Theor Appl Genet (1989) 78: 287–292

PEG- and electroporation-induced transformation in *Nicotiana tabacum*: influence of genotype on transformation frequencies

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Received January 23, 1989; Accepted April 12, 1989
Communicated by G. Wenzel

Summary. Experimental parameters for direct gene transfer with recombinant DNA encoding neomycin phosphotransferase II (NPTII) under control of eukaryotic expression signals were established. The introduced gene was shown by the growth of transformants on media containing kanamycin, by genomic blotting and by assaying NPTII activity. Leaf protoplasts from three green genotypes of varieties xanthii and petit havanna, and from four plastome-encoded albino genotypes of *Nicotiana tabacum* were analyzed with respect to cell division kinetics and yield of kanamycin-tolerant colonies after direct gene transfer. No clear correlation was found between the time of onset of cell division and transformation frequency.

Key words: Direct gene transfer – Electroporation – Genotype influence – Kanamycin resistance – *Nicotiana tabacum*

Introduction

Initial attempts to transform higher plants or cell lines with purified DNA used Ti-plasmid derived DNA sequences (Davey et al. 1980; Krens et al. 1982). The demonstration of vector-free transformation (Paszkowski et al. 1984) has initiated new activity in research on plant transformation (Hain et al. 1985; Lörz et al. 1985; Potrykus et al. 1985; Uchimiya et al. 1986) because of the potential of the method, in particular its general applicability to both dicotyledonous and monocotyledonous plants. Despite efforts to optimize conditions for direct gene transfer (Shillito et al. 1985; Meyer et al. 1985; Okada et al. 1986; Negrutiu et al. 1987), the technology is still at a relatively early stage of development and results obtained in different laboratories by varying experimental parameters are frequently contradictory. Also, the influence of genotype on transformation frequencies is still not completely understood (Köhler et al. 1987). Here we describe the influence of various experimental parameters and of the genotype on the yield of kanamycin-resistant colonies after direct gene transfer with DNA coding for the NPTII gene. Emphasis has been put on the analysis of the influence of the time of onset of cell divisions, since a substantial increase of transformation frequencies has been described with partially synchronized protoplasts (Meyer et al. 1985).

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Materials and methods

Plant material

Shoot cultures of the tobacco albino plastome mutants 'VBW', '339', '340' and '92V37' were obtained from Prof. Dr. E. Galun and Dr. D. Aviv (The Weizman Institute of Science, Israel) and maintained as described (Aviv and Galun 1985). Seeds of *Nicotiana tabacum* cv petit havanna genotypes 'SR1' and the corresponding wild-type ('PHWT') were received from Dr. A. Cséplö (Institute of Plant Physiology, Hungarian Academy of Sciences, Szeged). Line '102/2' of wild-type tobacco cv xanthii was derived as a protoclone via individual culture of a single protoplast (Koop and Schweiger 1985). Shoot cultures of green genotypes were maintained on B5 medium (Gamborg et al. 1968) containing 5×10^{-5} *M* $FeSO_4$ and 5×0^{-5} *M* Na_2EDTA, 100 mg/l meso-inositol, 0.1 mg/l thiamine-HCl and 0.1 mg/l pyridoxin-HCl. Shoot cultures were kept in a culture room at $25° \pm 2°C$ on 120 ml solidified medium in 720-ml glass jars with a metal lid, fitted with a foam plug for gas exchange. Day length was 16 h, light intensity was approximately 2,000–4,000 lx for the green varieties and approximately 800 lx for the plastome mutants. Lines '102/2' and 'VBW' were used for optimization experiments, whereas all lines were analyzed with respect to the influence of the genotype on transformation frequencies.

