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Enhancement of transformation rates in higher plants by low-dose irradiation: Are DNA repair systems involved in the incorporation of exogenous DNA into the plant genome?

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Abstract

Irradiation (X-ray; 5–15 Gy) of protoplasts treated with plasmid-DNA and PEG yielded higher transformation rates in comparison to non-irradiated protoplasts transformed by the same method. This could be demonstrated for four plant species. The irradiation doses used did not affect the total number of colonies regenerated without selection pressure, but resulted in 3–6-fold enhancement of hygromycin- or kanamycin-resistant colonies. Plant regeneration frequencies of transformed colonies derived from irradiated and non-irradiated protoplasts were similar in tobacco as well as in *Petunia*. Higher integration rates of foreign DNA as a consequence of an increased recombination machinery in irradiated cells may be responsible for the enhancement of the number of stably transformed colonies.

Introduction

In the past five years it has been demonstrated that direct gene transfer is an efficient method of transforming protoplasts of higher plants. Factors such as electroporation, carrier DNA, composition of incubation media, PEG concentration [36, 24], or nature of plasmid DNA and plant genotype [14] have been recognized to influence the number of transformed colonies. Despite the extensive use of DNA transfection, little is known about how transfection works. Therefore it is important to learn more about the mechanisms that govern uptake and integration of foreign DNA into plant cells.

In mammalian systems Loyter *et al.* [19] reported that DNA is taken up by almost all cells, but in the nucleus exogenous DNA could only be detected in small amounts. In animal and plant cells the frequency of transformation can be significantly raised when exogenous DNA is directly microinjected into the nucleus [41, 25]. These results and the observation that synchronized protoplasts in the S- or M-phase of the cell cycle can be efficiently transformed [23] have led to the assumption that the nuclear membrane might be a substantial barrier for the import and incorporation of foreign DNA [23].

Irradiation of plant cells, like in prokaryotes and other eukaryotes [7], results in single-strand breaks, base damage and, to a lesser extent, double-strand breaks [10]. Excision repair has been demonstrated in irradiated protoplasts of *Haplopappus, Nicotiana* and *Petunia* [11]. After UV irradiation of *Nicotiana sylvestris* leaf protoplasts repair replication could be detected [32]. It was suggested that a beta-type DNA-polymerase might be responsible since the incorporation of (^{3}H]-thymidine was resistant to the

DNA polymerase-alpha inhibitor aphidicolin. DNA repair has also been demonstrated in pollen, embryos and root tips [5, 12, 42]. It is now well documented that higher plants are able to repair damages caused by UV, ionizing radiation or chemicals by mechanisms similar to those described for bacteria and mammalian cells [38].

In this study, the effect of ionizing radiation on protoplasts transformed via direct gene transfer was determined. We worked with *Nicotiana tabacum, Petunia hybrida, Brassica nigra* and *Vigna aconitifolia* protoplasts to verify whether a low-dose irradiation has an effect on the number of stably transformed colonies.

Material and methods

Plant material

Protoplasts were isolated from *in vitro* shoot cultures of *Nicotiana tabacum* cvs. SR1 and W38, *Petunia hybrida* cv. Mitchell (haploid), *Brassica nigra* cv. 2051 and *Vigna aconitifolia* (moth bean) cvs. IPMCO-560 and IPMCO-88. Shoots were cultured (25 °C; 16 h light) on MS-agar medium lacking hormones. Moth bean seeds were obtained from S.E. Pawar of Bhabha Atomic Research Centre (Bombay, India).

Plasmid DNA

The kanamycin resistance-conferring plasmid pABD1 [26] was used to transform tobacco, *Petunia* and *Vigna* protoplasts. *Brassica* protoplasts were treated with plasmid pGL2, which was obtained by cloning the *Bam* HI fragment of the coding region of the Hygromycin® gene into the polylinker sequence of plasmid pDH51 (J. Paszkowski, personal communication). This multiple cloning site was introduced between the 35S promoter and terminator region of cauliflower mosaic virus. Plasmid pDH51 is fully described by Pietrzak *et al.* [28]. Plasmid isolation was carried out using a standard method [20]. Both plasmids, pGL2 and pABD1, were kindly provided by J. Paszkowski (ETH Zürich, Switzerland).

