Theor Appl Genet (1994) 88: 965–972

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Chromosome elimination in asymmetric somatic hybrids: effect of gamma dose and time in culture

Received: 15 December 1993 / Accepted: 12 January 1994

Abstract Mesophyll protoplasts of a kanamycin-resistant line of *Nicotiana plumbaginifolia* were gamma-irradiated and fused with mesophyll protoplasts of *N. tabacum* plants bearing the sulfur mutation. Hybrid calli were recovered by selection on media containing kanamycin. In one group of experiments, the degree of elimination of donor (*N. plumbaginifolia*) genetic material in the hybrid calli was assessed by dot-blot hybridization using a *N. plumbaginifolia*-specific repetitive-DNA sequence as a probe. The elimination of donor DNA was found to increase with increasing gamma dose for all doses tested (5–50 krad). Elimination of donor DNA was also found to continue in the calli for the first 12 months in culture. The degree of chromosome elimination was quite variable; for a 50-krad dose, some hybrids were recovered that retained less than 15% of the donor genome, whereas others retained nearly 50%. In a second set of experiments, the degree of donor-chromosome elimination was assessed from the fraction of hybrid calli that exhibited complementation of the Su phenotype due to retention of a wild-type Su allele of the donor. When *N. plumbaginifolia* protoplasts were inactivated by treatment with iodoacetate, rather than gamma irradiation, all the hybrid calli were green. However, when the donor protoplasts were inactivated by irradiation, the fraction of hybrid calli that were able to complement the Su mutation decreased with increasing gamma dose; for a 50-krad dose only 40% of the hybrid calli were green. From these data, the degree of radiation-induced donor-chromosome elimination was calculated and was found to agree closely with that measured by dot-blot hybridization. We conclude that radiation-induced elimination of donor chromosomes increases with gamma dose and time in culture in *N. tabacum* (+) *N. plumbaginifolia* hybrids, but that donor-chromosome elimination is an inherently variable process.

Key words Protoplast fusion · Irradiation
Asymmetric somatic hybrids · Chromosome elimination
Nicotiana

Communicated by P. Maliga

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Introduction

Asymmetric somatic hybridization is being explored as a means for transferring specific genes or chromosomes between sexually incompatible plant species. For asymmetric hybridization, protoplasts of a donor species are exposed to a lethal dose of X- or γ-radiation. Then, these irradiated protoplasts are fused with unirradiated protoplasts of the recipient species, and hybrids are usually recovered by selection for a nuclear-encoded trait of the donor. Irradiation causes fragmentation and elimination of donor chromosomes, but selection for a nuclear-encoded donor trait results in the recovery of hybrids that retain some donor genetic material. Donor-recipient fusions have been used to prepare asymmetric hybrids in a wide range of species combinations (see, for example, Gleba et al. 1988; Yamashita et al. 1989; Melzer and O'Connell 1992; Bauer-Weston et al. 1993; Puite and Schaart 1993). Many examples of fertile asymmetric hybrids have been reported, including cases involving species combinations in which fertile sexual or somatic hybrids have never been recovered (Dudits et al. 1987; Gleba et al. 1988; Bates 1990; Hinnisdaels et al. 1991). The fertility of asymmetric hybrids implies that it may be possible to introgress traits stably from a donor into a recipient species by asymmetric hybridization.

The published data on asymmetric hybrids indicates that the extent of radiation-induced chromosome elimination is very variable. In some cases only a single donor chromosome or chromosome fragment is detected in the hybrids

(Piastuch and Bates 1990; Hinnisdaels et al. 1991); in other cases more than 50% of the donor genome is retained (Wijbrandi et al. 1990; Wolters et al. 1991; McCabe et al. 1993). Because the goal of asymmetric hybridization is the selected transfer of desired traits, chromosomes, or chromosomal fragments, while the contribution of the donor genome is minimized, it is important to identify the factors that control chromosome elimination in donor-recipient fusions. Some physical, genetic, and cellular factors that could be involved in chromosome elimination in asymmetric hybrids include radiation dose, culture conditions, the method used for hybrid selection, and the ploidy and phylogenetic relatedness of the donor and recipient species. Assessing the relative importance of these factors is made difficult by the wide range of species combinations, radiation doses, culture conditions, and methods for hybrid analysis used in different laboratories. Considerable controversy exists over the importance of the most basic factor, radiation dose. Increasing radiation doses cause increasing amounts of chromosomal damage (Kiefer 1990); therefore, the extent of elimination of donor chromosomes would be expected to increase with radiation dose. Although two studies report an increase in donor-chromosome elimination with increasing radiation dose (Melzer and O'Connell 1992; Kovtun et al. 1993), numerous other studies reveal little increase in chromosome elimination beyond that induced by a relatively low, threshold radiation dose (Imamura et al. 1987; Yamashita et al. 1989; Wijbrandi et al. 1990; Wolters et al. 1991; Bauer-Weston et al. 1993; McCabe et al. 1993).

