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Lipofectin: direct gene transfer to higher plants using cationic liposomes

B. Spörlein and H.-U. Koop*

Botanical Institute of the Ludwig-Maximilian University, Menzinger Str. 67, W-8000 München 19, FRG

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Summary. It has recently been shown that lipofectin, a commercially available preparation of cationic liposomes is capable of animal and plant cell line transfection. Here, it is analyzed with respect to its toxicity for higher plant protoplasts and used for transient expression and stable transformation experiments with mesophyll protoplasts of *Nicotiana tabacum* and *Nicotiana plumbaginifolia*. Transient expression of the β-glucuronidase gene (*GUS*) under control of the CaMV-35S-promoter was lower than after introduction of the same gene by polyethylene glycol. By transferring the neomycin phosphotransferase gene (*NPTII*) and subsequent culture and regeneration under selection with kanamycin, stably transformed plants were recovered after using Lipofectin in various protocols with or without additional application of electroporation. Efficiencies of stable transformation were comparable to those achieved with PEG and/or electroporation. Confirmation of transformants included assaying the enzyme activity of the gene product, genomic blotting, and transfer of the resistant phenotype to the progeny produced from selfed primary transformants.

Key words: Cationic liposomes – Direct gene transfer – Electroporation – Plant transformation

Introduction

Among the various procedures for introducing isolated DNA into higher plant protoplasts (for a recent evaluation see: Potrykus 1990), liposome-mediated direct gene transfer is one of the most difficult (Caboche 1990). The problems lie in the preparation of liposomes and the reliable encapsulation of plasmids. Thus, although it has been proven a successful method in principle, this approach has found only limited attention. This might change since stable liposome preparations, which spontaneously bind to DNA and mediate its delivery into cells, are now commercially available. The successful application of Lipofectin preparation in animal systems (Felgner et al. 1987; Felgner and Ringold 1989) and first results with maize cell lines (Antonelli and Stadler 1990) have prompted us to attempt to investigate its suitability for tobacco protoplast transient expression and stable transformation systems, including plant regeneration and analysis of transmission to the progeny.

Materials and methods

Plasmids

Plasmids pRT100neo and pRT102gus (Töpfer et al. 1987) were used for stable integration and transient expression analysis, respectively.

Stable transformation

Conditions for growth of protoplast donor plants (*Nicotiana tabacum* cv petit havanna, SR1), isolation and culture of protoplasts, selection, plant regeneration, and Southern blot analysis were essentially as described by Tyagi et al. (1989). *NPTII* activity was assayed following the procedure of McDonell et al. (1987). Segregation in the progeny of selfed primary transformants was analyzed by germination of seeds from capsules randomly selected from five different transformants on solid medium containing 300 mg/l kanamycin sulfate. In each experiment, the number of transformants recovered from 2×10^5 treated protoplasts (*N. tabacum* SR1) was determined using a total of 8 µg plasmid and 40 µg carrier DNA in different volumes (0.5 ml, 1 ml, or 3 ml) of incubation medium (0.5 *M* mannitol, 30 m*M* $MgCl_2$) (compare Tyagi et al. 1989) and preincubation with DNA for 10 min.

In a first set of experiments (Table 2, A) transformation by electroporation (single pulse, 50 µs, 2 kV/cm), with or without

* To whom correspondence should be addressed

30 µl of lipofectin (GIBCO BRL, Eggenstein) suspension included in an incubation volume of 500 µl, was attempted and the transformation frequencies were compared to those found after treatment of protoplasts with the same amount of Lipofectin for 5 h without electroporation, followed by a washing step with 10 ml protoplast culture medium or dilution directly into the culture medium.

The second set of experiments (without electroporation) (Table 2, B) used incubation for 48 h with 10 µl lipofectin in 1 or 3 ml incubation or culture medium, again followed by washing with 10 ml culture medium or dilution directly into the medium used for protoplast culture.

For a negative control, protoplasts were incubated with DNA under the same conditions, but without electroporation and without lipofectin.

