amount of *N. plumbaginifolia* nuclear DNA in the hybrids was performed by cytological analysis (chromosome counts), and by measuring the amount of species-specific repetitive sequences from *N. plumbaginifolia*. The simultaneous application of the several different repetitive sequences to the analysis allowed us first, to increase the reliability of calculations and second, to assess the fate of the repeats in the hybrid genome.

## Materials and methods

### *Plant material and culture conditions*

*N. plumbaginifolia* nitrate reductase (NR)-deficient lines cnx20 and Nia26 (both 2n = 20) (Dirks et al. 1986) were propagated aseptically as cuttings on medium supplemented with ammonium succinate. The chlorophyll-deficient mutant V42 of *N. sylvestris* (2n = 24) (Negrutiu et al. 1983) was grown on MS-P medium (Murashige and Skoog 1962).

### *Protoplast isolation, fusion, selection and regeneration*

*N. plumbaginifolia* and *N. sylvestris* leaf sections were incubated in enzyme solution [0.1% cellulase Onozuka R10, 0.05% macerozyme Onozuka R10 (Serva), dissolved in 0.5 M sucrose]. After digestion, cell suspensions were passed through 100 µm nylon filters and the resulting protoplasts were exposed to various doses of gamma-rays, using a cobalt source. Fusion experiments were carried out either with *N. plumbaginifolia* or with *N. sylvestris* protoplasts that had been exposed to 0, 10, 100 or 1,000 Gy of irradiation in W5 solution (Medgyesy et al. 1980). Donor protoplasts irradiated with different doses were mixed with those of the non-irradiated recipient in a 1:1 ratio at a density of $10^5$ cells $ml^{-1}$. Fusion procedures were performed as described by Negrutiu et al. (1986). The suspension of fusion products was plated onto $NH_4$-$SK_3$ medium supplemented with ammonium succinate (Marton et al. 1982). After 1 month, colonies were transferred to selective modified MS-medium containing nitrates as a single source of nitrogen. After 2 months of cultivation, colonies that were able to regenerate stems were selected. Completely regenerated and rooted plants were analyzed.

### *Cytological analysis*

Root tips were fixed in 1:3 acetic ethanol for 12–16 h at 4 °C and stained in 1% acetic orcein for 24 h at room temperature. Squash preparations were examined using a Reichert Univar microscope (Reichert Jung, Austria).

The calculation of the putative percentage of the *N. plumbaginifolia* genome in a hybrid line utilized the formula:
$P = 0.26a/(0.26a + 0.175b) \times 100\%$, where
P = putative percentage of the *N. plumbaginifolia* genome, (%);
a = *N. plumbaginifolia* chromosome number;
b = *N. sylvestris* chromosome number;
0.26 = conventional average weight (pg) of one *N. plumbaginifolia* chromosome (0.26 = 5.21/20, where 5.21 = diploid genome weight (pg, Piastuch and Bates 1990) and 20 = chromosome number of *N. plumbaginifolia* diploid genome);
0.175 = conventional average weight (pg) of one *N. sylvestris* chromosome (0.26 = 4.20/24, where 4.20 = diploid genome weight (pg, Bennet and Smith 1976) and 24 = chromosome number of *N. sylvestris* diploid genome).

### *Cloning of repetitive DNA sequences*

*N. plumbaginifolia* DNA was isolated from leaves according to Shure et al. (1983). Caesium-chloride gradient centrifugation at 20 °C for 36 h was used for further purification of genomic DNA. Isolated DNA was digested with the restriction endonucleases *Hin*dIII and *Eco*RI (Amersham) and ligated into the cloning vector pUC19. The ligation mixture was used to transform *E. coli* strain TG1 (Aung and Hiller 1988). Clones containing repetitive DNA sequences were selected as previously described (Saul and Potrykus 1984). The cloned dispersed repetitive sequence pNP21 from *N. plumbaginifolia* (Piastuch and Bates 1990) was kindly supplied by Dr. George W. Bates, Florida State University, U.S.A.

Rapid isolation of plasmid DNAs utilized the alkaline-lysis method of Maniatis et al. (1982). Plant DNA inserts were excised from the plasmids with the appropriate restriction enzymes, separated in 1.5% agarose and adsorbed into DEAE-cellulose (Banner 1982). The probes were labelled with [$^{32}$P]-dATP using nick-translation.