Protoplast culture and selection of resistant colonies

Nicotiana tabacum

Protoplasts were isolated and cultured as described by Köhler *et al.* [14]. Resistant colonies were selected in the presence of 75 mg/l kanamycin. Plant regeneration was observed after transfer of resistant colonies to a solid regeneration medium (MS medium plus 0.5 mg/l BAP) containing 100 mg/l kanamycin.

Petunia hybrida

Protoplast culture and regeneration was carried out as described by Krüger-Lebus and Potrykus [17] with the following modifications. Small leaf pieces were incubated overnight in enzyme solution (0.5% cellulase "Onozuka R10", 0.1% Macerozyme R10, 0.04 M $CaCl_2$ in 0.6 M mannitol, pH 5.5). The washing and purification of the protoplasts was carried out as described by Schieder [34] using an osmoticum (0.05 M $CaCl_2$, 0.2% w/v MES 730 mOsm, adjusted with mannitol pH 5.8) and 0.6 M sucrose plus 0.2% w/v MES. Protoplasts were embedded in solidified (Sea Plaque) V47 medium [3] containing 2 mg/l 2,4-D and 0.5 mg/l BAP immediately after the transformation treatments. Selection was started after 6–7 days by transferring the cultures to a bead-type culture system [35] in 9-cm diameter Petri dishes. The liquid selection medium (V47: 0.2 mg/l 2,4-D, 0.05 mg/l BAP, 2% coconut water, 3% sucrose, 50 mg/l kanamycin) was replaced weekly. For regeneration, resistant colonies were transferred to NI medium containing 0.4 mg/l NAA, 1 mg/l BAP, 2 mg/l zeatin and 50–100 mg/l kanamycin. To enable rooting, shoots were transferred to MS medium without hormones.

Vigna aconitifolia

Protoplasts were isolated according to Köhler *et al.* [15]. For selection 75 mg/l kanamycin was added to the V47 culture medium [3]. After four weeks growing colonies were transferred to V47 solid medium supplemented with 100 mg/l kanamycin.

Brassica nigra

Protoplast isolation and culture was carried out according to Sacristan *et al.* [31]. Selection pressure

was established by adding 30 mg/l hygromycin to the culture media 7–10 days after the treatment of protoplasts with plasmid DNA and PEG. Hygromycin-resistant colonies were grown further on E medium [27] or on modified 2N medium [4] in the presence of hygromycin.

Transformation methods

Nicotiana tabacum *and* Brassica nigra

The incubation media and the PEG solution of Negrutiu *et al.* [24] were used to transform the protoplasts. Protoplasts suspended in W5 medium were kept on ice for 10 min and then centrifuged; the pellets were suspended in MaMgl medium [24]. 50 μg plasmid DNA (tobacco: pABD1; *B. nigra*: pGL2), 50 μg carrier DNA (calf thymus) and PEG (40%) at a ratio of one part to two parts DNA containing protoplast suspension were added. After incubation for 20 min, the suspension was diluted stepwise with sea water (630 mOsm). Protoplasts were washed twice and then incubated in culture medium at a concentration of $1-3 \times 10^5$/ml.

Petunia hybrida

Transformation was carried out according to Krüger-Lebus and Potrykus [17] with the following modifications. The protoplast incubation medium contained 20 mM $MgCl_2$ instead of 50 μg $CaCl_2$ circular plasmid DNA (pABD1) and 100 μg calf thymus DNA were added to 1 ml protoplast suspension (10^6 protoplasts/ml). After 1 min, 0.5 ml 40% PEG dissolved in osmoticum [17] were added to each sample and gently mixed. The samples were incubated for 10 min and the protoplasts were washed twice with osmoticum and finally resuspended in culture medium at a density of about $3-5 \times 10^4$/ml.

Vigna aconitifolia

The same transformation method [24] was used as for tobacco and *Brassica* protoplasts, but for the transformation of *Vigna* protoplasts 0.75 ml PEG (40%) was added to 1 ml protoplast suspension containing 50 μg plasmid DNA (pABD1) and 100 μg carrier DNA.

