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Transgenic Maize Plants by Tissue Electroporation

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In this paper, we describe the transformation of regenerable maize tissues by electroporation. In many maize lines, immature zygotic embryos can give rise to embryogenic callus cultures from which plants can be regenerated. Immature zygotic embryos or embryogenic type I calli were wounded either enzymatically or mechanically and subsequently electroporated with a chimeric gene encoding neomycin phosphotransferase (*neo*). Transformed embryogenic calli were selected from electroporated tissues on kanamycin-containing media and fertile transgenic maize plants were regenerated. The *neo* gene was transmitted to the progeny of kanamycin-resistant transformants in a Mendelian fashion. This showed that all transformants were nonchimeric, suggesting that transformation and regeneration are a single-cell event. The maize transformation procedure presented here does not require the establishment of genotype-dependent embryogenic type II callus or cell suspension cultures and facilitates the engineering of new traits into agronomically relevant maize inbred lines.

INTRODUCTION

Genetic transformation has become an important tool in the study of basic plant processes and in crop improvement. The development of genetic transformation techniques for the major cereal crops has been relatively slow, mainly as a consequence of their limited susceptibility to Agrobacterium and their poor capacity to regenerate fertile plants from protoplasts (Rhodes et al., 1988).

Recently, microprojectile bombardment using DNA-coated particles has been used to transform embryogenic maize cultures, which have subsequently been regenerated into fertile transgenic plants (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). These authors used derivatives of a particular maize inbred line, A188. This inbred has no agronomical value but is superior to most other maize inbreds in its capacity to regenerate plants at high frequency from embryogenic callus or cell suspension cultures. Particular callus cultures, the so-called type II callus, were a prerequisite for the initiation of cell cultures suitable for transformation (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). Type II callus is highly embryogenic, white or pale yellow, friable, and rapidly growing. Its establishment is very genotype dependent and is only achieved at low frequency (Vasil et al., 1984, 1985; Armstrong and Green, 1985). The cell culture properties of A188 can be transmitted through genetic crosses to recalcitrant inbreds (Hodges et al., 1986). Backcrossing, combined with selection in tissue culture in each generation, can lead to the development of agronomically relevant inbreds with tissue culture properties amenable to genetic transformation using microprojectile bombardment.

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It would be advantageous if fertile transgenic plants could be generated directly from elite maize inbred lines. Our goal was to develop a transformation technique that is less genotype dependent and which would eliminate the difficulty of establishing type II cell cultures. Therefore, we investigated whether immature zygotic embryos or type I callus could be used as target material in transformation experiments. Type I embryogenic callus is compact, nodular, and organized; it can be obtained readily and at high frequency from cultured immature zygotic embryos in a wide variety of maize inbreds and hybrids (Lu et al., 1982, 1983; Novak et al., 1983; Duncan et al., 1985; Tomes and Smith, 1985; Hodges et al., 1986).

In this paper, we describe DNA delivery by electroporation into maize immature zygotic embryos and into type I callus cultures. Transgenic embryogenic calli were obtained using the neomycin phosphotransferase (*neo*) gene as selectable marker. Transgenic maize plants were regenerated from these cultures and the inheritance of the introduced gene was studied over several generations.

RESULTS

Transient NPTII Expression in Electroporated Maize Tissues

Intact immature zygotic embryos of maize inbred lines H99 or Pa91 were electroporated in maize electroporation buffer (EPM) with plasmid pDE108 DNA, schematically shown in Figure 1, containing a chimeric cauliflower mosaic virus (CaMV) 35S–*neo* gene, using electroporation conditions as described

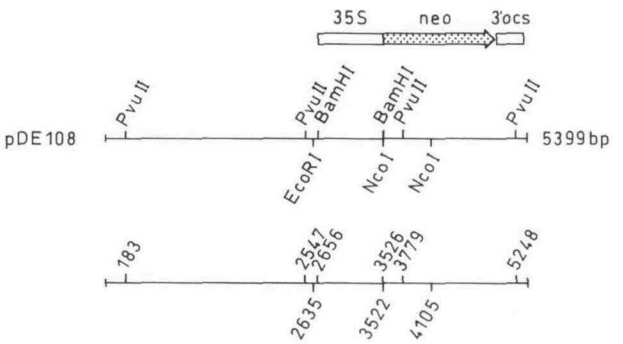

Figure 1. Plasmid pDE108.

Schematic representation of plasmid pDE108 linearized at the HindIII site. The chimeric *neo* gene is indicated. The coding region of the *neo* gene is represented by a stippled bar. The fragment containing the 3′ untranslated region of the octopine synthase gene (*3′ ocs*) is represented by an open bar. The arrow indicates the direction of transcription of the CaMV 35S promoter.

for leaf bases of rice seedlings (Dekeyser et al., 1990). Figure 2 shows that no detectable NPTII activity was observed when the embryos were assayed 4 to 6 days after electroporation. We reasoned that DNA delivery would require wounding of the embryos. This was achieved by an enzymatic treatment of the embryos in a 0.3% solution of macerozyme, an enzyme that degrades pectic substances for a short period varying from 1 to 3 min prior to electroporation. With this technique, NPTII activity could be detected reproducibly 4 to 6 days after electroporation with plasmid pDE108 DNA (Figure 2). NPTII activity was not significantly influenced either by increasing the DNA concentration from 10 to 20 μg per cuvette or by using linearized instead of covalently closed circular plasmid DNA. We also investigated whether transient expression was detectable in finely chopped embryogenic sectors of type I callus cultures, preplasmolyzed for 3 hr, and subsequently electroporated in the presence of plasmid pDE108 DNA. NPTII activity was detected in extracts of these tissues 4 to 6 days after electroporation (data not shown). Together, these data show that DNA can be electroporated into enzymatically wounded immature embryos and into mechanically wounded type I callus, and that the introduced gene is transiently expressed.

Influence of Wounding and Electroporation on Tissue Culture and Plant Regeneration

We analyzed the influence of enzymatical or mechanical wounding and electroporation on the capacity of immature embryos, as shown in Figure 3, and type I callus, as shown in Figure 4, respectively, to proliferate into embryonic callus and to regenerate plants. Routinely, untreated immature zygotic embryos of line Pa91 or H99 formed embryogenic type I calli at a frequency of ~100% upon in vitro culture. A short (1 to 3 min) enzymatic treatment and subsequent electroporation reduced this frequency to 50 to 90% (Figures 3B and 3C). An enzymatic treatment longer than 3 min drastically reduced type I callus formation.

