Theor Appl Genet (1990) 79:679–685

Effect of various irradiation treatments of plant protoplasts on the transformation rates after direct gene transfer

F. Köhler *, I. Benediktsson, G. Cardon[1], C.S. Andreo[2] and O. Schieder

Institute for Applied Genetics, Free University Berlin, Albrecht-Thaer-Weg 6, D-1000 Berlin 33
[1] Max-Planck-Institute for Plant Breeding Research, D-5000 Köln-Vogelsang, FRG
[2] National University of Rosario, CEFOBI, Suipacha 531, 2000 Rosario, Argentina

Received October 27, 1989; Accepted December 20, 1989
Communicated by G. Wenzel

Summary. In *P. hybrida* and *B. nigra* an enhancement of transformation rates (direct gene transfer) of about six- to seven-fold was obtained after irradiation of protoplasts with 12.5 Gy (X-ray). The effect of protoplast irradiation was similar in experiments where protoplasts were irradiated 1 h before transformation (X-ray/DNA) or 1 h after completion of the transformation procedure (DNA/X-ray). Increased X-ray doses up to 62.5 Gy resulted in further enhancement of percentages of transformed colonies, indicating a correlation between relative transformation frequencies (RTF) and the doses applied. Estimation of degradation rates of plasmid sequences in plant protoplasts yielded a reduction of plasmid concentration to 50% 8–12 h after transformation. In 1-day-old protoplasts, the level of plasmid fragments dropped to 0%–10% compared to 1 h after transformation. The results demonstrate that the integration rates of plasmid sequences into the plant genome may in part be governed by DNA repair mechanisms. This could be an explanation for the observed genotypic dependence of transformation rates in different plant species and plant genotypes. Gene copy number reconstructions revealed enhanced integration rates of plasmid sequences in transformed colonies derived from irradiated protoplasts.

Key words: *Brassica nigra* – Direct gene transfer – *Petunia hybrida* – Plasmid degradation rate – X-ray

Introduction

Transformation rates in direct gene transfer experiments are influenced by a range of chemical and physical parameters (Shillito et al. 1985; Negrutiu et al. 1987). In the past, contradictory results about the effect of carrier DNA, PEG concentration, electroporation, and the use of linear versus circular plasmid forms were published. Some of these diverse results may be explained by the use of different plant species. It has even been demonstrated that varieties of the same plant species express different transformation frequencies (Köhler et al. 1987a, b; Damm et al. 1989; Tyagi et al. 1989).

On the other hand, the occasionally weak reproducibility of transformation protocols may also be an indication that the fundamental processes in direct gene transfer are still not understood. Therefore, one has to ask which processes govern the uptake of DNA, the transport to the cell nucleus, and the integration of "foreign" DNA into the plant genome. The high transformation frequencies obtained in some plant species indicate that heterologous plasmid DNA is integrated very efficiently via illegitimate recombination. For that, a set of enzymes is required that is normally expressed during processes such as DNA synthesis and DNA repair. Freshly isolated protoplasts begin DNA replication after about 18–48 h, varying with plant genotypes (Cooke and Meyer 1981; Bergounioux et al. 1986, 1988). Tyagi et al. (1989) showed that there is no clear-cut correlation between the speed of initiation of cell division and the rate of transformation. Therefore, the cell cycle seems not to be a major factor influencing transformation rates.

In a previous publication (Köhler et al. 1989), we presented evidence that DNA repair processes may be involved in the integration of plasmid sequences into the plant genome. Low-dose irradiation (X-ray; 5–15 Gy) of protoplasts applied 1 h after completion of transformation procedure resulted in three- to six-fold enhancement in the number of transformed colonies. We assumed that in the nucleus, plasmid copies or fragments of it were

* To whom correspondence should be addressed

incorporated into the plant genome at a higher rate with protoplast irradiation compared to the nonirradiated controls. Increased transformation frequencies could be demonstrated in all plant species studied.

In this study, further studies are presented with *P. hybrida* and *B. nigra* protoplasts. We analyzed the effect of higher X-ray doses on transformation rates (dose-effect relationship) and here discuss the results from X-ray/DNA experiments (irradiation of protoplasts prior to transformation) in relation to DNA/X-ray experiments (irradiation of protoplasts after transformation). An experimental procedure is explained permitting an estimation of the gradual degradation of biologically functional plasmid sequences in the protoplasts of *P. hybrida* and *B. nigra*. These investigations support the view that the repair of introduced DNA damage enhances the integration rates of "foreign" DNA into the plant genome.