In this study we have examined the elimination of donor chromosomes in donor-recipient fusions between *Nicotiana tabacum* and *N. plumbaginifolia*. Chromosome elimination was assessed by two different approaches, one molecular and the other genetic. Both methods of analysis showed that chromosome elimination increases with radiation dose. Chromosome elimination was also found to continue during long-term callus culture.

Materials and methods

Plant material

The recipient species for all experiments was *N. tabacum* homozygous for the Sulfur mutation (genotype *Su/Su*). Kanamycin-resistant (Km^r), β-glucuronidase (GUS)-expressing, plants of *N. plumbaginifolia* were the donors. These *N. plumbaginifolia* plants had been transformed by *Agrobacterium* carrying the binary vector pBI121 (Clonetech, USA; Jefferson 1987) using the leaf-disc transformation procedure (Rogers et al. 1986). Km^r transformants with high levels of GUS expression were selfed and backcrossed, and the progeny were tested for Km^r and GUS expression. Only transformants carrying a single functional T-DNA insert were used as donors in fusion experiments. Plants used for fusions were propagated vitro on half-strength MS medium (Murashige and Skoog 1962) containing 1.5% sucrose.

Protoplast isolation, irradiation, fusion, culture, and hybrid selection

Leaves were cut into strips and digested overnight in a solution of 0.1% Cellulysin (Calbiochem, U.S.A.) +0.02% Pectolyase-Y23 (Kanematsu, Inc., Los Angeles, USA) +0.46 M mannitol in CPW salts (Frearson et al. 1973) at pH 5.[2] Live protoplasts were recovered by flotation on 18% sucrose and were washed twice with 0.46 M mannitol in CPW salts. For fusion, the protoplasts were pelleted and resuspended in 0.5 M mannitol +0.5 mM $CaCl_2$ at a density of 5×10^5 protoplasts/ml and mixed in a ratio of 1:3 (*N. plumbaginifolia* to *N. tabacum*). Protoplasts were fused electrically as described by Bates et al. (1987).

Prior to fusion, donor protoplasts were inactivated by irradiation with gamma rays from ^{137}Cs (370 rad/min). In experiments in which no radiation was applied, the donor protoplasts were inactivated by treatment with 2.5 mM iodoacetate in CPW salts +0.46 M mannitol for 30 min. Iodoacetate-treated protoplasts were washed three times with CPW salts +0.46 M mannitol prior to fusion. After fusion, 0.3 ml of the fused protoplast mixture was diluted with 0.5 ml of 0.5 M mannitol +1.2 ml of K_3 medium (Nagy and Maliga 1976) containing 0.48 M glucose, and the protoplasts were cultured at 27 °C, in the dark. After 4–6 days, 0.5 ml of TM-2 (Shahin 1985) was added, and 7–10 days after fusion 1.0 ml of TM-2 was added. At 11–14 days after fusion, 3.5 ml of TM-2 was added, and the cultures were split and transferred to the light (20–60 μE/m^2, 27 °C). Then, at 4-day intervals, the cultures were diluted and split three times by the addition of 3.5 ml of TM-2A (TM-2 without sugar and alcohols and with the hormone concentrations reduced by one half). Twenty-three to twenty-eight days after fusion, the cultures were pelleted, and the microcalli were resuspended in liquid MS medium (no hormones) and plated on solidified MS medium +0.2 mg/l benzyladenine (BA) with or without 100 mg/l of kanamycin. One to four weeks after plating, hybrid calli were identified by their ability to grow on kanamycin. Hybrid calli were subcultured every 4 weeks on MS +0.2 mg/l BA. *Nicotiana plumbaginifolia* calli will turn green and regenerate on this medium. Regenerated shoots were transferred to half-strength MS (no hormones) for rooting and in-vitro propagation.

