INCORPORATION AND MAINTENANCE OF RECOMBINANT-DNA PLASMID VEHICLES pBR313 AND pCR1 IN PLANT PROTOPLASTS

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1. Introduction

We have developed methods for inserting plasmid pBR313 of *Escherichia coli* and the large virulence-conferring plasmid pCK135 of *Agrobacterium tumefaciens* into cowpea protoplasts [1–3]. The uptake of pBR313 by barley protoplasts, which supports our findings, has been reported [4]. Both small and large plasmids can be recovered from the nuclei of cowpea protoplasts exposed briefly to plasmid DNA under proper conditions [2,3].

Although plasmid DNA replication and transcription, culminating in the synthesis of foreign gene products, have not been demonstrated in plant cells, the successful incorporation of plasmid DNA into protoplasts and their nuclei is the first step toward this demonstration.

We now provide evidence for the stable maintenance of gene-cloning plasmids in protoplasts of carrots, cowpea, periwinkle and turnips, and provide conditions most conductive for plasmid uptake by these plant cells.

2. Materials and methods

Plant material for obtaining protoplasts were from a suspension culture of carrot cells grown in Linsmaier-Skoog medium [5] and from periwinkle callus tissue (*Vinca rosea*) that were grown on Murashige-Skoog medium [6] and from young cowpea and turnip leaves that were harvested immediately prior to isolation from plants grown under conditions in [1]. All operations were carried out in a laminar flow hood. The leaf was surface-sterilized with 1% sodium hypochlorite for 5 min. The epidermis was scarified with autoclaved carborundum (320 mesh) as in [7], then rinsed with sterile distilled water. The leaf was cut into squares (ca. 2 × 2 cm) and placed in a flask containing 40 ml 0.45 M mannitol and vacuum was applied for 1 min. The flask was then shaken on a reciprocal shaker (60 strokes/min) to plasmolyze cells and to eliminate any carborundum on the leaf squares. The leaf squares were transfered to a solution containing 40 ml sterile filtered 2% Driselase (Kyowa Hakko Kogyo Co., Ltd.), 0.1% bovine serum albumin (Sigma) and 0.45 M mannitol, pH 5.2 and again vacuum infiltrated. In some cases, 1.5% cellulysin and 0.5% macerase (Cal Biochem) in 0.45 M mannitol, 0.1% bovine serum albumin was used in place of Driselase. In both cases the enzyme solutions were cleared with activated charcoal (0.5 g/100 ml), centrifuged to remove insoluble material, and adjusted to pH 5.2 prior to sterile filtration. The mixture was incubated for 1.5 h at 32°C. The debris was removed by filtration through 4 layers of sterile cheesecloth and the protoplasts were collected by centrifugation (200 × *g*, 2 min) and washed twice with 0.45 M mannitol. Carrot and periwinkle protoplasts were prepared in the same manner except the carborundum step was omitted.

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Microscope examination showed that 95% of these protoplasts were alive as judged by methylene blue staining and cytoplasmic streaming. The protoplasts remained alive for several days and began to lay down cell wall material and their numbers were measured with a haemacytometer. pBR313 and pCR1 are recombinant plasmids. pBR313 was derived from Col E1, pSF2124 and pSC101 [8] and pCR1, a kanamycin derivative of pML2 [9]. Radioactive (^3H, ^{14}C) and unlabelled plasmid DNAs, as covalently closed circular DNA (ccc-DNA), were prepared as in [1]. Plasmid pCR1 and Col E1 were prepared as in [9,10]. Electron microscopic examination of samples from each preparation confirmed that they were ccc-DNA. Preparations of pBR313 were composed of dimers and monomers as observed [1].

