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Fertile transgenic Indica rice plants obtained by electroporation of the seed embryo cells

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Abstract. We have obtained fertile transgenic plants of Indica rice variety IR36, by using electroporation to transfer the neomycin phosphotransferase II (*nptII*) gene into cells of mature embryos. Resistant calli were selected in the presence of 30 μg/ml G418. Nearly thirty transgenic plants were regenerated within three months after transformation. Many of them yielded seeds following self-pollination. Data from molecular analysis and enzyme assay proved that the foreign gene was stably integrated into the genome of resistant calli, R0 and R1 plants, and also expressed. Mendelian segregation of the *nptII* gene was observed in R1 progeny plants.

Abbreviations: NOS, nopaline synthase; NPTII and *nptII*, neomycin phosphotransferase II; OCS, octopine synthase; Km, kanamycin.

Introduction

The recalcitrance of cereal crops to *Agrobacterium*-mediated transformation has led to the development of various novel gene transfer methods (Potrykus 1990). The first reports on the recovery of transgenic cereal plants involved the use of direct DNA delivery methods such as electroporation (Rhodes *et al.* 1988; Toriyama *et al.* 1988; Zhang *et al.* 1988; Shimamoto *et al.* 1989) and PEG-mediated (Zhang and Wu 1988; Datta *et al.* 1990) gene transfer to protoplasts. However, the protoplast-to-plant system is strongly dependent on genotype. Most elite cereal varieties are very difficult to regenerate from protoplasts. Futhermore, the protoplast culture procedure is time-consuming and labor-intensive. In the past few years, variety-independent transformation methods of delivering DNA into intact cells via both electroporation (Morikawa *et al.* 1986; Dekeyser *et al.* 1990), which has been widely used to transform protoplasts of dicotyledons and monocotyledons since 1985 (Fromm *et al.* 1985; Langridge *et al.* 1985), and microprojectile bombardment (Klein *et al.* 1987; Klein *et al.* 1988) have been developed and applied to cereal transformation. Transgenic plants of important cereal crops such as rice, maize and wheat have been recovered by means of DNA-coated microprojectiles to transform embryogenic suspension cultures and calli of maize (Gordon-Kamm *et al.* 1990; Fromm *et al.* 1990), embryogenic calli of wheat (Vasil *et al.* 1992) and immature embryos of rice (Christou *et al.* 1991). Recently, transgenic maize plants by electroporation of immature embryos and type I calli was also reported (D'Halluin *et al.* 1992). In our laboratory, foreign genes have been successfully introduced into cells of rice mature embryo through electroporation, and transgenic plantlets from elite local rice cultivars including both Indica variety Sanerai and Japonica variety Nonghu No. 6 were obtained (Li *et al.* 1991).

Here we report the stable transformation of cells of mature embryo by electroporation, with subsequent recovery of fertile transgenic plants from Indica variety IR36.

Materials and methods

Plant material The seeds of the Indica rice (*Oryza sativa* L.) variety IR36, were kindly supplied by Zhongkai Agriculture Technology Institute, Guangzhou, China.

Plasmid The plasmid, pLGVneo2103, contains the chimeric gene with the NOS promoter, the *nptII* gene coding sequence which confers resistance to Km and G418, and a OCS polyadenylation sequence (Hain *et al.* 1985). Plasmid DNA was isolated and purified as previously described (Li *et al.* 1991).

Electroporator The High Performance Electroporation System-3 (HPES-3), designed and made in our laboratory (Patent: 91105038.8 (91.7.21); Int. Cl. C12M1/42, C12M15/02), was used in this experiment. Two kinds of output mode, both long-pulse and short-pulse, are set inside the device. It has six adjustable parameters, including voltage of pulse (Vp: 100-10,000V), number of pulses (Np: 1-2048, increase by 12 degrees), duration of pulse (Td: 1-200 μs, fixable on 62 μs), rest time of pulse (Tr: 0.125-256 s, increase by 12 degrees), number of cycles (Nc: 1-98, 99 equals limitless) and distance between the anode and sample mixture surface (H: 0-10 mm).

