TRANSGENIC RICE PLANTS AFTER DIRECT GENE TRANSFER INTO PROTOPLASTS

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We have regenerated whole plants of rice (*Oryza sativa* L.) derived from protoplasts, which had been electroporated with plasmid DNA possessing a chimeric gene encoding aminoglycoside phosphotransferase II (APH(3′)II). Transformed calli were selected on the basis of tolerance to the antibiotic, G418. Several plants were regenerated possessing functional APH(3′)II activity due to the integration of intact foreign DNA into their genome.

Direct DNA transformation into cereal protoplasts using polyethylene glycol treatment or electroporation has been reported for wheat[1], Italian ryegrass[2], maize[3], *Panicum* sp.[4] and rice[5]. The system for plant regeneration from rice protoplasts has been extensively studied in several laboratories[6–10]. The combination of these two methods provided us with the means to regenerate transgenic rice plants.

RESULTS AND DISCUSSION

Rice protoplasts isolated from suspension cells were electroporated in the presence of unlinked circular-form plasmids, pCN (5.4 kb), and pBI221 (5.6 kb). pCN contains the cauliflower mosaic virus (CaMV) 35S promoter and an aminoglycoside phosphotransferase II gene (APH(3′)II), which confers resistance to the antibiotics, kanamycin and G418 (Fig. 1). The plasmid pBI221, consisting of pUC19, the CaMV 35S promoter, the *E. coli* β-glucuronidase (GUS) gene[11] and the terminator of the nopaline synthase gene, is the product of Clontech Lab. Inc. (CA, USA), and was used for determining effective conditions of electroporation by monitoring transient expression of the GUS gene[11].

The plating efficiency of electroporated protoplasts was about 0.5% after three weeks. The dividing cells were selected by incubation in G418 (20 μg/ml)-containing medium for 2 weeks, and a transformation frequency of 4×10^{-6} (based on the number of initial cells) was obtained. No G418-resistant colonies formed in the control. Calli larger than 1 mm in diameter were transferred to regeneration medium (details in *Experimental Protocol*) for 1–2 months. Of nineteen G418-resistant independent colonies, five plants were regenerated. These plants were neither albino, nor bleached in the presence of 50 μg/ml kanamycin sulphate (Fig. 2). APH(3′)II activity was detected in the leaf extracts of transformed plants, whereas no such activity was seen in the control, excluding possible endophytic contaminants (Fig. 3).

The use of G418 instead of kanamycin is more effective for selecting transformants in rice, because many calli unable to regenerate green plants were observed after selection by kanamycin (unpublished results). G418 com-

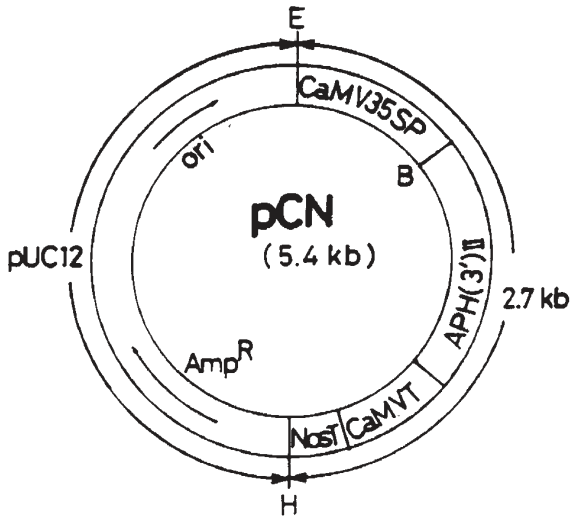

FIGURE 1 Physical map of pCN. CaMV 35SP: 35S promoter of cauliflower mosaic virus[21]. APH(3′)II: aminoglycoside phosphotransferase II structural gene from Tn5[22]. CaMVT: terminator of CaMV[21]. NosT: terminator of nopaline synthase[23]. Amp^R: ampicillin resistant gene. Ori: origin of replication. B: BamHI. E: EcoRI. H: HindIII.