Plasmids and bacterial strains

The plasmid 'pUCNK1 (HindIII)', containing the NPTII gene from Tn5 under control of eukaryotic promotor and polyadeny-

lation signals (pNos, nopaline synthase promotor and ocspA, octopine synthase polyadenylation signal), was obtained from Prof. Dr. L. Willmitzer, Berlin. Plasmid 'pRT100neo', carrying the same gene and the same polyadenylation signal but the CaMV 35S-promotor (Töpfer et al. 1987), was a gift of Dr. R. Töpfer, Cologne. Plasmid 'pKm2', containing the same gene under prokaryotic expression control (Beck et al. 1982), was supplied by Prof. Dr. H. Schaller, Heidelberg. Plasmid 'pUC-NK1 (HindIII)' was primarily used for optimization experiments, 'pRT100neo' for the analysis of the genotype influence on transformation frequencies and 'pKM2' served as the source for a hybridization probe in genomic blots. All plasmids were propagated in *E. coli* K12/RR1/M15.

Protoplast isolation

Protoplasts were isolated from leaves of 3- to 4-week-old plants as described earlier (Koop and Schweiger 1985), except that 1% cellulase, 1% macerozyme and 1% polybuffer 74 (LKB-Pharmacia) were used. Protoplasts were washed by repeated flotation on 0.5 *M* sucrose and then pelleted by centrifugation at $35 \times g$ for 3–5 min. They were finally suspended in 0.5 *M* mannitol at a density of $1-2 \times 10^6$/ml.

Transformation procedures

About 0.25 ml protoplast suspension was taken in a 2-ml Nunc tube and 25 µl DNA solution, containing plasmid DNA (4, 8 or 40 µg) and carrier DNA (calf thymus DNA type I, Sigma, sonicated to a size below 2,000 bp 20, 40 or 200 µg), was added. After 5–10 min, 0.25 ml of 24% or 16% PEG (MW 6,000, Sigma) containing $MgCl_2$ (30 m*M*) and 0.4 *M* mannitol was added. The protoplast suspension was incubated for either 10 or 30 min at room temperature. If desirable, a heat shock at 44 °C for 5 min was given, before adding PEG. Electroporation, when desired, was performed in the presence or absence of PEG by injecting single rectangle DC-pulses (50 µs, 1, 1.5 or 2 kV/cm) into the protoplast/DNA mixture. The pulse was produced by a 'TA 750 Electrotransfection' device (Krüss, Hamburg), using a pipette chamber with 0.5 mm electrode distance. After treatment for transformation, samples were transferred to 2.5 ml of liquid culture medium, which was identical with the protoplast isolation medium, except that sucrose was replaced by 0.4 *M* glucose and it contained 0.5 mg/l 2.4-D.

Protoplast culture, selection of transformed lines and regeneration of plants

Petri dishes containing protoplasts were incubated in darkness at 25 °C for 1 week, followed by diffuse light of 16/8 h light/dark cycle. Kanamycin sulfate (75 mg/l) (Sigma) was added to the medium after 3–4 weeks of culture. Glucose concentration in the culture medium was gradually reduced and finally replaced by 3% sucrose. After 3–4 additional weeks, resistant colonies were transferred to solid medium supplemented with 3% sucrose and 0.05 mg/l 2,4-D. For regeneration of shoots, resistant calli were transferred to Murashige and Skoog's medium (1962) containing 1 mg/l BAP and 100 mg/l kanamycin sulfate. For root formation, shoots were inoculated on the same medium as used for shoot cultures but with 100 mg/l kanamycin sulfate.