Plasmid DNA uptake by cowpea protoplasts were performed under the conditions in [1]. The conditions optimal for DNA uptake for carrot and turnip protoplasts are described below. Protoplasts ($10-13 \times 10^6$) were pipetted into sterilized plastic petri dishes (9 cm diam.) containing the basal salts of White's medium [11], 300 μg/ml loridine (Lilly), 1 μg/ml 2,4-D, 25 units nystatin and 0.45 M mannitol (total vol. 15 ml). Plasmid pBR313 [^3H]DNA was added (0.5 μg DNA/10^6 protoplasts) alone, in combination with poly (L-ornithine) HBr (Miles-Yeda, 13 900 daltons) (5 μg/μg DNA), or protamine sulfate (10 μg/μg DNA), or at concentrations specified in the text. In the latter two cases, the polycationic compounds were incubated 1 h on ice with plasmid DNA prior to addition of the protoplasts. Aliquots (2 ml) of protoplasts were taken at various time intervals, counted with a hemacytometer, and washed three times with 0.45 M mannitol-White's basal salts medium and resuspended in the same medium and incubated at room temperature for an additional 24 h. After this time cells were pelleted by centrifugation ($200 \times g$, 5 min) and lysed in 0.5 ml lysis buffer (20 mM Tris–HCl, pH 8.0, 5 mM Na_2-EDTA and 0.1% SDS. The lysate was incubated for 20 min at 37°C. Radiolabeled material was precipitated with 10% trichloroacetic acid (TCA) and the precipitate collected on glass fiber filters (Whatman GF/C pre-wetted with 10% TCA), washed with 95% ethanol, dried and counted in a Beckman or Packard liquid scintillation spectrometer (Models LS-233 and 2425, respectively).

Nuclei were isolated from protoplasts that were first incubated with deoxyribonuclease I (100 μg/ml) and were obtained as in [1]. Nuclear DNA was either counted directly as TCA-precipitable radioactivity or by analysis by velocity sedimentation in a 5–20% sucrose gradient containing 50 mM Tris–Cl, pH 8.0, 0.5 M NaCl and 5 mM EDTA. Centrifugation was performed in a Beckman SW50.1 rotor at 45 000 rev/min at 20°C for 70 min. The sucrose gradient was fractionated as in [1].

3. Results

3.1. *Enhanced uptake of pBR313 by polycations and Zn^{2+}*

Carrot protoplasts in 0.45 M mannitol are able to take up pBR313 [^3H]DNA. This uptake is greatly enhanced by at least 10-fold in the presence of protamine sulfate and Zn^{2+} and 5-fold in the presence of protamine sulfate and Ca^{2+} (table 1). Protamine sulfate or Zn^{2+} alone causes increased uptake of less than 2-fold. Also poly (L-ornithine) with Zn^{2+} or Zn^{2+} and Ca^{2+} greatly enhanced plasmid uptake.

Similarly, uptake of pBR313 DNA by protoplasts of periwinkle callus tissue and turnip leaf mesophyll is enhanced with poly (L-ornithine) and Zn^{2+}.

3.2. *Kinetics of plasmid DNA uptake*

The effects of the protamine sulfate and poly (L-ornithin) on the uptake of pBR313 DNA by turnip leaf mesophyll protoplasts as a function of time is shown in fig.1. In this experiment each polycationic reagent was mixed with plasmid DNA and incubated for 1 h at 0°C prior to addition to the protoplast. After combination with the protoplasts, samples were taken at various time intervals during incubation at 23°C and washed three times with 0.45 M mannitol in White's medium and incubated for an additional 24 h before the amount of TCA-precipitable counts were taken.

Figure 1 shows that there is an immediate enhancing effect on plasmid uptake by both polycationic reagents. However, with time a continued decrease in the relative uptake of plasmid was observed in the presence of protamine sulfate. On the other hand, uptake of plasmid DNA continued with poly (L-ornithine). The apparent decrease in the presence of protamine

Table 1
Enhanced uptake of pBR313 [^{3}H]DNA by protoplasts in presence of polycations and metal cations

Protoplasts	cpm
Carrots[a]	
Control	282
Protamine sulfate (10 μg/μg DNA)	305
Protamine sulfate (10 μg/μg DNA) + Zn^{2+} (5 mM)	3291
Protamine sulfate + Ca^{2+} (5 mM)	1832
Protamine sulfate + Zn^{2+} + Ca^{2+} (5 mM ea.)	1814
Poly (L-ornithine) (5 μg/μg DNA)	340
Poly (L-ornithine) + Zn^{2+} (5 mM)	1524
Poly (L-ornithine) + Zn^{2+} (5 mM ea.)	1555
Control	203
Protamine sulfate (50 μg/μg DNA)	474
Protamine sulfate + Zn^{2+} (5 mM)	5048
Poly (L-ornithine) (10 μg/μg DNA)	241
Poly (L-ornithine) + Zn^{2+} (5 mM)	4127
Poly (L-ornithine) + Ca^{2+} (5 mM)	1098
Poly (L-ornithine) + Zn^{2+} + Ca^{2+} (5 mM ea.)	5249
Periwinkle[b]	
Control	1594
Poly (L-ornithine) (6.5 μg/ml)	2539
Zn^{2+} (10 mM)	2707
Poly (L-ornithine) (6.5 μg/ml) + Zn^{2+} (10 mM)	16 439