Electroporation Dehusked rice seeds were sterilized with 0.5% paracetic acid for 15 min, subsquently rinsed repeatedly with distilled water. They were then placed on MS proliferation medium (Li *et al.* 1991). Thirty-six hours later, the coleoptiles appeared through the broken pericarps. The embryos were separated, and cut into two longitudinal sections (half-embryos). The half-embryos were then put into the electroporation chamber. To every 30 half-embryos was added 100 μl Hepes buffer (Li *et al.* 1991) containing 20 μg/ml pLGVneo2103 and 50 μg/ml calf thymus DNA. Electroporation was carried out under the following condition: long-pulse output, Vp = 10 kV, Np = 2^7-2^9, Td = 60-80 μs, Tr = 1-4 s, Nc = 10-30, h = 5 mm. As control, half-embryos were also electroporated in the absence of plasmid DNA.

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Table 1. Recovery of fertile transgenic IR36 plants from half-embroys electroporated with pLGVneo2103

Exp.	No. of half-embryos electroporated	No. of half-embryos with G418^R callus	No. of calli tested for regeneration	No. of plantlets regenerated	No. of R0 plants reaching maturity	No. of R0 plants with *nptII* gene	No. of R0 plants with NPTII activity	No. of fertile transgenic R0 plants
1	150	44	20	14	12	10	10	7
2	150	41	20	17	15	11	8	6
3	150	36	20	9	8	8	5	3
Total	450	121	60	40	35	29	23	16

Selection of transformed calli and plant regeneration The electroporated half-embryos were left on MS proliferation medium for 2 days, and then incubated on the same medium containing 30 μg/ml G418 (Sigma). They were transferred to fresh selection medium two weeks later. After approximately 4 weeks of selection in the dark, the G418-resistant calli initiated from half-embroys were transferred onto N6 regeneration medium (Li *et al.* 1991) without antibiotic. Regeneration was performed under fluorescent illumination for 10 h photoperiod. The regenerated plantlets were hardened off when 8-10 cm high, subsequently transferred to soil in pots. In the greenhouse the plants grew to maturity and set seed.

Progeny test for resistance to Km Dehulled seeds harvested from self pollinated transgenic plants were washed extensively with water, then placed on filter paper drenched with water for 36 h. Viable seeds were picked out and subsequently germinated in the nutrient solution (Feldmann and Marks 1987) containing 50 μg/ml Km (Sigma). Four days later, seedlings were cultured in sand with Km-free nutrient solution. After 7 days, the numbers of green and white seedlings were counted. Green seedlings were transferred to soil.

Southern blot and dot blot analysis Genomic DNA was isolated according to Paszkowski *et al.* (1984). DNA analyses were done as described by Sambrook *et al.* (1989). For Southern blot analysis, approximately 5 μg of DNA was undigested or digested with restriction endonucleases, electrophoresed through 0.8% agarose and transferred to nylon membrane. For dot blot analysis, undigested DNA samples were spotted onto nylon membranes in 5 μl aliquots (approximately 2 μg DNA). The probe was a ^{32}P-labeled 1.6 kb PstI internal fragment of the *nptII* gene from pLGVneo2103.

NPTII enzyme assay NPTII activity was determined by the dot assay as reported previously (Wang and Li 1989). Crude extracts of protein were prepared as follows: Callus or leaf tissue (100-200 mg) was ground in equivalent amounts w/v(100-200 μl) of extraction buffer. As positive control, *E. coli* strain HB101 containing pLGVneo2103 was sonicated for 30 s in bacterial extraction buffer. Following centrifugation, the supernatants were assayed for NPTII activity. Protein content was estimated using dye binding method of Bradford (1976). Dot radioactivity was determined by scintillation counting.