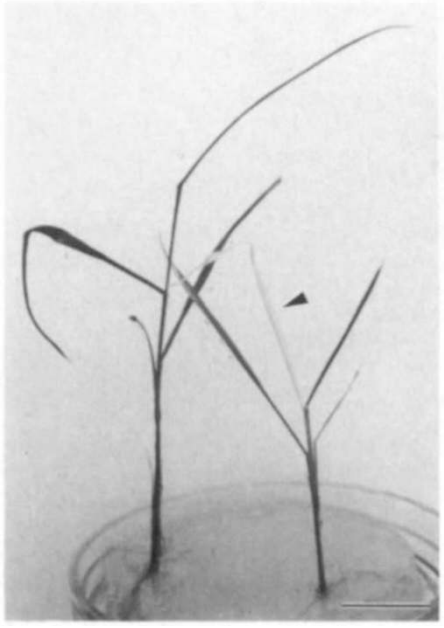

FIGURE 2 A transgenic plant of rice regenerated from G418-resistant callus after electroporation of plasmid DNA into protoplasts (left), and a control plant (right). Plantlets were placed in B5 medium[16] supplemented with 50 μg/ml kanamycin sulphate for 3 weeks. The transgenic plant developed green leaves, demonstrating the plant's resistance to kanamycin; a newly developed leaf of the control plant was bleached by the kanamycin sulphate (an arrow). Scale: 2 cm.

pletely inhibited the growth of normal cells. GUS activity was not detected in these five transgenic plants, probably due to unlinked incorporation of pCN and pBI221 into the rice genome. In this respect, linearized plasmids need to be used as reported for tobacco[12].

Southern blot analysis of DNA from the transgenic plants expressing APH(3′)II activity was performed using the chimeric APH(3′)II gene as a probe (Fig. 4). The probe hybridized to a 2.7 kb fragment of EcoRI/HindIII-digested DNA, which corresponds to the intact chimeric APH(3′)II gene of pCN (Fig. 1). Other bands seem to be fragments of a chimeric gene integrated into host DNA. A single or a few copy of DNA were considered to be inserted into the host genome of each plant. Observation of chromosome number in root tips of each plant showed one diploid, three triploid and one unidentified (data not shown). Genetic tests need to be done to examine the manner of the foreign gene integration and inheritance.

We have demonstrated that the appropriate protoplast culture system in combination with a standardized DNA-delivery method are sufficient to obtain transgenic rice plants. Most recently, regeneration of transgenic plants from maize protoplasts by electroporation has been reported[13]. Thus at present, two major monocot crop transformations seem to be feasible. We are now applying this system to the molecular genetic manipulation of rice plants.

EXPERIMENTAL PROTOCOL

Plasmid construction. BamHI and BglII fragments of pH1K1[14] containing APH(3′)II and CaMVT were inserted into a promoter terminator cassette CaMV35SP/NosT of pUC12.

DNA transfer and selection of transformants. Protoplasts were isolated from anther-derived cell suspensions of rice (*Oryza sativa* L. cv. Yamahoushi)[15], and electroporated according to Fromm et al.[3], with some modification, as follows. Protoplasts (2×10^5) and circular-form plasmid DNAs (10 μg each) were suspended in 0.6 ml of a buffer consisting of 0.5 mM 2-[N-Morpholino]ethanesulfonic acid (pH5.8), 7 mM KCl, 4 mM $CaCl_2 \cdot 2H_2O$ and 6.5% mannitol in a plastic cubette (inter-electrode distance was 0.4 cm). An electrical pulse was delivered either from a 22 μF capacitor charged at 750 v/cm (hand-made product) or from a 125 μF capacitor charged at 500 v/cm (Gene-Pulser, Bio-Rad, CA, USA). The resistance-capacitance (RC) time-constants were 4 msec and 20 msec, respectively. After 10 min at 4°C, followed by 10 min at room temperature, electroporated-protoplasts were transferred to a petri-dish (5 cm in diameter) containing 2.5 ml B5 medium[16] supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 5% mannitol. After 2 weeks, 1 ml NO_3 medium (B5 medium without ammonium sulphate) supplemented with 2 mg/l 2,4-D and 3% glucose was added. After 3 weeks, the medium was replaced by NO_3 medium lacking glucose, and containing 2 μg/ml G418 sulphate (Schering Co., NJ, USA). One month after electroporation, surviving microcalli were transferred to NO_3 medium containing 20 μg/ml G418 sulphate and 1% agarose (Sigma type I). After another 2 weeks, growing calli were transferred onto N6 medium[17] containing 0.2 mg/l indole-3-acetic acid, 1 mg/l kinetin and 1% agarose (regeneration medium).