[a] Protoplasts (267 000/ml) in 2 ml 0.45 M mannitol were incubated at 23°C for 20 min in the presence of the polycation and/or metal cation and washed twice with 0.45 M mannitol. They were treated with DNAase I (100 μg/ml), pH 7 in the presence of 10 mM $MgCl_2$ in 0.45 M mannitol for 15 min at 23°C. They were then washed twice with a solution of 0.45 M mannitol containing 10 mM EDTA, pH 8.0, before they were lysed and the TCA-insoluble precipitate counted for radioactivity

[b] Protoplasts (780 000/ml) in 4 ml solution containing 0.25 M sucrose, 14 mM Tris–Cl, pH 8.0, and the respective polycation and cation. Incubation was at room temperature for 20 min. Cells were washed twice with 0.25 M sucrose and then incubated with DNAase I (150 μg/ml) in the presence of 10 mM Tris–Cl, pH 7.0, 0.25 M sucrose, 10 mM $MgCl_2$ for 15 min at 23°C. They were then washed with 0.25 M sucrose, 0.25 M EDTA, pH 8.0, before they were lysed and the TCA-insoluble precipitate counted for radioactivity

sulfate is not due to decreasing proportion of living cells in the protoplast sample.

3.3. *Effect of pH and polycations on plasmid uptake*

Protoplasts prepared from carrot callus tissue were incubated with pBR313 [^{3}H]DNA in the presence of polycationic reagents at the pH values given in table 2. The data show that protamine sulfate together with Zn^{2+} gives the greatest effect on pBR313 DNA uptake. It supports the data of fig.1 which showed that protamine sulfate itself enhanced uptake on short incubation periods (30 min). The effect of protamine

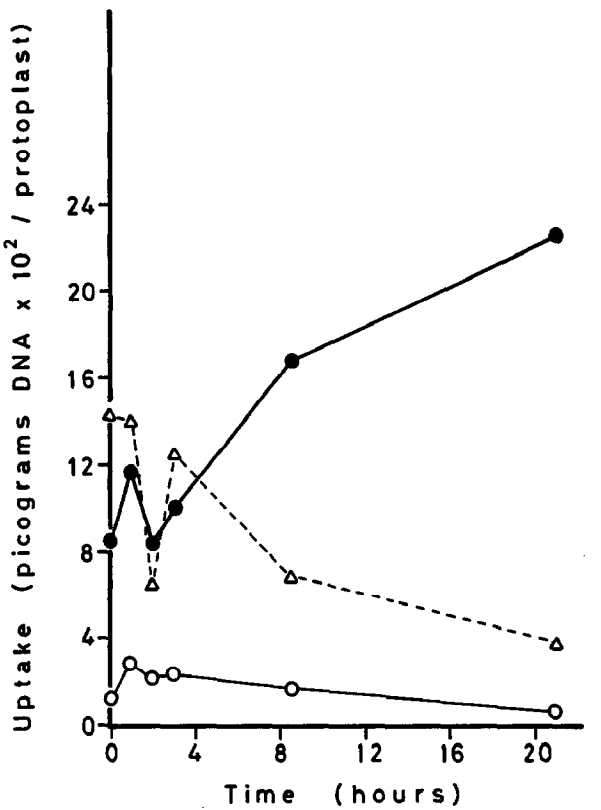

Fig.1. Uptake of pBR313 [^{3}H]DNA by turnip leaf mesophyll protoplasts as a function of time. pBR313 [^{3}H]DNA was mixed with either poly (L-ornithine) (5 μg/μg DNA) (●——●) or protamine sulfate (10 μg/μg DNA) (△– – –△) and incubated for 1 h on ice prior to addition to the protoplasts at a dosage of 0.5 μg DNA/10^6 cells. Samples of protoplasts (2 ml or 820 000 cells/ml), incubated at 23°C, were taken at the time intervals indicated in the figure. They were washed three times in 0.45 M mannitol in White's medium, resuspended in fresh 0.45 M mannitol in White's basal salts medium, and incubated for an additional 24 h at 23°C. After incubation, the cells were pelleted by centrifugation (250 × *g*, 5 min) and lysed in 0.5 ml 0.1% SDS, 20 mM Tris–Cl, pH 8.0, 5 mM Na_2EDTA, 20 min, 37°C. Radioactivity of the TCA-precipitable material was then determined as in section 2. Control (○——○).