Transient expression

Nicotiana plumbaginifolia LR 400 (Czeplö and Maliga 1982) mesophyll protoplasts were used for transient expression analysis using the *GUS* gene (Jefferson 1987). In each experiment, 10^6 protoplasts (*N. plumbaginifolia*) were incubated for 2 days in 2.5-ml culture medium containing 40 µl of crude plasmid DNA (1 mg/ml bidistilled water) (Birnboim and Doly 1979) and 60 µl or 180 µl lipofectin (Table 4). For a negative control, incubation of protoplasts was performed omitting DNA and lipofectin. For a positive control, protoplasts were treated with 12% PEG 6,000 (Merck) in 0.4 *M* mannitol and 30 m*M* $MgCl_2$ for 30 min, washed into culture medium, and similarly treated.

After 2 days culture, protoplasts were pelleted for 10 min at $50 \times g$, lysed in 100 µl lysis buffer (Jefferson 1987), and centrifuged in an Eppendorf centrifuge. Forty microliters of the supernatant, equivalent to 90 µg of protein (determination of protein content after Bradford 1976) was incubated in 100 µl lysis buffer containing 1 m*M* 4-methylumbelliferyl-glucuronide as a substrate. The enzyme reaction was stopped by the addition of Na_2CO_3 (Jefferson 1987), and fluorimetric determinations (TK100 fluorimeter, Hoefer Scientific, San Francisco) were made after 1 or 18 h of incubation as indicated for the individual experiments.

Analysis of toxicity

Leaf protoplasts of *N. tabacum* SR1 were incubated in 500 µl 0.5 *M* mannitol in the presence of varying amounts of lipofectin for 5 or 48 h, respectively, transferred to culture medium by dilution with 5 vol. or washing with 20 vol. of culture medium, respectively, and viability was microscopically determined 2 days after protoplast isolation. Colony formation was determined after 8 days of culture.

Results and discussion

The main aim of our investigations was to test, in principle, the applicability of stable cationic liposomes spontaneously interacting with DNA, and negatively charged plasma membranes for transformation studies in higher plants, in general, and for tobacco mesophyll protoplasts, in particular (compare Antonelli and Stadler 1990). Although we have included the analysis of the influence of a number of experimental parameters on transformation efficiencies, optimizing all conditions was not the purpose of our experiments, and a more thorough study including a number of plant species and different genotypes will be needed to evaluate this approach compared to other transformation procedures. However, the following conclusions can already be drawn from this study.

Table 1. Analysis of the toxicity of lipofectin for leaf protoplasts of *Nicotiana tabacum* SR1

Duration of treatment (h)	5	48	5		48	
Concentration (µg/ml) of lipofectin	Survival (%) after 2 days		Divisions (%) after 8 days of culture (I: first, II: multiple divisions)			
			I	II	I	II
(A) Treatment followed by dilution with 5 vol. of culture medium						
0	81	68	62	25	59	8
10	67	47	50	21	55	8
20	40	34	50	13	56	6
60	20	16	*	*	*	*
(B) Treatment followed by washing with 20 vol. of culture medium						
0	76	67	93	75	91	52
10	74	50	93	76	95	46
20	52	39	86	60	90	36
60	22	16	31	2	*	*

* No survival

Toxicity of lipofectin

For animal cell cultures, toxicity of lipofectin at concentrations higher than 100 µl per 3 ml culture medium has been reported (Felgner et al. 1987). The concentration used for maize protoplasts was 25 µl/ml (Antonelli and Stadler 1990). For mesophyll protoplasts of *Nicotiana* species the same concentration range seems to be valid (Table 1). Survival is still 50% after incubation with 10 µg/ml for as long as 48 h. This value is identical to the survival rate after PEG treatment in our experiments, and was routinely used for adjusting electroporation conditions by Shillito et al. (1985). Thus, lipofectin can be regarded as a moderately toxic chemical component. Its toxicity does not prohibit its use for transformation experiments. Furthermore, simple dilution with culture medium at the end of the incubation period is a sufficient means of reaching nontoxic conditions.

