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Genetics

Stable transformation of tobacco by electroporation: Evidence for plasmid concatenation

(gene transfer/*Nicotiana tabacum*/integration/Mendelian inheritance)

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ABSTRACT **Electroporation (electric field-mediated DNA transfer) of tobacco protoplasts in the presence of the linearized plasmid pMON200 has led to the formation of transgenic plants. Defined electric shocks were delivered by capacitive discharges with readily available, low-cost electrical components. This transformation procedure is simple and efficient and may suggest a quick method for determining the appropriate electric fields for new cell systems. An optimal transformation frequency of 2.2×10^{-4} (based on the number of cells subjected to the shock) was obtained with a single 2000-V/cm, 250-μs-duration capacitive discharge. Calli transformed to kanamycin resistance have been regenerated into whole plants. Southern blots of DNA from the transgenic plants demonstrate the integration of the selectable marker gene (neomycin phosphotransferase) at single or multiple genomic sites. In some cases, the plasmid appears to be integrated intact; in others, it is rearranged. The blots also provide evidence of plasmid recircularization and/or the formation of head-to-head and head-to-tail concatemers in most of the plants analyzed. Although some plants apparently have multiple integration sites, analysis of progeny obtained by self-fertilization of the transgenic plants indicates that the kanamycin-resistance marker is inherited as a single dominant gene.**

Investigations of gene expression have been facilitated by the development of procedures to induce the uptake, integration, and expression of foreign DNA. Because the totipotency of some plants makes it possible to regenerate intact organisms from single cells, transformation can be used to study the tissue-specific and developmental regulation of plant gene expression (1, 2). Gene transfer has been achieved by a variety of physical and chemical techniques in yeast (3), invertebrate (4), and mammalian (5, 6) systems. The principal procedure for the stable transformation of plants involves the use of the phytopathogen *Agrobacterium tumefaciens* harboring modified Ti plasmids (7). Genes inserted into the T-DNA region of this plasmid are efficiently transferred to the host plant genome. Integrations usually occur at single sites, resulting in the transfer of unrearranged copies, either singly or as tandem repeats. Unfortunately, *Agrobacterium*'s host range is limited to dicots and certain monocots in the Liliales (8). In addition, DNA transfer by *Agrobacterium* may not lend itself well to studies of early events leading to integration, transient expression assays of promoter activity, or "targeted" DNA integration.

For these reasons, free-DNA transfer procedures are being actively developed for plants. Two procedures have been used. One involves exposing protoplasts to DNA in the presence of polyethylene glycol (PEG) and divalent cations (9–11). In the second procedure, protoplasts are treated with liposome-encapsulated DNA (12). Both procedures successfully transform plant cells, including species that are not hosts for *Agrobacterium*. However, the efficiency with which transformed plants are recovered is generally low (about 10^{-5}).

The electroporation (electric field-mediated DNA transfer) studies described here evolved from our work on protoplast electrofusion (13, 14). Electroporation has been used to transform bacterial (15) and mammalian (6) cells with high efficiency ($>10^{-4}$) and should be equally effective with plant cells. Recently, Fromm *et al.* (16) reported the introduction of foreign genes into maize protoplasts by electroporation, and Shillito *et al.* (17) reported high-efficiency transformation of plants when electroporation was used in combination with PEG, Mg^{2+}, and a heat shock treatment. We report here the efficient transfer of foreign genes into tobacco protoplasts by electroporation alone. The regenerative potential of tobacco protoplasts, in contrast with those of maize, enabled us to study the integration and inheritance of these foreign genes in intact plants. The procedure we developed extends that of Potter *et al.* (6), is rapid and simple, and utilizes defined high-voltage pulses. Transgenic plants recovered in these experiments contain from one to a few copies of the transfected DNA, which is generally inherited in a Mendelian fashion. Most of the plants analyzed so far display complex restriction patterns upon Southern blotting. However, from the data, we suggest that part of this complexity is due to inter- and intramolecular ligations of the transfected DNA, which precede its integration. This phenomenon is reminiscent of the recombination events that occur during transfection of mammalian (5) and invertebrate (4, 18) cells with free DNA.