Results

Selection and analysis of transformed calli

Half-embryos were electroporated with pLGVneo2103 DNA. After 2 days on non-selective MS proliferation medium, the electroporated half-embryos were transferred to the same medium supplemented with 30 μg/ml G418. One or two weeks later, calli appeared at the wound positions of approximately 27% of half-embryos. After subculture for 2-3 weeks, the calli proliferated to reach 2-4 mm in diameter. At least 25% of the calli was embryogenic (Fig. 1B). Meanwhile, averagely 31% of half-embryos turned pale white and died, because of the damage caused by electrical shock. Besides, the rest of half-embryos turned brown and failed to survive on the selective medium (Fig. 1A, right). The results of three independent experiments are summarized in Table 1. No resistant calli developed in the control samples electroporated without plasmid (Fig. 1A, left).

G418-resistant calli were propagated on non-selective MS medium for subsequent analysis, or transferred to N6 medium for regeneration.

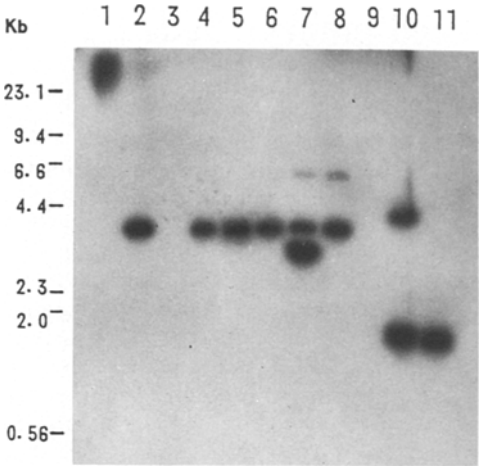

Figure 2. Southern hybridization analysis of one G418-resistant rice callus line (C_1, C_2, C_3) from each of three experiments. The probe was a ^{32}P-labeled 1.6 kb PstI fragment of pLGVneo2103. Lane 1, undigested DNA from six-month-old callus line C_1. Lane 2, pLGVneo2103 digested with EcoRI and HindIII as copy number standard, corresponding to one copy per diploid rice genome. Lanes 3, 9, DNA from non-transformed callus digested with EcoRI/HindIII (lane 3) and PstI (lane 9), respectively. Lanes 4-6, EcoRI/HindIII double-digested DNA from callus line C_1 at two (lane 4), four (lane 5) and six (lane 6) months after transformation. Lanes 7, 8, EcoRI/HindIII digested DNA from six-month-old callus line C_2 (lane 7) and C_3 (lane 8). Lanes 10, 11, PstI digested DNA from six-month-old callus line C_2 (lane 10) and C_3 (lane 11), respectively.

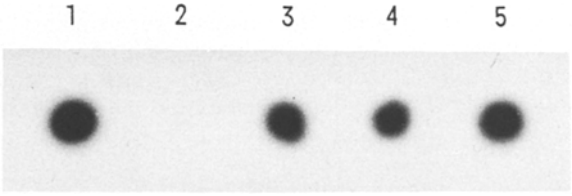

Figure 3. NPTII enzyme dot assay in transgenic rice callus lines. Dot 1 was bacterial extract form *E. coli* HB101 as positive control. Dot 2 was extract form non-transformed callus as negative control. Dots 3-5 represent extracts of transgenic callus lines C_1, C_2 and C_3 from three experiments.

Stable transformation of G418-resistant callus was confirmed by Southern analysis. Genomic DNA isolated from one G418-resistant callus line from each of the three experiments, was undigested or digested. The undigested DNA hybridized with the ^{32}P-labeled 1.6 kb

PstI fragment from pLGVneo2103 gave a signal only in the high molecular weight region, indicating the integration of *nptII* gene into chromosomal DNA (Fig. 2, lane 1). After restriction digestion with PstI, the expected 1.6 kb band corresponding to the *nptII* gene coding sequence of pLGVneo2103 was observed (Fig. 2, lanes 10,11). Double-digestion with EcoRI and HindIII, generated the 3.3 kb hybridization fragment corresponding to the *nptII* chimeric gene of pLGVneo2103 in all the three callus lines (Fig. 2, lanes 4-8). Thus, in these three G418-resistant callus lines, at least one copy of the introduced *nptII* gene was integrated into rice genomic DNA. DNA isolated from non-transformed callus did not show any hybridization signal with the probe (Fig. 2, lanes 3, 9). The above-mentioned G418-resistant callus lines were analysed for the expression of *nptII* gene by NPTII enzyme dot assay. Protein extracts from these three callus lines showed NPTII activity. Some differences were seen in the levels of NPTII activity between different lines (Fig. 3, dots 3-5). Protein extract from control callus did not show NPTII activity (Fig. 3, dot 2). Therefore, the foreign *nptII* gene has been stably both integrated and expressed in G418-resistant callus.