Isolation of DNA and Southern blot analysis

Plasmid DNA was isolated according to Birnboim and Doly (1979) and further purified by CsCl ethidium bromide gradient centrifugation (Maniatis et al. 1982). DNA from tobacco leaf or callus was isolated, essentially as described by Rogers and Bendich (1985), but phenol/chloroform extraction and ethanol precipitation steps were introduced both before and after digestion with restriction enzymes. The DNA was dissolved in TE buffer (1 m*M* EDTA, 10 m*M* TRIS, pH 8.6), run on 0.8% agarose slab gels and then transferred to nitrocellulose filters (BA 85, Schleicher and Schüll, Dassel), according to Southern. The hybridization probe, the 1,180-bp EcoRI-SalI fragment of 'pKm2' representing the NPTII gene from Tn5 (Beck et al. 1982), was radioactively labelled using the 'Multiprime' DNA labelling system (Amersham, Braunschweig), according to the instructions of the supplier, to give approximately 5×10^8 cpm/µg DNA. Prehybridization and hybridization were for 12 and 48 h, respectively, at 42 °C in 50% formamide, 5 × SSC (1 × SSC: 0.15 *M* NaCl, 0.015 *M* Na-citrate, pH 6.8), 5 × Denhardt buffer (Maniatis et al. 1982) and 200 µg calf thymus DNA, and the filters were washed for several hours in 50% formamide, 5 × SSC and 0.5% SDS at 55 °C.

NPTII-assay

The enzymatic assay for neomycin phosphotransferase II was done according to Reiss et al. (1984) as modified by Schreier et al. (1985).

Determination of transformation and cell division frequencies

Transformation frequencies were determined as 'absolute transformation frequencies' (Negrutiu et al. 1987), i.e. the number of resistant colonies per treated protoplasts. Mean values were calculated from two parallels. In general, determinations were made by at least two independent experiments. A few samples gave transformation frequencies approximately one order of magnitude higher than all the others. Since these results could not be reproduced, they were not included in the evaluation. Cell division frequencies were determined microscopically.

Results and discussion

Characterization of transformants

Colonies resistant to kanamycin were obtained from protoplasts of all genotypes tested. All tissues derived from plantlets regenerated from these colonies contain DNA sequences homologous to the gene used for transformation (Fig. 1), when tested by Southern analysis. NPTII activity was also detected (Fig. 2) in all 37 lines analyzed after random selection from resistant colonies or plants. Undigested DNA displays the introduced sequences only in the high molecular weight range, which confirms integration into the plant genome. The presence of multiple bands in genomic blots after terminal restriction endonuclease digestion (Fig. 1) indicates that the foreign DNA has been incorporated at more than one site of the host DNA. Similar observations have also been reported by others (e.g. Hain et al. 1985; Riggs et al. 1986).

NPTII produced by transformed calli and plants of both plastome mutants and wild-type tobacco is similar in activity and electrophoretic mobility to the enzyme found in bacteria carrying the NPTII gene from Tn5 under prokaryotic expression control (Fig. 2). No such signal was found in control plants regenerated from untreated protoplasts.

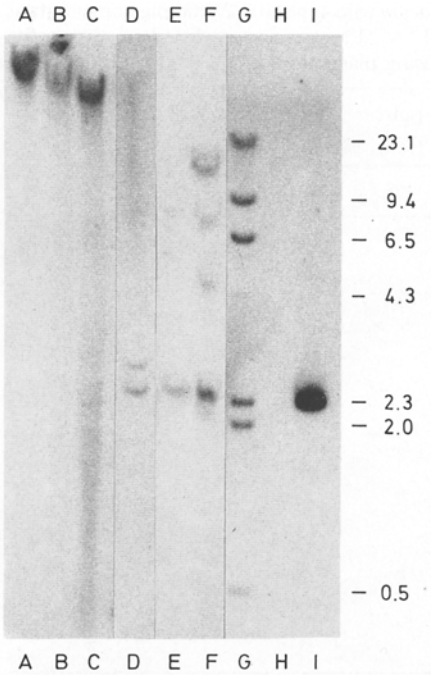

Fig. 1. Genomic blot analysis of *Nicotiana tabacum* transformed with plasmid 'pUCNK1 (HindIII)'. *A–C* – undigested DNA, *D–I* – DNA digested with HindIII. Fifteen micrograms of DNA was loaded into *tracks A* through *F* and *H*. *Track I* contained 25 pg DNA. *A, B* and *D, E* represent DNA isolated from callus of line '102/2', whereas *tracks C* and *F* were loaded with DNA from a regenerated plantlet of line 'VBW'. *Track G:* ^{32}P-labelled DNA of phage λ; *track H:* DNA from a non-transformed shoot of line '102/2'; *track I:* DNA from plasmid 'pUCNK1'. The 1180-bp EcoRI-SalII fragment of 'pKM2' was used as a probe after labelling (5×10^8 cpm/μg DNA) with the 'Multiprime' labelling system (Amersham, Braunschweig). Length standards (kbp) as derived from *track H* are indicated on the right margin