Irradiation

About one hour after completion of the transformation treatment, protoplasts, suspended in culture medium, were irradiated at a rate of 12 Gy/min (X-ray; Phillips MGCO1). Protoplasts were irradiated at 90 kV for 25–75 s, corresponding to 5–15 Gy.

Slot blot analysis

Genomic DNA was isolated from callus material of *B. nigra* and from regenerated *N. tabacum* plants using a minipreparation method [22]. In both cases, plant material was cultured under selection pressure for 4–5 months before DNA was isolated. The *Bam* HI fragment of pGL2 containing the coding sequence of the hygromycin resistance gene and the *Eco* RV fragment of pABD1 for detection of the NPT II gene sequence [26] were biotinylated by nick translation using a nick translation system with biotinylated nucleotides of BRL. After slot blotting (Bio-dot SF slotting apparatus; Bio-Rad), hybridization and visualization of biotinylated DNA were carried out as specified by the suppliers (Bio-Rad and BRL).

Estimation of transformation rates

Transformation rates are expressed as percentages of antibiotic-resistant colonies. In each single experiment the total number of colonies derived from irradiated and non-irradiated protoplasts were calculated from control cultures grown without any selection pressure. This was necessary because of different colony formation rates from experiment to experiment indicating the general "tissue culture variability". The number of resistant colonies was calculated from cultures that were treated in the same way as the controls concerning transformation and irradiation. In contrast to the controls, a selection pressure was established after 6–7 days.

Results

Protoplasts of the four plant species were irradiated one hour after completion of the transformation procedure. Doses of 5–15 Gy (χ-rays) did not affect the colony formation rate of the cultures in four plant species. The number of colonies was determined about 4 weeks after isolation of the protoplasts. Otherwise, the plating efficiency of irradiated protoplasts, determined as the percentage of dividing cells after 7–10 days, was slightly reduced (2–10%) compared to non-irradiated cultures. In tobacco, a reduction of colony number of 40–50% was observed after protoplast irradiation with doses of 30–40 Gy. Irradiation with 90 Gy in tobacco and 200 Gy in *Brassica nigra* did not result in colony formation (data not shown).

Nicotiana tabacum

In general, high transformation rates were observed for both tobacco cultivars tested. The use of incubation media according to Negrutiu *et al.* [24] yielded transformation rates in non-irradiated controls of 1.69% (Table 1) iwht "W38" protoplasts, which implies a marked enhancement compared to results published [14].

In three independent experiments, protoplasts treated with pABD1 plasmid DNA/PEG, washed and resuspended in liquid culture medium, were irradiated one hour after the transformation procedure. One third of the cultures were irradiated with 6 Gy, another third were irradiated with 15 Gy and the remaining dishes were not irradiated. On the average, 30–45 dishes each containing 2.5 ml (10^5 protoplasts/ml) were treated that way in one single experiment.

In all experiments, the transformation rates of irradiated protoplasts were higher than those of the non-irradiated controls. 2.85% resistant colonies could be regenerated from protoplasts of tobacco cultivar "W38" irradiated with 6 Gy. The dose of 15 Gy resulted in 5.5% kanamycin-resistant colonies whereas only 1.69% of the colonies derived from non-irradiated protoplasts grew in the presence of 75 mg/l kanamycin (Table 1).

Table 1. Effect of X-irradiation on the transformation rate of *Nicotiana tabacum* "W38". 10^6 protoplasts were treated with 50 μg pABD1 DNA and 50 μg calf thymus DNA or only with 100 μg calf thymus DNA (control). X-irradiation (0, 6 and 15 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6–7 days by using culture media supplemented with 75 mg/l kanamycin. Results are from 3 independent experiments.

	% Kanamycin resistant colonies (number of Kan® colonies)		
	0.0 Gy	6.0 Gy	15.0 Gy
100 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)	0.0 (0)
50 μg pABD1/50 μg carrier DNA per 10^6 protoplasts	0.0 (0)	1.03 (151)	4.00 (588)
	2.22 (352)	3.05 (484)	4.10 (651)
	2.85 (970)	4.47 (1522)	8.47 (2884)
Mean value	1.69	2.85	5.52
Standard deviation	1.50	1.73	2.55
Degree of comparison by irradiation*	1.0	1.7	3.3

* The mean value of transformation rates of non-irradiated cultures were equated with 1.0 and other mean values were calculated accordingly.