Regeneration and analysis of R0 plants

Compact embryogenic G418-resistant calli were transferred to N6 regeneration medium under light. Approximately two weeks later, the first somatic embryos were visible, subsequently developed shoots and roots (Fig. 1C). After three weeks, plantlets reached 8-10cm in height (Fig. 1D). A total of 40 plantlets were regenerated from three experiments. Thirty-five of these plants survived transfer to soil and grew to maturity in the greenhouse (Fig. 1E). The phenotypic characteristics of these R0 plants were similar to those of the control plants regenerated from non-transformed calli or derived from seeds.

Genomic DNA from all R0 mature plants was digested with EcoRI and HindIII, and subjected to Southern blot analysis. Twenty-nine of the thirty-five plants contained sequences that hybridized to the *nptII* gene probe, indicating stable integration of this foreign gene into the rice genome (Fig. 4A). Transgenic plant R0-17 was chosen to determine the integration pattern of *nptII* gene in detail. Hybridization of undigested DNA with the probe in the high molecular weight fragment demonstrated integration of the *nptII* gene into genomic DNA (Fig. 4B, lane 1). When cut with PstI, the 1.6kb hybridization band together with two other bands (One band was close to another band.) was observed (Fig. 4B, lane 3). Digestion with EcoRI, which cut once within pLGVneo2103, produced a faint band the size of the plasmid (7.6kb). Four additional bands were also found (Fig. 4B, lane 5). Digestion with EcoRI and HindIII, resulted in the expected 3.3kb band and another band (Fig. 4B, lane 7). Southern blot analysis of the R0-17 plant indicated that several copies of the *nptII* gene were integrated at multiple loci of the genome of this rice plant. There was no hybridization to control material (Fig.