Table 2
Effect of pH and polycationic compounds on the uptake of pBR313 DNA by carrot protoplasts[a]

	pH			
	6	7	8	9
Control	90[b]	40	47	35
Protamine sulfate (PS)	234	158	179	460
PS + Zn^{2+} (as 5 mM $ZnCl_2$)	131	472	258	252
PS + Ca^{2+} (as 5 mM $CaCl_2$)	98	119	142	204
PS + Zn^{2+} + Ca^{2+}	106	233	207	173
Poly (L-ornithine) (PLO)	248	175	177	129
PLO + Zn^{2+}	181	287	68	298
PLO + Ca^{2+}	198	251	229	209
PLO + Zn^{2+} + Ca^{2+}	212	202	46	34

[a] Protoplasts were incubated with plasmid DNA and polycation for 30 min at 23°C, washed twice with 0.45 M mannitol, treated with DNAase I (100 μg/ml) in 0.45 M mannitol, 0.01 M Tris–Cl, 0.01 M $MgCl_4$, pH 7.0 for 15 min. 37°C and washed three times with 0.45 M mannitol–0.01 M Na_2EDTA, pH 8.0. The cells were lysed with 1% SDS, 15 min, 37°C and 100 mg salmon sperm DNA was added before the addition of TCA. Radioactivity of the TCA-insoluble precipitate was counted

[b] cpm corrected for background

sulfate alone is increased with increased pH of the incubation medium. However, together with Zn^{2+}, plasmid uptake enhancement by protamine sulfate was best achieved at pH 7.0. Added Ca^{2+} has lesser effects than Zn^{2+}. The DNA uptake enhancing effect of poly (L-ornithine) was highest at pH 6.0 but this shifted to pH 7.0 in the presence of Zn^{2+}. Again Ca^{2+} had lesser effects than Zn^{2+}.

3.4. *Fate of plasmid DNA*

Besides pBR313, plasmid pCR1 (8.6 M daltons) is also taken up by cowpea protoplasts in the presence of poly (L-ornithine) and Zn^{2+}. This plasmid is cleaved into linear molecules of about 1.2 M daltons (fig.2). The cleavage to small molecules of less than 1.2 M daltons is reduced somewhat when the initial incubation medium contained poly (L-ornithine) in 5 mM $CaCl_2$ at pH 10.5. About 10% of pCR1 DNA population suffered only two cuts under these conditions. However, pCR1 DNA had received an average of 6 cleavages.

When cowpea protoplasts were allowed to take up Col E1 DNA under similar conditions (poly (L-ornithine) + Zn^{2+}), this plasmid was also cleaved to linear molecules of about 1.4 M daltons (fig.3) and therefore underwent an average of 2 cuts. Cowpea protoplasts treated with KCN did not prevent cleavage of Col E1 although it decreased the uptake of the plasmid.