Materials and methods

Plant material

Shoot cultures of *Brassica nigra* cv 2051 and *Petunia hybrida* cv Mitchell (haploid) were cultured in vitro on MS agar medium (Murashige and Skoog 1962) lacking hormones.

Plasmids

The kanamycin-resistance-conferring plasmids pABD1 (Paszkowski et al. 1984) and pHP 23 (Paszkowki et al. 1988) were used to transform *P. hybrida* protoplasts. In pABD1, the *NPT II* gene is under control of the CaMV 19S promoter, whereas plasmid pHP23 carries the *NPT II* gene under the transcriptional control of the CaMV 35S and a part of the 19S-promoter. Direct gene transfer experiments with *B. nigra* were carried out with the hygromycin-resistance-conferring plasmid pGL2 containing the coding region of the hygromycin phosphotransferase gen (*HPT*) and the 35S promoter. Plasmids were isolated according to Maniatis et al. (1982). All plasmids were kindly provided by J. Paszkowski (ETH Zurich).

Protoplast isolation and transformation

Isolation and purification of mesophyll protoplasts were carried out with a standard method (Schieder 1984). The procedure of direct gene transfer for *Petunia* protoplasts was based on the method of Krüger-Lebus and Potrykus (1987). *Brassica* protoplasts were treated with carrier DNA, plasmid DNA and PEG (final concentration: 13%) according to Negrutiu et al. (1987). The methods have been described in detail by Köhler et al. (1989).

Protoplast culture, selection, and regeneration of plants

Brassica nigra. Methods have been described in Sacristan et al. (1989). Protoplasts were cultured in M1 medium (Li and Kohlenbach 1982), embedded in M1 seaplaque medium after 7 days, and selected in the presence of liquid K3 medium (Glimelius 1984) containing 30 mg/l hygromycin B. Resistant colonies were transferred on 2N medium (Chuong et al. 1987) supplemented with 30 mg/l hygromycin B.

Petunia hybrida. Protoplasts were cultured as described by Köhler et al. (1989) in V47 medium (Binding 1974). Kanamycin-resistant colonies were selected by the addition of 50 mg/l kanamycin sulphate. Larger calli (2–3 mm) were transferred for regeneration to NT-1 medium containing 0.4 mg/l NAA, 1 mg/l BAP, and 2 mg/l zeatin (Nagata and Takebe 1971). Shoots were rooted on hormone-free NT-1 medium.

Irradiation of protoplasts

X-ray/DNA experiments. Freshly isolated *B. nigra* protoplasts were suspended in W5 medium (Negrutiu et al. 1987) or, in the case of *Petunia* protoplasts, in a $MgCl_2$/mannitol/MES-solution (Köhler et al. 1989). After irradiation with the respective dosage they were then treated with DNA/PEG as described.

DNA/X-ray experiments. After completion of the transformation process the protoplasts, suspended in culture medium, were then irradiated at different intervals (1–48 h) at a rate of 12.5 Gy/min (X-ray; Phillips MGC01). Standard irradiation dose was 12.5 Gy. In other experiments, protoplasts were irradiated 1 h after transformation with increasing doses from 2 to 62.5 Gy corresponding to 10–300 s of irradiation.

Determination of transformation frequencies

The ratio between the number of antibiotic-resistant colonies and the total number of colonies regenerated without a selection pressure is shown as percentage of transformed colonies (relative transformation frequency: RTF). Absolute transformation frequencies (ATF), the number of resistant calli per protoplasts treated, were calculated in some experiments. Since transformation rates in repeated experiments varied up to a factor of ten in some cases, the effect of irradiation on transformation rates was evaluated as follows. In each independent experiment, the transformation frequency of the control (nonirradiated sample treated in the same way as the irradiated protoplasts) was equated with 1.0. The transformation frequencies of different irradiation samples were then calculated accordingly (irradiation factor: IF). This allowed a direct comparison of irradiation effect in repeated experiments independent of the different levels of transformation frequencies of controls (nonirradiated cultures).

$$\text{Irradiation factor (IF)} = \frac{\text{RTF (\%) of irradiated sample}}{\text{RTF (\%) of control (nonirradiated)}}$$

In independent experiments a comparison of the effect of different irradiation treatment is based on the calculated irradiation factors.

