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Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts

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Summary. DNA from a bacterial plasmid containing the T-DNA border sequences of *Agrobacterium tumefaciens* was transferred into the nucleus or the cytoplasm of tobacco mesophyll protoplasts by microinjection. Following culture in hanging drops, some of these protoplasts produced calli containing the foreign DNA sequences. Evidence for the presence of the injected plasmid DNA in these calli was provided by Southern hybridization analysis. The results demonstrated that random portions of the bacterial plasmid were linked to plant DNA and that integration did not occur at the T-DNA borders present on the injected plasmid. The average number of integrated copies ranged from less than one to 1–2 per tobacco genome. The frequency of integration averaged 14% with intranuclear injections compared to 6% with cytoplasmic injections. With further refinement, the use of microinjection may allow the introduction of many different types of genetic elements into plants.

Key words: Gene transfer – Plant cell transformation – Plant tissue culture – Recombinant DNA

Introduction

Transformation of animal cells by microinjection has been used successfully for many years (Jaenisch and Mintz 1974; Rusconi and Schaffner 1981; Wagner et al. 1981; Rubin and Spradling 1982). The production of transgenic mice (Palmiter et al. 1983) and transgenic rabbits, sheep, and pigs (Hammer et al. 1985) by microinjection is stimulus to apply these techniques to the modification of plant species. Early attempts at plant cell microinjection were hampered by either the rigid plant cell wall or by the fragility of plant protoplasts because suitable methods of immobilizing plant cells or protoplasts for injection were not developed.

More recently methods of immobilizing plant protoplasts for microinjection have been published (Steinbiss and Stabel 1983; Griesbach 1983; Lawrence and Davies 1985; Morikawa and Yamada 1985). However, efficient transforamtion of plant cells has yet to be demonstrated by microinjection. Several factors may contribute to this lack of success, but perhaps one important factor has been the inability to easily target specific cellular compartments for injection. For example, the frequency of transformation following nuclear injection of mammalian cells or eggs has been demonstrably higher than that following cytoplasmic injections (Capecchi 1980; Yamaizumi et al. 1983; Hammer et al. 1985; Brinster et al. 1985). For this reason, we developed a holding pipette method (modified from that used with mammalian embryos, Wagner et al. 1981) for immobilizing protoplasts during injection. This allows manipulation of the protoplasts for optimal orientation of the nucleus relative to the injection pipette.

We report here that plant cells can be genetically transformed by microinjection of DNA into protoplasts using the holding pipette method. Approximately 14% of the intranuclearly injected tobacco mesophyll protoplasts after microculture into calli contained sequences homologous to the injected DNA. Transformation rates at that level coupled with the ability to culture only the microinjected cells, as described below, will allow transformation strategies to be developed that do not depend upon selectable markers.

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Materials and methods

Figure 1 is a generalized diagram of the experimental procedures detailed below.

Plant material. Protoplast donor plants of *Nicotiana tabacum* cv. Xanthi nc were grown in glass jars under aseptic conditions as described elsewhere (Facciotti and Pilet 1979). Apical shoots were placed into 100 ml of agar medium (0.6% Gibco Phytagar MS medium containing 30.0 g/l sucrose, 1.0 mg/l IAA and 0.15 mg/l kinetin, adjusted to pH 5.55 prior to autoclaving). The cultures were kept at $23 \pm 2°$ C under a 12 h dark/light regime. Young leaves were removed from 2–3 week old plants, the main veins discarded, and the leaf blades infiltrated with a 6% sorbitol solution containing 0.04% pectinase (Pectolyase Y-23, Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) and 0.4% cellulase (Onozuka RS, Yakult Pharmaceutical Industry Co. Ltd., Nishinomiya, Japan). After 2–3 h incubation, the macerate was passed through a 52 μm nylon filter. The protoplasts were pelleted by centrifugation at 50 g, washed twice with 6% sorbitol solution, and then suspended at a density of $1–2 \times 10^5$/ml in modified MS medium (0.5X MS Gibco 510–1118, plus 5.0 g/l sucrose, 71.0 g/l sorbitol) with 3.0 mg/l NAA and 1.0 mg/l BAP as indicated by Caboche (1980). Preculturing of the protoplasts prior to injection was performed in 15 cm Parafilm-sealed petri dishes at $23 \pm 2°$ C in the dark for 1–3 days.