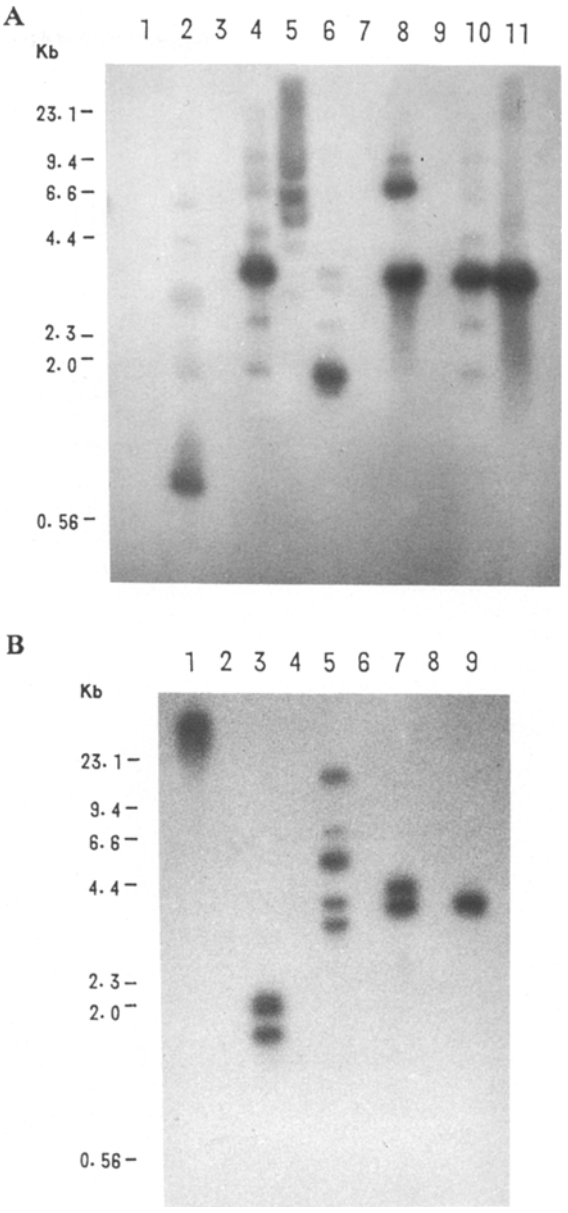

Figure 4. Southern blot analysis of R0 rice plants regenerated from G418-resistant calli, hybridized to a ^{32}P-labeled probe of the *nptII* gene from pLGVneo2103. (A) Genomic DNA from ten R0 plants (lanes 2-11) and a non-transformed plant (lane 1) was digested with EcoRI and HindIII; (B) Genomic DNA from transgenic plants R0-17 (lanes 1,3, 5,7) and control plant (lanes 2,4,6,8) was undigested (lanes 1,2) or digested with PstI (lanes 3,4), EcoRI (lanes 5,6) and EcoRI/HindIII (lanes 7,8), respectively. Copy number reconstruction using EcoRI/HindIII digested pLGVneo2103 DNA corresponds to one copy per diploid rice genome (lane 9).

4A, lane 1; Fig. 4B, lanes 2,4,6,8). Protein extracts from leaves of each of 29 transgenic plants were examined for NPTII activity. Twenty-three plants had NPTII activity. Activity levels varied extensively among these plants, and a very low level of background activity was also detected in the control (Fig. 5, Table 2). Therefore, of 35 R0 plants reaching maturity, 23 R0 plants not only contained the exogenous *nptII* gene, but also expressed it.

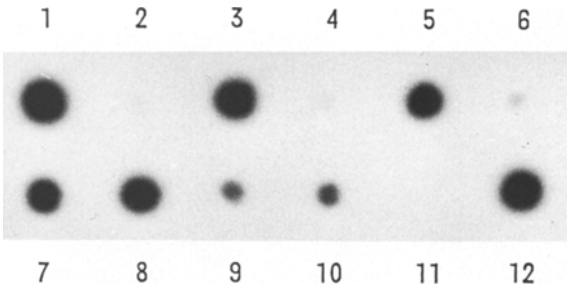

Figure 5. NPTII enzyme dot assay of leaf tissue extracts from transgenic rice R0 plants. Dot 1, *E. coli* HB101 as positive control. Dot 2, non-transformed plant as negative control. Dots 3-12, transgenic plants R0-1 to R0-10.

Table 2. NPTII activity in transgenic rice R0 plants and control

Samples	NPTII activity (cpm/μg)
Controls	
E. coli HB101	2807.5
Non-transformed plant	0.3
Transformed plants	
R0-1	1942.6
R0-2	0.5
R0-3	1215.2
R0-4	37.1
R0-5	962.7
R0-6	1554.3
R0-7	218.9
R0-8	306.4
R0-9	0.2
R0-10	2135.0

Sixteen fertile transgenic plants yielded seeds following self-pollination (Fig. 1F).

Analysis of progeny (R1 plants)

Germinating seeds derived from 12 transgenic R0 plants which contained NPTII activity were tested for Km resistance. Part of the results of the segregation of Km resistance are presented in Table 3, which show that the ratio of resistant to sensitive progeny is consistant with a 3:1 Mendelian segregation ratio (Fig. 1G). Resistant green seedlings were transferred to soil. All of these R1 plants reached maturity in the field and produced normal amounts of seeds.

Table 3. Transmission of Km resistance to the rice progeny

Progeny of	No. of seeds tested	No. of resistant seeds	No. of sensitive seeds	χ^2
R0-1	56	41	15	0.095
R0-6	63	47	16	0.005
R0-14	42	33	9	0.286
R0-19	50	37	13	0.027
R0-25	46	35	11	0.029

χ^2 tests indicate good agreement with segregation ratios of 3:1.