Southern blot analysis of plant DNA

Genomic DNA of regenerated, kanamycin-resistant *Petunia* plants (transformed with pABD1) was isolated using the CTAB method (Murray and Thompson 1980) and further purified by CsCl gradient centrifugation. Ten micrograms of high-molecular-weight plant DNA were cut with EcoRV or BstEII and separated on a 0.8% agarose gel. DNA was transferred to a Hybond filter and hybridized with a random-primed labelled probe. The labelled fragment was a 1.5-kb PstI fragment of the *NPT* gene derived from pGLV11neo (Hain et al. 1985). EcoRV restriction of DNA should yield a 1.2-kb band containing the intact *NPT II* gene, whereas DNA restricted by BstEII, which does not cut the plasmid, should show a transformant-specific hybridization pattern (Paszkowski al. 1984).

Slot blot analysis

Genomic DNA was isolated from callus material of *B. nigra* according to the method of Paszkowski et al. (1984). Undigested DNA (1.5 µg) was fixed on a nitrocellulose membrane by "slot blotting" (Bio-Dot SF slot format microfiltration apparatus;

Bio-Rad). The membrane was probed with a ^{32}P-labelled (Nick translation) BamH1 fragment of plasmid pGL2 representing the coding sequence of the *HPT* gene. Different amounts of plasmid pGL2 (5–250 pg) were also spotted for gene-copy-number reconstructions (haploid genome of *B. nigra*: 1.5 pg). A quantitative evaluation was done by counting the slots in a scintillation counter (Beckmann 3081).

Results

In all independent experiments described below, protoplasts, which were treated with 100–150 µg carrier DNA but not with plasmid DNA (irradiated and nonirradiated protoplasts), did not produce any antibiotic-resistant colonies. Resistant colonies were selected in *B. nigra* and further cultivated by addition of 30 mg/l hygromycin to the culture media. Using 50 mg/l kanamycin sulphate in the culture medium of *Petunia* protoplasts allowed a clear-cut selection of resistant colonies and regeneration of transgenic plants.

Are the enhanced transformation rates due to induced changes in the plasmid structure?

As the irradiation of protoplasts 1 h after the transformation procedure (Köhler et al. 1989) includes irradiation of plasmids located within the protoplasts, one should consider the possibility that plasmids linearized or otherwise changed by irradiation may lead to higher transformation rates compared to nonirradiated protoplasts treated with circular plasmid forms.

To demonstrate that irradiation has its effect at the cell level only, protoplasts were irradiated prior to or after transformation. A representative experiment with both *Petunia*- and *B. nigra*-protoplasts is shown in Table 1. For each plant species, three independent experiments (according to Table 1) were carried out (Fig. 1). The effect of irradiation on the number of stable transformed colonies was calculated for each single experiment. The DNA/X-ray results (irradiation 1 h after transformation) with an irradiation factor of 5.8 for *Petunia* protoplasts and 6.0 for *B. nigra* protoplasts agree with earlier results (Köhler et al. 1989). Irradiation of protoplasts 1 h before transformation (X-ray/DNA) resulted in slightly enhanced irradiation factors (6.5 and 6.6) compared to the DNA/X-ray experiments but these differences are statistically not significant. A more than sixfold increase in the X-ray/DNA experiments means that irradiation influences factors in the plant cells leading to the observed higher transformation rates. This irradiation influence must be active for at least 1 h since that was the period between irradiation and transformation.

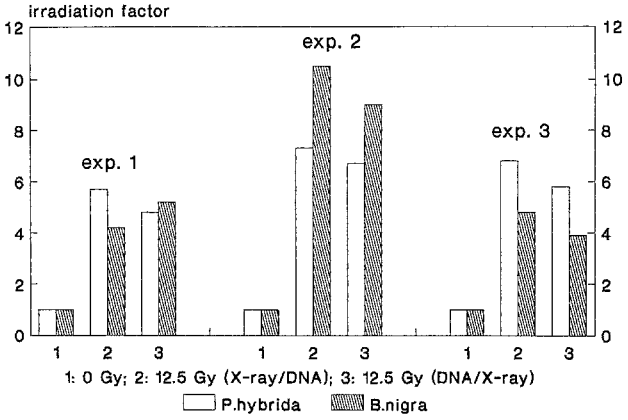

Fig. 1. Effect of protoplast irradiation on transformation rates in *P. hybrida* and *B. nigra*. Protoplasts were exposed to X-rays (12.5 Gy) 1 h before (*2*) or 1 h after transformation (*3*). Three independent experiments carried out according to Table 1 are shown. Relative transformation frequencies (RTF) are expressed as irradiation factors (IF): IF = RTF (%) of irradiated cultures (2 or 3)/RTF (%) of nonirradiated cultures (1). IFs of control cultures (*1*): 1.0