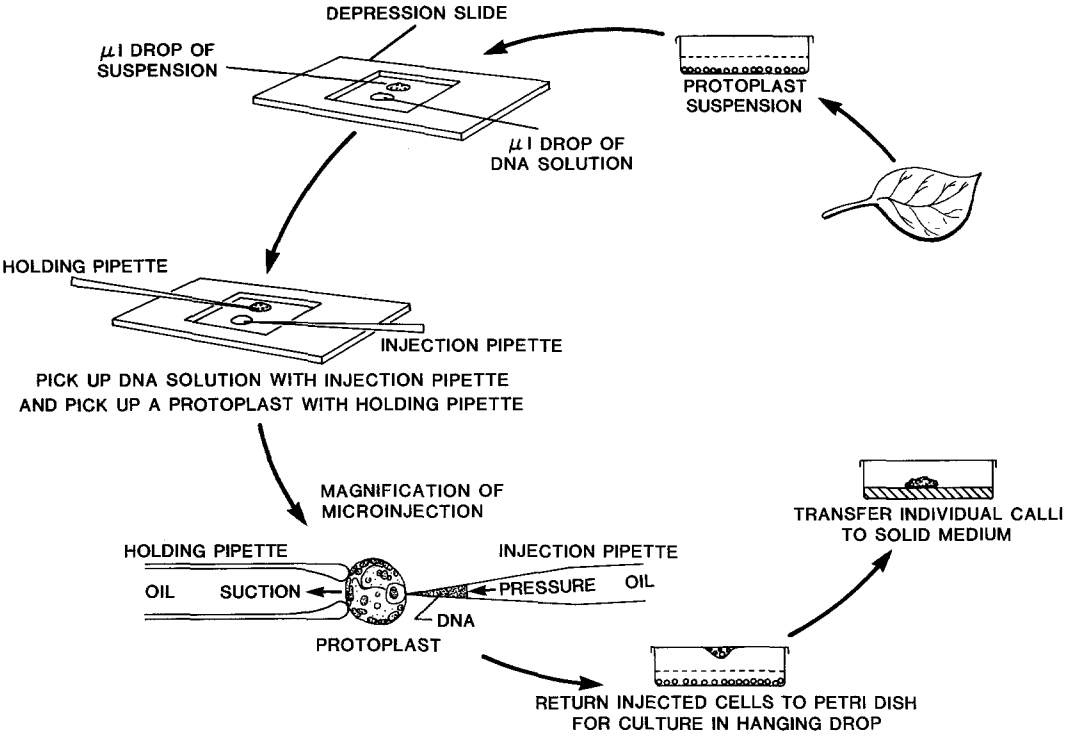

Fig. 1. Diagram of the experimental procedure (described in Materials and methods) for microinjection of plant protoplasts using the holding pipette method of immobilization

Plasmid construction. pCGN561 (Fig. 2) is a 29.3 kb (kb = kilobase pairs) plasmid containing bacterial DNA sequences between T-DNA borders (regions of an *Agrobacterium* Ti plasmid which have been implicated in the integration of T-DNA into plant chromosomes). pCGN561 was constructed as follows. The left border contained in bp (bp = base pairs) 602–2212 (*Hin*dIII-*Sma*I) and the right border contained in bp 13362–15208 (*Xho*I-*Eco*RI) of pTiA6 (Barker et al. 1983) were each converted into a *Hin*dIII-*Eco*RI fragment by cloning into mp9. These two borders were then combined to form a *Hin*dIII fragment of approximately 3.5 kb which was inserted into the *Hin*dIII site of pVCK102 (Knauf and Nester 1982) to create pCGN506 (Knauf VC, Facciotti D, Gardner RC, Houck CM (1986) A binary *Agrobacterium* vector system based on an octopine synthase promoter cassette, Manuscript in preparation). pCGN561 was derived from pCGN506 by replacing the small *Eco*RI-*Bam*HI fragment of pCGN506 with an *Eco*RI-*Bam*HI fragment consisting of the pACYC184 origin of replication (*Eco*RI-*Hin*dIII) and the APH(3′)II gene from Tn*5* (*Hin*dIII-*Bam*HI) conferring kanamycin resistance in bacteria. Preparations of pCGN561 DNA, containing predominantly covalently closed and open circular forms, were suspended in 1 × TE (10 mM Tris, pH 8.0; 1 mM EDTA) at a concentration of 0.03 mg/ml (approximately 1000 copies/picoliter) for injection.