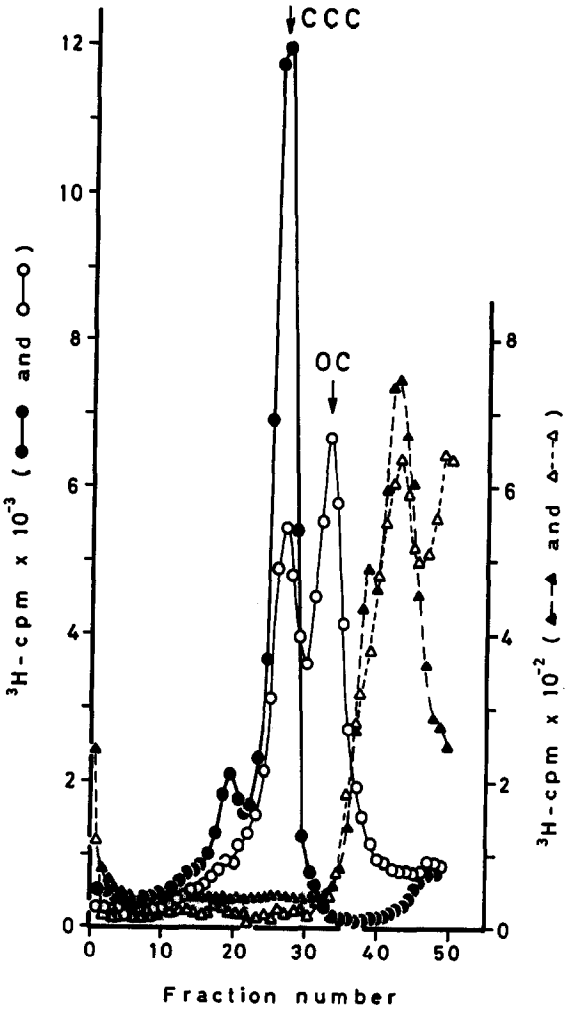

Fig.2. Fate of pCR1 DNA in cowpea leaf protoplasts. Protoplasts (1.2 × 10^6 cells/ml) were incubated with pCR1 [^{3}H]DNA (1.3 × 10^5 cpm) in the presence of poly (L-ornithine) and Zn^{2+} at 25°C (△——△) or in 0.05 M glycine–NaOH buffer, pH 10.5 containing 5 μg/ml poly (L-ornithine), 0.4 M mannitol and 0.05 M $CaCl_2$ at 37°C (▲——▲). DNA was extracted after 30 min from DNAase-treated protoplasts and analyzed by sucrose density gradient centrifugation as in [1]. Reference gradients of donor pCR1 [^{3}H]DNA (●——●) and spontaneously nicked pCR1 [^{3}H]DNA (○——○) were run in parallel.

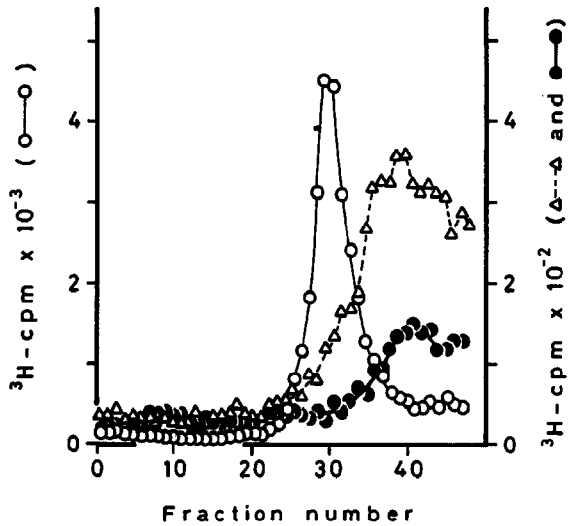

Fig.3. Fate of Col E1 DNA in cowpea protoplast nuclei. Protoplasts (2.5 X 10^6 cells) were incubated with Col E1 [^{3}H]DNA (1.5 X 10^5 cpm) in the presence (●——●) or absence (Δ– –Δ) of 10 mM KCN that was preincubated with poly (L-ornithine) and Zn as in fig.1 legend. Nucleic acids were obtained after 30 min incubation as described in section 2. The protoplasts were then incubated with DNAase I (100 μg in 5 mM $MgCl_2$, pH 8.0) for 15 min, 37°C and washed twice with 5 mM morpholinopropane sulfonic acid, pH 6.5, 10 mM Na_2EDTA and 2.5% Triton X-100, and the nuclear DNA extracted and analyzed by sucrose density centrifugation as in [1]. Col E1 [^{14}C]DNA was added as a marker in the gradient (o——o).