Table 1. Protoplasts were treated with 50 µg plasmid DNA (pHP23: *P hybrida*; pGL2: *B. nigra*), 100 µg carrier DNA and 13% PEG. Resistant colonies were counted 4–6 weeks after transformation. X-ray/DNA samples: protoplast were irradiated 1 h before transformation. DNA/X-ray samples: protoplast were irradiated 1 h after transformation

Irradiation treatment	Treated protoplasts	Total no. calli	Resistant calli	RTF (%)	ATF ($\times 10^{-4}$)
P. hybrida			(Kan.^{R}-calli)		
0.0 Gy	1×10^6	7.4×10^4	37	0.05	0.37
12.5 Gy[a]	1×10^6	6.5×10^4	221	0.34	2.21
12.5 Gy[b]	1×10^6	7.0×10^4	203	0.29	2.03
B. nigra			(Hyg.^{R}-calli)		
0.0 Gy	1×10^6	2.4×10^4	42	0.175	0.42
12.5 Gy[a]	1×10^6	2.4×10^4	176	0.733	1.76
12.5 Gy[b]	1×10^6	2.4×10^4	217	0.904	2.17

[a] X-ray/DNA exp.
[b] DNA/X-ray exp.
RTF – Relative transformation frequency
ATF – Absolute transformation frequency

Relationship between irradiation dose and transformation frequency

The question posed was whether increasing doses of X-ray resulted in further increase of transformation rates compared to the results of low-dose irradiation. Doses of 5–15 Gy did not affect the colony regeneration rate in *N. tabacum*, *V. aconitifolia* and *B. nigra* (Köhler et al. 1989).

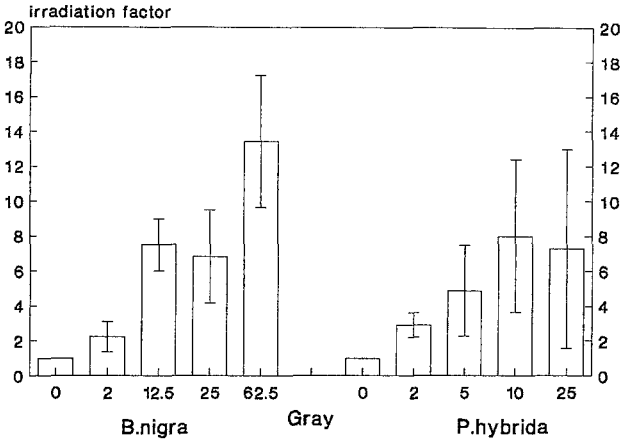

Fig. 2. Effect of increasing X-ray doses on transformation rates in *P. hybrida* and *B. nigra*. The mean values (± standard error) of irradiation factors (Fig. 1) were calculated from three experiments

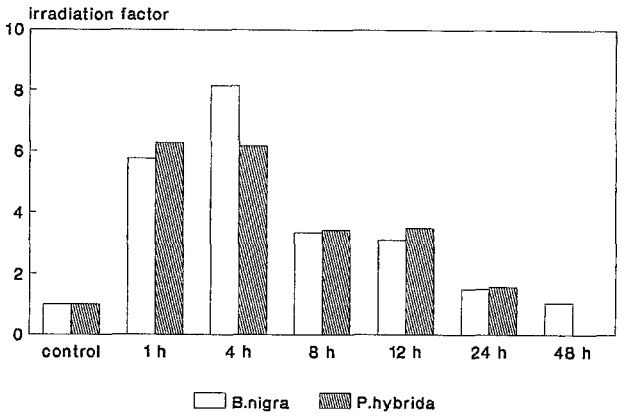

Fig. 3. Effect of different periods between transformation and irradiation (12.5 Gy) on irradiation factors (Fig. 1). Protoplasts of *B. nigra* were treated with plasmid pGL2, carrier DNA, and PEG. Irradiation was carried out 1, 4, 8, 12, 24, and 48 h after completion of transformation procedure. Protoplasts of *P. hybrida* were treated with plasmid pHP23, carrier DNA, and PEG. Irradiation was carried out 1, 4, 8, 12, and 24 h after completion of transformation procedure