Micromanipulation. Slides were prepared as described by Crossway and Houck (1985). Each slide held a drop of protoplasts in medium and a separate drop of the DNA solution. Slides used for controls (noninjected protoplasts) were prepared in the same manner, omitting the DNA drop. Injection pipettes (tip external diameter approximately 0.2 μm as measured by scanning electron microscopy) were pulled from R6 glass capillary tubing (Drummond Sci. Co., Broomall, PA, USA) on a pipette puller (Ultrafine, Frederick Haer and Co., Brunswick, ME, USA). Holding pipettes (5–10 μm internal diameter) were pulled from Leitz 520–119 glass capillary tubing and fire polished on a microforge (DeFonbrune MF-80).

Microinjections were performed as described by Wagner

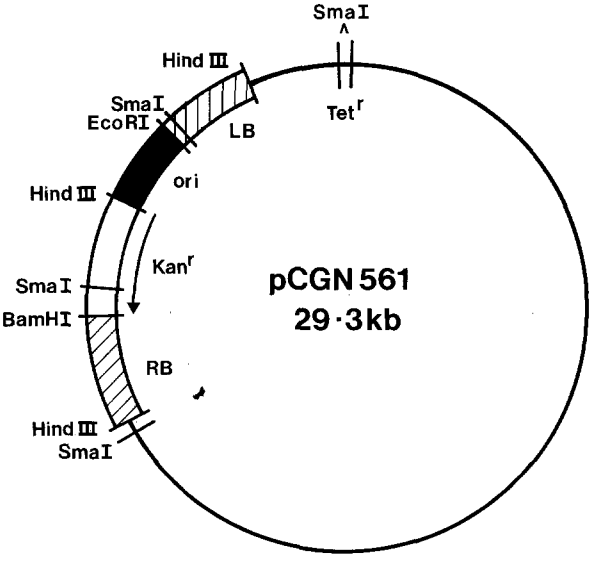

Fig. 2. Restriction endonuclease map of the injected plasmid, pCGN561. Construction of the plasmid is described in Materials and methods. pRK290 DNA, ——; pACYC184 origin of replication (*ori*), ■; APH(3′)II gene from Tn*5* (Kan^r), □; right border and left border from the T-DNA of an *Agrobacterium* Ti plasmid (RB and LB), ▨. *Hin*dIII digestion of pCGN561 yields three fragments: one containing the LB and *ori*, ~3.1 kb; one containing the RB and APH, ~3.3 kb; and one containing pRK290 DNA, ~22.9 kb

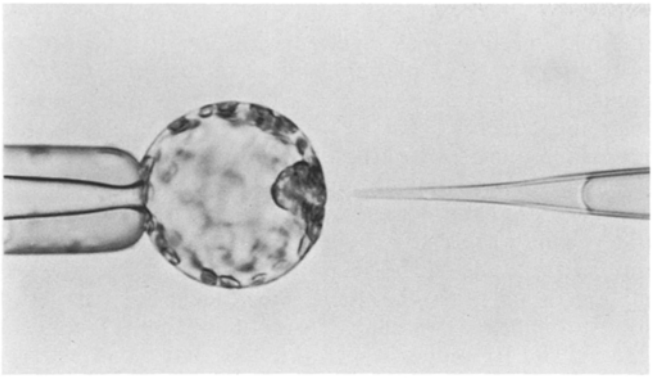

Fig. 3. Microinjection into the nucleus of a tobacco mesophyll protoplast. The protoplast (~50 μm diameter) is held by slight suction on the holding pipette (left). Estimation of quantity injected is made by observation of the meniscus between the DNA solution and oil in the tip of the injection pipette (right)