### *Southern and dot-blot hybridization*

DNA was extracted from the somatic hybrid cultures according to the method of Dellaporta et al. (1983). For Southern blots, apporoximately 5 µg of genomic DNA was digested with 15–20 U of restriction endonucleases for 4 h, size-fractionated on 0.8% agarose gels overnight and transferred to "Kapron" membrane (Hiu, Kalur, USSR). For dot-blot hybridization, genomic and plasmid DNAs were denatured by boiling for 10 min. Serial dilutions of plasmid DNA (2, 4, 6, 8, 10 ng) and genomic DNAs of *N. plumbaginifolia*, *N. sylvestris* (25, 50, 100, 150, 200 ng) and hybrids (50, 200 ng) were spotted onto "Kapron" membrane, washed with 0.5 M NaOH, 1.5 M NaCl and neutralized in 0.5 M Tris-HCl (pH 7.5), 1 mM EDTA. Hybridizations were carried out as described by Church and Gilbert (1984). The filters were

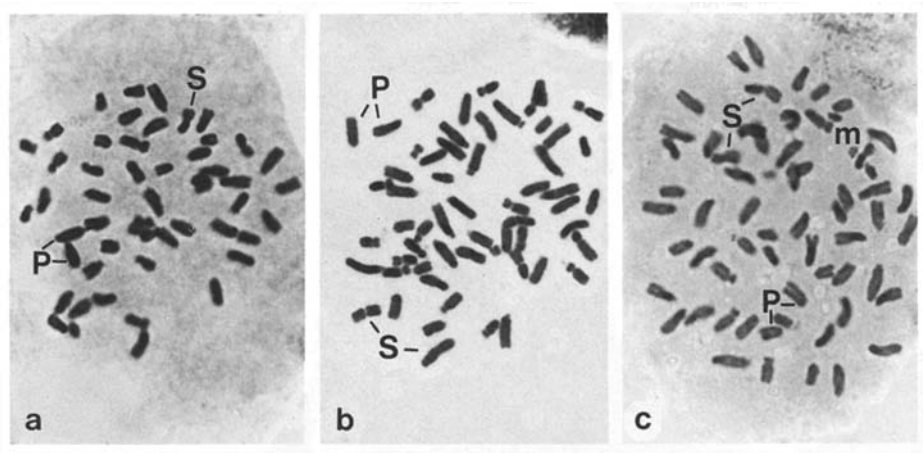


**Fig. 1 a–c.** Chromosome spreads of asymmetric somatic hybrids between *N. plumbaginifolia* and *N. sylvestris*. Mesophyll protoplasts of *N. sylvestris* (donor) were treated with 10 Gy (**a**), 100 Gy (**b**), 1000 Gy (**c**) of gamma-rays prior to cell fusion with non-irradiated protoplasts of *N. plumbaginifolia* (recipient). *P*, *S* indicate chromosome material from *N. plumbaginifolia* and *N. sylvestris* respectively; *m* indicates minichromosomes

**Table 1.** Description of the hybrid lines obtained by fusion of protoplasts from *N. sylvestris* V42 (recipient) with gamma-irradiated mesophyll protoplasts from *N. plumbaginifolia* Nia26 (donor)

| Dose (Gy) | Code | No. of V42 chrom. | No. of Nia26 chrom. | % of Nia26 genome in hybrid[a] | % of repeats in hybrid genome | |
|---|---|---|---|---|---|---|
| | | | | | NPR18 | NPR9 |
| – | oct/3z[b] | 44–45 | 20 | 40.1 | 49.6 | 40.8 |
| 10 | 1NV/1 | 24 | 20 | 55.4 | 56.3 | 53.6 |
| 10 | 1NV/3 | 24 | 20 | 55.4 | 56.1 | 59.5 |
| 10 | 1NV/15 | 24 | 20 | 55.4 | 61.9 | 57.1 |
| 10 | 1NV/30 | 24 | 20 | 55.4 | 59.2 | 58.1 |
| 10 | 1NV/32 | 24 | 20 | 55.4 | 62.2 | 55.9 |
| 10 | 1NV/21 | 23–24 | 20–21 | 56.5 | 58.4 | 110.4 |
| 100 | 10NV/1 | 45 | 16 | 34.6 | 34.0 | 33.6 |
| 100 | 10NV/2 | 46 | 14 | 31.1 | 40.1 | 38.8 |
| 100 | 10NV/5 | 30 | 15–16 | 43.9 | 46.6 | 49.6 |
| 100 | 10NV/10 | 48 | 14–16 | 32.3 | 33.0 | 35.6 |
| 1000 | 100NV/4 | 34 | 5–6 | 19.4 | 37.2 | 51.6 |
| 1000 | 100NV/7 | 46–48 | 9–10 | 22.9 | 33.8 | 80.9 |
| 1000 | 100NV/9 | 40–41 | 3–4 | 13.5 | 37.0 | 104.7 |
| 1000 | 100NV/10 | 44 | 2 | 7.2 | 15.2 | 64.9 |