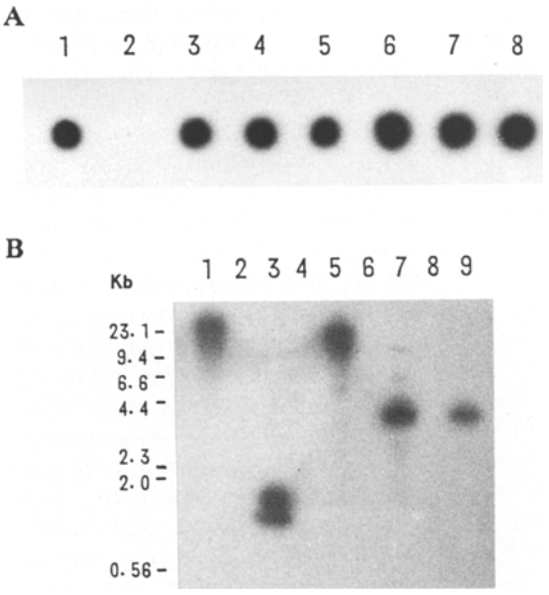

Figure 6. Molecular analysis of R1 rice plants, hybridized with a ^{32}P-labeled *nptII* gene fragment from pLGVneo2103. (A) Dot blot analysis of undigested DNA from six Km-resistant R1 plants and a control. Dots 3-5, three R1 plants derived from the transgenic plant R0-1. Dots 6-8, three R1 plants derived from the transgenic plant R0-3. (B) Southern blot analysis of a transgenic R1 plant (lanes 1,3,5,7) and non-transformed plant (lanes 2,4,6,8). Genomic DNA was undigested (lanes 1,2) or digested with PstI (lanes 3,4), EcoRI (lanes 5,6) and EcoRI/HindIII (lanes 7,8), respectively. Lane 9 was pLGVneo2103 digested with EcoRI and HindIII, corresponding to one copy per diploid rice genome.

Three R1 resistant plants derived from each of 12 R0 plants were analysed by dot blotting for the presence of *nptII* gene. Undigested DNA from all of 36 R1 plants hybridized to the probe (Fig. 6A). One R1 plant was further analysed by Southern blotting to confirm integration of the foreign *nptII* gene into the genome. Uncut DNA showed hybridization only in the high molecular weight DNA (Fig. 6B, lane 1). Digestion with PstI generated the 1.6 kb fragment (Fig. 6B, lane 3). Digestion with EcoRI resulted in hybridization signals mainly larger than 7.6 kb, likely due to incomplete digestion (Fig. 6B, lane 5). When cut with EcoRI and HindIII, the predicted 3.3kb band was observed (Fig. 6B, lane 7). In contrast DNA from control plant failed to hybridize to the probe (Fig. 6A, dot 2; Fig. 6B, lanes 2,4,6,8). NPTII activity was detected in all of

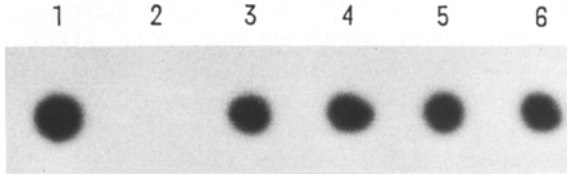

Figure 7. Dot assay for NPTII activity in leaf extracts from transgenic rice R1 plants. Dot 1, *E. coli* HB101. Dot 2, non-transformed plant. Dot 3, transgenic plant R0-1. Dots 4-6, three transgenic R1 plants derived from R0-1.

36 R1 resistant plants, the activity levels were comparable with levels in R0 plants from which the R1 plants derived (Fig. 7, dots 3-6). Leaf extract from control plant did not show NPTII activity (Fig. 7, dot 2). These results demonstrate the stable transmission and expression of the introduced *nptII* gene among transformant progeny.