et al. (1981). Protoplasts were picked up individually on the holding pipette by gentle suction and turned so that the nucleus was readily accessible to the injection pipette (Fig. 3). For intranuclear injections, the pipette was inserted into the nucleus and successful injection was assessed by observation of slight nuclear swelling. For cytoplasmic injections, the pipette was inserted into the cytoplasm near the nucleus thereby dislodging some of the surrounding chloroplasts. Approximately 2 picoliters (estimated by meniscus displacement assuming the volume of a cone for the pipette tip) of DNA solution was injected into each protoplast. Following microinjection, the injection pipette was removed and the protoplast was placed at the bottom of the medium drop by releasing suction on the holding pipette. After all the protoplasts in the drop were injected, the slide was transferred to a laminar flow hood for culture preparation.

Hanging drop culture. A culture procedure was developed that allows for the propagation of microinjected protoplasts separate from noninjected protoplasts. The procedure is based upon the hanging drop culture technique of Gleba (1978). Normal protoplast culture densities (10^4–10^6 per ml) were obtained by using very small (≤ 0.25 μl) drops of medium for the culture of small numbers of injected protoplasts (see Crossway et al. 1985). The individual calli produced from single injected protoplasts were later subcultured separately until enough callus material was obtained for DNA isolation.

Using a dissecting scope, the injected protoplasts were picked up from the slide with a transfer pipette (see Crossway and Houck 1985) excluding as much medium as possible. They were then deposited on the lid of a petri dish forming a hanging drop when the lid was inverted over a protoplast culture. The size of the hanging drop was dependent on the number of protoplasts injected and the amount of medium transferred from the pipette with the protoplasts. Control hanging drops were made in a separate petri dish using approximately the same number of noninjected protoplasts from control depression slides. The Parafilm-sealed cultures were then incubated at $23 \pm 2°$ C in the dark.

Growth of the hanging drop culture was monitored by observation of the cells in the drops with a dissecting microscope. As the microcalli grew, the drops were diluted with additional medium to replenish nutrient supplies and/or to reduce the osmolarity. For the first 1–2 weeks of culture, the microcalli were maintained in the medium in which they were cultured prior to injection. Additional medium was added occasionally if the microcalli were growing rapidly. After 1–2 weeks, the drop was gradually diluted with the same medium containing only 0.2 mg/l NAA. Every 1–2 weeks, the drop was diluted with this medium except that the osmolarity was lowered in steps (56 g/l sorbitol followed by 20 g/l sorbitol). When the calli reached about 1.0 mm diameter in size (usually after 1.5–2 months of culture), they were transferred to agar (0.6% Gibco Phytagar) plates containing the same medium. To obtain sufficient material for several DNA isolations (5–10 g), the calli were cultured for at least 6 months from the date of injection.

DNA isolation and hybridization. Plant DNA was isolated according to the miniprep procedure described by Dellaporta et al. (1983) with the following modifications. Samples of approximately 2 g of powdered frozen callus tissue were extracted in a boiling extraction buffer (100 mM Tris, pH 8; 50 mM EDTA; 10 mM β-mercaptoethanol). After the isopropanol precipitation, the DNA was redissolved in 400 μl 1 × TE. RNase A was added to 10 μg/ml. After 10 min incubation at 37° C, Proteinase K (BRL, Rockville, MD, USA) was added to a final concentration of 250 μg/ml and incubation was continued for 1 h. Following the phenol and chloroform-isoamyl alcohol extractions, the DNA samples were brought to 0.2 M Na Acetate, pH 5.5, and precipitated by adding two volumes of absolute ethanol. The DNA was redissolved in 1 × TE and stored at 5° C until use.