[a] The percentage of the *N. plumbaginifolia* genome in a hybrid line has been calculated using the cytological analysis data (see Materials and methods)

[b] Line oct/3z is a product of symmetric cell fusion between mesophyll protoplasts of *N. plumbaginifolia* cnx20 and *N. sylvestris* ALC without applying gamma-irradiation (Famelaer et al. 1989)

Underlined figures indicate lines in which NPR18 or NPR9 are represented in quantities greater than expectation

washed in 0.1 × SSC, 1% SDS at 65 °C for 60 min. After autoradiography, the dots were cut from the membrane and counted in a liquid scintillation analyzer. Background radioactivity was subtracted from the values obtained.

## Results and discussion

### *Asymmetric somatic hybrids between N. sylvestris and N. plumbaginifolia*

Interspecific hybrids between *N. sylvestris* and *N. plumbaginifolia*, obtained after irradiation of *N. sylvestris* with 10 and 100 Gy, were morphologically normal, with a phenotype resembling the recipient (*N. plumbaginifolia*). Hybrids obtained after irradiation of *N. sylvestris* with 1,000 Gy had a reduced morphogenetic potential; their stems and roots regenerated only slowly (during 4–5 months) and they had twisted leaf blades. After a year of cultivation, the morphology of the leaf blades had returned to normal.

Somatic hybrids, obtained after fusion of *N. plumbaginifolia* protoplasts irradiated with 10 or 100 Gy and intact protoplasts of *N. sylvestris*, resembled tetraploid *N. plumbaginifolia* morphologically, although cytological and molecular analyses have confirmed their hybrid nature (Table 1). All hybrid clones in these combinations were morphologically normal and fertile. Hybrids obtained using the highest irradiation dose (1,000 Gy) produced poor shoots, regenerated slowly, and were sterile.

### *Chromosome analysis of hybrids*

Chromosome numbers are shown in Tables 1 and 2 (columns 3 and 4). Karyological analysis of hybrid combinations with irradiated *N. sylvestris* demonstrated a wide range of variation among recombinant forms (Table 2, Fig. 1 a–c). As a rule, the hybrids combined a complete or incomplete diploid genome (donor) and a diploid or tetraploid set of *N. plumbaginifolia* chromosomes (recipient). Hybrids obtained after 1,000 Gy of irradiation could be divided into two groups, according to the presence of either two (100VC/2, oct100/7) or three

**Table 2.** Description of the hybrid lines obtained by fusion of protoplasts from *N. plumbaginifolia* cnx20 (recipient) with gamma-irradiated mesophyll protoplasts from *N. sylvestris* V42 (donor)

| Dose (Gy) | Code | No. of V42 chrom. | No. of Nia26 chrom. | % of Nia26 genome in hybrids[a] | % of repeats in hybrid genome | | |
|---|---|---|---|---|---|---|---|
| | | | | | NPR18 | NPR9 | pNP21 |
| 10 | 1VC/1 | 24 | 28 | 63.5 | 73.6 | 68.0 | |
| 10 | 1VC/2 | 24 | 32 | 66.5 | 105.6 | 66.3 | 71.0 |
| 10 | 1VC/3 | | | | 141.5 | 71.4 | 69.4 |
| 100 | 10VC/1 | 24 | 34–36 | 68.4 | 76.3 | 72.8 | 78.0 |
| 100 | 10VC/2 | 20 | 36 | 72.8 | 94.4 | 74.0 | 68.0 |
| 100 | 10VC/7 | 12 | 40 | 83.2 | 80.0 | 83.7 | 79.1 |
| 100 | 10VC/9 | 23–24 | 20–24 | 57.2 | 60.5 | 58.4 | 61.3 |
| 500 | oct50/7[b] | 8–10 | 56–58 | 90.4 | 94.3 | 83.0 | 85.9 |
| 500 | oct50/8[b] | 10 | 40 | 85.6 | 79.4 | 95.0 | 85.2 |
| 1000 | 100VC/2 | 4–5 | 35–38 | 92.2 | 150.0 | 91.1 | 97.3 |
| 1000 | 100VC/3 | 8 | 52–53 | 90.7 | 91.1 | 83.7 | 89.0 |
| 1000 | oct100/4[b] | 8 | 55–56 | 91.0 | 84.3 | 92.1 | |
| 1000 | oct100/6[b] | 9 | 58–59 | 90.5 | 85.7 | 90.7 | |
| 1000 | oct100/7[b] | 7 | 40 | 89.5 | 137.1 | 89.5 | 80.0 |