On the average, the number of colonies dropped to 83% and 27% by treatment of *Petunia* protoplasts with 10 and 25 Gy, respectively. A reduction of colony regeneration rate in *B. nigra* to about 25% was reached after irradiation of protoplasts with 62.5 Gy corresponding to 5 min irradiation time. Figure 2 shows the effect of doses up to 25 Gy and 62.5 Gy, respectively, for *Petunia* and *B. nigra* protoplasts. These doses were elected as maxima since they resulted in a drop of colony regeneration to one-fourth. For calculating the percentages of antibiotic-resistant colonies (RTF), the total number of colonies regenerated without any selection pressure was accounted for each dose applied. In an average of three independent experiments there was a correlation between the irradiation doses and the relative transformation rates expressed as irradiation factors. In *Petunia*, irradiation of protoplasts 1 h prior to transformation with 2, 5, and 10 Gy led to enhancement of transformation frequencies to 2.9, 4.9, and 8.0 times respectively. Doses of 25 Gy did not yield a further increase in transformation rates (factor: 7.3). In *B. nigra* an irradiation dose of 62.5 Gy resulted in relative transformation frequencies of 0.6%–2.9%. An average irradiation factor (IF) of 13.4 was calculated (Fig. 2). Using lower doses (12.5 Gy; 25 Gy) irradiation factors of 7.5 and 6.8 were determined.

Estimation of degradation rate of biologically active plasmid forms in plant protoplasts

The results shown here allow the conclusion that more plasmid sequences are integrated into the plant genome after protoplast irradiation compared to the controls. By prolonging the period between the transformation process and irradiation, it should be possible to estimate the relative amount of plasmids or plasmid fragments in the nucleus at the moment of irradiation. For that purpose, protoplasts were treated with plasmid DNA, carrier DNA, and PEG, washed, suspended in culture medium, and irradiated after 1, 4, 8, 12, and 24 (48) h with 12.5 Gy. In each independent experiment and for each irradiation dose the total number of colonies and the number of antibiotic-resistant colonies was counted and RTF (%) and IF were calculated (Fig. 3). In both plant species, there was no clear difference between irradiation applied 1 or 4 h after transformation (irradiation factors: 6–8). Irradiation of protoplasts after 8–12 h resulted in IFs that were reduced by half when compared to the IFs of earlier irradiation dates. A decrease of IFs close to the level of nonirradiated control cultures was observed from protoplast cultures that were irradiated 24 (–48) h after completion of the transformation process. Irradiation of protoplasts at different points (1–48 h) did not affect the colony regeneration rate compared with cultures exposed to X-ray 1 h after transformation. Double irradiation treatments (1 + 24 h) of *B. nigra* protoplasts yielded IF-values similar to the IFs of the single irradiation treatments after 1 h (data not shown).

Plant regeneration and Southern blot analysis: Petunia hybrida

About 2% of selected, kanamycin-resistant *Petunia* calli regenerated shoots on regeneration medium. Shoots were rooted on hormone-free medium. Eight different *Petunia* clones bloomed after growing in the greenhouse, but none of them set seeds. To confirm the transformation on the molecular level, EcoRV- and BstII-cut genomic DNA of the transformants were analyzed (Fig. 4).