Ten microgram samples of DNA were digested with a 2.5-fold excess of the *Hin*dIII restriction enzyme (BRL). After a 3 h digestion at 37° C, samples were subjected to electrophoresis in 0.7% agarose in Tris-borate-EDTA buffer (TBE; Maniatis et al. 1982) at 20 V overnight. The plasmid hybridization probe, pCGN561, was digested with *Hin*dIII before nick-translation (Shewmaker et al. 1985). For the APH(3′)II-specific probe, a 1.0 kb *Eco*RI fragment was isolated from pCGN546 (Facciotti et al. 1985), a pUC7 recombinant containing the 1.0 kb *Bgl*II-*Sma*I fragment [APH(3′)II gene] of Tn*5*. Southern analyses were performed as described (Maniatis et al. 1982) except filters were prehybridized and hybridized at 50° C and washed at 60° C to reduce heterologous DNA hybridization. Autoradiography was performed using Kodak X-Omat AR film and Dupont Cronex Lightening-Plus intensifying screens at −70° C.

Results

Intranuclear injections

A summary of cell viabilities, callus formation, and transformation following intranuclear microinjection of pCGN561 into tobacco protoplasts is shown in Table 1. Cell viabilities following injection averaged 86%, while plating efficiencies in these experiments averaged 50%. Transformants were obtained in 4 out of 5 experiments and the average frequency of transformation (see below) in these experiments was 14% (# transformants/# surviving calli X 100).

When DNA extracted from calli produced from intranuclear injections was digested with *Hin*dIII and probed with

Table 1. Summary of pCGN561 intranuclear microinjection experiments with tobacco mesophyll protoplasts

Exp. #	# Injected protoplasts	# Alive day 3	# Calli produced	# Containing 561 DNA
1	14	11	2	1
2	21	19	1	0
3	25	24	24	3
4	20	15	15	2
5	33	28	14	2
Total	113	97	56	8

the entire pCGN561 plasmid, 8 out of 56 calli surviving from 5 independent experiments showed hybridization with plasmid sequences (Tables 1 and 3, Fig. 4). By comparison with reconstructions, four of these transformants were estimated to contain an average of 1–2 copies of the plasmid sequence per genome while the other four contained less than 1 copy per genome (Table 3). When the same hybridization filters were washed to remove the plasmid probe and rehybridized with the APH(3′)II-specific probe, three samples showed hybridization with the APH(3′)II sequence (Table 3).

Cytoplasmic injections

A summary of cell viabilities, callus formation, and transformation following cytoplasmic microinjection of pCGN561 into tobacco protoplasts is shown in Table 2. Cell viabilities following injection averaged 54%, while plating efficiencies in these experiments averaged 17%. These averages may reflect inexperience with the microculture procedure because more recent experiments in our laboratory do not show significant differences in viabilities or plating efficiencies between nuclear and cytoplasmic injection experiments. Transformants were obtained in 3 out of 9 independent experiments and the average frequency of transformation in these experiments was 6% (see below).

When DNA extracted from calli produced from cytoplasmic injections was digested with *Hin*dIII and probed with the pCGN561 plasmid, only 2 of the three transformants (out of 53 surviving calli) showed hybridization with plasmid sequences (Tables 2 and 3). By comparison with reconstructions, both of these transformants were estimated to contain approximately 1 copy of the plasmid sequences per genome (Table 3 and Fig. 4). When these hybridization filters were washed to remove the plasmid probe and rehybridized with the APH(3′)II probe, only one sample showed hybridization with the APH(3′)II sequence. This third transformant was estimated to contain about 1 copy of the APH(3′)II sequence per genome. The same sample, however, had not hybridized before with the pCGN561 probe. It is possible that only a small portion of the 4.5 kb *Hin*dIII fragment contains any homology to pCGN561 and is therefore not detectable using that probe.

Integration patterns

As can be seen in Table 3 and Fig. 4, the restriction endonuclease patterns of the integrated sequences differed among the transformed calli. In the intranuclear injection experiments at least 6 distinct events can be discerned from the restriction patterns, and 3 different events were observed in the cytoplasmic injection experiments. Thus, the transformation frequencies stated above are valid minimum estimates given the sensitivity of our screening method.