[a] The percentage of the *N. plumbaginifolia* genome in a hybrid line has been calculated using the cytological analysis data (see Materials and methods)

[b] Lines oct50 and oct100 were obtained by fusion of protoplasts from *N. plumbaginifolia* cnx20 with gamma-irradiated mesophyll protoplasts from *N. sylvestris* ALC (Famelaer et al. 1989)

Underlinded figures indicate lines in which NPR18 is represented in quantities greater than expectation

(100VC/3, oct100/4, oct100/6) recipient diploid chromosome complements in addition to 1–5 chromosomes from *N. sylvestris* and 3–7 reconstructed chromosomes of indeterminate origin.

Hybrids obtained after irradiation of *N. plumbaginifolia* were more homogeneous cytologically than those obtained after irradiation of *N. sylvestris* (Table 1). After donor irradiation with 10 Gy, all regenerants were symmetric hybrids that possessed diploid chromosome sets from each of the parents. Regenerant plants selected after treatment with 100 Gy combined incomplete diploid and tetraploid parental genomes. Application of 1,000 Gy led to a significant loss of *N. sylvestris* chromosomes. In this case, only 2–10 *N. sylvestris* chromosomes, including minichromosomes, remained in the regenerants, in addition to a complete tetra- or hexaploid genome from *N. plumbaginifolia*.

The putative percentage of the *N. plumbaginifolia* genome in any one hybrid line was calculated, taking into account their chromosome numbers and the conventional average weight of each *N. plumbaginifolia* or *N. sylvestris* chromosome (see Materials and methods, Tables 1 and 2, column 5). It needs to be borne in mind that the values obtained give only a rough estimate of the amount of the *N. plumbaginifolia* genome in the hybrid plants. This is so because the differences in size of the different chromosomes in *N. plumbaginifolia* and *N. sylvestris* is not taken into account. Hence, in order to obtain data which is more reliable, we have cloned several species-specific repetitive sequences of *N. plumbaginifolia* and used them, together with the dispersed repeat which was isolated earlier, to calculate the relative contribution of the *N. plumbaginifolia* genome to the asymmetric somatic hybrids.

### *Isolation and characterization of repetitive DNA sequences from N. plumbaginifolia*

Repetitive sequences were selected from a partial library of 300 recombinant clones after colony hybridization with $^{32}$P-labelled genomic DNA from *N. plumbaginifolia*. Rehybridization of the same colonies with total *N. sylvestris* DNA identified those repeats that were species-specific. Two of them, which hybridized only with total *N. plumbaginifolia* DNA, were selected for further investigation. The plant inserts of these plasmids (NPR9 and NPR18) had no homology with pNP21 (Piastuch and Bates 1990) and gave different hybridization patterns on Southern blots from those of other repetitive sequences previously isolated from *N. plumbaginifolia* (Seeckaert and Jacobs 1988).

NPR9 was used as a probe for Southern hybridization to restriction digests of total DNA from *N. plumbaginifolia* (Fig. 2a). Distinct bands were obtained after hybridization with *Bam*HI-digested DNA (Fig. 2a, lane 3), whereas after *Hin*dIII digestion, the hybridization signal was located mainly near the origin (Fig. 2a, lane 1).

*Bam*HI, *Bam*HI + *Eco*RI, and *Bam*HI + *Hin*dIII digestions of *N. plumbaginifolia* DNA were probed with NPR18 (Fig. 2b, lanes 1–3). In each case, the probe hybridized to a smear throughout the whole track. This kind of hybridization pattern is typical of dispersed repetitive sequences (Mouras et al. 1987; Piastuch and Bates 1990).