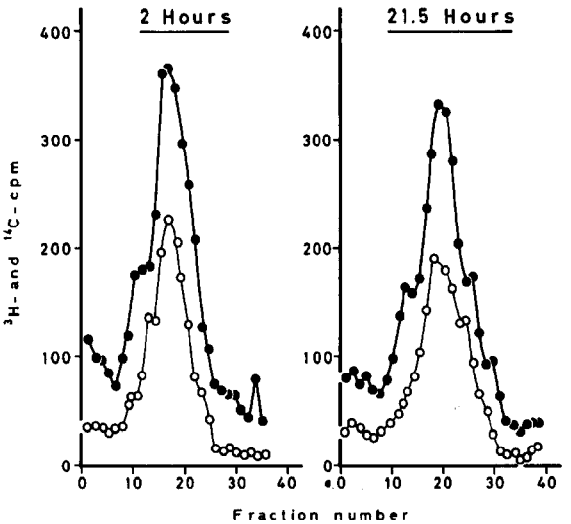

Fig.4. Fate of pBR313 in turnip protoplasts. Protoplasts were incubated with pBR313 [^{3}H]DNA (0.5 μg DNA/10^6 protoplasts) that was preincubated with poly (L-ornithine) (5 μg/μg DNA) and 5 mM $ZnCl_2$ for 1 h on ice. Samples were taken at various time intervals, washed three times with 0.45 M mannitol in White's basal salts medium, resuspended in fresh medium and incubated for 24 h at 23°C. Cells collected by centrifugation were lysed with SDS, processed for DNA extraction, mixed with pBR313 [^{14}C]DNA and DNA analyzed by sucrose density gradient centrifugation [1]. (●——●) pBR313 [^{3}H]DNA; (o——o) pBR313 [^{14}C]DNA.

pBR313 underwent an average of 2 cleavages in the nuclei of cowpea protoplasts [1]. We now show that pBR313 remains stably stored in the nuclear fraction of turnip leaf mesophyll protoplasts for at least 45.5 h. In this experiment turnip protoplasts (12.3 X 10^6) were incubated for 21.5 h with pBR313 [^{3}H]DNA (0.5 μg DNA/10^6 protoplasts) that was preincubated with poly (L-ornithine) (5 μg/μg DNA) and $ZnCl_2$ (5 mM) for 1 h on crushed ice. Aliquots (2 ml) were taken at various time intervals. The cells were washed three times, and resuspended in 0.45 M mannitol in White's medium for 24 h, 23°C. The protoplast samples were further incubated at 23°C; at various times, nuclei were isolated and lysed with 0.1% SDS in buffer (20 mM Tris–Cl, pH 8.0, 5 mM Na_2 EDTA) at 37°C for 20 min. The lysed nuclear fraction was mixed with pBR313 [^{14}C]DNA and analyzed by velocity sedimentation in a 5–20% linear sucrose density gradient. Data from two time points are illustrated in fig.4. Note that after 2 h or 21.5 h incubation at 23°C there was no degradation of pBR313 DNA.

4. Discussion

In support of [1–3], our present work shows carrot, periwinkle, and turnip protoplasts besides cowpea leaf protoplasts are able to take up pBR313 DNA. Our present studies also confirm that this uptake is greatly enhanced with the polycationic reagents: protamine sulfate or poly (L-ornithine). However, stable incorporation of plasmid is best achieved with poly (L-ornithine) (fig.1,4). Protamine sulfate seems to elicit the enhancement only for a short time (30 min). Further enhancement of plasmid uptake is achieved by the addition of Zn^{2+}. Protamine sulfate together with Zn^{2+} elicited the largest uptake response

but as shown in fig.1, the uptake enhancement is short-termed. On the other hand, poly (L-ornithine) plus Zn^{2+} also enhanced uptake. This uptake was best achieved at pH 7.0 (table 2) and the incorporated plasmid remains stably stored. Besides pBR313 plasmids, pCR1 and Col E1 are also taken up by protoplasts. They undergo some degradation into linear molecules and the number of cleavages depends on the plasmid type: in cowpea leaf mesophyll protoplasts pCR1 (8.6 M daltons) is cleaved into an average of 6 segments, Col E1 (4.2 M daltons) is cleaved into 3 segments, and earlier studies [1] showed that pBR313 (5.8 M daltons) is cleaved twice to also form 3 segments. Also the number of cleavages that these plasmids suffer depends on the plant from which protoplasts were derived. As discussed above, pBR313 is cleaved into 2–3 segments in leaf mesophyll protoplasts of cowpea whereas the same molecules undergo no degradation in leaf mesophyll protoplasts of turnip. In these protoplasts the plasmid is stably incorporated for relatively long periods of time (almost 2 days).

It is uncertain at this time whether these limited degradations are due to specific cleavages or to random cuts in the plasmid molecule. Hopefully the cleavages will turn out to be specific so that inserted genes of chimera plasmids remain protected in the plant protoplasts and thus have the potential for gene expression. Our present studies indicate that the protoplasts of turnip confer little damage to the introduced plasmid and therefore these cells could serve as effective model systems for molecular genetics of recombinant DNA in an eukaryotic cell.

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