MATERIALS AND METHODS

Plasmids and Plant Materials. Seeds of *Nicotiana tabacum* cv. Xanthi were the generous gift of Roy Chaleff (E. I. du Pont de Nemours & Co.). The plasmid pMON200 and seeds of *Nicotiana plumbaginifolia*, transformed by *Agrobacterium* carrying pMON200, were kindly provided by Robert Fraley and Robert Horsch through Monsanto Corp. pMON200 contains the neomycin phosphotransferase II (NPTII) gene linked to plant regulatory signals and confers antibiotic (kanamycin and G418) resistance to plants (19).

Protoplast Isolation, Electroporation, and Selection. Mesophyll protoplasts were isolated from leaves of *N. tabacum* as described (13). The protoplasts were resuspended at a density of 5×10^5 cells per ml in ice-cold 0.5 M mannitol containing 20 μg of pMON200 per ml linearized with *Eco*RI. Aliquots (0.25 ml) of the protoplast/DNA suspension were pipetted into the electroporation chamber (described below), which was held on ice in a laminar-flow hood.

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Abbreviations: kb, kilobase(s); nF, nanofarad(s); NPTII, neomycin phosphotransferase II; NOS, nopaline synthase; R–C, resistance–capacitance.

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Electrical pulses were delivered by capacitive discharges. When multiple pulses were given, the cells were allowed 1 min to recover between pulses. Following electroporation, the protoplasts were diluted with 0.75 ml of K3S medium containing 0.45 M mannitol (13) and cultured by standard procedures (20). Transformants were recovered by selection in kanamycin at 100 μg/ml by the agarose-bead technique (21). After 4–6 weeks of selection, kanamycin-resistant calli were transferred to solid medium for further growth and regeneration (20). Only the final rooting medium lacked kanamycin.

Electroporation Equipment. Electrical pulses were delivered by standard high-voltage capacitors [5, 10, 15, 25, 50 nanofarads (nF)] charged with a 2000-V electrophoresis power supply and then discharged across the plates in the electroporation chamber by means of a relay [Potter & Brumfield (Newark Electronics, Jacksonville, FL) type PRD3DYO or type JML 1110-81]. Pulse lengths (discharge times) were determined with a voltage divider and a recording oscilloscope. The electroporation chamber consists of a Plexiglas box (open at the top) of the following dimensions: 1 cm deep, 1 cm long, 0.5 cm across. The long walls of the chamber are lined by two stainless steel plates (0.5 cm apart), which serve as electrodes. The chamber was sterilized with ethanol.

DNA Isolation and Analysis. Plasmid DNA, prepared by the alkaline extraction procedure (22), was further purified by CsCl gradient centrifugation. Plant DNA was isolated from leaf tissue as described by Dellaporta *et al.* (23). For Southern blot analysis, aliquots of plant DNA were restricted with enzymes (Bethesda Research Laboratories and Pharmacia) according to the manufacturers' specifications. Fragments were separated by electrophoresis on 1% agarose gels and transferred to nitrocellulose by the Southern (24) procedure or by the procedure of Chomczynski and Qasba (25), which involves alkaline transfer to GeneScreen*Plus* (New England Nuclear). Prehybridization and hybridization were carried out by standard procedures (22).

RESULTS

Determination of the dc Fields Appropriate for Transformation. To determine the optimal fields, we examined the relationship between pulse-induced cell death and transformation frequency (Table 1). Resuspending the protoplasts in ice-cold mannitol somewhat decreases viability (control versus the 0-nF treatment). All of the dc pulses tried led to a further decrease in viability. This cell death had both a rapid and a progressive component. Immediately after short dc pulses (5, 10, 15 nF), most of the protoplasts were viable. However, cell death was more pronounced 24 hr later. Large pulses (25, 50 nF), which killed 70–80% of the cells (compared with controls) within 2 hr of the treatment, resulted in few protoplasts surviving after 24 hr. Giving multiple pulses or increasing the pulse voltage above 2000 V/cm further decreased viability. Voltages below 2000 V/cm were not studied.

The maximum transformation efficiency (2.2×10^{-4}, based on the number of protoplasts treated) was obtained with a single 10-nF, 2000-V/cm pulse. No kanamycin-resistant colonies were recovered in controls (protoplasts not treated with DNA or treated with DNA but not given electrical pulses) in six separate experiments. Pulses that were larger or longer than 10 nF, 2000 V/cm (or multiple pulses) reduced transformation frequency. However, adding feeder cells to the cultures following electroporation increased the recovery of resistant colonies for all treatments, except the single 10-nF pulse. Possibly the reason that large or long dc pulses reduced the transformation frequency is that the ensuing cell death caused the cell density to fall below that necessary for efficient protoplast culture.