Stable transformation

Resistant colonies were recovered after applying kanamycin selection conditions, which in previous experiments had never led to "false positives" (Tyagi et al. 1989) and stably transformed plants were regenerated after treatment of protoplasts with DNA and lipofectin under all conditions investigated (Table 2 A, B). *NPTII* activity was found in all five tested regenerated plants

Table 2. Influence of different parameters on recovery of resistant colonies after transformation of *Nicotiana tabacum* cv petit havanna, SR1 mesophyll protoplasts. Application of a particular experimental parameter is indicated by the symbol '+'

	Volume of incubation (ml)	Lipofectin concentration (μg/ml)	Duration of incubation (h)	Pulse (50 μs, 2 kV cm^{-1})	Washing after incubation	Resistant colonies selected from 2×10^5 treated protoplasts
Control	1	–	48	–	–	–
(A) Treatment including electroporation						
	0.5	60	–	+	–	2
	0.5	60	–	+	–	30
	0.5	60	5	–	–	1
	0.5	60	5	–	+	3
(B) Treatment without electroporation						
	1	10	48[a]	–	–	23
	1	10	48[b]	–	–	29
	3	3.3	48[a]	–	+	3
	3	3.3	48[b]	–	+	13

[a] Incubation in mannitol
[b] Incubation in culture medium
All samples were incubated with 8 μg plasmid and 40 μg carrier DNA for 10 min prior to the addition of lipofectin or application of a pulse. Note that, due to different incubation volumes, different DNA concentrations were used

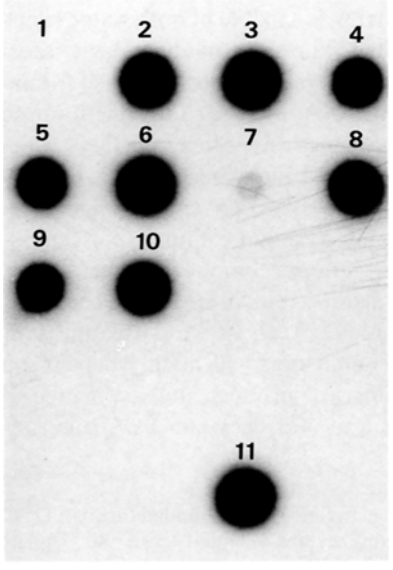

Fig. 1. Dot-assay of *NPTII* activity (McDonell et al. 1987) in enzyme extracts for leaves of untransformed *Nicotiana tabacum* SR1 (*1*) and nine randomly selected independent transformants (*2* through *10*) derived from the treatment of protoplasts with lipofectin in the presence of plasmid pRT100neo (Töpfer et al. 1987). Enzyme extract from *E. coli*, harboring the *NPTII* gene, was used as a positive control (*11*). Fifteen microliters of enzyme extract containing 40 μg of protein was used for each sample

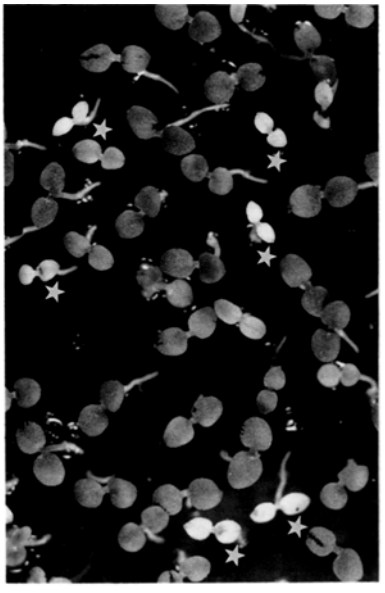

Fig. 2. Segregation of phenotpyes (resistance to 300 mg/l kanamycin sulfate) in seeds from a selfed transformant produced by treatment of *Nicotiana tabacum* SR1 protoplasts with lipofectin in the presence of plasmid pRT100neo (Töpfer et al. 1987). Wild-type seedlings are marked by *asterisks*

selected at random from a total number of 90 (Fig. 1). Southern analysis confirmed the transgenic state of regenerated plants (results not shown). The gene introduced by transformation is transferred to the progeny, and segregation of phenotypically resistant and nonresistant seedlings was found at ratios close to those expected for integration of a single functional gene copy per genome in the progeny of selfed primary transformants (Fig. 2, Table 3).