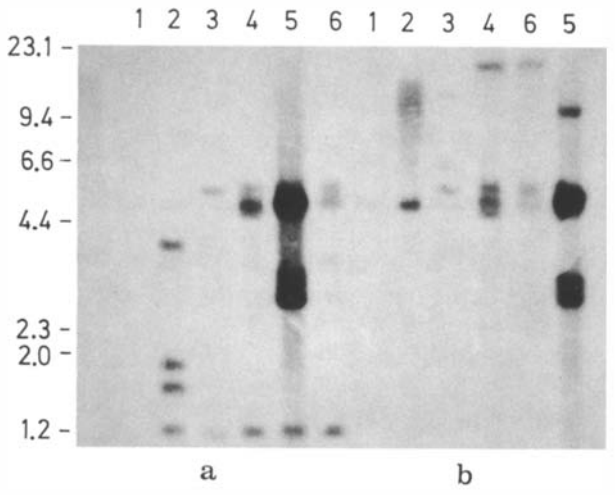

Fig. 4 a and b. Southern blot analysis of *Petunia hybrida* transformed with plasmid pABD1. Ten micrograms of genomic DNA was hybridized with a 1.5-kb PstI fragment (random labelling) of the *NPT II* gene. **a** DNA digested with EcoRV. **b** DNA digested with BstEII. *Lane 1:* DNA from nontransformed *Petunia* levels. *Lane 2–6:* DNA from leaves of regenerated, transformed *Petunia* plants. *Lane 2:* plant derived from nonirradiated protoplasts. *Lanes 3–6:* plants derived from irradiated protoplasts (10 Gy). Markers were produced by lambda/HindIII fragments

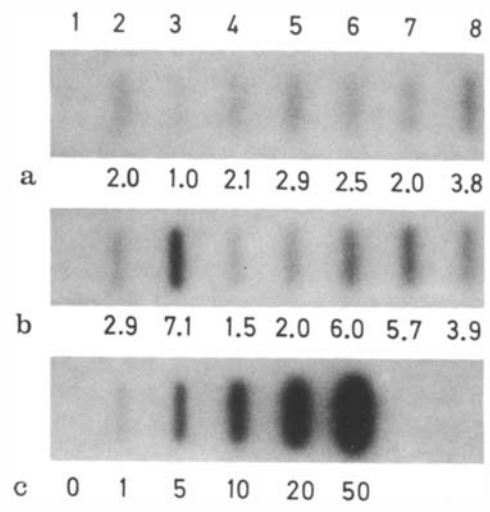

Fig. 5 a–c. Slot blotting: hybridization of the BamH1 fragment of plasmid pGL2 with copy-number reconstructions (**c**) and 1.5 µg genomic DNA of *B. nigra* (**a, b**). *a1–b1:* DNA of untransformed colonies. *a2–a8:* DNA of hygromycin-resistant colonies derived from nonirradiated protoplasts. *b2–b8:* DNA of hygromycin-resistant colonies derived from irradiated (12.5 Gy) protoplasts. *c1–c6:* 0–250 pg of plasmid pGL2 representing 0, 1, 5, 10, 20, and 50 copies per haploid genome. Numbers below the slots indicate the copy number integrated into the genome. Calculations base on measuring radioactivity of filter strips in a scintillation counter

EcoRV restriction of DNA resulted in the expected 1.2-kb band (EcoRV fragment of pABD1; Paszkowski et al. 1984). Bigger fragments indicate integration of parts of the *NPT II* coding sequence, possibly resulting from deletion of one of the EcoRV sites. BstEII does not cut pABD1 and gives a transformant-specific hybridization pattern indicating integration of plasmid sequences into the plant genome. Fragments of various size and number (BstEII: 2–6, Fig. 4) were obtained which could be explained as multiple integration of pABD1 sequences. DNA of non-transformed leaves did not hybridize with the probe DNA (lane 1; Fig. 4).

A more detailed Southern blot analysis of transformed *Petunia* clones is in progress to verify whether there are differences in the hybridization pattern of transformants derived from irradiated and nonirradiated clones (I. Benediktsson and F. Köhler, in preparation).

Slot blot analysis

A slot blot analysis was done to evaluate gene copy numbers in hygromycin-resistant calli of *B. nigra* derived from irradiated and nonirradiated protoplasts (Fig. 5). Undigested genomic DNA was hybridized with the BamH1 fragment of plasmid pGL2. Quantitative estimation of HPT sequences by counting (cpm) the slots in the scintillation counter revealed gene copy numbers of 1–4 in transformed colonies derived from nonirradiated protoplasts. From seven clones tested, five clones showed 2–3 HPT sequences per haploid genome. Genomic DNA of transformed colonies derived from irradiated *B. nigra* protoplasts (12.5 Gy) contained 2–8 gene copies. In four of seven clones, 4–7 gene copies were detected.

Discussion

In a previous publication, we could demonstrate that low-dose irradiation of plant protoplasts (four plant species tested) yielded enhanced transformation frequencies (Köhler et al. 1989). The intention of this study was to present further evidence that DNA repair mechanisms may be involved in the integration of "foreign" DNA into the plant genome.