Dot-blot determination of the amount of *N. plumbaginifolia* DNA in the hybrids

DNA was isolated from calli and plants by a modified CTAB procedure (Saghai-Mahoof et al. 1984). DNA yields were determined fluorometrically with Hoechst dye 33258 (Labarca and Paigen 1980) and a TKO 100 minifluorometer (Hoefer Scientific Instruments, USA). Genomic DNA samples of 31.25–250 ng were used for dot-blot hybridizations with a BRL dot blotter (Cat. Series 155, Gibco-BRL Life Technologies, Inc., Bethesda Md., USA). The probe used was produced by isolation of the 1.5-kb cloned insert from plasmid pNP21 (Piastuch and Bates 1990) and labeled with ^{32}P by the random primer procedure (Feinberg and Vogelstein 1983). Plasmid pNP21 contains a 1.5-kb *Hin*dIII fragment of *N. plumbaginifolia* DNA, which is a dispersed repeat sequence present as 1600 copies per haploid nuclear genome of *N. plumbaginifolia* (Piastuch and Bates 1990). This cloned repeat sequence does not hybridize to DNA of *N. tabacum*. Dot blots were prepared, hybridized, and washed as described by Piastuch and Bates (1990). Hybridization was visualized and quantified by scanning with a Betascope Blot Analyzer (Model 603, Betagen Corp., USA). The relative amount of *N. plumbaginifolia* DNA in the hybrids was calculated from a comparison of the slopes of the linear range of hybridization of pNP21 to a dilution series of genomic DNA of the hybrids and genomic DNA of *N. plumbaginifolia*. Calculations were normalized to the hybridization value expected for a symmetric hybrid of *N. tabacum* (+) *N. plumbaginifolia*.

GUS assays

GUS activity in the *N. plumbaginifolia* transformants was quantified by the fluorometric assay described by Jefferson (1987). Qualitative assessment of GUS expression in hybrid calli and plants was carried out by the placement of 1-mm^2 tissue samples in wells of a microtiter plate, crushing of the tissues in 50–100 μl of extraction buffer (Jefferson 1987) containing 100 μM of 4-methyl umbelliferyl glucuronide, and incubation of the samples at 30 °C for 2 h to overnight. Fluorescence was detected by viewing of the microtiter plates under ultraviolet light.

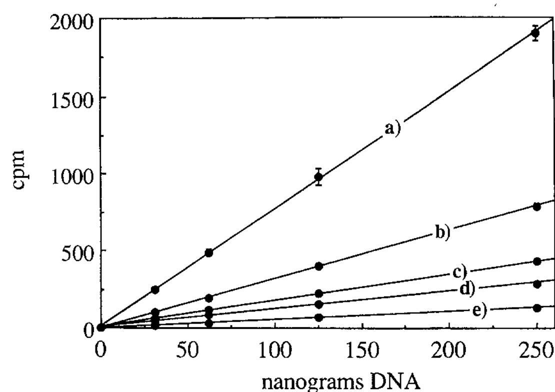


Fig. 1 Example of dot-blot hybridization data obtained when DNA from *N. plumbaginifolia* and representative asymmetric hybrids is probed with the *N. plumbaginifolia*-specific clone pNP21. DNAs applied to the blot were (*a*) *N. plumbaginifolia*; (*b*) a 1:1 genomic mixture of *N. plumbaginifolia* + *N. tabacum* DNAs; (*c*)-(*e*) three different asymmetric hybrids recovered from donor-recipient fusions in which donor protoplasts were irradiated, prior to fusion, with 5 krad, 10 krad, and 50 krad of gamma rays, respectively. For this analysis, DNA was isolated from the hybrids 6 months after fusion. *Error bars* indicate the range of triplicate samples; cpm, counts per min hybridized

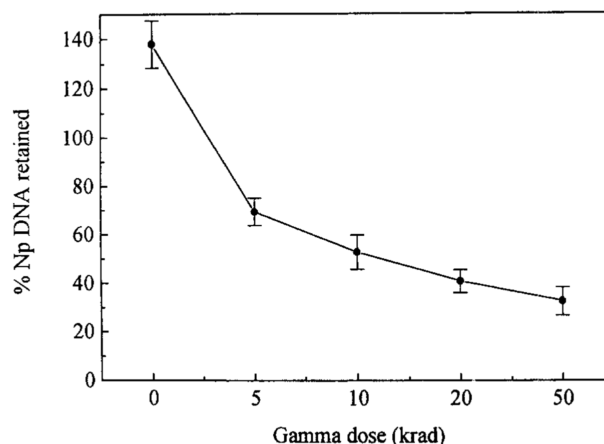


Fig. 2 Effect of radiation dose on the elimination of donor DNA in donor-recipient fusions between *N. tabacum* and *N. plumbaginifolia*. The *N. plumbaginifolia* protoplasts were irradiated prior to fusion with protoplasts of *N. tabacum*. The amount of *N. plumbaginifolia* DNA in the hybrids was assessed by dot-blot hybridization of DNA isolated from the hybrids 6 months after fusion. The data are presented as the percentage of *N. plumbaginifolia* DNA retained in the hybrids compared with that expected for a 1:1 symmetric hybrid between *N. tabacum* and *N. plumbaginifolia*. Each point represents the average of data obtained from 8 to 12 independent hybrids. The *error bars* represent standard errors of the mean