Table 1. Relationship of dc pulse-induced cell death and the frequency of transformation to kanamycin resistance

Treatment	% cell survival		Resistant colonies recovered	Transformation frequency ($\times 10^{-5}$)
	2 hr	24 hr		
Control	80	80	0	0
0 nF + DNA	67	63	0	0
5 nF	64	51	5	4
10 nF	62	36	28	22.4
15 nF	52	22	8	6.4
25 nF	20	1	0	0
50 nF	12	2	0	0
10 nF × 2	26	12	5	4
10 nF × 4	17	1	0	0
10 nF, 3000 V/cm	22	11	0	0
10 nF, 4000 V/cm	12	4	0	0
10 nF + feeder	ND	ND	29	23
10 nF × 2 + feeder	ND	ND	18	14.4
15 nF + feeder	ND	ND	17	13.6
15 nF × 2 + feeder	ND	ND	20	16

dc electrical pulses were given as capacitive discharges and are represented as nF. A 10-nF discharge had a resistance–capacitance (R–C) time constant of 250 μs. Except where indicated, single pulses were given with a starting voltage of 2000 V/cm. Control represents protoplasts that were washed in culture medium (rather than 0.5 M mannitol) and were placed into culture without being shocked or exposed to DNA. For all other samples, the protoplasts were washed in 0.5 M mannitol and then resuspended in ice-cold 0.5 M mannitol containing 20 μg of linearized pMON200 per ml. The 0-nF sample represents protoplasts so treated but not given an electric shock. Multiple pulses, given 1 min apart, are indicated as × 2 (two) and × 4 (four). Feeder indicates that protoplasts given dc pulses were cultured in the presence of untreated protoplasts (density, 5×10^4 cells per ml). Cell survival was determined by Evans blue exclusion (26). Resistant colonies were recovered by selection with 100 μg of kanamycin per ml. Transformation frequencies are based on the number of cells in each sample at the start of the experiment (1.25×10^5). ND, not determined.

Taken together, the data support the suggestion that dc pulses that produce significant cell death (about 50% kill) are optimal for transformation. This observation may provide a rapid method for determining the optimal field parameters for new systems.

Recovery of Kanamycin-Resistant Plants. Kanamycin-resistant calli, identified after 4 weeks of selection, were subcultured as individual calli until large enough to regenerate. Calli chosen for regeneration were selected at random. A total of 53 plants were regenerated from 18 different kanamycin-resistant calli. All but 2 of these plants were morphologically normal; most proved to be fertile. We retested whole plants for resistance to 200 μg of kanamycin per ml using the leaf callus assay (27). Twenty-six plants were tested; all were able to form rapidly growing callus. Callus formation from leaf sections from untransformed plants was completely inhibited by 50 μg of kanamycin per ml.

Analysis of Genomic Plant DNA. The plasmid used for transfection, pMON200, contains two genes that can be expressed in plant tissues: a chimeric gene, consisting of the NPTII gene under control of the 5′ and 3′ regulatory sequences of the nopaline synthase gene (NOS), and an intact NOS gene (Fig. 1*A*).

Genomic DNAs were digested with *Hin*dIII and subjected to Southern analysis with the 1.3-kb *Eco*RI/*Bam*HI fragment (chimeric gene) as a probe (Fig. 1*B*). Some transformants exhibited a single band at positions of greater than unit length (9.5 kb), consistent with integration of the unrearranged plasmid(s) at a single site. In other transformants, major bands of less than unit length were observed, indicative of DNA rearrangements prior to or during integration. In