Transformation frequency

Frequencies of transformation are comparable to those found by PEG and/or electroporation using the same protoplast culture and selection conditions (Tyagi et al.

Table 3. Segregation of phenotype (resistance to 300 mg/l kanamycin sulfate) in seeds from randomly selected fruit capsules from five selfed primary transformants of *Nicotiana tabacum* SR1

Transformant number	Total number of seeds (%)	Seeds failing to germinate (%)	Number of green seedlings (%)	Number of white seedlings (%)
1	178 (100)	20 (11)	126 (71)	32 (18)
2	665 (100)	30 (5)	499 (75)	136 (20)
3	443 (100)	26 (6)	317 (72)	100 (22)
4	247 (100)	–	194 (79)	53 (21)
5	183 (100)	–	140 (77)	43 (23)

Table 4. Levels of transient expression of β-glucuronidase after different transformation treatments of mesophyll protoplasts of *Nicotiana plumbaginifolia* LR 400

	DNA	Lipofectin concentration (μg/ml)	PEG	Enzyme activity 1 h	Enzyme activity 18 h
Negative control	–	–	–	0.09	0.19
Positive control	+	–	+	135	1,040
Sample 1	+	20	–	0.7	74
Sample 2	+	60	–	3	120

Samples were treated with 40 μg plasmid DNA (if applicable) in 0.5 ml incubation medium. Enzyme activity is given as the amount (pmol) of the reaction product formed during incubation of the *GUS* assay for 1 and 18 h, respectively. The positive control was treated with 12% PEG for 30 min, as indicated in the 'Materials and methods' section, and then washed into culture medium. Samples 1 and 2 were incubated in the presence of lipofectin diluted into the culture medium

1989). Higher efficiencies are found after electroporation in the presence of lipofectin (Table 2, A), suggesting an additive effect of both treatments. This observation has not been reported before. Prolonged incubation with the DNA/liposome mixture (Table 2, B) also increases transformation rates, which is in accordance with similar results with animal cells (Felgner et al. 1987; Felgner and Ringold 1989). No difference is found using incubation or culture medium during liposome treatment, or between samples diluted directly into culture or in which the incubation mixture was removed by washing prior to culture. Thus, lipofectin can be used in a very simple procedure simply by including the DNA and the liposomes in the culture medium. Further efforts should be directed towards defining the most efficient combination of DNA amount, lipofectin concentration, and duration of treatment. So far we have not been able to achieve higher transformation frequencies than with other approaches (see, however, Antonelli and Stadler 1990). The ease of operation and the fact that conditions used in this investigation might still be far from optimal suggest that, at least in a number cellular systems, lipofectin might prove superior to other methods of direct gene transfer.

Transient expression

Transient expression of genes introduced by lipofectin is also observed (Table 4). The level of expression increases with lipofectin concentration; it is, however, much lower than the level observed after PEG treatment. Using *Nicotiana tabacum* SR1 protoplasts in comparable experiments did not lead to any detectable *GUS* activity. Higher levels of transient expression in *N. plumbaginifolia* are in accordance with Negrutiu et al. (1987, 1990). It is not possible from our experiments to deduce the efficiency of lipofectin in transient expression studies involving different experimental conditions and/or different protoplasts.

Conclusions

Lipofectin, a commercially available and stable preparation of cationic liposomes, is capable of both stable transformation and transient expression by direct gene transfer into tobacco mesophyll protoplasts. Complex formation of liposomes and DNA and their introduction into protoplasts occur spontaneously. Since the DNA does not have to be encapsulated into liposomes, this procedure is significantly simpler than conventional liposome-mediated transformation. It might stimulate more application of this transformation technique in higher plants. The efficiency of the method is increased by its combination with other techniques such as PEG treatment or electroporation. Its significance will mainly depend on the cost of this commerical product and the degree to which the transformation efficiency can be further improved.

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