A similar enhancement of the transformation rate was observed for protoplasts of tobacco cultivar "SR1" (Table 2). The mean proportions of kanamycin-resistant colonies were estimated to be 1.7% for non-irradiated protoplasts, 3.9% for protoplasts irradiated with 6 Gy, and 3.5% for protoplasts irradiated with 15 Gy.

The results of the experiments with the lines "SR1" and "W38" are based on about 1000 kanamycin-resistant colonies derived from non-irradiated protoplasts and about 13000 resistant colonies derived from irradiated protoplasts, respectively. Details on the exact numbers of resistant colonies in the single experiments are given in Tables 1 and 2. Differences between numbers of resistant colonies and calculated transformation rates are based on rounding off the percentages of resistant colonies. Further, the total number of colonies grown in the absence of any selection pressure varied in the experiments as the number of resistant colo-

Table 2. Effect of X-irradiation on the transformation rate of *Nicotiana tabacum* "SR1". 10^6 protoplasts were treated with 50 μg pABD1 DNA and 50 μg calf thymus DNA or only with 100 μg calf thymus DNA (control). X-irradiation (0, 6 and 15 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6 – 7 days by using culture media supplemented with 75 mg/l kanamycin. Results are from 3 independent experiments.

	% Kanamycin-resistant colonies (number of Kan® colonies)		
	0.0 Gy	6.0 Gy	15.0 Gy
100 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)	0.0 (0)
50 μg pABD1/50 μg carrier DNA per 10^6 protoplasts	0.09 (16)	1.35 (240)	0.34 (61)
	0.40 (150)	2.45 (925)	2.55 (965)
	4.60 (945)	7.94 (1630)	7.62 (1565)
Mean value	1.70	3.91	3.50
Standard deviation	2.52	3.53	3.73
Degree of comparison by irradiation*	1.0	2.3	2.1

* See footnote to Table 1.

nies (Tables 1 and 2). This holds as well for the results shown in Tables 3 – 6.

Resistant colonies, selected on an agarose bead-type culture system, grew further after transfer to a solid medium containing 100 mg/l kanamycin. There was no difference in plant regeneration capability between the colonies derived from irradiated and those derived from non-irradiated protoplasts. Transformed plants did neither express phenotypic differences irrespective of irradiation dose used. Genetic analysis of the regenerated plants concerning the inheritance of the introduced marker gene, will be performed to check whether there is any difference between transformed plants regenerated from irradiated or non-irradiated protoplasts.

Transformation of regenerated plants was confirmed by slot blot analysis (Fig. 1). DNA was isolated from plants which grew 3 – 5 months on media containing 100 mg/l kanamycin.

Petunia hybrida

Transformation of haploid *Petunia* protoplasts was carried out with the kanamycin resistance-conferring plasmid pABD1. In incubation medium [17], $CaCl_2$ was replaced by $MgCl_2$ which resulted in 2 – 3-fold higher transformation rates (G. Cardon, unpublished). In non-irradiated cultures, 0.02 – 0.09% of the regenerated colonies (mean of five independent experiments: 0.05%) were able to grow in the presence of 50 mg/l kanamycin (Table 3), Krüger-Lebus and Potrykus [17] obtained transformation frequencies of 0.01 – 0.4% in nine independent experiments.

Irradiation of protoplasts with 8 Gy reduced the plating efficiency to 98% but did not affect the rate of colony formation. Transformation rates were calculated to be 0.18 – 0.60% (mean 0.32%) for irradiated cultures, which means a more than 6-fold increase. For the same number of protoplasts, 300

Table 3. Effect of X-irradiation on the transformation rate of *Petunia hybrida* "Mitchell". 10^6 protoplasts were treated with 50 μg pABD1 DNA and 100 μg calf thymus DNA or only with 150 μg calf thymus DNA (control). X-irradiation (0 and 8 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6 – 7 days by using culture media supplemented with 50 mg/l kanamycin. Results are from 5 independent experiments.