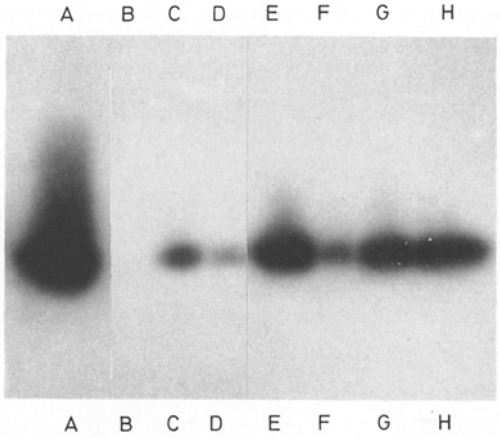

Fig. 2. Neomycin phosphotransferase II activity in *Nicotiana tabacum* transformed with plasmid 'pUCNK1 (HindIII)'. As a control, enzyme activity was also assayed from extracts of bacteria harboring a plasmid containing the gene for kanamycine resistance (*track A*). *Track B:* extract from a non-transformed shoot of line '102/2'; *tracks C–E:* extracts from callus of three different transformants of line '102/2'; *tracks F–H:* extracts from regenerated shoots of line 'VBW'. The figure represents only the qualitative detection of NPTII activity. Therefore, intensities of bands do not reflect different levels of gene expression

Transformation frequencies

The absolute transformation frequencies ranged from 10^{-6} to 10^{-3} (Table 1, Fig. 4). Even higher frequencies, up to 1.7×10^{-2}, could be estimated if samples were included in the evaluations in which individual resistant colonies were difficult to distinguish from each other. Petri dishes of such samples contained so many colonies that a lawn of cells was soon produced. Evidently, these colonies were all truly resistant, i.e. they kept growing after transfer to solid selection medium. Since selection was initially performed in liquid medium, at least some of the resistant colonies could be derived through separation of cells after division. Thus, the possibility that single transformation events could lead to varying numbers of resistant colonies precludes accurate determination of transformation efficiency. Since such petri dishes occurred only sporadically and unpredictably, these samples were not used for calculation of transformation frequencies.

Factors influencing transformation frequencies in lines '102/2' and 'VBW' PEG. Higher transformation frequencies compared to '102/2' protoplasts were found in 'VBW' protoplasts (Table 1 A). This result confirms that different lines may give different transformation frequencies (Köhler et al. 1987), and stresses the need to optimize conditions for each material (Negrutiu et al. 1987). Surprisingly, a further increase (five- to seven-fold) was possible when 'VBW' protoplasts were treated with PEG for a shorter period (10 min instead of the 30 min treatment used as a standard duration by Paszkowski et al. 1984 and Lörz et al. 1985). These protoplasts seem to be more sensitive to PEG than the '102/2' samples, and many damaged protoplasts can be observed after a 30-min treatment. Negrutiu et al. (1987) have also used different duraitons of PEG treatments by varying the interval between addition of PEG and the DNA. In their report they have not, however, determined whether a PEG treatment shorter than 20 min might be beneficial.