The initial quality of immature embryos was a critical factor in the establishment of embryogenic type I callus. We observed that maize plants grown under suboptimal conditions produced cobs whose embryos were very poor in type I callus formation. Enzymatic wounding of the embryos reduced their capacity to form type I callus even further.

Mechanical wounding and subsequent electroporation did not significantly affect the growth of treated type I calli when plated on proliferation medium (Figure 4B). Although a slimy type of tissue proliferated frequently from the plated aggregates, subculturing of embryogenic sectors readily led to embryogenic callus cultures.

Selection of Stably Transformed Calli and Regeneration of Kanamycin-Resistant Plants

The above data indicate that DNA can be delivered into enzymatically wounded immature embryos and into mechanically wounded type I calli, and that the capacity of both tissues to proliferate into embryogenic type I calli is not significantly affected. We then investigated whether this procedure allowed the selection of stably transformed cell lines from which fertile transgenic plants could be regenerated.

Figure 3A shows immature embryos that were electroporated with 10 μg of linearized pDE108 DNA per cuvette and transferred immediately to selective medium. Embryos electroporated without DNA showed only some swelling and did not proliferate into callus on substrate containing 200 mg/L kanamycin (Figures 3D and 3E). Embryos electroporated in the presence of linearized pDE108 plasmid started forming type I callus within 2 weeks after transfer to selective medium (Figures 3F and 3G). Figure 4A shows type I callus that was

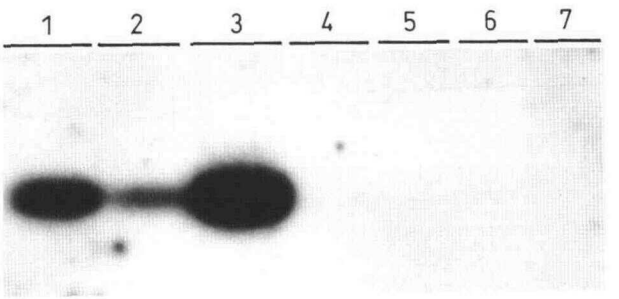

Figure 2. Transient Expression in Immature Zygotic Embryos.

NPTII assay on 50 μg of protein extract of different samples of ~30 immature embryos 5 days after electroporation with linearized pDE108 plasmid DNA. Lanes 1, 2, and 3, enzymatically treated immature embryos; lanes 4, 5, 6, and 7, nonenzymatically treated immature embryos. Exposure time, 20 hr.

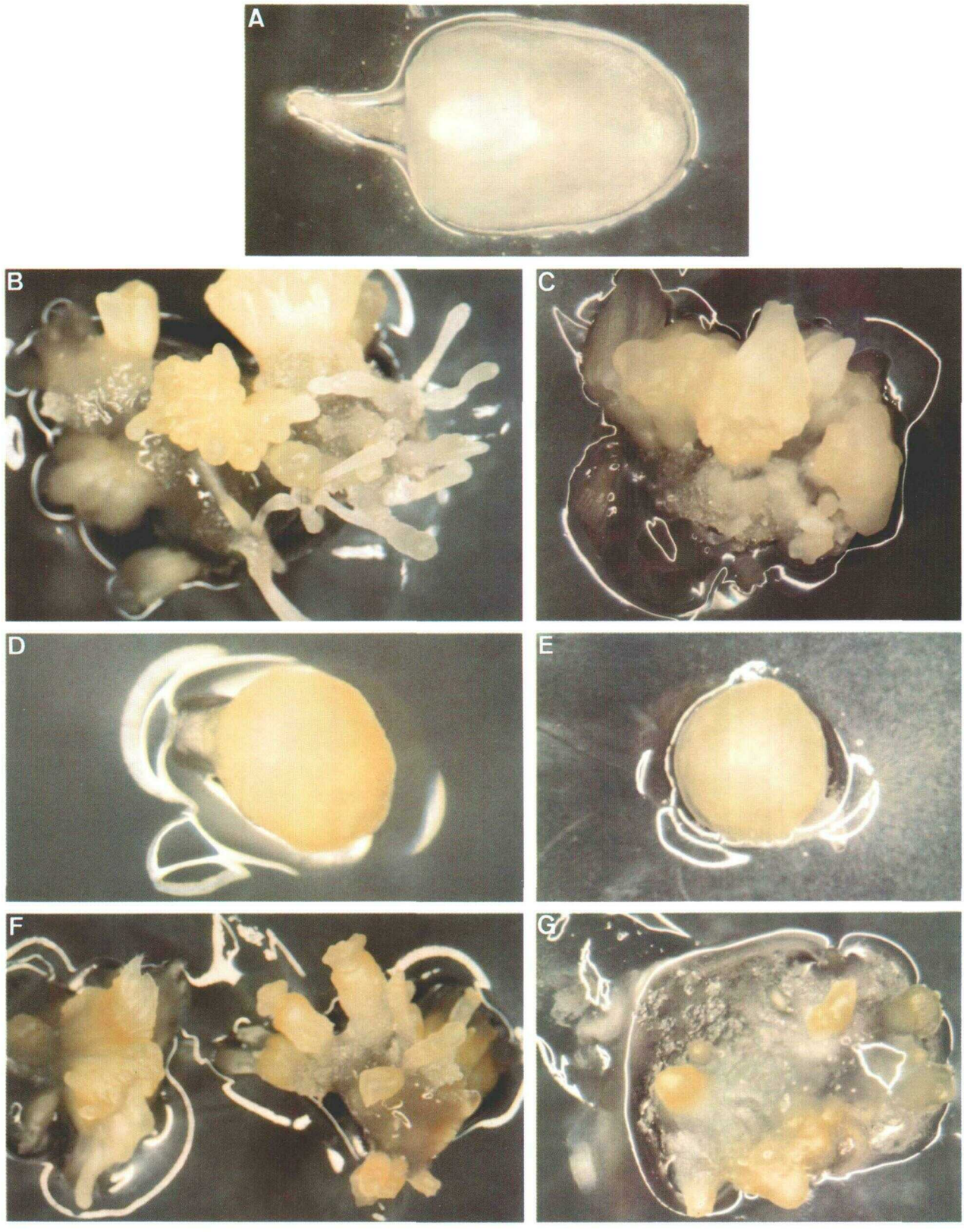

Figure 3. Electroporation of Enzymatically Treated Immature Embryos of Line H99.