Results

Effect of radiation dose on donor-DNA retention by the hybrids

The amount of donor DNA in the hybrids was assessed by dot-blot hybridization using the *N. plumbaginifolia*-specific repetitive DNA clone pNP21 (Piastuch and Bates 1990) as a probe. Figure 1 shows the results of a typical dot blot of DNA of *N. plumbaginifolia* and several asymmetric hybrids probed with pNP21. The hybrid samples represent DNA isolated from Km^r calli 6 months after irradiation and fusion. Also included in this figure is the hybridization obtained for a measured mixture of *N. tabacum* and *N. plumbaginifolia* DNAs. The amount of *N. plumbaginifolia* DNA in the hybrids was determined by a comparison of the degree of hybridization of the probe to DNA of the hybrids with its hybridization to DNA of *N. plumbaginifolia*. A serial dilution of the target DNA was always applied to the blots to ensure that comparisons were made for the range in which the degree of hybridization increased linearly with the amount of target DNA.

Nicotiana tabacum (2N=48) contains 9.67 pg of DNA per diploid nucleus (Galbraith et al. 1983), and *N. plumbaginifolia* (2N=20) contains 5.21 pg per diploid nucleus (Galbraith et al., personal communication). Thus, a symmetric hybrid of *N. tabacum* (+) *N. plumbaginifolia* would be expected to give a dot-blot value 35% of that obtained for *N. plumbaginifolia* DNA alone. Reconstructions gave actual dot-blot values of 37.5% (on average) for 1:1 genomic equivalent mixtures of *N. tabacum* + *N. plumbaginifolia* DNA. We have normalized all our dot-blot data to this measured value for a 1:1 mixture. Thus, the 5-krad hybrid shown in Fig. 1 contains 62.4% of the *N. plumbaginifolia* genome expected for a symmetric hybrid, and the 10-krad and 50-krad hybrids contain 42.1% and 17.9%, respectively.

Figure 2 presents compiled dot-blot data and shows the amount of *N. plumbaginifolia* DNA present in hybrid calli as a function of the radiation dose applied to the donor protoplasts prior to fusion. The hybridization data plotted here are for DNAs isolated from hybrids 6 months after fusion. The 0-krad samples represent DNA from hybrids in which the donor protoplasts were inactivated by iodoacetate treatment rather than by irradiation. As reflected by the standard errors plotted in Fig. 2, there is considerable variation in the amount of *N. plumbaginifolia* in the hybrids. Nonetheless, there is a progressive decrease in the average amount of *N. plumbaginifolia* DNA in the hybrids with increasing radiation dose. We have applied the Newman-Keuls multiple-comparison test to the data shown in Fig. 2 to determine their statistical significance. The results of this test (shown in Fig. 3) indicate that, at the 0.05 confidence level, the 0-krad group differs from all the other groups, and the 5-krad group differs significantly from the 20-krad and 50-krad groups. However, several of the other group comparisons are significant at the 0.1 level. Specifically, there is a 92% probability that the 5-krad and 10-krad groups are different and a 90% probability that 10-krad and 50-krad doses result in different amounts of chromosome elimination. The difference between the 10-krad and 20-krad groups also approaches significance (P=0.79). The test of our data indicates a 60% chance that the 20-krad and 50-krad groups differ.

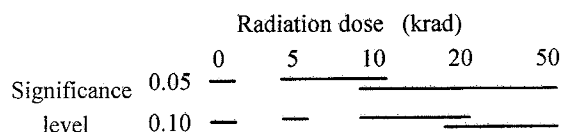


Fig. 3 Results of the Newman-Keuls multiple-comparison test to determine the statistical differences between the amounts of donor DNA eliminated in response to different radiation doses. The significance was determined for critical values of both 0.05 and 0.1. Treatments grouped together by a *continuous underline* are not significantly different

Table 1 Percentage of *N. plumbaginifolia* DNA in individual hybrid calli during extended callus culture. Hybrids are from irradiation with 5 krad (5K-1 to 5K-7), 10 krad (10K-1 to 10K-6), 20 krad (20K-1 to 20K-7), and 50 krad (50K-1 to 50K-6) of gamma rays. DNA samples were isolated from individual calli after 6 months and again after 12 months in culture and were analyzed by dot-blot hybridization using pNP21 as the probe. The results are expressed as the percentage of *N. plumbaginifolia* DNA retained