	% Kanamycin-resistant colonies (number of Kan® colonies)	
	0.0 Gy	8.0 Gy
150 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)
50 μg pABD1/100 μg carrier DNA per 10^6 protoplasts	0.09 (156)	0.36 (607)
	0.02 (20)	0.22 (263)
	0.02 (38)	0.24 (468)
	0.04 (65)	0.60 (1042)
	0.06 (166)	0.18 (518)
Mean value	0.05	0.32
Standard deviation	0.03	0.17
Degree of comparison by irradiation*	1.0	6.4

* See footnote to Table 1.

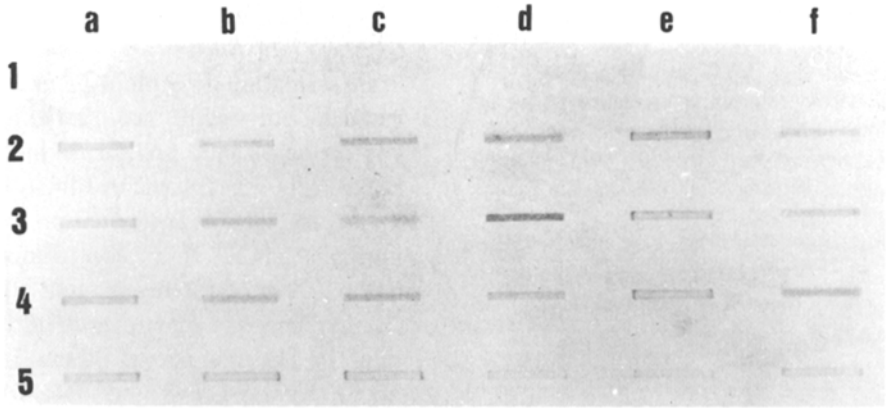

Fig. 1. Slot blot analysis of DNA isolated from *Nicotiana tabacum* "W38" plants probed with the biotinylated *Eco* RV fragment of pABD1 containing the NPT II gene. Row 1: DNA of regenerated plants derived from protoplasts treated only with carrier-DNA (controls). Rows 2–5: DNA of kanamycin-resistant plants derived from protoplasts treated with plasmid DNA and irradiated with 0 Gy (a, b), 6 Gy (c, d) and 15 Gy (e, f).

resistant colonies were observed for non-irradiated protoplasts whereas 2035 kanamycin-resistant colonies derived from irradiated protoplasts.

Protoplasts from control treatments (150 μg carrier DNA; irradiated and non-irradiated) did not produce any kanamycin-resistant colonies (Table 3).

Like in tobacco, plant regeneration was independent of the presence of kanamycin in the culture medium. Southern blot analysis of DNA from transformed plants confirmed the insertion of the NPT II gene (data not shown). Detailed Southern analysis of kanamycin-resistant plants derived from irradiated and non-irradiated plants is in progress. These studies should show whether the numbers of integrated gene copies and the integration patterns differ with regard to protoplast irradiation (G. Cardon *et al.*, in preparation).

Vigna aconitifolia

Using non-irradiated protoplasts treated with pABD1 DNA, transformation rates of 1.28% (line 560) and 0.38% (line 88) were obtained (Tables 4 and 5). This genotypic dependence of transformation rates has been observed previously for the two *Vigna* lines "560" and "909" [14, 15]. Per 10^6 protoplasts (irradiated or non-irradiated) treated with DNA and PEG, about 2500 colonies were formed without selection pressure. In three experiments 95 and 24 resistant colonies grew on selection medium using non-irradiated protoplasts of line "560" and line "88", respectively. Like in the other plant species,

Table 4. Effect of X-irradiation on the transformation rate of *Vigna aconitifolia* "560". 10^6 protoplasts were treated with 50 μg pABD1 DNA and 100 μg calf thymus DNA or only with 150 μg calf thymus DNA (control). X-irradiation (0, 5 and 10 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6–7 days by using culture media supplemented with 75 mg/l kanamycin. Results are from 3 independent experiments.

	% Kanamycin-resistant colonies (number of Kan® colonies)		
	0.0 Gy	5.0 Gy	10.0 Gy
150 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)	0.0 (0)
50 μg pABD1/100 μg carrier DNA per 10^6 protoplasts	0.92 (25) 1.17 (30) 1.74 (40)	2.00 (49) 1.94 (56) 2.52 (60)	3.92 (95) 3.13 (82) 3.24 (78)
Mean value	1.28	2.15	3.43
Standard deviation	0.42	0.32	0.43
Degree of comparison by irradiation*	1.0	1.7	2.7

* See footnote to Table 1.