Higher concentrations of plasmid DNA along with higher concentrations of carrier DNA were found to increase the frequency of transformation, both in 'VBW' and '102/2' protoplasts (Table 1 A). At first glance, this increase of transformation frequency, with concentrations of plasmid DNA higher than 10 μg/ml and carrier DNA higher than 50 μg/ml, appears to be at variance with experiments on *Nicotiana tabacum* cv petit havanna SR1 reported by Shillito et al. (1985). However, these authors have increased either plasmid or carrier DNA

Table 1. Influence of different parameters on transformation frequencies of *Nicotiana tabacum* wild-type '102/2' and plastome mutant 'VBW'. Application of a particular experimental parameter is indicated by the symbol '+'. Frequencies are listed separately for independent experiments using the same conditions. Transformations were performed using plasmid 'pUCNK1 (HindIII)'

	PEG: concentration (%)		PEG: duration (min)		DNA: plasmid; carrier (μg/ml)			DC-pulse (kV/cm)			Heatshock 44 °C 5 min	Transformation frequencies ($\times 10^{-5}$)
	8	12	10	30	8; 40	16; 80	80; 400	1	1.5	2		
A PEG only		+		+	+						+	1.6, 1.3, 1.0
line '102/2'		+		+	+							5.2, 3.6, 4.2
		+		+			+				+	9.3, 10.4
		+	+			+						10.2
line 'VBW'		+		+	+						+	5.6
		+	+		+						+	42.8
		+		+			+				+	11.5
		+	+				+				+	66.4
B Electroporation						+		+				0.6
line 'VBW'						+			+			0.4
						+				+		7.2, 5.2
	+		+			+		+				4.4, 12.0
	+		+			+			+			2.2, 3.6
	+		+			+				+		−*
	+			+		+		+				−*
		+	+			+		+				−*

* No colonies were regenerated after these treatments

concentrations, whereas both DNA concentrations were raised simultaneously in the present study.

We have also tested the effect of a heat shock on transformation as recommended by Shillito et al. (1985). No promotive effect was found – a slight decrease was even detected for '102/2' protoplasts. In a number of experiments this treatment proved deleterious to the cells, in particular if combined with electroporation. Therefore, it was later omitted from the transformation protocol. No apparent effect of heat shock on transformation frequencies was also reported by Negrutiu et al. (1987) for *Nicotiana plumbaginifolia*.

Electroporation. Transformation rates were studied by electroporation with different combinations of a DC-pulse, with various PEG concentrations and durations of PEG treatment prior to the pulse (Table 1 B). Electroporation with a single rectangular pulse of 50 μs and 1–2 kV/cm field strength in the absence of PEG leads to the recovery of resistant colonies at frequencies comparable to those achieved with PEG alone. PEG treatment plus electric pulse proved detrimental in our hands, if: (a) PEG concentrations higher than 8% were applied, (b) PEG treatment for 30 min rather than 10 min was applied, or (c) the field strength was higher than 1.5 kV/cm. Thus, in order to use a DC-pulse in the presence of PEG, it was essential to reduce PEG concentration, incubation time and pulse heigth to ensure protoplast survival.

Influence of genotype

Experimental conditions. Since significant differences in transformation frequencies were found between the two lines used, experiments were performed to analyze the influence of the genotype in more detail. Seven different genotypes, including '102/2' and 'VBW' studied above, were compared with respect to their transformability under identical conditions. Since PEG concentration and incubation time appeared to be critical factors for the survival of protoplasts and, implicitly, for transformation frequencies, these experiments were exclusively done by electroporation, i.e. without PEG. This approach seemed reasonable, because recovery of resistant colonies should be in the same range as with PEG alone (see above). Furthermore, electroporation is a straightforward procedure and under the conditions used is not causing the loss of protoplasts. Note that a single DC-pulse of 2kV field strength and 50 μs duration was used, which ensures survival of more than 80% of the protoplasts. This is in contrast to conditions employed by Shillito et al. (1985). These authors, using exponentially decaying rather then rectangular pulses, adjusted the field strength to survival of only 50% of the protoplasts.