(A) Enzymatically treated immature embryo after electroporation.
(B) and **(C)** Embryos electroporated with pDE108 DNA, cultured for 3 weeks on nonselective substrate.
(D) and **(E)** Embryos electroporated without DNA, cultured for 3 weeks on substrate containing 200 mg/L kanamycin.
(F) and **(G)** Embryos electroporated with DNA, cultured for 3 weeks on substrate containing 200 mg/L kanamycin.

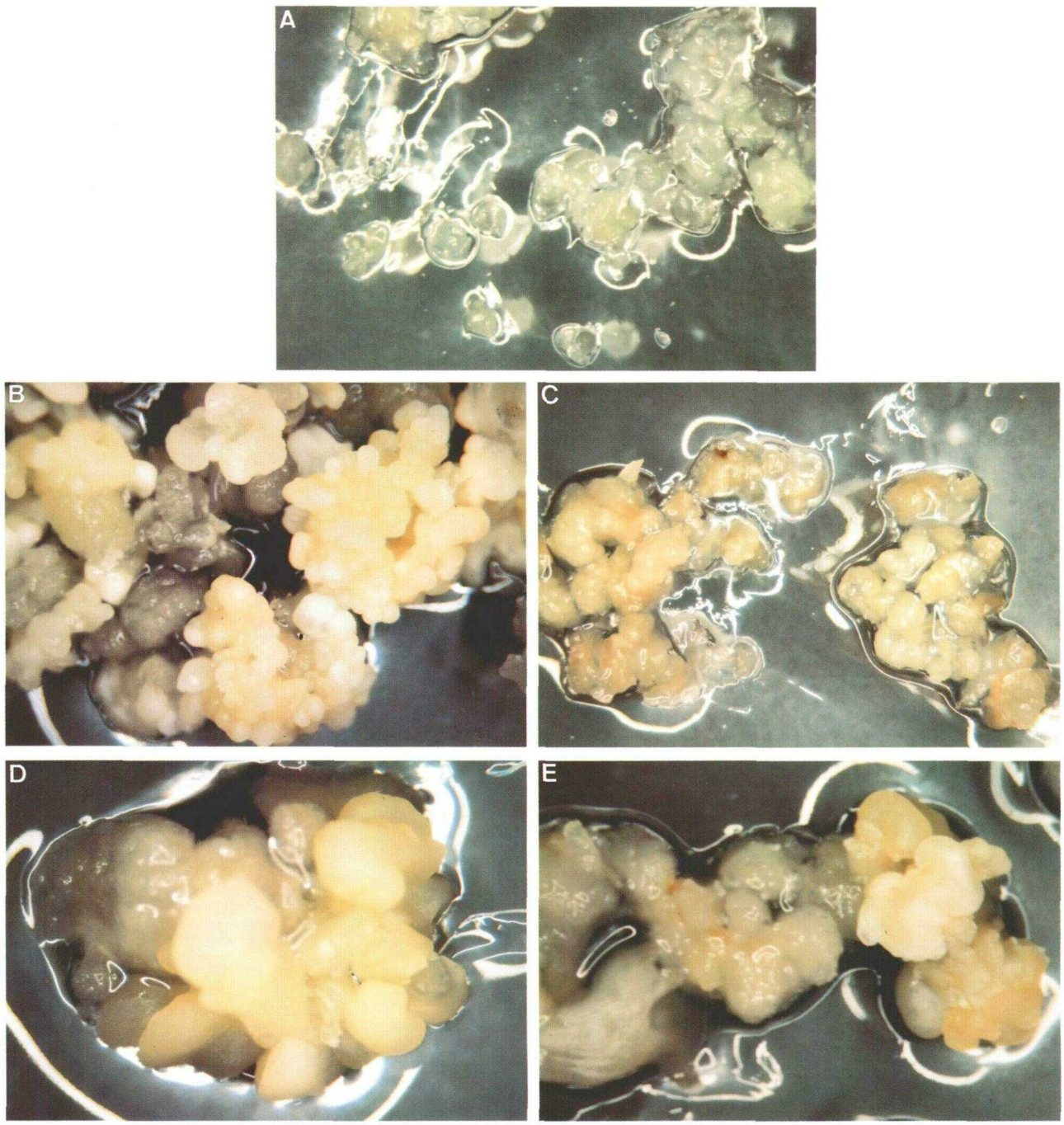

Figure 4. Electroporation of Mechanically Wounded Type I Callus of Line Pa91.

(A) Finely chopped type I callus after electroporation.
(B) Type I callus electroporated with pDE108 DNA, cultured for 5 weeks on nonselective substrate.
(C) Type I callus electroporated without DNA, cultured for 5 weeks on substrate containing 200 mg/L kanamycin.
(D) and **(E)** Type I callus electroporated with pDE108 DNA, cultured for 5 weeks on substrate containing 200 mg/L kanamycin.

electroporated with pDE108 DNA, as described for immature embryos, and immediately plated as small aggregates onto selective substrate. Type I calli electroporated without DNA did not proliferate into embryogenic tissue (Figure 4C), whereas proliferation from type I callus electroporated in the presence of DNA became apparent 4 weeks after electroporation (Figures 4D and 4E).

Proliferating calli from both immature embryos and type I callus were subcultured on selective medium for 6 to 8 weeks. Subsequently, kanamycin-resistant calli were transferred to nonselective medium containing a high concentration of cytokinin for 10 to 14 days to induce germination of somatic embryos. The embryogenic tissues were then transferred to a hormone-free medium to allow development into green plantlets, which were transferred to soil 2 to 4 weeks later.

Table 1 summarizes the results from 10 independent transformation experiments. The number of immature embryos that showed proliferation of embryogenic callus on selective substrate varied from 4 to 28%. The frequency at which finely chopped type I calli showed proliferation is expressed as the number of embryogenic calli obtained from the total amount of finely chopped tissue plated as small aggregates. Each selected callus was derived from either a separate embryo or a single aggregate of type I calli. Thus, each selected callus line represents an independent transformation event. The number of selected callus lines from which plants could be regenerated varied dramatically from one experiment to the other. Approximately 90% of plants recovered from kanamycin-resistant calli expressed the *neo* gene, as evidenced by NPTII gel assays (data not shown). The number of plants that regenerated from a selected callus line varied from one to 30. Optimally, 12 weeks were required to obtain transgenic plants from the start of the experiment to their transfer to the greenhouse.