Callus #	% Np DNA retained	
	6 Months	12 Months
5K-1	41	27
5K-2	90	70
5K-3	48	45
5K-4	85	94
5K-5	58	73
5K-6	82	84
5K-7	80	82
10K-1	53	52
10K-2	31	29
10K-3	56	36
10K-4	28	19
10K-5	52	29
10K-6	82	47
20K-1	55	31
20K-2	39	33
20K-3	29	21
20K-4	45	26
20K-5	68	38
20K-6	23	15
20K-7	54	42
50K-1	37	36
50K-2	26	26
50K-3	29	14
50K-4	21	19
50K-5	23	12
50K-6	65	48

The average amount of *N. plumbaginifolia* DNA in the 0-krad hybrids is 139%, rather than the 100% expected for a symmetric hybrid. The raw data show that these hybrids fall into two groups, those with dot-blot values around 100% and those with values around 160%. Chromosome counts were made for several plants regenerated from these hybrids. Plants regenerated from hybrid calli with dot-blot values of 100% had chromosome counts of 68, the expected value for a symmetric hybrid. Plants from hybrids with higher dot-blot values were mixoploid and had chromosome numbers ranging from 80 to 96. Plants regenerated from gamma-fusion calli had chromosome counts lower than 68 and had a good correspondence between their dot-blot values and chromosome counts. For example, root tips of a plant regenerated from a 5-krad callus with a 43% dot-blot value had a chromosome count of 56, and a 10-krad callus with a dot-blot value of 47% regenerated a plant with a chromosome count of 57.

Effect of time in culture on the elimination of donor chromosomes

To learn whether radiation-induced chromosome elimination continues during long-term callus culture, DNA samples were isolated from individual hybrid calli after 6 and 12 months in culture and were analyzed by dot-blot hybridization. As shown in Table 1, the amount of donor-chromosome elimination varies greatly from callus to callus, but the trend is for individual calli to lose *N. plumbaginifolia* DNA over time. Both the paired-sample *t*-Test and the Wilcoxon paired-sample test show that this decrease is significant ($P < 0.05$) for the 10-krad, 20-krad, and 50-krad calli, but is not significant for the 5-krad calli. After 12 months in culture the average dot-blot values for the 10-krad, 20-krad, and 50-krad calli had decreased by 28%, 34%, and 23%, respectively, from their six-month value. We have analyzed a small number of samples after 18 months in culture and have obtained no evidence for further elimination of *N. plumbaginifolia* DNA (data not shown).

Segregation of genetic markers of the donor in hybrid calli: the Su-complementation assay for chromosome elimination

As an independent approach to the evaluation of the effect of radiation dose on chromosome elimination, we asked whether two independent genetic markers in the donor plants will segregate in the hybrid calli. The rationale for this experiment is the idea that we can follow the elimination of donor chromosomes in a group of hybrids by following the segregation of two genetic markers present on different donor chromosomes. By this approach, the effectiveness of different radiation treatments can be assessed by the identification of hybrids that express one marker gene of the donor followed by measurement of the fraction of these hybrids that express the second donor marker. In this assay, the loss of the second marker is assumed to be due to the loss of the donor chromosome carrying it.

The two genetic markers used in this experiment were the Su locus and a Km^r-GUS T-DNA insert. In all our fusions, the recipient was *N. tabacum* homozygous for the nuclear-encoded Sulfur mutation (Evans et al. 1983), and the donor was *N. plumbaginifolia* wild type for the Su locus (normal green) and carrying a single Km^r-GUS insertion from *Agrobacterium*. It has previously been demonstrated that the Su mutation of tobacco can be complemented by the corresponding wild-type allele in interspecific somatic hybrids of *Nicotiana* (Evans et al. 1983).

Also, the presence/absence of the Su mutation can be assessed in culture in calli. If the wild-type Su allele in *N. plumbaginifolia* complements the allele in *N. tabacum* and is on a chromosome different from that carrying the Km^r-GUS transgene, then the segregation of the green/Su and Km^r-GUS markers in hybrid calli should provide a measure of the elimination of donor chromosomes. This experiment can be done in two ways: following irradiation and fusion, hybrid calli can be selected on media containing kanamycin and scored for their ability to green, or unselected hybrid calli can be identified by their ability to green and then scored for GUS activity.

In a preliminary experiment, both approaches were tried using *N. plumbaginifolia* protoplasts irradiated with a dose of 20 krad. When hybrid calli were allowed to green without being selected for expression of Km^r, they ranged in color from dark green to various shades of light green to yellow (sulfur). This variation in color made positive identification of hybrid calli difficult, but of 34 clearly-green calli, 18 (53%) were found not to express GUS. This segregation of the Su and GUS markers suggests they are located on different chromosomes in *N. plumbaginifolia*. Further evidence for the independent segregation of these markers is provided by the observation that, in seven calli in which both green and yellow sectors were visible, there was no correlation between a given sector's color and whether or not it expressed GUS (data not shown). In the reciprocal experiment, hybrid calli were recovered by selection for Km^r and were scored for their ability to green. Thirty-three percent of Km^r calli (19 of 58) were sulfur colored, and the remaining 39 calli (67%) were some shade of green. These data also indicate that the marker loci, Su and Km^r-GUS, were segregating independently in the calli.