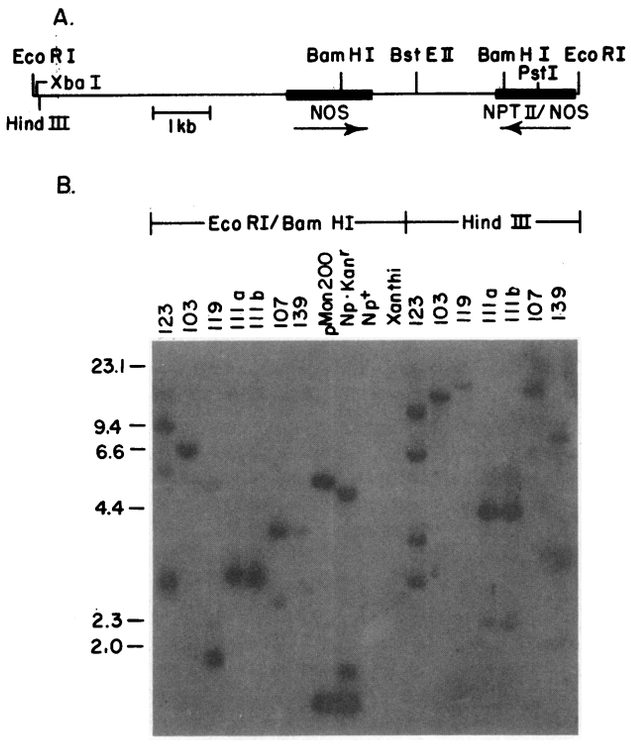

FIG. 1. (*A*) Diagram of pMON200 (19), linearized with *Eco*RI. The plasmid [9.5 kilobases (kb)] contains a chimeric NPTII/NOS gene and an intact NOS gene. A polylinker, located 5′ to the transcription start site of the chimeric gene, contains restriction sites for *Hin*dIII and *Xba* I. Bold lines represent the coding region of genes; arrows indicate the direction of transcription. (*B*) Southern blot hybridization of transgenic plants. DNA was isolated from a number of transformants; 10-μg aliquots were restricted with *Hin*dIII or *Eco*RI and *Bam*HI and loaded on each lane. Wild-type *N. plumbaginifolia* (Np^+) and *N. tabacum* cv. Xanthi (Xanthi) are shown as untransformed controls. Np·Kan^r represents DNA from *N. plumbaginifolia* plants transformed with pMON200 via *A. tumefaciens* (27). The digests were transferred to a nylon membrane (25) and probed with a nick-translated 1.3-kb *Eco*RI/*Bam*HI fragment of pMON200. Lanes containing DNA from plant 139 were inadvertently underloaded. Size standards are λ *Hin*dIII fragments represented on the ordinate as kilobase pairs.

addition, plant 123 gave rise to multiple bands, suggesting more than one integration site.

Restriction of genomic DNA with *Eco*RI and *Bam*HI was expected to release a 1.3-kb fragment corresponding to the NPTII/NOS chimeric gene. This band is observed in restricted plasmid DNA (pMON200) and in genomic DNA from *N. plumbaginifolia* plants transformed with pMON200 via *Agrobacterium* (Np·Kan^r in Fig. 1*B*; see also ref. 27). Untransformed *N. plumbaginifolia* (Np^+) and *N. tabacum* show no hybridization to this probe. As seen in Fig. 1, none of the plants regenerated from electroporated protoplasts contains the expected 1.3-kb *Eco*RI/*Bam*HI band. Because *Eco*RI was used to linearize the plasmid prior to electroporation, this observation probably means that the free ends of the plasmid were modified before or during integration and were no longer susceptible to digestion by *Eco*RI. Complete digestion of the samples is inferred from the appearance of the 1.3-kb band in the Np·Kan^r lane.

The *Eco*RI/*Bam*HI digests of pMON200 and DNA from the Np·Kan^r plants also exhibit a second, less intense band (at about 5 kb) due to hybridization of the probe to the 5′ regulatory region of the intact NOS gene. This cross-hybridization to the NOS gene may account for the minor bands seen in the *Eco*RI/*Bam*HI digests of plants 107, 119, and 123. This inference has not yet been tested with a probe specific for the NOS gene. However, nopaline was detected in leaf extracts of some of the transgenic plants by paper chromatography (28), indicating that the NOS gene is present and expressed (data not shown).