Table 1. Summary of 10 Transformation Experiments

Inbred	No. of Explants[a]	Selected Embryogenic Calli (%)[b]	Shoot Regenerating Calli[c]	Regenerated Plants[d]	Transformed Plants[e]
Immature Embryos					
H99	675	186 (28)	8	78	70
H99	125	17 (14)	2	3	3
Pa91	75	4 (5)	2	10	9
H99	90	4 (4)	1	3	2
H99	150	23 (15)	2	8	6
H99	325	85 (26)	0	0	0
Type I Callus					
Pa91	25	41	7	31	27
Pa91	10	25	1	1	1
Pa91	10	13	2	3	3
Pa91	10	9	0	0	0

[a] The number of immature embryos or the number of cuvettes containing ~150 mg finely chopped type I callus.

[b] The number of embryos or type I calli aggregates showing proliferation after ~2 months on substrate with 200 mg/L kanamycin. Numbers in parentheses represent percent of total explants that showed proliferation of embryogenic callus.

[c] The number of kanamycin-resistant callus lines from which plants could be regenerated.

[d] The number of plants obtained from the regenerating callus lines.

[e] The number of regenerated plants that expressed the *neo* gene, assessed by kanamycin dot assays or by NPTII gel assays.

Phenotype of R_0 Plants and Inheritance of the *neo* Gene

Over a 4-month period, 148 R_0 plants were regenerated from 31 independent kanamycin-resistant calli and transferred to the greenhouse. The majority (>95%) of kanamycin-resistant plants survived transfer to soil, flowered, and produced viable pollen. As shown in Figure 5, most (>90%) plants developed normally and formed a normal tassel and ear. Seed set was obtained either by selfing or cross-pollination. Selfed R_0 plants produced 20 to 100 seeds per ear, whereas cross-pollination to wild-type plants yielded 100 to 200 seeds per ear. Sometimes R_0 plants showed characteristics typical for tissue culture–induced stress, such as reduced stature and pistillate flowers on the tassel. In such cases, 10 to 100 viable seeds were produced on the tassel.

Progeny from independently transformed R_0 plants were analyzed to determine the inheritance of the *neo* gene. NPTII activity was assessed by a localized application of a kanamycin solution (dot assay). Figure 5D shows that plants expressing the *neo* gene had no symptoms, whereas the newly formed leaves of nontransformed plants bleached and turned white. The dot assay allowed us to monitor the segregation of NPTII activity in large numbers of progeny. Table 2 presents segregation data of R_1 progeny from nine transformed R_0 plants. The segregation data obtained were compared with the expected frequencies in a chi-square test. The results are not significantly different from a 1-to-1 segregation in crosses and a 3-to-1 segregation in selfings. These data indicate that the NPTII activity was encoded by, and transmitted as, a single, dominant allele. In addition, progeny from 36 other primary regenerants were analyzed. The *neo* gene segregated in 34 of 36 progeny as a single, dominant allele. Two progeny revealed a higher number of sensitive plants than expected. NPTII enzyme assays, performed on some of the progeny, confirmed the segregation data obtained by the kanamycin dot assay.

To further test the inheritance of NPTII activity in the R_2 and R_3 generations, R_1 and R_2 plants expressing the *neo* gene were selfed and crossed to nontransformed plants. Transgenic plants were used as either the female or male parent. The data in Table 2 show that the *neo* gene was stably transmitted to the R_2 and R_3 generations in a Mendelian manner through both male and female gametes.