For estimation of the fraction of *N. plumbaginifolia* genome corresponding to NPR9- and NPR18-homologous sequences, different dilutions of the repeats (2–10 ng) and *N. plumbaginifolia* (25–200 ng) DNAs were spotted onto "Kapron" membranes and hybridized with NPR9- and NPR18-repetitive sequences. The copy number of the repeats per *N. plumbaginifolia* haploid genome was then calculated, taking into account the length of the repetitive sequences and assuming the *N. plumbaginifolia* haploid genome (1C) weight to be 5.21/2 = 2.6 pg. It was determined that NPR18 was a moderately repetitive sequence occupying about 0.4% of the nuclear genome and having approximately 2,900 copies per haploid genome. NPR9 is a part of highly repetitive DNA sequence occupying approximately 3% of the total genome and has about 12,000 copies per haploid genome.

NPR9 and NPR18 showed extremely low signals after Southern hybridization with *N. sylvestris* DNA (Fig. 2, lanes a2 and b4), compared to hybridizations with *N. plumbaginifolia* (Fig. 2, lanes a1, b3). Thus, we suggest that these repeats are suitable for use as species-specific markers in asymmetric hybrid analysis.

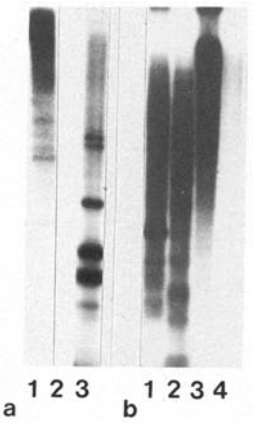


**Fig. 2a, b.** Characterization of the species-specific repetitive sequences NPR9 and NPR18. Total DNAs of *N. plumbaginifolia* and *N. sylvestris* were digested with various restriction endonucleases, separated on an 0.8% agarose gel and probed with NPR9 (**a**) and NPR18 (**b**); *N. plumbaginifolia* DNA was restricted with *Hin*dIII (*lane a1*), *Bam*HI (*lanes a3, b3*), *Bam*HI + *Eco*RI (*lane b2*), *Bam*HI + *Hin*dIII (*lane b1*), *N. sylvestris* DNA was digested with *Bam*HI (*lanes a2, b4*)

*Use of repeated sequences for quantitative analysis of hybrids*

Fig. 3a, b shows some examples of dot-blot hybridization in which dilutions of total genomic DNA from hybrids and parental plants were hybridized with NPR9 and NPR18. The radioactivities of the individual dots were quantified by scintillation counting and plotted in relation to DNA amounts. The intensity of hybridization between *N. plumbaginifolia* and the two hybrids in relation to DNA sample concentration is shown in Fig. 4a, b. The calibration plots were used to compare the intensity of the hybridization signal generated by equal amounts of *N. plumbaginifolia* and hybrid DNAs. The percentage of *N. plumbaginifolia* repetitive sequences in each of the somatic hybrids was calculated assuming the quantity of the repeated sequence in the *N. plumbaginifolia* genome to be 100% (Tables 1 and 2, columns 6–8). The relative estimated quantity of dispersed repeats is considered to reflect the proportion of the total *N. plumbaginifolia* genome in asymmetric hybrids (Imamura et al. 1987; Piastuch and Bates 1990). In the opinion of Piastuch and Bates (1990), 10% discrepancies between the chromosome counts and dot-blot data may be related either to experimental errors in dot-blotting or to an undetected nonrandom distribution of the repetitive sequences in the plant genome. In order to increase the reliability of our calculation, two different dispersed repeats (NPR18 and pNP21) and the highly repetitive sequence NPR9 were applied to somatic hybrid analysis.

The results of dot-hybridization with the dispersed repeat NPR18, together with the cytological analysis data, show that in fusion experiments with irradiated *N. plumbaginifolia*, increasing the irradiation dose leads to a loss of *N. plumbaginifolia* nuclear DNA in the somatic hybrids (Table 1). The fact that previous investigators did not detect a correlation between donor irradiation dose and the degree of elimination of donor nuclear DNA (Dudits et al. 1987; Imamura et al. 1987; Gleba et al. 1988; Piastuch and Bates 1990; Wolters et al. 1991) may be related either to the low number of asymmetric hybrids analyzed and the narrow range of irradiation doses used or, in examples of somatic hybridization between distant relatives, to parental genome incompatibility. A correlation between irradiation dose and donor genome elimination has, however, been detected in somatic hybrids between species of *Lycopersicon* using RFLP analysis (Wijbrandi et al. 1990b; Melzer and O'Connell 1992).