Table 5. Effect of X-irradiation on the transformation rate of *Vigna aconitifolia* "88". 10^6 protoplasts were treated with 50 μg pABD1 DNA and 100 μg calf thymus DNA or only with 150 μg calf thymus DNA (control). X-irradiation (0, 5 and 10 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6 – 7 days by using culture media supplemented with 75 mg/l kanamycin. Results are from 3 independent experiments.

	% Kanamycin-resistant colonies (number of Kan® colonies)		
	0.0 Gy	5.0 Gy	10.0 Gy
150 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)	0.0 (0)
50 μg pABD1/100 μg carrier DNA per 10^6 protoplasts	0.49 (10)	0.69 (20)	0.98 (23)
	0.43 (9)	1.00 (21)	1.04 (25)
	0.21 (5)	0.63 (12)	1.11 (28)
Mean value	0.38	0.77	1.04
Standard deviation	0.15	0.20	0.07
Degree of comparison by irradiation*	1.0	2.0	2.7

* See footnote to Table 1.

enhancement of transformation rates was observed in each single experiment after irradiation of protoplasts. A 10 Gy irradiation dose resulted in an about 2.7-fold increase of transformed colonies in both lines, and a 5 Gy dose resulted in a 1.7-fold increase in line 560 and a 2-fold increase in line 88 (Tables 4 and 5). These results of the irradiation experiments are based on 420 and 129 transformed colonies in the lines "560" and "88", respectively. Irradiated and non-irradiated protoplasts which were treated only with carrier DNA/PEG, did not form colonies in the presence of 75 mg/l kanamycin.

Brassica nigra

Transformation of *B. nigra* protoplasts via direct gene transfer was demonstrated by Köhler *et al.* [16] and Golz *et al.* [9]. Resistant colonies were obtained by using the hygromycin resistance-conferring plasmid pGL2 for transformation. However, treatment of protoplasts with pABD1 plasmid, allowing kanamycin selection in tobacco, *Petunia* and *Vigna*, was not successful.

Hygromycin-resistant colonies were selected in the presence of 30 mg/l hygromycin. Non-transformed protoplasts and colonies did not survive this concentration. Colony formation on selection medium was also not observed from irradiated protoplasts (5 and 10 Gy), which were treated with carrier DNA but not with plasmid-DNA (Table 6).

Protoplast irradiation increased the transformation rates from 0.12% (non-irradiated) to 0.3% (5 Gy) and 0.67% (10 Gy). The number of transformed colonies derived from irradiated protoplasts was 3 – 6-fold higher in each single experiment, compared to the number of resistant colonies developed from non-irradiated protoplasts. The total number of colonies grown in the absence of selection pressure varied from 1250 to 30600 per 10^6 protoplasts in three independent experiments. The numbers of transformed colonies in each single experiment are shown in Table 6.

Table 6. Effect of X-irradiation on the transformation rate of *Brassica nigra* "2051". 10^6 protoplasts were treated with 50 μg pGL2 DNA and 50 μg calf thymus DNA or only with 100 μg calf thymus DNA (control). X-irradiation (0, 5 and 10 Gy) was carried out one hour after completion of transformation procedure. Selection was started after 6 – 7 days by using culture media supplemented with 30 mg/l hygromycin. Results are shown from 3 independent experiments.

	% Hygromycin-resistant colonies (number of Hyg® colonies)		
	0.0 Gy	5.0 Gy	10.0 Gy
100 μg carrier DNA per 10^6 protoplasts	0.0 (0)	0.0 (0)	0.0 (0)
50 μg pGL2/50 μg carrier DNA per 10^6 protoplasts	0.17 (51)	n.d.	0.65 (199)
	0.11 (16)	0.36 (53)	0.68 (100)
	0.07 (9)	0.23 (28)	n.d.**
Mean value	0.12	0.30	0.67
Standard deviation	0.05	0.09	0.02
Degree of comparison by irradiation*	1.0	2.5	5.6

* See footnote to Table 1.
** n.d. = not determined.