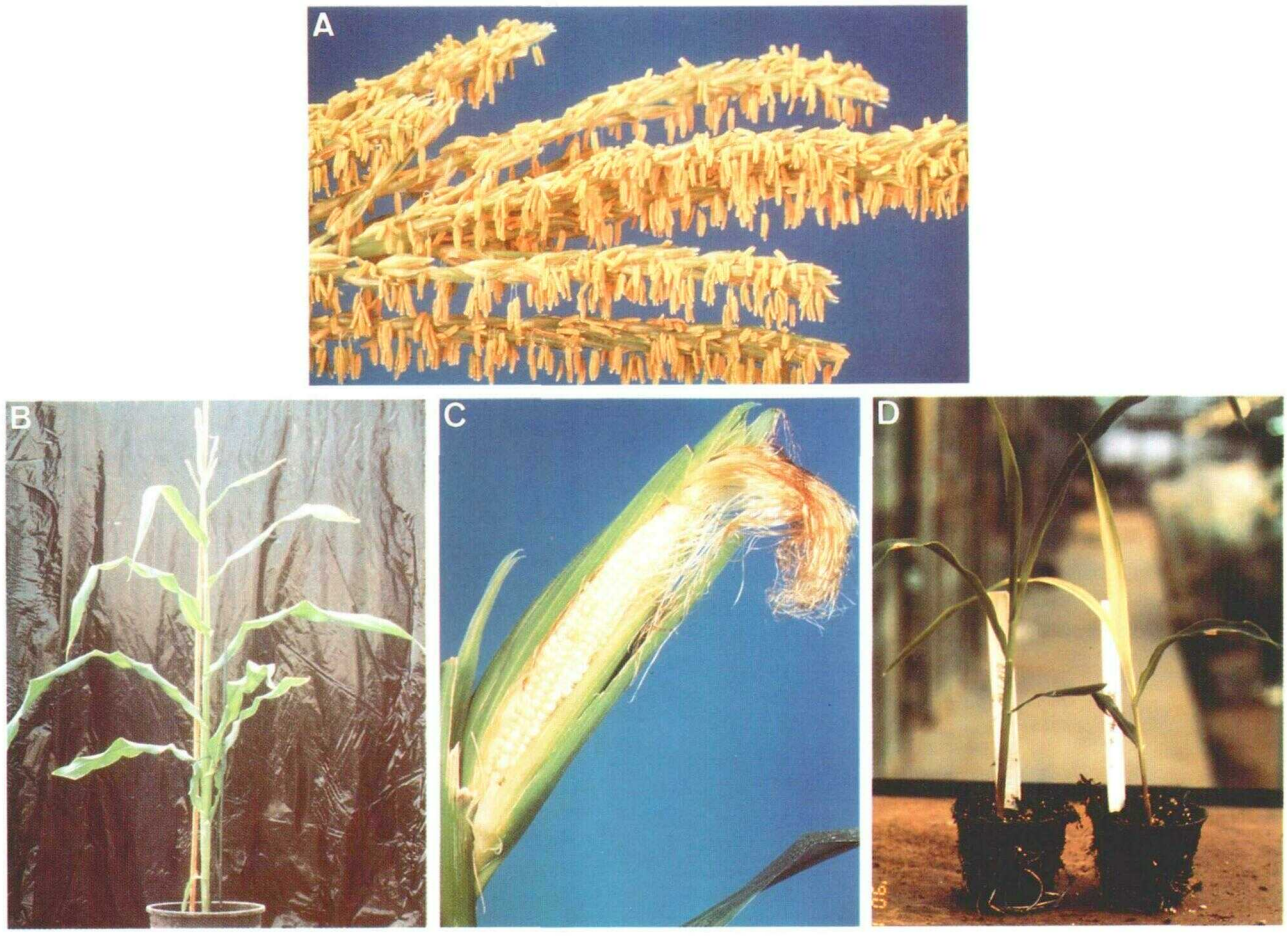

Figure 5. Phenotype of R_0 Plants.

(A) Tassel.
(B) Flowering plant.
(C) Ear.
(D) Kanamycin dot assay of a transgenic plant expressing the *neo* gene (left) and a nontransformed plant (right), 8 days after kanamycin application.

Molecular Analysis of Transgenic Plants

Figure 6 presents a DNA gel blot hybridization analysis of genomic DNA of several primary transformants digested with BamHI and hybridized with a 583-bp NcoI fragment from the *neo* gene. Both relatively simple and complex integration patterns were observed. One copy of the *neo* gene was present in transformants P4, H4, and H3. Transformants P1, H6, and H1 revealed two hybridizing bands and thus contain two copies of the *neo* gene. A 4.5-kb hybridizing fragment is present in all three plant DNAs. This 4.5-kb fragment is most likely generated from two pDE108 plasmid copies inserted in a head-to-tail configuration. The other hybridizing fragment constitutes plasmid–plant junction DNA which is of a different size in each transformant.

The inserted DNA in transformant P1 was analyzed in more detail. Figure 7 shows a DNA gel blot hybridization of BamHI- and EcoRI-digested P1 DNA using the complete pDE108 plasmid as a probe. The BamHI lane now shows three hybridizing bands: a 4.5-kb fragment, which agrees with a head-to-tail junction fragment, and two plasmid–plant junction fragments of 17 and 2.6 kb. The 870-bp fragment that is internal to pDE108 (Figure 1) and which is only visible after long exposure time is not shown on Figure 7. Because EcoRI cleaves pDE108 only once, a head-to-tail configuration would reveal a band with the exact size of pDE108. Such a 5.4-kb fragment is indeed present, together with two plasmid–plant junction fragments of 7.9 and 7.7 kb. The exact linkage of the plasmid copies that had been linearized at the HindIII site prior to DNA delivery was determined. A polymerase chain reaction (PCR) was performed

Table 2. Segregation of NPTII Activity in R_1, R_2, and R_3 Progeny of Transgenic Maize Plants[a]

Plant[b]	NPTII + [c]	NPTII − [c]	P[d]	χ^{2e}
R_1				
Selfing				
P1	2	1		
P2	26	19	0.18	1.77
P3	28	12	0.77	0.06
P4	29	23	0.06	3.44
P5	26	7	0.77	0.08
H5	13	2	0.65	0.21
P6	27	21	0.08	2.95
Male				
P1	47	32	0.26	1.25
P2	20	28	0.54	0.38
P3	31	19	0.31	1.01
P4	32	30	0.99	0.00
P5	9	8	0.99	0.00
H5	19	17	0.99	0.00
P6	22	27	0.84	0.04
H6	36	35	0.86	0.28
Female				
H4	27	15	0.27	1.21
R_2				
Selfing				
HP99 × H6	66	11	0.30	1.09
P1 × P1	32	11	0.99	0.00
H99 × P1	39	9	0.62	0.24
H99 × P2	38	10	0.81	0.06
Ms71 × P4	33	15	0.64	0.20
Male				
Pa91 × H6	23	25	0.99	0.00
H99 × P2	20	26	0.67	1.75
Ms71 × P4	23	25	0.99	0.00
Female				
H99 × H6	20	21	0.83	0.05
Pa91 × H6	26	22	0.83	0.04
H99 × P1	38	42	0.87	0.02
H99 × P2	31	17	0.21	1.53
Ms71 × P4	22	26	0.84	0.04
R_3				
Selfing				
(H99 × H6) × (H99 × H6)	30	4	0.24	1.39
Male				
(H99 × H6) × H99	32	32	0.86	0.03
(H99 × P1) × H99	22	25	0.99	0.00

[a] Transgenic R_0 plants or kanamycin-resistant R_1 or R_2 plants were either selfed or used as male or female parent in crosses with wild-type plants of H99, Pa91, or Ms71.

[b] Nomenclature: H and P, R_0 plants, see Figure 6; (H99 × H6), R_1 plant from the cross using H99 as female and H6 as male; (H99 × H6) × H99, R_2 plant from the cross using (H99 × H6) as female and H99 as male.

[c] With or without NPTII activity.

[d] P = χ^2 probability with 1 degree of freedom.

[e] χ^2 = chi-square values with Yates (continuity) correction.

to amplify the junction fragment carrying the linearization site. Sequence analysis of the amplified fragment proved the head-to-tail configuration. The DNA sequence at the junction revealed that the 5′ protruding nucleotides at the HindIII cleavage site plus one base pair were absent (data not shown).

BamHI-digested DNA of transformant H5 revealed four hybridizing bands when probed with the *neo* gene (Figure 6). The 4.5-kb fragment is again indicative for two plasmid copies in a head-to-tail configuration. The 3.8-kb fragment could be derived from two plasmid copies inserted in a tail-to-tail configuration, because it is twice the size of the 1873-bp BamHI–HindIII fragment of pDE108, which contains the *neo* gene. The two remaining hybridizing fragments probably constitute plasmid–plant junction DNA.

