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 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 spfF, neomycin phosphotransferase II; OCS, octopine synthase; Km, kanamycin.

Introduction

The recalcitrance of cereal crops to Agrobacterium-mediating 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. Furthermore, 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 microparticle 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, pLVGneo2103, 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).

Electroporation 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 modes, both long-pulse and short-pulse, are set inside the device. It has six adjustable parameters, including voltage of pulse (Vp; 100-1000V), number of pulses (Np; 1-2048, increase by 12 degrees), duration of pulse (Td; 1-800 μs, fixed at 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, subsequently 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 pericarp. 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 pLVGneo2103 and 50 μg/ml saff thymus DNA. Electroporation was carried out under the following condition: long-pulse output, Vp = 10 kV, Np= 3-29, 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-embryos electroporated with pLGVneo2103

<table>
<thead>
<tr>
<th>Exp.</th>
<th>No. of half-embryos electroporated</th>
<th>No. of half-embryos with G418(^8) callus</th>
<th>No. of calli tested for regeneration</th>
<th>No. of plantlets regenerated</th>
<th>No. of R0 plants reaching maturity</th>
<th>No. of R0 plants with nptII gene</th>
<th>No. of R0 plants with NPTII activity</th>
<th>No. of fertile transgenic R0 plants</th>
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<tr>
<td>1</td>
<td>150</td>
<td>44</td>
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<td>14</td>
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<td>Total</td>
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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 \(\mu\)g/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-embryos were transferred onto N\(\delta\) regeneration medium (Li et al. 1991) without antibiotic. Regeneration was performed under fluorescent illumination for 10 h photoperiod. The regenerated plantlets were hardened on the same medium containing 30 \(\mu\)g/ml G418 (Sigma). They were transferred to fresh selection medium two weeks later. After approximately 8 weeks of selection in the dark, the G418-resistant calli initiated from half-embryos were transferred onto N\(\delta\) regeneration medium (Li et al. 1991) without antibiotic. Regeneration was performed under fluorescent illumination for 10 h photoperiod. The regenerated plantlets were hardened on the same medium containing 30 \(\mu\)g/ml G418 (Sigma). They were transferred to fresh selection medium two weeks later. After approximately 8-10 cm high, subsequently transferred to soil in pots. In the greenhouse the plants grew to maturity and set seed.

Table 2.

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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 60 \(\mu\)g/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 \(\mu\)g 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 \(\mu\)l aliquots (approximately 3 \(\mu\)g DNA). The probe was a \(^{32}\)P-labeled 1.6 kb PstI internal fragment of the nptII gene from pLVG-neo2103.

NPTII enzyme activity. 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 \(\mu\)l) 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 \(\mu\)g/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, average 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 N\(\delta\) medium for regeneration.

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

Figure 2. Southern hybridization analysis of one G418-resistant rice callus line (C\(\text{C}_1, C\text{C}_2, C\text{C}_3\)) from each of three experiments. The probe was a \(^{32}\)P-labeled 1.6 kb PstI fragment of pLVGneo2103. Lane 1, undigested DNA from six-month-old callus line \(C\text{C}_1\). Lane 2, pLVG-neo2103 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 \(C\text{C}_1\) 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 \(C\text{C}_1\) (lane 7) and \(C\text{C}_1\) (lane 8). Lanes 10, 11, PstI digested DNA from six-month-old callus line \(C\text{C}_1\) (lane 10) and \(C\text{C}_1\) (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 \(C\text{C}_1, C\text{C}_2, C\text{C}_3\) from three experiments.