Cell division kinetics. The genotypes did not differ significantly in their plating efficiencies, since in all lines used, microcolonies were formed from more than 80% of the

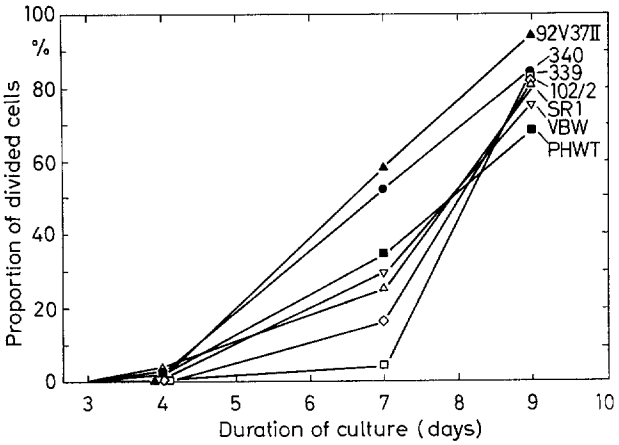

Fig. 3. Frequencies of initial cell division in protoplast-derived cells from various genotypes of *Nicotiana tabacum* during the first 9 days of culture. Genotypes are listed on the right

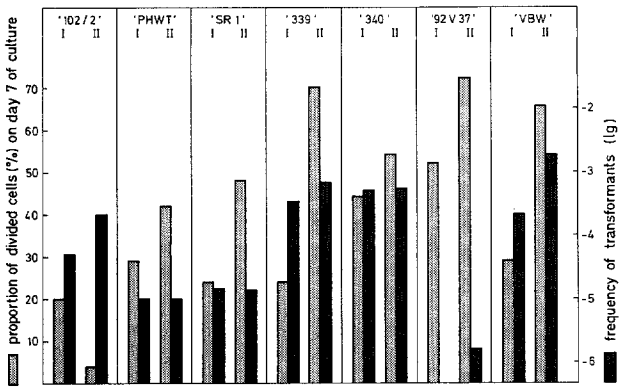

Fig. 4. Frequencies of divided cells (%) after 7 days of culture and transformation frequencies after electroporation in the presence of plasmid 'pRT100neo' (80 µg/ml plus 0.4 mg/ml carrier-DNA) and selection on medium containing 100 mg/l kanamycin sulfate for 8–10 weeks in different genotypes of *Nicotiana tabacum*. Genotypes are indicated at the top, numbers I and II represent different experiments. Note that transformation frequencies are given logarithmically

protoplasts that survived the transformation treatment. The lines differed considerably, however in their cell division kinetics. First dvisions were found on day 4 of culture in most genotypes (Fig. 3), division frequencies varied to the greatest extent – from less than 5% ('102/2') to more than 50% ('92V37') – on day 7, and all lines showed more than 50% cell divisions after 9 days of culture. Thus, cell division frequencies on day 7 of culture have been used as a measure for the speed of cell division (Fig. 4).

Influence of speed of cell division on transformation frequencies

Aphidicolin, an inhibitor of mitosis in tobacco protoplasts, inhibits the alpha-like DNA polymerase; 2,6-dichlorobenzonitrile (DB) inhibits cytokinesis by inhibition of cell wall formation. By using both inhibitors together, Meyer et al. (1985) achieved partial synchronization of division in *Nicotiana tabacum* cv petit havanna SR1 protoplast-derived cells. As a consequence of this synchronization, an increase of transformation frequencies as much as two orders of magnitude has been observed by these authors. On the other hand, Negrutiu et al. (1987) and I. Potrykus (personal communication) could not detect any influence of DB or aphidicolin applied separately or simultaneously on transformation frequencies in *N. tabacum* or *N. plumbaginifolia*.