Because we found it more convenient to select for hybrids on the basis of kanamycin resistance and then to score the hybrid calli for greening, we used this approach to examine the effect of various radiation doses on donor-chromosome elimination. The results, shown in Fig. 4, indicate that increasing radiation doses result in a progressive reduction of the fraction of hybrid calli that are able to green. The shape of the radiation dose-response curve in Fig. 4 is flatter than that of Fig. 2. This difference between the two assays is probably not the result of differences in chromosome elimination. Instead it probably reflects the fact that both members of the homologous pair of *N. plumbaginifolia* chromosomes carrying the wild-type Su allele must be eliminated in order to form a sulfur-colored callus, whereas every *N. plumbaginifolia* chromosome that is lost is detectable by dot blotting.

The percentage of *N. plumbaginifolia* DNA eliminated can be estimated from the Su-complementation-assay data in Fig. 4 if one assumes that the Su locus of *N. plumbaginifolia* behaves like an average locus and that loss of the ability to green is due to elimination of both homologues carrying this locus. For example, after a 50-krad dose, 60% of the hybrid calli were sulfur colored (i.e., 40% were green). Thus the probability of losing any one *N. plumbaginifolia* chromosome is $\sqrt{0.6}=0.77$, and the chance of retaining only one chromosome is 1–0.77=0.23. From these probabilities, and the binomial expansion coefficients for n=20 (*N. plumbaginifolia* has 20 chromosomes), it is possible to make a theoretical calculation of the number of *N. plumbaginifolia* chromosomes retained in individual calli in a population of hybrid calli. This calculation indicates that a 50-krad dose will result in the elimination, on average, of 15.4 *N. plumbaginifolia* chromosomes. Similar calculations have been made on the greening data from the other radiation doses, and the results of these calculations are shown in Table 2. As seen from this table, the degrees of chromosome elimination calculated from the Su-complementation assay agree closely with those measured by dot-blot hybridization is except for the 50-krad dose.

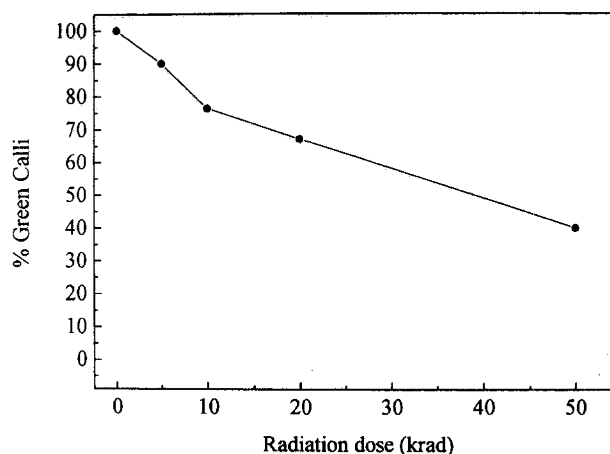


Fig. 4 Fraction of *N. tabacum* (+) *N. plumbaginifolia* hybrid calli that are able to turn green as a function of the radiation dose applied to the *N. plumbaginifolia* protoplasts prior to fusion

Table 2 Comparison of the extent of radiation-induced chromosome elimination as measured by dot-blot hybridization and by complementation of the Su locus. The data are expressed as the percentage of the *N. plumbaginifolia* genome retained in the hybrids. The Su-complementation values presented here were calculated from the fraction of greenish hybrid calli (Fig. 4) as described below[a]

Radiation dose (krad)	Percentage of donor genome retained	
	Dot-blot assay	Su-complementation assay
5	69.5	68
10	53	52
20	40	43
50	32.5	22.5

[a] To calculate the distribution of donor chromosomes in the hybrids from the Su-complementation-assay data in Fig. 4, the probability of elimination of any one donor chromosome (p) was taken as the square root of the fraction of sulfur-colored calli for each radiation dose, and the probability of retention of any one donor chromosome (q) was taken as 1-p. The distribution of donor chromosomes is then the binomial expansion $(p+q)^{20}$. The average number of donor chromosomes retained per callus was then calculated from the binomial expansions for each radiation dose

Chromosome elimination in regenerated plants

To determine the effect of plant regeneration on chromosome elimination, we isolated DNA from each callus when a shoot was removed for rooting and again from the rooted plant when it had grown larger (1–2 months later). In each plant-callus pair, the dot-blot values for the calli and plants were similar. For example, a 50-krad callus with a 32% dot-blot value regenerated a plant with a 40% value; two 20-krad callus and plant pairs gave callus and plant values of 21% and 19%, and 37% and 37%, respectively; a 10-krad pair gave values of 47% and 54%; and a 5-krad pair gave values of 95% and 96%. The differences between dot-blot values for individual plant-callus pairs are not statistically significant (P>0.3).