The observed restriction endonuclease patterns suggest that only portions of the plasmid had integrated into the tobacco genome. Only intranuclear transformant 3–1 has a pattern which suggests that almost all of the pCGN561 plasmid was incorporated into the tobacco genome. In our transformants, all of the APH(3′)II-hybridizing bands are slightly larger than the 3.3 kb *Hin*dIII fragment expected from pCGN561. This result can be explained by elimination of one of the plasmid *Hin*dIII sites either through failure of the DNA containing that site to integrate or through disruption of the *Hin*dIII site by the recombination event. Similarly, the restriction endonuclease patterns observed

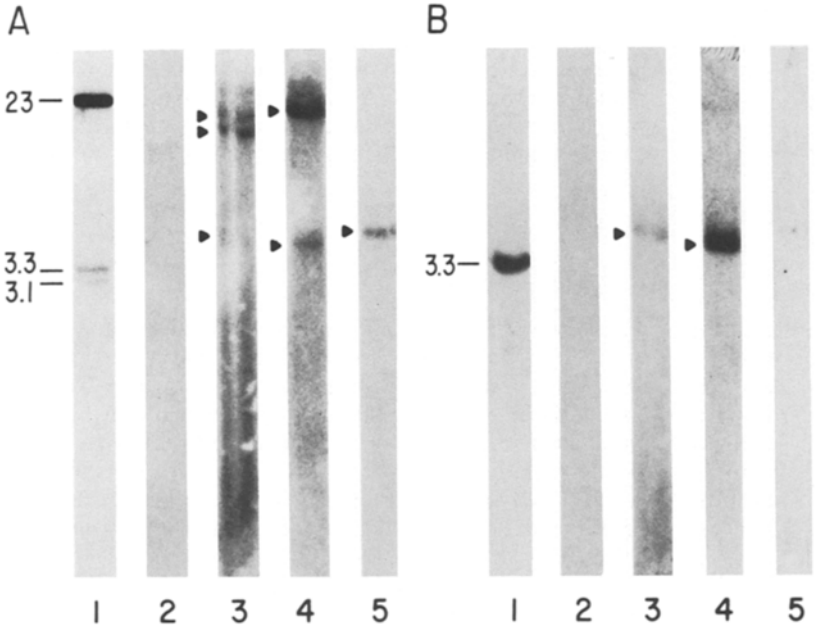

Fig. 4A, B. Southern hybridization analysis of DNA isolated from calli produced from microinjection of tobacco mesophyll protoplasts. Conditions for hybridizations are described in Materials and methods. **A** *Hin*dIII-digested DNA samples (~10 μg DNA/lane) hybridized with the pCGN561 plasmid probe. **B** The same filters as in **A** after washing to remove the plasmid probe and rehybridization with the APH(3′)II probe. Lanes 1–5 are the same samples in both panels: 1. pCGN561 reconstruction based on genome size, 0.5 copy/genome, with molecular weights of the hybridizing bands in kb, 2. Noninjected control, 3. Intranuclearly injected sample 1–1 (Table 3), 4. Intranuclearly injected sample 3–1 (Table 3), 5. Cytoplasmicly injected sample 4–1 (Table 3). *Arrows* indicate hybridizing bands in microinjected samples

Table 2. Summary of pCGN561 cytoplasmic microinjection experiments with tobacco mesophyll protoplasts

Exp. #	# Injected protoplasts	# Alive day 3	# Calli produced	# Containing 561 DNA
1	26	10	1	0
2	31	22	13	1
3	43	20	7	1
4	33	21	5	1
5	38	14	5	0
6	32	18	3	0
7	31	15	2	0
8	40	28	13	0
9	30	16	4	0
Total	304	164	53	3

Table 3. Summary of Southern analyses of transformed calli produced from pCGN561 microinjection of tobacco mesophyll protoplasts

Type of injection	Exp. # [a]	Trans formed callus #	Band size (kb) [b]		Approx. # copies per genome
			561 probe	APH(3′)II probe	
Nuclear	1	1 [c]	11.5	N.D. [d]	~1
			10.0	N.D.	
			3.6	3.6	
	3	1 [c]	23.0	N.D.	1–2
			3.5	3.5	
		2	3.6	3.6	<1
		3	5.4	N.D.	<1
			1.6	N.D.	
	4	1	5.2	N.D.	<1
		2	4.9	N.D.	<1
	5	1	2.8	N.D.	1–2
		2	2.6	N.D.	1–2
Cytoplasmic	2	1	N.D.	4.5	~1
	3	1	3.2	N.D.	~1
			2.5	N.D.	
			1.4	N.D.	
	4	1 [c]	3.8	N.D.	~1