Discussion

Direct DNA delivery based on the protoplast recipient system is now employed routinely to produce transgenic plants of cereals (Davey *et al.* 1991). Nevertheless, the reproducible plant regeneration procedure from protoplasts was established only for a minority of varieties. IR36 is an elite Indica rice cultivar of global importance, from which recovery of plants regenerated from protoplasts has not yet been reported. We have studied the tissue culture of thirty-two rice cultivars (including Indica and Japonica varieties). The results demonstrated that almost all rice cultivars could be regenerated from mature embryo cells through somatic embryogenesis even though the frequencies of embryogenic callus formation and regeneration varied among cultivars (unpublished data). Therefore, we have attempted to use the cells of mature embryo as new recipient system for transformation. Following successful introduction of foreign genes into elite local cultivars including Indica variety Sanerai and Japonica variety Nonghu No. 6 (Li *et al.* 1991), in this paper we showed the recovery of transgenic plants and their progeny of IR36, suggesting that this transformation protocol is reproducible.

Among various genotype-independent gene transfer techniques developed lately, microprojectile bombardment is a relatively effective method. Transgenic cereal plants were recovered using microprojectiles to transfer genes into cells of embryogenic suspension culture and callus of maize (Gordon-Kamm *et al.* 1990; Fromm *et al.* 1990), embryogenic cells of wheat (Vasil *et al.* 1992). However, the long periods of time required for the establishment of embryogenic callus and suspension culture led to phenotypic abnormalities of regenerated plants, and reduced their fertility. The above-mentioned transgenic R0 plants from maize and wheat failed to produce seeds from self pollination. Nonetheless, fertility problems were not observed after microprojectile bombardment with immature embryos of rice (Christou *et al.* 1991). Comparably, with electroporation of less culture-dependent tissue such as maize immature embryos or type I calli (D'Halluin *et al.* 1992), and rice mature embryos reported here, transgenic rice and maize plants could be obtained through a simple culture protocol within a short period of time, subsequently yielding selfed seeds. Therefore, tissue electroporation is a simple and promising alternative for cereal transformation. Moreover, our procedure offers the major adventage that it does not depend on the availability of flowering plants, because of using mature embryos instead of immature embryos as recipient material for transformation.

Molecular analysis of R0 plants regenerated from G418-resistant calli demonstrated that not all plants were transformed, only 29 of 35 mature R0 plants contained the *nptII* gene from pLGVeno2103. We had shown that the rice plant regeneration from Km-resistant callus originated from single cell (Li *et al.* 1991). Therefore, it is possible that part of G418-resistant calli from electroporated half-embryos were chimera with transformed and non-transformed cells. Owing to protection by proximate transformed cells on the same explant, non-transformed cells could survive and proliferate under selective pressure, and subsequently develop to somatic embryos and plants. The same phenomenon has been described by other authors (Fromm *et al.* 1990; Christou *et al.* 1991). Fromm *et al.* (1990) solved the problem of non-transgenic maize plants regenerated from chimeric transgenic calli by using selection during regeneration. However, we had reported earlier that at the stages of somatic embryogenesis and plantlet regeneration from transformed rice calli, the removal of selective agent is necessary. Considering the possibility that some R0 plants might carry but not express the *nptII* gene, kanamycin was not involved in the plantlet growth stage in this paper, in contrast to our previous experiments (Li *et al.* 1991). Differing from the results of Fromm *et al.* (1990), most of R0 plants regenerated from G418-resistant calli in this experiment contained the foreign *nptII* gene. This indicated that G418 selection at the callus proliferation stage was quite efficient.