The discrepancy (10–20%) between the cytological data and the results of dot-hybridization with NPR18 (Table 1, 1,000 Gy) may be explained by the presence of minichromosomes, which are sometimes difficult to detect cytologically, but will nevertheless contribute to the hybridization signal with NPR18. Because DNA for these lines has been isolated from tissue culture, whereas

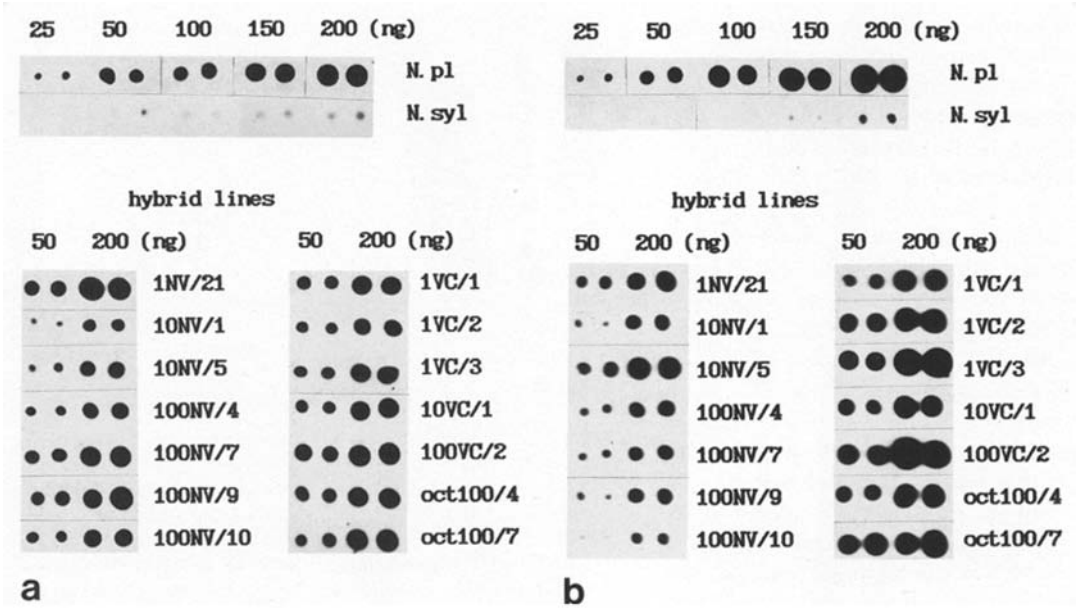


**Fig. 3a, b.** Dot-blot hybridization of genomic DNAs from *N. plumbaginifolia* (*N. pl*), *N. sylvestris* (*N. syl*) and somatic hybrids probed with NPR9 (**a**), NPR18 (**b**)

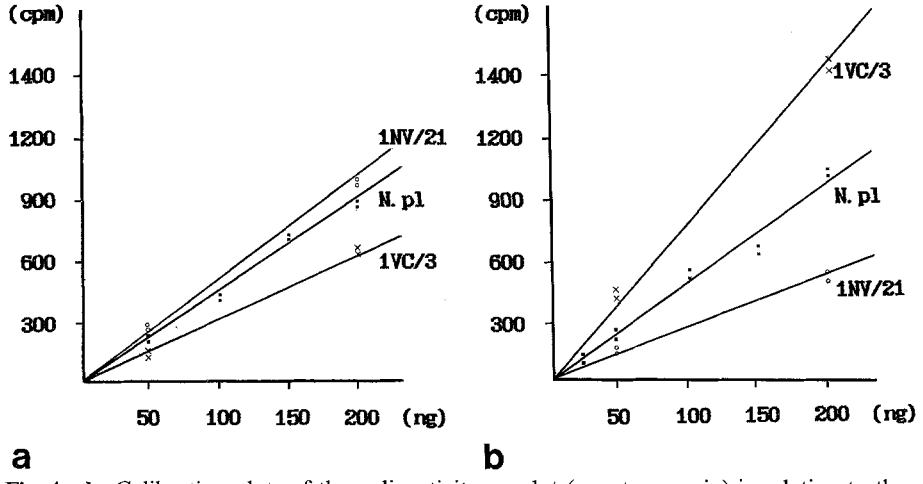


**Fig. 4a, b.** Calibration plots of the radioactivity per dot (counts per min) in relation to the amounts (ng) of genomic DNAs from *N. plumbaginifolia* and hybrids 1NV/21 and 1VC/3. **a** Dots were cut out of the filter in Fig. 3a. **b** Dots were cut out of the filter in Fig. 3b. Radioactivity per dot was measured in a liquid scintillation analyzer. Background radioactivity was subtracted from the values obtained

cytological analysis has been performed on hybrid roots of regenerants, the elimination of irradiated donor chromosomes during the regeneration of hybrid lines is also possible.