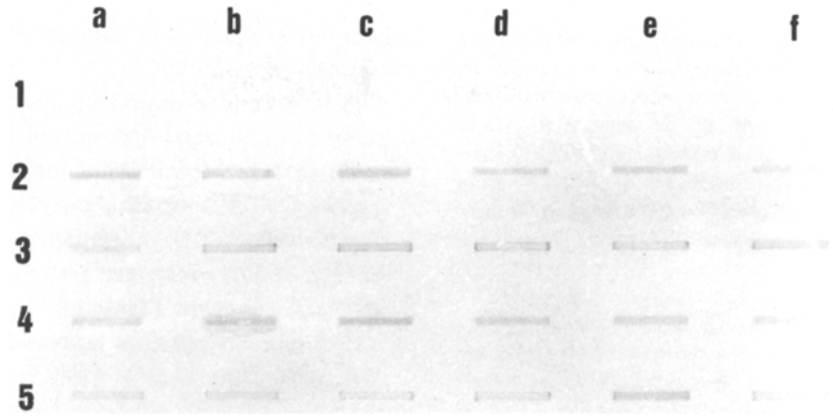

Fig. 2. Slot blot analysis of DNA isolated from *Brassica nigra* colonies probed with the *Bam* HI fragment of pGL2 containing the hygromycin-resistance gene. Row 1: DNA of colonies derived from protoplasts treated only with carrier DNA (controls). Rows 2–5: DNA of hygromycin-resistant colonies derived from protoplasts treated with plasmid DNA and irradiated with 0 Gy (a, b), 5 Gy (c, d) and 10 Gy (e, f).

Plant regeneration from hygromycin-resistant colonies did not take place. Figure 2 demonstrated that in each of the tested colonies the antibiotic gene is present. The slot blot analysis was carried out using callus material grown 4–5 months on selection medium.

Discussion

In the literature highly diverse doses of ionizing radiation have been reported resulting in cell killing. The colony formation rate of the four plant species tested was not affected by doses of 5–15 Gy. On the other hand, cultured lymphoma cells irradiated with 1.5 Gy had a survival rate of only 25% [1]. High radiation resistance has been demonstrated for cultured plant cells. Doses of up to 200 Gy did not affect the growth of haploid *Antirrhinum* suspensions [21] nor the fresh weight of *Phaseolus* calli [2]. 50% survival was recorded for a *Haplopappus* cell suspension exposed to 30 Gy [8]. To our knowledge, the highest radiation resistance of cultured plant cells has been demonstrated for *Catharanthus roseus*. After treatment of cell suspensions with 1000 Gy the rate of survival of the colonies was 20% [6].

Beside the fact that the conditions were different at the period of irradiation (e.g. composition of culture media, temperature) in the given examples, it is obvious that cells of different species strongly differ in radiation tolerance. The molecular or biochemical cause of the different response of cultured cells to ionizing radiation is unknown.

In all experiments carried out with *N. tabacum, B. nigra, P. hybrida* and *V. aconitifolia* an enhancement of transformation frequencies by X-irradiation was observed. The number of stably transformed colonies was 2–6-fold higher after irradiation of the protoplasts depending on irradiation dose used (5–15 Gy) and plant species (Tables 1–6).

Although the reproducibility of the transformation rates, mainly in tobacco, was less for non-irradiated protoplasts, the influence of irradiation on transformation rates was detectable in each of the 20 experiments carried out. The partly large standard deviations shown in Tables 1–6 are mainly based on the variability of the transformation rates already expressed in non-irradiated cultures. This is indicated by the different numbers of resistant colonies in the single experiments (Tables 1–6). The differences between irradiated and non-irradiated cultures were observed in experiments yielding relatively low numbers of transformants, as well as in experiments that yielded relatively high numbers of transformants.

Therefore, the reproducibility of these experiments with respect to the effect of irradiation on transformation rate seems to be shown. We did not

test higher radiation doses; therefore, doses of 20 Gy or more may result in a stronger enhancement of transformation rate than is shown in Tables 1–6. On the other hand, the total number of colonies would decrease if larger doses were used.