Acknowledgments

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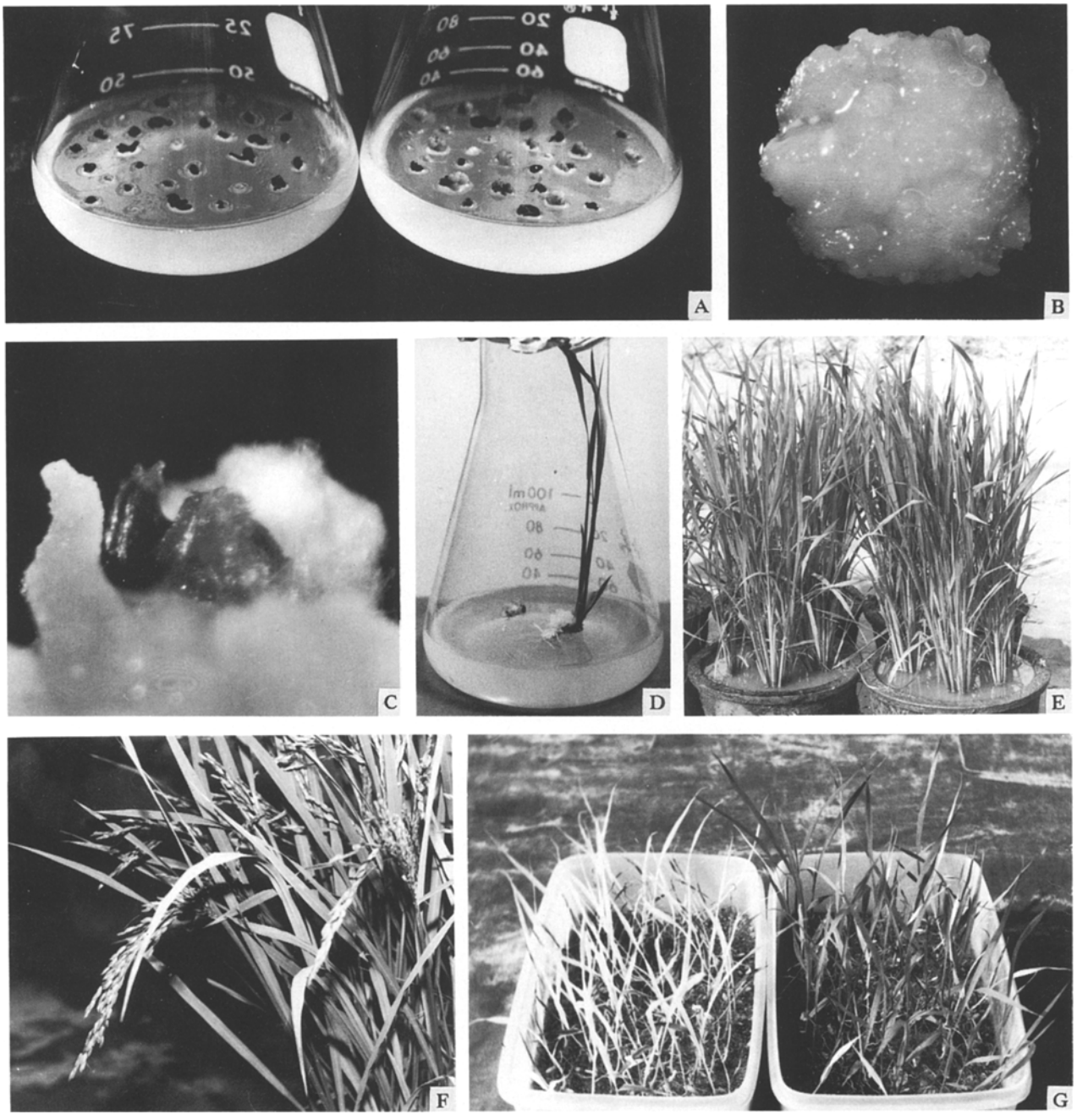

Figure 1. Production of transgenic Indica rice (IR36) plants and their progeny. (A) Electroporated half-embryos on MS proliferation medium supplemented with 30μg/ml G418, showing G418-resistant callus proliferation from cells of mature embryo electroporated with pLGVneo2103 (right) and not in control (left); (B) Embryogenic G418-resistant callus; (C) shoot and root developing from transformed embryogenic callus; (D) Transgenic plantlets regenerated from transformed callus; (E) Mature R0 plants; (F) Fertile transgenic R0 plants that set selfed-seeds; (G) Test of Km resistance in progeny, showing a approximate 3:1 segregation of the *nptII* gene in R1 seedlings derived from transgenic R0 plant (right) and not in control (left).