The other transformants P5, P3, P2, P6, and H2 have a complex integration pattern and the copy number could not be precisely ascertained. Intense bands of 4.5 and 3.8 kb are clearly visible, indicating that plasmid concatamers exist in head-to-tail and tail-to-tail configurations. Some of the hybridizing bands are smaller than what would be expected if only intact copies of pDE108 were present. These fragments thus clearly indicate that some plasmid DNA has undergone deletions and/or rearrangements. Transformants P2 and P5 have a nearly identical integration pattern, indicating that they originated from the same transformation event.

The fact that for the majority of transformants the *neo* gene segregated as a single, dominant allele suggests that they contain only one active copy of the *neo* gene, or that copies of the *neo* gene are closely linked. From four transformants with complex integration patterns (P2, P3, P5, and P6), four NPTII-positive and four NPTII-negative R_1 plants were analyzed by DNA gel blot hybridization. NPTII-negative R_1 plants did not hybridize to plasmid pDE108, whereas the complex integration patterns were stably inherited in NPTII-positive R_1 plants (data not shown). These data indicate that the inserted DNA sequences are integrated at the same or closely linked loci. The inheritance of the inserted DNA sequences in transformant P1 was followed over three generations. Figure 7 shows that the kanamycin-resistant progeny of three generations had integration patterns that were indistinguishable from that of the R_0 plant.

DISCUSSION

Cell Competence for Transformation and Regeneration

The establishment of embryogenic, friable, type II cell cultures is no longer a prerequisite for the genetic transformation of maize. All published data suggested that such cell lines were the only source of totipotent cells for genetic transformation. We have shown that DNA can be delivered by electroporation into preconditioned immature zygotic embryos or into type I callus. The preconditioning involves a mild enzymatic treatment of immature embryos or cutting and preplasmolysis of

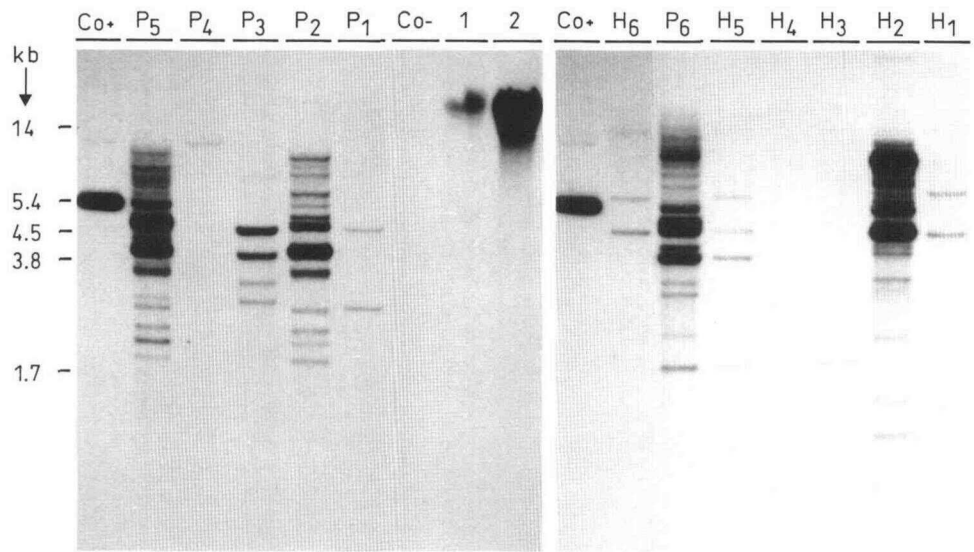

Figure 6. DNA Hybridization Analysis of Maize Transformants.

Genomic DNA was digested with BamHI and hybridized with a 583-bp ^{32}P-labeled NcoI fragment of the *neo* gene. P and H refer to transformants of lines Pa91 and H99, respectively. P5, P4, P3, P2, and P1, transformants from type I callus electroporation; H6, P6, H5, H4, H3, H2, and H1, transformants from immature embryo electroporation; lanes 1 and 2, undigested genomic DNA of H6 and P2, respectively; Co−, nontransformed control of Pa91; Co+, 95 pg (10-copy reconstruction) of plasmid pDE108. DNA linearized with HindIII.

type I callus. It is unclear whether this preconditioning makes transformation-competent cells accessible to the DNA and/or whether it induces competence for DNA uptake in the target cells.

We have shown that stably transformed callus lines can be established upon culturing DNA electroporated tissues on selective media. Stably transformed lines were subcultured as embryogenic calli and plants were regenerated under standard conditions. The transgene was inherited by the progeny as a single, dominant allele. The inserted DNA sequences segregated as single units in the transgenic lines, indicating that these sequences were integrated at the same or closely linked loci.

The number of kanamycin-resistant callus lines from which plants could be regenerated varied considerably from one experiment to the other. The quality of immature embryos was best in spring, probably as a consequence of high light and moderate temperature, whereas high temperature in summer or low-intensity light in winter dramatically reduces the response of immature embryos in transformation experiments.

Do Transgenic Maize Plants Originate from Single Cells?

Histological and ultrastructural examination has shown that the scutellum of maize immature zygotic embryos cultured for 3 days contain a broad subepidermal region on the abaxial surface with meristematic cells, whereas the adaxial scutellum cells enlarge and degenerate (Vasil et al., 1985; Fransz and Schel, 1990). It is not known whether regenerating shoots originate from a single cell within the scutellum. Similarly, it is unclear whether plantlets regenerated from type I callus are derived from a single cell. If regeneration occurs from multicellular structures composed of transformed and nontransformed

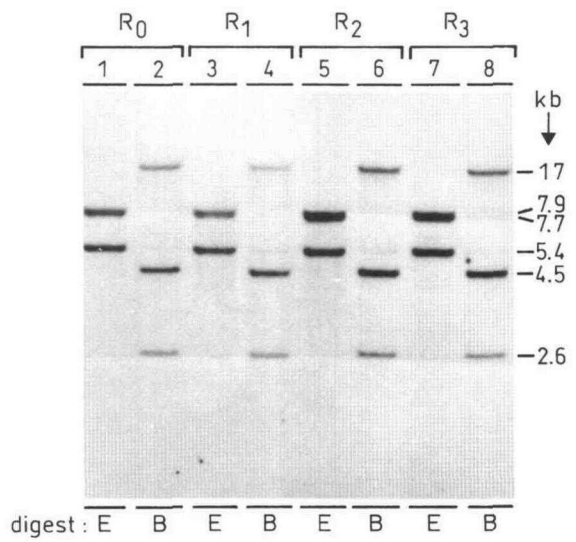

Figure 7. DNA Hybridization Analysis of Primary Transformant P1 (R_0) and Kanamycin-Resistant Plants from the R_1, R_2, and R_3 Generations.