Discussion

We conclude that the elimination of donor chromosomes is radiation-dose dependent over the range of 5 to 50 krad. This conclusion is based on the consistent decrease in the retention of donor genetic material with each increase in radiation dose, as measured by both the dot-blot hybridization assay and the Su-complementation assay (see Fig. 2 and Table 2). The lack of statistically significant differences in dot-blot values between some of the doses is probably due to the small sample sizes used and the callus-to-callus variation. Our chromosome-elimination data take the form of an exponential decay, as would be expected theoretically for a radiation-dose inactivation curve (Lea 1947), and an exponential relationship means that a demonstration of statistically-significant effects is difficult at high radiation doses, where differences between treatment groups are small.

Kovtun et al. (1993) examined donor-recipient fusions of *N. plumbaginifolia* (+) *N. sylvestris* and found a radiation-dose-dependent increase in the elimination of donor chromosomes for doses of 1, 10, and 100 krad applied to donor protoplasts. They assessed chromosome elimination by means of chromosome counts and dot-blot hybridization using a donor-specific repetitive DNA probe. By comparing the dot-blot values obtained for individual hybrids probed with different repetitive DNA probes, they found some instances in which individual repeat sequences had been amplified in the asymmetric hybrids. Amplification of repeat sequences that are homologous to the probe reduces the utility of dot-blot hybridization for the detection of radiation-induced chromosome elimination. Kovtun et al. (1993) also tested the cloned repeat used as a probe in our study (pNP21) and did not find amplification of this particular sequence. Although we cannot rule out the possibility that amplification of donor repeat sequences has occurred in some of our hybrid calli, the correlation of our dot-blot and Su-complementation assays for chromosome elimination suggest this is not a serious problem. It is interesting to note, however, that at the highest radiation dose we tried (50 krad) the dot-blot assay measures less chromosome elimination than the greening assay.

Melzer and O'Connell (1992) report that chromosome elimination in asymmetric hybrids of *Lycopersicon esculentum* (+) *L. pennellii* increases with radiation dose for doses of 5, 25, 50, and 100 krad. They assessed chromosome elimination by means of mapped RFLP markers. Their data show that for a given radiation dose the degree of donor chromosome elimination can vary considerably from hybrid to hybrid but that there is a clear trend for increased elimination with increasing radiation dose. We also find considerable hybrid-to-hybrid variation in the degree of chromosome elimination.

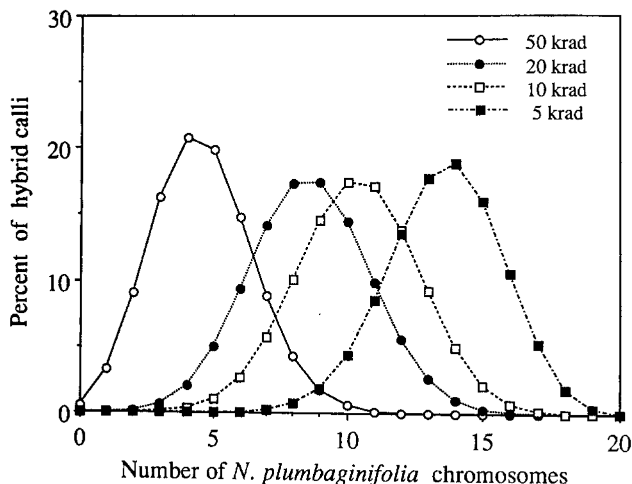
A number of studies have reported no increase in donor-chromosome elimination with increasing radiation dose. In some cases this lack of a dose-response effect can probably be attributed to the testing of an insufficiently wide range of radiation doses or to the small numbers of hybrids analyzed (see, for example, Imamura et al. 1987; Famelaer et al. 1989). The use of chromosome counts for assessing the degree of donor-chromosome elimination may also result in an underestimation of genome loss because of the presence of fragmented donor chromosomes. For example, Yamashita et al. (1989) reported that the number of *Brassica campestris* chromosomes eliminated in *B. oleracea* (+) *B. campestris* asymmetric hybrids was the same for radiation doses of 30 and 80 krad. However, isozyme analysis of these same hybrids suggested that the higher radiation dose caused greater loss of donor marker genes. The method used for the selection of asymmetric hybrids may also influence the degree of radiation-induced chromosome elimination. Wolters et al. (1991) found no difference in the elimination of *Solanum tuberosum* DNA from *L. esculentum* (+) *S. tuberosum* asymmetric hybrids when radiation doses of 5 and 50 krad were compared. These hybrids were identified by screening for correction of a maternally-inherited recipient trait (albinism), so that the degree of donor-chromosome elimination may have been influenced by the need for appropriate nuclear-cytoplasmic interactions in this case.