[a] Experiment number refers to Tables 1 and 2
[b] Expected sizes with *Hin*dIII-digested pCGN561 DNA are 22.9, 3.3, and 3.1 kb with the 561 probe and 3.3 kb with the APH(3′)II probe
[c] Figure 4 contains Southerns of these transformants
[d] N.D. = Not detected

with the pCGN561 plasmid probe can be most simply explained by a small portion of the plasmid integrating in the majority of our transformants. For this reason, little information regarding rearrangement prior to integration can be obtained from these data. However, the pattern observed for intranuclear transformant 3–1 is clearly consistent with minimal rearrangement prior to integration as at least one of the two pCGN561-hybridizing bands is of the expected size for *Hin*dIII digested DNA. Only intranuclear transformant 1–1 requires either rearrangement or multiple integrations to explain the observed pattern. The observed integration patterns along with the low copy numbers do not support concatamerization of the plasmid DNA prior to integration.

If both of the T-DNA borders were used to mediate integration of the microinjected DNA, the fragment transferred would always include the APH(3′)II gene and the pACYC origin of replication flanked by the 25 bp repeats from the T-DNA borders (Fig. 2). From the internal *Hin*dIII site to the border repeat, the APH(3′)II-containing portion would be 2.2 kb and the *ori*-containing portion would be 2.8 kb. When the DNA is digested with *Hin*dIII, the actual size of the fragments seen will be greater than or equal to these sizes as the external *Hin*dIII sites will be derived at random from the flanking plant DNA. Two bands (or more if there were multiple integrations) of variable size should be detected with the pCGN561 probe and one of these fragments should always be detected with the APH(3′)II probe. In our experiments (Table 3), the APH(3′)II-containing portion of the plasmid is present only in 4 out of 11 transformants. Furthermore, 7 of the 11 transformants contain only a single hybridizing fragment. Our data, thus, are clearly not consistent with use of both T-DNA borders for integration of microinjection DNA.

We cannot, however, rule out use of a single border for integration in at least some of the transformants based on our data. Restriction endonuclease patterns or band sizes cannot be predicted for integration mediated by only the left or right border because one end would presumably be derived at random. But, one can compare the observed patterns to the plasmid map (Fig. 2) to determine if the observed pattern is consistent with integration using one or the other border. A number of the observed integration patterns are consistent with integration using either border but several of the observed patterns are inconsistent with use of one or the other border. For example, nuclear sample 3–3 and cytoplasmic sample 3–1 could not be obtained using either the left or the right border unless one postulates rearrangement or multiple integrations. When viewed in total, our results are most simply explained by random integration of portions of the injected plasmid without use of the T-DNA borders.

Discussion

Microinjection has been used successfully for transformation of animal cells for a number of years. This method of direct DNA introduction may have advantages over the current systems used for transformation of plant species. To test the feasibility of transforming plant cells via microinjection, we looked for integration of foreign DNA in callus produced from tobacco mesophyll protoplasts that had been microinjected with a bacterial plasmid. As this plasmid also contained T-DNA borders (regions of an *Agrobacterium* Ti plasmid which are important for integration of T-DNA into plant chromosomes), we also looked for mediation of integration by the border sequences. Because evidence from animal cell microinjection studies indicated a much higher efficiency of transformation in nuclear injections compared to cytoplasmic injections, we looked to see if this was true for plant cells.

We have shown in a number of different experiments (Tables 1 and 2) that integration of foreign DNA can be achieved following microinjection of plant protoplasts. Our results (Table 3 and Fig. 4) suggest incorporation of portions of the injected plasmid in predominantly single copy

events. These results are similar to those seen when circular plasmids were injected into animal cells (Gordon and Ruddle 1981; Stewart et al. 1982) and contrast with animal cell microinjections of linear DNA (Constantini and Lacy 1981; Palmiter et al. 1983; Hammer et al. 1985; Brinster et al. 1985) where the number of integrated copies is often high due to concatamerization.