Genomic DNA was digested with BamHI (B) and EcoRI (E) and hybridized with ^{32}P-labeled pDE108 plasmid.

cells, chimeric plants would occur at high frequency. The fact that the *neo* gene was inherited in a Mendelian fashion in progeny of transgenic plants indicates that the primary transformants were not chimeric for the transgene. Thus, our data strongly suggest that single cells in immature embryos or type I callus can be transformed and regenerated into plants. Histological analysis of enzymatically treated immature embryos or mechanically wounded type I callus electroporated with reporter genes, such as the β-glucuronidase gene or regulatory genes controlling anthocyanin biosynthesis, should provide more precise information on which cells from the scutellum of the embryos or from type I callus are susceptible to gene transfer by electroporation.

Advantages of Tissue Electroporation

Electroporation of organized and easily regenerable tissue, such as type I callus or immature embryos, allowed us to generate transgenic maize plants. In rice, immature embryos have also been successfully used as starting material for transformation by electric-discharge particle bombardment (Christou et al., 1991, 1992). Prior to this report, transgenic maize plants had only been obtained by using type II callus–derived cell cultures. These cultures were initiated from immature embryos at least 6 to 12 months prior to the transformation experiment, and transformants often showed phenotypic abnormalities and reduced fertility, most likely as a consequence of the long tissue culture period (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). The method presented here only requires a short tissue culture period; under optimal conditions, rooted transformed plants could be transferred to the greenhouse 3 months after transformation experiments with immature embryos. Fertility problems were not observed and embryo rescue on R_0 plants was not required to recover progeny.

Previous reports on maize transformation have indicated that the tissue culture characteristics of A188, a maize inbred line of no agronomic value, were transferred to important breeding lines by crossing and backcrossing. Many backcrosses were required to obtain agronomically relevant lines with adequate tissue culture capabilities. We focused our transformation experiments on two public inbred lines, H99 and Pa91, both of which are highly regenerable from type I callus cultures (Hodges et al., 1986). As type I callus derived from cultured immature zygotic embryos can be obtained in a wide variety of maize lines, it should be possible to apply the tissue electroporation procedure to a wide variety of maize lines.

METHODS

Plant Material

Maize plants of the public inbred lines H99 and Pa91 were grown in the greenhouse in 20-L pots containing slow-release fertilizer. Growth conditions were at 25°C and 16-hr light of ~20,000 lux (daylight supplemented by sodium vapor and mercury halide lamps); temperature was reduced to 15 to 20°C at night. Immature zygotic embryos (1 to 1.5 mm in length) were excised from ears 10 to 14 days after pollination and plated with their embryonic axis in contact with the substrate. Type I callus was initiated from immature embryos in the dark at 23°C on Mah1VII substrate: N6 medium (Chu et al., 1975) supplemented with 100 mg/L casein hydrolysate, 6 mM L-proline, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1 mg/L 2, 4-D, and 2% sucrose solidified with 1.6 g/L Phytagel (Sigma), and supplemented with 0.75 g/L $MgCl_2$, pH 5.8.

Plasmids

Plasmid pDE108 carries a chimeric cauliflower mosaic virus (CaMV) 35S-*neo*-3′ *ocs* gene (Figure 1) (Denecke et al., 1989). Plasmid DNA was purified on Qiagen (Qiagen Inc.) columns and resuspended in 10 mM Tris-HCl, pH 7.9, and 0.1 mM EDTA at a concentration of 1 mg/mL. The plasmid DNA was linearized at the unique HindIII site prior to electroporation.

Electroporation

Immature Embryos

Excised immature embryos of H99 or Pa91 were enzymatically treated for 1 to 3 min with an enzyme solution containing 0.3% macerozyme (Kinki Yakult, Nishinomiya, Japan) in CPW salts (Frearson et al., 1973) supplemented with 10% mannitol and 5 mM Mes, pH 5.6. The embryos were then carefully washed with a N6aph solution (macro- and microelements of N6 medium supplemented with 6 mM asparagine, 12 mM proline, 1 mg/L thiamin-HCl, 0.5 mg/L nicotinic acid, 100 mg/L casein hydrolysate, 100 mg/L inositol, 30 g/L sucrose, and 54 g/L mannitol). After washing, 100 to 150 embryos were transferred into a disposable microcuvette (1938 PS microcuvettes; Kartell, Binasco, Italy) containing 200 μL maize electroporation buffer (EPM [80 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes, and 0.425 M mannitol, pH 7.2]). Ten or 20 μg of plasmid DNA was added per cuvette and coincubated with the enzyme-treated embryos. After 1 hr, the cuvettes were transferred to an ice bath. After a 10-min incubation on ice, the electroporation was carried out by discharging one pulse with a field strength of 375 V/cm from a 900 μF capacitor. The pulse strength, capacitance, and electroporation apparatus are as described by Dekeyser et al. (1990). Immediately thereafter, 200 to 400 μL of fresh liquid N6aph substrate was added and the cuvettes were incubated for 10 min on ice prior to transfer of the embryos onto selective medium.

Type I Callus

Embryogenic tissue was dissected from developing type I callus of Pa91 that had been cultured on Mah1VII substrate for a period of ~2 months with subculture intervals of 14 to 20 days. The embryogenic tissue was cut in pieces ~1.5-mm thick in EPM buffer without KCl. After ~3 hr of preplasmolysis in this buffer, the callus pieces were transferred to cuvettes containing 200 μL of EPM supplemented with 80 mM KCl. Approximately 150 mg of callus fragments was transferred to each cuvette. Subsequent conditions were as for electroporation of immature embryos.