Plasmid Ligation Events. The complex restriction patterns seen in Fig. 1*B* may be the result of plasmid rearrangements. However, another interpretation is possible. In mammalian (5) and invertebrate (4, 18) cells, intra- and intermolecular plasmid ligation events have been observed after transfection with linear or circular plasmid DNAs. To investigate this possibility, we digested genomic DNAs with enzymes that would produce characteristic fragments (containing the plasmid–plasmid junctions) should such ligations have occurred. These digests were probed with a probe specific for the NPTII gene. Three possible ligation events that would be detectable by this procedure are recircularization, head-to-head ligation of plasmid monomers, and head-to-tail ligation of monomers. (We have arbitrarily designated as the head the end of the plasmid containing the chimeric NPTII/NOS gene.) As seen in Fig. 2, recircularized monomers and head-to-tail concatemers could release a 6.3-kb fragment upon digestion with *Bam*HI. The presence of *Bst*EII fragments of unit length (9.5 kb) is consistent with head-to-tail concatemer formation. Likewise, the presence of either a 2.6-kb *Bam*HI fragment or a 6.1-kb *Bst*EII fragment is consistent with formation of a head-to-head concatemer. In addition, a 5.9-kb *Bam*HI fragment could be released by partial digestion.

The samples in Fig. 3 were actually digested with pairs of enzymes (*Bam*HI/*Xba* I or *Bst*EII/*Hin*dIII), as we expected this treatment to release certain small fragments such as the 1.3-kb *Bam*HI/*Xba* I fragment shown in Fig. 2. These fragments were not obtained, possibly because of degradation of the plasmid's free ends prior to ligation or because of DNA methylation. In any case, the following inferences can be made about ligation events based on the data in Fig. 3. Plant 101 displays the 6.3-kb *Bam*HI fragment expected from integration of a recircularized plasmid or a head-to-tail concatemer. Additional evidence for head-to-tail concatemers is provided by the unit length *Bst*EII fragments in plants 108, 121, and 143. Four of the seven plants examined display the 2.6-kb *Bam*HI fragment expected from integration of a portion of a head-to-head concatemer (arrow in Fig. 3). Plants 108 and 123 give rise to a 5.9-kb *Bam*HI fragment, which

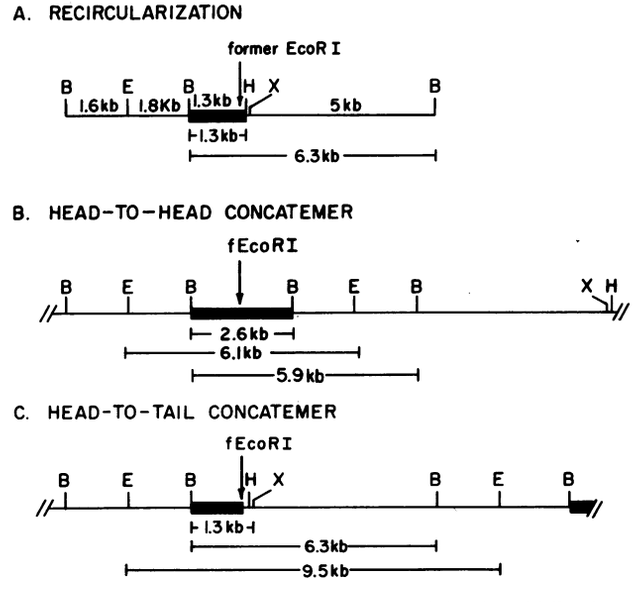

FIG. 2. Schematic representation of possible plasmid ligations. B, *Bam*HI; E, *Bst*EII; H, *Hin*dIII; X, *Xba* I.

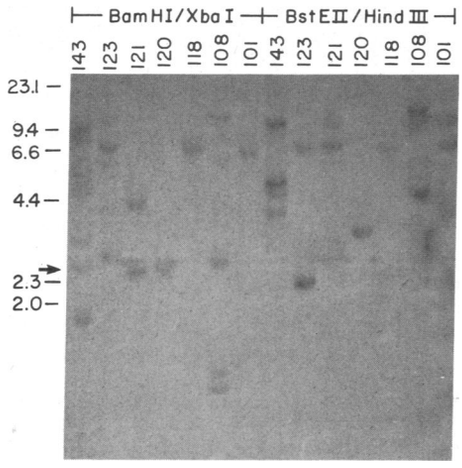

FIG. 3. Southern blot hybridization of concatenation events. Samples were digested with either *Bam*HI/*Xba* I or *Bst*EII/*Hin*dIII, separated by electrophoresis, and transferred to nitrocellulose (24). The filter was probed with a nick-translated 0.57-kb *Bam*HI/*Pst* I fragment specific to the NPTII coding region. DNA from untransformed tobacco plants did not hybridize to this probe (data not shown). The arrow indicates the position of the 2.6-kb *Bam*HI fragment, which is indicative of head-to-head plasmid ligation. Ordinate values are λ *Hin*dIII size standards (in kilobase pairs).

could be released by partial digestion of a head-to-head concatemer.