In the course of the experiments described here, transformation was also performed after culture for several days in the presence of DB. Thus, the foreign DNA was applied during the period immediately preceding cell division. Pretreatment with DB did not result in the recovery of a higher number of resistant colonies (data not shown). This would have to be anticipated, if the time between the introduction of foreign DNA and DNA replication influenced transformation efficiencies, as indicated by the results achieved with synchronized cultures. In order to further elucidate a possible influence of the cell cycle stage upon transformation rates, cell lines which differed in their speed to initiate cell division were compared, assuming that early cell division reflects early S- and M-phases. Transformation frequencies of seven different lines in at least two independent experiments gave no clear-cut correlation between the speed of initiation of cell division and the rate of transformation (Fig. 4). This implies that slowly dividing protoplast preparations do not necessarily lead to low transformation yields ('102/2' II), nor does fast division necessarily lead to high yields ('92V37' II, 'SR1' II). It is worth noting that the speed of cell division of a given genotype can vary between different experiments, without any influence on transformation frequency ('PHWT', 'SR1', '339'). Conversely, transformation frequencies may differ between genotypes with similar division kinetics (compare 'SR1' II with '340'). These experiments strongly suggest that different susceptibilities of genotypes to transformation by direct gene transfer (Köhler et al. 1987; Negrutiu et al. 1987) are not caused by the different time span between transformation treatment and first mitosis. This would mean that physiological parameters different from cell cycle stage are at least partly responsible for the observed effects. These parameters remain to be determined.

Acknowledgements. ST was supported by DFG (He 693), AKT was a visiting Academic Fellow under the special gene technology program of the German Academic Exchange Service. The experiments were funded through DFG SFB No. 184/A2. The authors are indebted to colleagues who supplied plant material or plasmids, as indicated in 'Materials and methods', and those

who made data available to us prior to publication. The skillful technical assistance by Mrs. M. Grimm is gratefully acknowledged.

References

Aviv D, Galun E (1985) An in vitro procedure to assign pigment mutations in *Nicotiana* to either the chloroplast or the nucleus. J Hered 76:135–137

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Davey MR, Cocking EC, Freeman J, Pearce N, Tudor I (1980) Transformation of *Petunia* protoplasts by isolated *Agrobacterium* plasmids. Plant Sci Lett 18:307–313

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

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 (1987) Stable transformation of moth bean *Vigna aconitifolia* via direct gene transfer. Plant Cell Rep 6:313–317

Koop HU, Schweiger HG (1985) Regeneration of plants from individually cultivated protoplasts using an improved microculture system. J Plant Physiol 121:245–257

Krens FA, Molendijk L. Wullems GJ, Schilperoort RA (1982) In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296:72–74

Lörz H, Baker B, Schell J (1985) Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199:178–182

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

Meyer P, Walgenbach E, Bussmann K, Hombrecher G, Saedler H (1985) Synchronized tobacco protoplasts are efficiently transformed by DNA. Mol Gen Genet 201:513–518

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol Biol 8:363–373

Okada K, Takebe I, Nagata T (1986) Introduction of functional RNA into plant protoplasts by electroporation. Mol Gen Genet 205:398–403

Paszkowski J, Pisan B, Shillito RD, Hohn T, Hohn B, Potrykus I (1984) Direct gene transfer to plants. EMBO J 3:2717–2722

Potrykus I, Shillito RD, Saul MW, Paszkowski J (1985) Direct gene transfer, State of the art and future potential. Plant Mol Biol Rep 3:117–128

Reiss B, Sprengel HW, Schaller H (1984) A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30:211–218

Riggs CD, Bates GW (1986) Stable transformation of tobacco by electroporation: Evidence for plasmid concatenation. Proc Natl Acad Sci USA 83:5602–5606

Rogers DO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76

Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant protoplasts. EMBO J 4:25–32

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

Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiß HH (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acid Res 15:5890

Uchimiya H, Fushimi T, Hashimoto H, Harada H, Syono K, Sugawara Y (1986) Expression of a foreign gene in callus derived from DNA treated protoplasts of rice (*Oryza sativa* L). Mol Gen Genet 204:204–207