In a first set of experiments the consequence of temporal differences between irradiation and transformation was analyzed. No significant difference in X-ray/DNA experiments (irradiation of protoplast before transformation) to DNA/X-ray experiments (irradiation of protoplasts previously treated with DNA; Fig. 1) was obtained. Therefore, the observed higher transformation rates cannot be based on linearization of plasmids through irradiation. It was important to demonstrate this because highly contradictory results have been published regarding the effect of different plasmid forms on transformation frequencies (Schocher et al. 1986; Negrutiu

et al. 1987; Ballas et al. 1988; Damm et al. 1989; Wilson et al. 1989).

In X-ray/DNA experiments (*P. hybrida*) and in DNA/X-ray experiments (*B. nigra*), increasing X-ray doses were tested. A good correlation has been found between the doses applied and the transformation frequencies obtained (Fig. 2). For mouse cells it has been shown that there is a correlation between the spontaneous repair synthesis and the radiosensitivity of different cell types. A linear-dose-effect relationship was established between irradiation (2–100 Gy) and the DNA repair synthesis (Korr et al. 1989). Similar studies with our plant material are in progress.

With both plant species experiments were carried out in which the period between transformation procedure and irradiation was prolonged from 1 to 24 (48) h (Fig. 3). No differences were observed in transformation frequencies with the irradiation points 1 and 4 h (irradiation factors: 6–7). An effect of irradiation 8–12 h after transformation was also clearly observed, but irradiation factors dropped to 3–4. In protoplast cultures irradiated 1–2 days after transformation, the percentage of antibiotic-resistant colonies was in the range of one- to twofold higher than in the control cultures (nonirradiated). This could be interpreted to mean that 8–12 h after transformation, the plasmid sequences inside the cells are degraded to about 50% of the introduced sequences. Another 12 h later most of the plasmid fragments were biologically inactive. This estimation seems to be valid for both *Petunia* and *B. nigra* protoplasts. Further, this result agrees with transient expression (CAT) experiments using *Petunia hybrida* protoplasts (Ballas et al. 1988). Southern blot analysis revealed that the "disappearance rate" of different topological plasmid forms (supercoiled, relaxed, linear) in protoplasts appears to be similar. Two hours after transformation with supercoiled or linear plasmid DNA, about the same amount of plasmid sequences was present in nuclei of transformed protoplasts. In comparison to that, only about 40% and 10%, respectively, of the initial plasmid concentrations were detected 7 and 20 h after transformation. These data are based on isolation of nuclei of transfected protoplasts at the various times and on measuring the radioactivity of bands (representing the plasmid forms) cut out from the nitrocellulose filter (Ballas et al. 1988). Transient expression experiments with the tobacco/CAT system also indicated a rapid decay of biologically active forms of both the DNA transfected and the CAT-mRNA synthesized within the first hours (Pröls et al. 1988).

Genomic DNA from five regenerated, transgenic *Petunia* plants was analyzed by Southern blot. All transformants tested possessed the intact *NPT II* gene as a 1.2-kb segment. Comparing gene copy number, reconstruction from transformed *B. nigra* colonies revealed enhanced integration of HPT sequences into plant genome in colonies derived from irradiated protoplasts (seven clones tested). On the average, 2.3 gene copies (standard error: 0.9) were present in transgenic lines from nonirradiated protoplasts, whereas 4.2 gene copies (standard error: 2.2) were detected in lines from irradiated protoplasts (Fig. 5). This might be an indication of enhanced integration events per protoplast due to irradiation. On the other hand, no correlation was observed between gene copy numbers in transgenic *P. hybrida* lines derived from irradiated and nonirradiated protoplasts (I. Benediktsson and F. Köhler, in preparation).

One important step in the transformation process of higher plant cells is the integration of DNA sequences into the plant genome. The results presented here and in a previous publication (Köhler et al. 1989) may indicate that repair mechanisms are involved in this integration step. Without irradiation of protoplasts, an activation of repair processes could result from the maceration of plant tissue and from the transformation procedure itself. Both processes represent strong stress factors. The amount of active repair enzymes after protoplast isolation may be one factor for interpreting the different transformation frequencies observed in different plant genotypes (Köhler et al. 1987a, b; Damm et al. 1989; Tyagi et al. 1989).

Acknowledgements. We thank K. Afsar for excellent technical assistance.