Based on the complexity of their digestion patterns, and by comparison to the hybridization intensities of measured amounts of pMON200 (data not shown), some of the plants (108, 121, 123, 143) appear to contain two to five copies of the plasmid. This inference is also supported by the observation of the simultaneous presence of portions of head-to-head and head-to-tail concatemers in plants 108, 143, and possibly 123. None of the plants examined contains the 6.1-kb *Bst*EII fragment expected from integration of a head-to-head concatemer. Instead, four of the plants display a 6.5-kb fragment upon digestion with *Bst*EII and *Hin*dIII. We cannot account for the consistent appearance of this fragment by any simple ligation events.

Different plants were analyzed in the experiments in Figs. 1 and 3 by happenstance rather than design. However, when the samples shown in Fig. 1 were reanalyzed with the double enzyme digests of Fig. 3, similar patterns were observed. For example, plant 111 gives a single *Bam*HI/*Xba* I band at 2.6 kb and the 6.5-kb *Bst*EII/*Hin*dIII band. Plant 107 gives bands at 9.5 kb and 6.5 kb upon digestion with *Bst*EII/*Hin*dIII and bands at 2.6 kb and 4.0 kb upon digestion with *Bam*HI/*Xba* I.

Taken together, the data provide evidence for plasmid ligation in most samples. Some of the unexplained bands must be plasmid–genomic junction sites. However, the complexity of the hybridization patterns points to the additional element of plasmid rearrangement.

Inheritance of Kanamycin Resistance in Transgenic Plants. Seeds collected from self-pollinated plants were germinated and grown in the light on P medium (13) containing 1% sucrose and 100 μg of kanamycin per ml. This procedure provides a rapid test of the sexual transmission and stability of the NPTII gene (7, 11). In the presence of kanamycin, sensitive plants never form secondary roots or true leaves, and, after 4 weeks, their cotyledons have bleached. Resistant plants exhibit normal development.

Most of the transgenic plants tested so far show ≈3:1 (resistant:sensitive) transmission ratios, indicative of a single dominant gene inherited in a Mendelian fashion. For example, plant 101, 116:37 or 3.1:1; plant 103, 89:26 or 3.4:1; plant 108, 89:27 or 3.3:1; plant 109, 189:66 or 2.9:1; plant 120, 90:30 or 3.0:1. An interesting variation is exhibited by plants 121, 123, and 143, which give ratios closer to 2:1 (plant 121, 121:57 or 2.1:1; plant 123, 140:62 or 2.3:1; plant 143, 92:38 or 2.4:1). Such ratios are consistent with the inheritance of a homozygous lethal mutation. However, more complicated explanations are also possible. Results of the Southern blots in Figs. 1 and 3 indicate that plant 123 probably contains more than one copy of the plasmid. If one copy had been inserted normally but the other had been inserted in such a way as to create a homozygous lethal mutation, then the plant would exhibit non-Mendelian inheritance for kanamycin resistance. Backcrosses to wild-type plants and Southern blot analyses of the progeny will probably be necessary to distinguish these possibilities.

Comparison of offspring of different plants shows that they can vary in the degree of resistance (based on the growth of seedlings in the presence and absence of kanamycin). Some plants showed almost no growth inhibition by kanamycin, whereas others, although capable of chlorophyll production and organ formation when grown in kanamycin, were quite stunted. This qualitative assessment of resistance does not correlate with the pattern of inheritance or the number of copies of plasmid that were integrated. For example, plant 108, which probably contains several copies of plasmid (see Fig. 3), shows weak resistance, whereas offspring of plant 101, which appears to contain a single plasmid insert, display a high degree of resistance to kanamycin. This variation may be due to position effects with respect to the site of integration or it may reflect plasmid modification.

DISCUSSION

Although *Agrobacterium* provides a highly efficient vehicle for the stable transfer of foreign DNA into plant cells, free-DNA transfer has the advantage of having no host range limitation and requires no special DNA sequences other than a selectable marker gene. Prior to our work with electroporation, two other free-DNA transfer techniques were described: fusion of liposomes loaded with DNA (12) and a combination Ca^{2+}/PEG treatment (10, 29) or Ca^{2+}/polyvinyl alcohol treatment (30). In addition, free-DNA transfer is effective with species not susceptible to infection by *Agrobacterium* (10, 31).