APH(3′)II assay. Leaves (10–20 mg) were homogenized in 50 μl buffer and 30 μl of each extract was electrophoresed through a polyacrylamide gel and assayed for APH(3′)II activity by the method of Schreier et al.[18]

Analysis of DNA. DNA was prepared from leaves according to

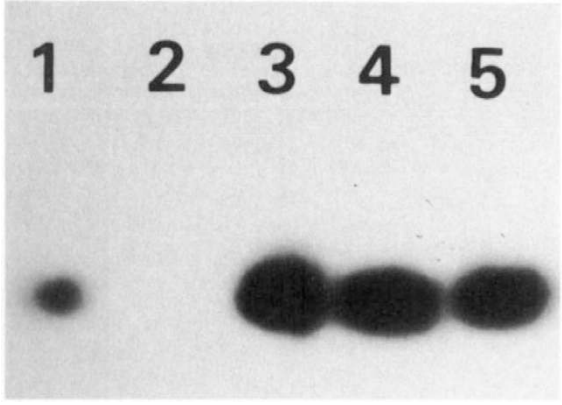

FIGURE 3 APH(3′)II in the leaves of regenerated plants. Lane 1: Transformed callus of rice (T16)[24], served as a positive control for APH(3′)II activity. Lane 2: A plant regenerated from protoplasts without electroporation, showing no APH(3′)II activity. Lanes 3–5: Transgenic plants obtained by electroporation, showing APH(3′)II activity.

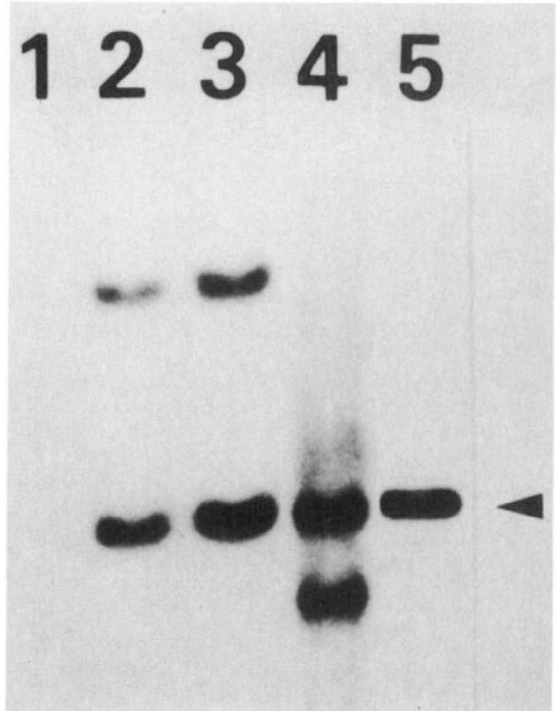

FIGURE 4 Southern blot hybridization of DNA prepared from leaves of normal and transgenic plants. The EcoRI/BglII fragment of pH1K1[14] was used as a probe for APH(3′)II gene. Lane 1: DNA of a control plant, which did not hybridize to the probe DNA. Lanes 2–4: DNA from each transgenic plant. Lane 5: One copy reconstruction of the intact chimeric APH(3′)II gene (2.7 kb) of pCN (Fig. 1) digested with EcoRI and HindIII (an arrow). Lambda DNA digested with HindIII was used for size estimations.

Rogers and Bendich[19]. DNA (4 μg) was digested with EcoRI and HindIII, electrophoresed, blotted onto a nitrocellulose filter, and then hybridized with multi-translated (Amersham) probe DNA[20].

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