Of the eleven transformants recovered in our experiments, only four contained the APH(3′)II-hybridizing fragment (Table 3). This might be expected with injection of a large plasmid (pCGN561 is 29.3 kb). As the majority of our transformants have integrated less than 8 kb of the plasmid sequence, it is not too surprising that only about one-third of them contain the APH(3′)II-hybridizing fragment. Steinbiss et al. (1985) have shown that passage of plasmids greater than 5 kb through submicron injection pipettes results in significant breakage. The predominance of small integrants in our experiments might be explained by this and the increased efficiency of integration of linear molecules compared to circular molecules (Boyd 1985; Brinster et al. 1985).

As described in the Results section, the restriction endonuclease patterns of our transformants are inconsistent with use of the T-DNA borders to mediate integration. Our results are similar to those where naked DNA uptake was used to produce transformants from plasmids containing T-DNA borders (Krens and Schilperoort 1984). Thus, both of these studies support a requirement for a specific interaction between the bacterium or its products and the borders in effecting DNA transfer and/or integration.

The high frequency of transformation seen here with intranuclear injection of plant protoplasts is similar to that observed with animal eggs (Wagner et al. 1981; Brinster et al. 1981; Hammer et al. 1985; Brinster et al. 1985). Although the numbers involved do not permit a good statistical comparison, our transformation frequencies suggest that there is about a 2–3 fold increase in transformation with intranuclear injections compared to cytoplasmic injections of plant protoplasts. This differs from the up to 100-fold differences that have been observed in analogous experiments with animal cell injections (Capecchi 1980; Yamaizumi et al. 1983; Hammer et al. 1985; Brinster et al. 1985). The relatively high frequency that we obtained in our cytoplasmic injection experiments was unexpected but is not dissimilar to exceptional animal cell cytoplasmic injection experiments (Brinster et al. 1985).

Although we routinely detect an estimated 0.1 copy per tobacco genome equivalent (7.8 picograms/2C, Bennett and Smith 1976) in reconstruction experiments, our screening method would give a low estimate of the number of transformants if some samples escaped detection because only a small percentage of their cells contained pCGN561 DNA. One callus (nuclear 1–1 in Table 3) which was positive on first analysis was re-examined following subculture. Only one of the six subcultures from this callus showed significant hybridization to the plasmid probe (data not shown). Several explanations for such a result can be advanced. The first, although remote because of the culture procedure employed in this study, would be cross-contamination of a transformant with a nontransformed cell(s) during the initial microculture steps. Other possibilities include instability of the integrated foreign DNA during cell culture or integration of the injected DNA after cell division has taken place. The latter is the postulated mechanism for the low frequency of chimeras (mosaics) that have been shown by outbreeding of mice obtained from microinjection of eggs (Stewart et al. 1982; Brinster et al. 1985).

Hanging drop culture allows growth of injected cells in isolation from noninjected cells. This may obviate the need for selectable markers which have recently been developed for plant systems (Herrera-Estrella et al. 1983; Bevan et al. 1983; Fraley et al. 1983). Furthermore, such markers may not be desirable in plants grown for commercial purposes. The transformation frequencies obtained here allow one to simply screen for the presence of the desired gene by DNA dot hybridization as is routinely done in mammals (Brinster et al. 1985).

This report suggests that transformation frequencies for plants via microinjection are similar to those reported for animals. If gene expression in transgenic plants obtained via microinjection also proves comparable to that in animals, microinjection will be an efficient means for studying gene expression in plant species and for producing genetically improved plants. With microinjection, a variety of genetic elements (chloroplasts, mitochondria, nuclei, and chromosomes) in addition to naked DNA should also become vehicles for plant transformation (see Crossway et al. 1985). The application of this technology to plant species not amenable to *Agrobacterium*-mediated transformation would allow the improvement of cereal crops by genetic engineering techniques. This would require adaptation of the technology to regenerable material with cell walls such as suspension cultures or pollen grains. As there are other methods of transforming plant protoplasts with DNA, these other applications may be the most important use of plant cell microinjection.

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