Electroporation appears to offer two advantages over other free-DNA transfer procedures. The technique is rapid and simple, requiring a minimal amount of preparation and handling of the cells and DNA. Second, electroporation may permit higher transformation efficiencies. With other procedures, efficiencies of 4×10^{-5} to 1×10^{-6} have been obtained for plants (9, 12, 29, 31). Using electroporation, Fromm *et al.* (16) obtained transformation frequencies of 8×10^{-5}. We obtained frequencies as high as 2.2×10^{-4}, similar to the maximal value obtained by Hain *et al.* (30) with the Ca^{2+}/polyvinyl alcohol treatment. However, the data of Hain *et al.* appear to be based on the number of dividing colonies at the start of selection, which is only 30% of the initial protoplast population. Our value is based on the total number of protoplasts treated. We did not measure plating efficiencies for electroporated protoplasts; however, electroporation treatments yielding optimal transformation left 36% of the protoplasts viable (Table 1). Assuming that 60% of the surviving cells eventually divide, which is typical of tobacco protoplasts (13), our maximal transformation efficiency is about 10^{-3}. Although this value is lower by a factor of ≈10 than that generally obtained with *Agrobacterium* (27) or the combination PEG/heat shock/electroporation treatment of Shillito *et al.* (17), it is nonetheless sufficient for easy recovery of transformed cells.

In seeking optimal conditions for transformation, we assumed that transformation by electroporation would be maximal with field pulses similar to or slightly in excess of those that are optimal for electrofusion. This appears to be the case. Optimal transformation was obtained with 2000-V/cm capacitive discharges that had a R–C decay constant of 250 μs. Electrofusion of tobacco protoplasts is maximal with 1000-V/cm, 50-μs square wave pulses (14) or 1000-V/cm, 1-ms (R–C) capacitive discharges (32). Fromm *et al.* (16, 33) introduced genes into maize protoplasts by electroporation using lower-voltage, longer pulses (500 V/cm, 4 ms). We have not reproduced their conditions, nor did they investigate the use of high-voltage, short pulses. An additional difference between their experiments and ours is their use of supercoiled as opposed to linear DNA. What effect the form of the DNA may have on the efficiency of electroporation and the types of transformants obtained is unknown.

From the Southern blotting and inheritance data, it appears that the transfected DNA is stably integrated into plant chromosomes. Although identification and analysis of the junction fragments may be required to prove this contention, the long-term, stable maintenance of free plasmid DNA does not appear to occur in animal cells (5) and, to our knowledge, has never been reported for plant cells. The 3:1 inheritance ratios obtained suggest that most of the transgenic plants contain a single plasmid integration site. If multiple integrations were involved, either some of the copies must be silent or they must be closely linked.

Evidence for plasmid recircularization or concatemer formation was obtained for most of the plants examined. These events appear to be associated with other, as yet unknown, rearrangements or modifications of the transfecting plasmid. In every case, the *Eco*RI site used to linearize the plasmid was lost or modified. Moreover, none of the plants displayed all of the restriction fragments expected from integration of either a head-to-head or a head-to-tail concatemer. This result may indicate that only a portion of the concatenated plasmid was retained by the plants or it may indicate plasmid rearrangement. Interestingly, six of nine samples digested with *Bst*EII and *Hin*dIII displayed an unexpected 6.5-kb band. This observation suggests that, in addition to ligation, the plasmid has undergone some sort of regular rearrangement.

Plasmid ligation appears to be random; evidence of head-to-head and head-to-tail concatemers is seen. This observation is reminiscent of results obtained by microinjection of sea urchins (4) and nematodes (18) but contrasts with the results obtained for mammalian cells in which concatemers are in the form of head-to-tail arrays (34). Transformation of plants by *Agrobacterium* can also lead to the integration of head-to-tail tandem arrays (7). Such arrays could form through plasmid recircularization followed by amplification or by homologous recombination, either before or after integration (34). However, random head-to-head and head-to-tail arrays are unlikely to have formed either by this mechanism or by recombination near a previously integrated copy. This circumstance suggests that the plasmid ligations observed here occurred prior to integration.

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