Selection and Regeneration of Transformants

Immature Embryos

The embryos were transferred immediately after electroporation to selective Mahi1VII substrate (Mah1VII supplemented with 0.2 M mannitol and 200 mg/L kanamycin) and cultured in the dark at 23°C. After ~14 days, the embryos were transferred to Mah1VII substrate (without mannitol) supplemented with 200 mg/L kanamycin. The embryos were further subcultured in the dark on this substrate for 6 to 8 weeks with subculturing intervals of ~3 weeks. For regeneration, the developing embryogenic tissue was isolated and transferred to MS medium (Murashige and Skoog, 1962) supplemented with 5 mg/L 6-benzylaminopurine for line H99 and 5 mg/L zeatin for line Pa91 and cultured at 23°C with a daylength of 16 hr. Fluorescent lamps ("lumilux white" and "natural"; Osram, Munich, Germany) were used with a light intensity of 2000 lux. The embryogenic tissue was maintained on this medium for 10 to 14 days and subsequently transferred to MS medium without hormones and 6% sucrose. Developing shoots were transferred to half-strength MS medium with 1.5% sucrose for further development into plantlets. These plantlets were transferred to soil and grown to maturity in the greenhouse.

Type I Callus

Immediately after electroporation the callus pieces were transferred to Mahi1VII substrate with 200 mg/L kanamycin and cultured in the dark at 23°C. The tissue of one cuvette was plated in random orientation onto one Petri dish of 9-cm diameter. Fourteen days later, the callus pieces were subcultured on the same selective substrate but without mannitol (Mah1VII). The further regeneration protocol was as described for immature embryos.

Neomycin Phosphotransferase II Assays

Gel Assay

Neomycin phosphotransferase II (NPTII) activity was detected by the in situ gel assay according to the method of Reiss et al. (1984).

Dot Assay

An incision was made with a pair of scissors up to the midvein in leaves of ~4-week-old plants, and a 2% kanamycin solution containing 0.2% SDS was applied with a cotton wrap. Plants were assessed 8 to 10 days after kanamycin application.

DNA Gel Blot Hybridization

Total plant DNA was isolated as described by Dellaporta et al. (1983). The DNA was digested, separated by electrophoresis on a 1% agarose gel, transferred to nylon Hybond-N+ membranes (Amersham), and hybridized with ^{32}P-radioactive probes that were labeled as described by Amersham (Megaprime) or Pharmacia (T7 Quick Prime).

Polymerase Chain Reaction

DNA was prepared according to Dellaporta et al. (1983). For polymerase chain reaction (PCR) analysis, 500 ng of DNA was heat denatured at 95°C for 5 min prior to the start of the PCR cycles. The complete PCR mixture contained 5 μL DNA (500 ng); 15 μL H_2O and 30 μL Mastermix (5 μL amplification buffer, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin); 1 μL 10 mM each dATP, dCTP, dGTP, and dTTP; 0.2 μL *Thermus aquaticus* DNA polymerase (5 units per μL); 0.5 μL each primer (10 pmol/μL); and H_2O (added to 30 μL). The primers used were 5′-CAGTGACGACAAATCGTTGGGC-3′ (position on pDE108, 2916→2937) and 5′-AATACGCAAACCGCCTCTCC-3′ (position on pDE108, 3372←3391). The PCR cycle was 1 min at 95°C, 1 min at 53°C, and 2 min at 72°C, for a total of 35 cycles. Sequencing according to Maxam and Gilbert (1980) was performed on an agarose gel–purified 476-bp fragment.

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REFERENCES

Armstrong, C.L., and Green, C.E. (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta **164**, 207–214.

Christou, P., Ford, T.L., and Kofron, M. (1991). Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio./Technol. **9**, 957–962.

Christou, P., Ford, T.L., and Kofron, M. (1992). The development of a variety-independent gene-transfer method for rice. TIBTECH **10**, 239–246.

Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y., and Bi, F.Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. Peking **18**, 659–668.

Dekeyser, R.A., Claes, B., De Rycke, R.M.U., Habets, M.E., Van Montagu, M.C., and Caplan, A.B. (1990). Transient gene expression in intact and organized rice tissues. Plant Cell **2**, 591–602.

Dellaporta, S., Wood, J., and Hicks, J. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1**, 19–21.

Denecke, J., Gosselé, V., Botterman, J., and Cornelissen, M. (1989). Quantitative analysis of transiently expressed genes in plant cells. Methods Mol. Cell. Biol. **1**, 19–27.

Duncan, D.R., Williams, M.E., Zehr, B.E., and Widholm, J.M. (1985). The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta **165**, 322–332.

Fransz, P.F., and Schel, J.H.N. (1990). Cytodifferentiation during the development of friable embryogenic callus of maize (*Zea mays*). Can. J. Bot. **69,** 26–33.

Frearson, E.M., Power, J.B., and Cocking, E.C. (1973). The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol. **33,** 130–137.

Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., and Klein, T.M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio./Technol. **8,** 833–839.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R., Jr., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., and Lemaux, P.G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell **2,** 603–618.

Hodges, T.K., Kamo, K.K., Imbrie, C.W., and Becwar, M.R. (1986). Genotype specificity of somatic embryogenesis and regeneration in maize. Bio./Technol. **4,** 219–223.

Lu, C., Vasil, I.K., and Ozias-Akins, P. (1982). Somatic embryogenesis in *Zea mays*. L. Theor. Appl. Genet. **62,** 109–112.

Lu, C., Vasil, V., and Vasil, I.K. (1983). Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (*Zea mays* L.). Theor. Appl. Genet. **66,** 285–289.

Maxam, A., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65,** 499–560.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Novak, F.J., Dolezelova, M., Nesticky, M., and Piovarci, A. (1983). Somatic embryogenesis and plant regeneration in *Zea mays* L. Maydica **28,** 381–390.

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

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D., and Detmer, J.J. (1988). Genetically transformed maize plants from protoplasts. Science **240,** 204–207.

Tomes, D.T., and Smith, O.S. (1985). The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. Theor. Appl. Genet. **70,** 505–509.

Vasil, V., Vasil, I.K., and Lu, C. (1984). Somatic embryogenesis in long term callus cultures of *Zea mays* L. (Gramineae). Am. J. Bot. **71,** 158–161.

Vasil, V., Lu, C., and Vasil, I.K. (1985). Histology of somatic embryogenesis in cultured immature embryos of maize (*Zea mays* L.). Protoplasma **127,** 1–8.

Walters, D.A., Vetsch, C.S., Potts, D.E., and Lundquist R.C. (1992). Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. Plant Mol. Biol. **18,** 189–200.