For most somatic hybrids obtained after irradiation of *N. plumbaginifolia* with 10 and 100 Gy, the result of dot-hybridization with NPR9 and NPR18 was in agreement with the cytological analysis (Table 1). This suggests that NPR9 is located on all, or almost all, *N. plumbaginifolia* chromosomes. However, the somatic hybrid line 1NV/21 of this combination was characterized by a two-fold increase in the amount of NPR9 (Table 1, Figs. 3, 4) relative to the value expected from cytological analysis and the result of dot hybridisation with NPR18. After irradiation of *N. plumbaginifolia* with 1,000 Gy, all hybrid lines showed a two- to nine-fold increase in NPR9 (Table 1, Fig. 3). These results suggest firstly, that the repeated sequence NPR9 has been amplified in the somatic hybrids, and secondly, that the degree of this amplification is correlated with the irradiation dose administered to the donor prior to cell fusion.

Plant cell cultivation and somatic hybridization can induce gene amplification (Xiao et al. 1987; Gleba et al. 1988; Hauptmann et al. 1988). Repetitive DNA se-

quences from rice have been shown to be amplified in cell culture (Zheng et al. 1987; Cuzzoni et al. 1990). Evidence for amplification of repeated DNA sequences has also been given for wheat × rye hybrids regenerated from immature embryos (Lapitan et al. 1988). There are many examples of amplification, sometimes resulting in "megachromosomes", in sexual hybrids between *Nicotiana* species (Gerstel and Burns 1966, 1976; Reed and Burns 1989; Reed et al. 1989). These examples can involve euchromatin or heterochromatin, suggesting that the amplified sequences could be either clustered (heterochromatic) or dispersed (euchromatic) repeated sequences.

Since an increase in copy number of NPR9 has not been observed for hybrids obtained without irradiation (Table 1, hybrid line oct/3z), we suggest that amplification of the repeated sequence has been induced by irradiation of the donor genome rather than by cultivation or the somatic hybrid of *N. plumbaginifolia*. This suggestion is supported by the fact that the increase in copy number of NPR9 is more pronounced with increasing doses of irradiation. Thus, for low radiation doses, the incidence of amplification is restricted to line 1NV/21, while amplification of NPR9 has been detected for all lines obtained after irradiating *N. plumbaginifolia* with 1,000 Gy (Table 1).

For all hybrid lines using irradiated *N. sylvestris* the results of dot-hybridization with NPR9 and pNP21 agree largely with cytological analysis (Table 2). However, the results of dot-blotting with the dispersed repeat NPR18 do not always coincide with the expected content of *N. plumbaginifolia* DNA in hybrids as determiend by cytological analysis. Clones 1VC/2, 1VC/3, 100VS/2 and oct100/7 showed amplification of NPR18 (Table 2). After hybridization with NPR18, DNA from these hybrids generated stronger hybridization signals than did *N. plumbaginifolia* DNA (Figs. 3, 4). It is interesting that hybrid line 1VC/3 which showed the most prominent amplification had a reduced morphogenetic potential and we were unable to obtain roots for cytological analysis from this line. The fact that there is no increase in NPR18 copy number for the somatic hybrid line oct/3z obtained without irradiation (Table 1) suggests that amplification of NPR18 may have been induced in the hybrid by the influence of the irradiated *N. sylvestris* protoplast on the non-irradiated *N. plumbaginifolia* genome. These data provide the first evidence that irradiation of one of the partners used for asymmetric somatic hybridization may lead to serious changes in the genome organization of the second (non-irradiated) parent. The technology of "gamma-hybridization" is thus quite a complex process, the consequences of which require careful investigation.

Our research shows that repeated sequences are a genetically unstable part of the nuclear genome and may serve as indicators of various influences on the plant cell. This fact needs to be taken into account, and caution exercised, in the interpretation of somatic hybrid analysis using repetitive DNA sequences.

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