References

Ballas N, Zakai N, Friedberg D, Loyter A (1988) Linear forms of plasmid DNA are superior to supercoiled structures as active templates for gene expression in plant protoplasts. Plant Mol Biol 11:517–527

Bergounioux C, Perennes C, Miege C, Gadal P (1986) Male sterility effect upon protoplast division in *Petunia hybrida*. Protoplasma 130:138–144

Bergounioux C, Perennes C, Brown SC, Sarda C, Gadal P (1988) Relation between protoplast division, cell cycle stage and, nuclear chromatin structure. Protoplasma 142:127–136

Binding H (1974) Regeneration von haploiden und diploiden Pflanzen aus Protoplasten von *Petunia hybrida*. Z Pflanzenphysiol 74:327–356

Chuong PV, Pauls KP, Beversdorf WD (1987) Plant regeneration from *Brassica nigra* (L.) Koch stem protoplasts. In Vitro Cell Develop Biol 23:449–452

Cook R, Meyer Y (1981) Hormonal control of tobacco protoplast nucleic acid metabolism during in vitro culture. Planta 152:1–7

Damm B, Schmidt R, Willmitzer L (1989) Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. Mol Gen Genet 217:6–12

Glimelius K (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol Plant 61:38–44

Hain R, Stabel P, Czernilofsky AP, Steinbiß HH, Herrera-Estrella L, Schell J (1985) Uptake, integration, expression, and genetic transmission of a selectable chimaeric gene by plant protoplasts. Mol Gen Genet 199:161–168

Köhler F, Golz C, Eapen S, Kohn H, Schieder O (1987a) Stable transformation of moth bean *Vigna aconitifolia* via direct gene transfer. Plant Cell Rep 6:313–317

Köhler F, Golz C, Eapen S, Schieder O (1987b) Influence of plant cultivar and plasmid-DNA on transformation rates in tobacco and moth bean. Plant Sci 53:87–91

Köhler F, Cardon G, Pöhlmann M, Gill R, Schieder O (1989) 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? Plant Mol Biol 12:189–199

Korr H, Koeser K, Oldenkott S, Schmidt H, Schulze B (1989) X-ray dose effect relationship on unscheduled DNA synthesis in mouse brain cells studied in vivo. Radiat Environ Biophys 28:13–26

Krüger-Lebus S, Potrykus I (1987) A simple and efficient method for direct gene transfer to *Petunia hybrida* without electroporation. Plant Mol Biol Rep 5:289–294

Li L, Kohlenbach HW (1982) Somatic embryogenesis in quite a direct way in cultures of mesophyll protoplasts of *Brassica napus* L. Plant Cell Rep 1:209–211

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Murashige T, Skoog F (1962) Revised media for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Nagata T, Takebe I (1971) Plating of isolated tobacco mesophyll protoplasts on agar medium. Planta 99:12–22

Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions. Plant Mol Bioll 8:363–373

Paszkowski J, Shillito R, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722

Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7:4021–4026

Pröls M, Töpfer R, Schell J, Steinbiß HH (1988) Transient gene expression in tobacco protoplasts: I. Time course of CAT appearance. Plant Cell Rep 7:221–224

Sacristan MD, Gerdemann-Knörck M, Schieder O (1989) Incorporation of hygromycin resistance in *Brassica nigra* and its transfer to *B. napus* through asymmetric protoplast fusion. Theor Appl Genet 78:194–200

Schieder O (1984) Isolation and culture of protoplasts: Datura. In: Vasil (ed) Cell culture and somatic cell genetics of plants, vol 1. Academic Press, Orlando, pp 350–355

Schocher RJ, Shillito RD, Saul MW, Paszkowski J, Potrykus I (1986) Co-transformation of unlinked foreign genes into plants by direct gene transfer. Bio/Technol 4:1093–1096

Shillito RD, Saul M, Paszkowski J, Potrykus I (1985) High efficiency direct gene transfer to plants. Bio/Technol 3:1099–1103

Tyagi S, Spörlein B, Tyagi AK, Herrmann RG, Koop HU (1989) PEG- and electroporation-induced transformation in *Nicotiana tabacum*: influence of genotype on transformation frequencies. Theor Appl Genet 78:287–292

Wilson SM, Thorpe TA, Moloney MM (1989) PEG-mediated expression of GUS and CAT genes in protoplasts from embryogenic suspension cultures of *Picea glauca*. Plant Cell Rep 7:704–707