The experiment in which we assessed chromosome elimination using two genetic markers (the Su-complementation assay) not only reinforces our dot-blot assay results but, additionally, these data also suggest that the elimination of donor chromosomes may be largely random. Consequently, the data can be used to predict the callus-to-callus variability in donor-chromosome elimination observed in our dot-blot assays. When the Su-complementation-assay data were transformed so that they could be compared with the dot-blot data, the two methods for assessing radiation-induced chromosome elimination gave similar results (Table 2). Two assumptions were made in the transformation of the Su-complementation-assay data. First, we assumed that *N. plumbaginifolia* has two homologous chromosomes bearing the allele that complements the Su mutation and that both of these homologues must be lost to form a sulfur-colored hybrid callus. Second, we assumed that the loss or retention of the *N. plumbaginifolia* Su locus would resemble the behavior of an average *N.*

plumbaginifolia locus. The agreement between the Su-complementation-assay data and the dot-blot data provide evidence that these assumptions are warranted. If they are valid, then our results suggest that radiation-induced chromosome elimination is, on average, a random process and that each individual donor chromosome (except the one being selected for) may have a similar probability of undergoing elimination. However, physical and genetic considerations make it unlikely that all donor chromosomes will behave in this way. For example, the retention of specific donor chromosomes may be required for compatible interactions between organelles and other cellular components of the two species in the hybrid calli. In addition, *N. plumbaginifolia* is somewhat unusual in that all its chromosomes are approximately the same size. In plants with chromosomes of different sizes, the larger chromosomes would be expected to undergo more radiation-induced breakage and therefore might be eliminated more readily than smaller chromosomes.

On the basis of our Su-complementation-assay data and the assumptions described above, it is possible to make a theoretical calculation of the number of donor chromosomes retained in a population of hybrid calli for a particular radiation dose. The results of this calculation for 5-, 10-, 20-, and 50-krad doses are shown in Fig. 5. The calculation predicts a decrease in the number of *N. plumbaginifolia* chromosomes retained with increasing radiation dose, but with considerable callus-to-callus variation. For example, it predicts that 12% of the calli recovered following a 50-krad irradiation will retain just one or two donor chromosomes (5–10% of the donor genome) but 13% would still have seven or more donor chromosomes (35–40% of the donor genome). Our dot-blot hybridization values show just such callus-to-callus variation in the amount of donor DNA retained. After a 5-krad dose, the dot-blot values for the hybrids ranged from 90% to 41% of that expected for a symmetric hybrid, and for a 50-krad dose they ranged from 65% to 17.9%. This callus-to-callus variation in chromosome elimination has also been observed by many other laboratories working with asymmetric hybrids (Wolters et al. 1991; Melzer and O'Connell 1992; Kovtun et al. 1993; Puite and Schaart 1993), and is probably a general feature of asymmetric hybridization.

Fig. 5 Theoretical distributions of the number of *N. plumbaginifolia* chromosomes retained in individual hybrid calli as a result of the irradiation of the *N. plumbaginifolia* protoplasts prior to fusion. These distributions were calculated from the Su-complementation-assay data in Fig. 4, as described in Table 2



The data in Table 1 indicate that the majority of donor-chromosome elimination occurs during the first 6 months after fusion, but chromosome elimination continues for at least the first 12 months of callus culture. These observations suggest that chromosome elimination may be maximized by the use of high radiation doses and by the delay of plant regeneration until the calli are nearly a year old. Even under these conditions, our data show that most of the asymmetric hybrids will retain 30% or more of the donor genome. To obtain, with reasonable frequencies, asymmetric hybrids containing only one or two donor chromosomes, it may be necessary to supplement irradiation with other chromosome-destabilizing treatments. Potentially useful approaches might include treatment of donor protoplasts with agents that induce micronucleation (Ramulu et al. 1993) or treatment with chemicals that induce double-strand breaks in DNA.

Acknowledgements This work was supported by US-Israeli Binational Agricultural Research and Development Fund grant #IS-1631-89 to A.Z. and G.W.B.

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