One simple explanation for the high transformation rates obtained after irradiation could be that plasmid molecules were linearized by irradiation resulting in higher numbers of transformed colonies. Shillito *et al.* [36] reported that linear DNA is 2.5–10-fold more efficient than circular DNA when electroporation is used for direct gene transfer. Rabussy *et al.* [30] found similar levels of transient gene expression with both linear and circular DNA. In *Petunia* and in tobacco, the use of linear plasmid DNA (*Sma* I digestion of pABD1), in combination with carrier DNA, did not yield higher transformation rates compared than did treatment of protoplasts with circular plasmid and carrier DNA. Further, enhancement of transformation rates was also observed when irradiation of the protoplasts was carried out one hour before transformation (M. Pöhlmann, unpublished data).

Irradiation of protoplasts one hour after transformation implies that plasmid molecules or fragments thereof must exist in a biologically active form in the cells for at least one hour. Preliminary results show that irradiation carried out 24 h after transformation does not increase the number of transformed colonies. That supports the results obtained with transient gene expression in tobacco protoplasts. Measuring time courses of CAT appearance, Pröls *et al.* [29] suggested that a substantial inactivation of the transferred DNA occurs within the first hours.

We did not observe any difference in colony growth, plant regeneration frequency or *in vitro* phenotypic appearance of regenerated, transformed plants in tobacco and *Petunia* between cultures derived from irradiated and non-irradiated protoplasts. Maize callus material irradiated with 8 Gy showed an increase in growth rate and a 20% increase in embryogenic callus. Irradiation did not affect plant height of regenerated plants, but 98% of the plants were sterile [40]. Until now, our regenerated plants have been kept *in vitro*, hence it is not possible to decide whether similar genetic damages like sterility have been originated by irradiation.

In all cases, irradiation was applied about one hour after completion of transformation, which implies that, before irradiation was carried out, exogenous plasmid DNA and PEG had been washed away. Therefore, a higher uptake rate of plasmid DNA by the protoplasts induced by irradiation can be excluded.

Ayad and Fox [1] demonstrated repair synthesis of DNA after a X-ray treatment of lymphoma cells with 1.5–4.0 Gy. ^{3}H-labelled DNA uptake and integration was higher in irradiated cells. They present good evidence that repair processes were implicated in integration of exogenous DNA. It has been suggested on the basis of the enhancement of transformation observed in human cells by UV irradiation of transfecting plasmids and cells that UV damage might play a role in stimulating recombination between plasmid and recipient cell DNA [37, 39].

The increased numbers of stably transformed colonies after protoplast irradiation may be the result of a higher integration rate of the marker genes. We speculate that DNA molecules present in the nuclei at the moment of irradiation are integrated into the genome to a larger extent than in the situation of non-irradiated protoplasts. The presumed higher rate of integration of plasmid molecules into the genome seems to indicate that the recombination machinery of the cells has been activated by irradiation.

That recombination events play an important role in integration of foreign DNA is well documented. The use of carrier DNA resulted in a 3-fold increase of transformation rates [36]. Jongsma *et al.* [13] have demonstrated that carrier DNA is not neutral in function and may play an active role in stabilizing and fusing plasmid DNA sequences. Plasmids are frequently degraded and crumbled into smaller fragments which in some cases are linked with carrier DNA to form larger molecules prior to integration [13]. Further, the overall pattern of integration suggests that recombination with plant genomic DNA happens at random sites in the plasmid DNA. Recombination between plasmid sequences, interactions with carrier DNA, and the creation of larger molecules called transgenomes in the nuclei, have also been reported for DNA-mediated gene transfer in mammalian cells [33].

Ionizing radiation causes immediate single-strand breakage, strong inhibition of replicon initiation and only a small blockage of replication at lower doses [18]. Induced strand breaks with subsequent repair processes could thus enhance the recombination events resulting in increased integration rates of foreign DNA and, therefore, leading to the increased numbers of stably transformed colonies after protoplast irradiation.

It has been suggested that the bottleneck for plant cell transformation is mainly concerned with passing all physical barriers and not with the event of integration [19, 23, 13]. The results of the irradiation experiments seem to indicate that the integration rate of foreign DNA also represents a strongly limiting step in transformation of higher plant cells.

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