Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts


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ABSTRACT

Transgenic rice plants have been regenerated by somatic embryogenesis from cell suspension derived protoplasts electroporated with plasmid carrying the NPTII gene under the control of the 35S promoter from cauliflower mosaic virus. Heat shock of protoplasts prior to electroporation maximised the throughput of kanamycin resistant colonies. Omission of kanamycin from the medium for plant regeneration was essential for the recovery of transgenic rice plants carrying the NPTII gene. This report of the production of kanamycin resistant transgenic rice plants establishes the use of protoplasts for rice genetic engineering.

ABBREVIATIONS

NPTII, neomycin phosphotransferase; SDS, sodium dodecyl sulphate.

INTRODUCTION

A major constraint in the production of transgenic plants in most of the cereals is the inability to induce plant regeneration from protoplasts treated with DNA. To date, plant regeneration from transformed protoplast-derived tissues has been achieved only in Zea mays (Rhodes et al. 1986). The experimental approach used most extensively involves DNA uptake into protoplasts by treatment with polyethylene glycol or electroporation. This technique has been used to study transient gene expression (Fromm et al. 1985; Ou-Lee et al. 1986; Werr and Lorz 1986; Hauptmann et al. 1987; Junker et al., 1987) and has also resulted in the production of stably transformed tissues of gramineous species, including Lolium multiflorum (Potrykus et al. 1985b), Oryza sativa (Uchimiya et al. 1986; Yang et al. 1988a,b), Panicum maximum (Hauptmann et al. 1988), Saccharum spp. (Chen et al. 1987), Triticum monococcum (Lorz et al. 1986; Hauptmann et al. 1988), and Zea mays (Fromm et al. 1986).

Our studies have established a reproducible procedure for the regeneration of fertile plants from rice protoplasts (Oryza sativa L. v Taipei 309) with a regeneration efficiency adequate for assessments of transgenic plant production (Abdullah et al. 1986; 1988). Recently, we have reported that electroporation is the most efficient procedure for the production of kanamycin resistant rice tissues following DNA uptake into rice (Taipei 309) protoplasts (Yang et al. 1988b). We have now combined these optimum conditions for transformation using electroporation with those for plant regeneration to produce transgenic rice plants resistant to kanamycin.

MATERIALS AND METHODS

Protoplast isolation

Protoplasts were isolated using a routine procedure (Abdullah et al. 1986) from an established (more than 10 months old) cell suspension of Oryza sativa L. v Taipei 309. The suspension was initiated from leaf base callus and designated line LB3. Some preparations of freshly isolated protoplasts were heat shocked at 45°C for 5 min, followed by 10 sec on ice (Thompson et al. 1986b) prior to plasmid uptake by electroporation.

Plasmid constructs

E. coli HB101 was transformed by the calcium chloride procedure (Mandel and Higa 1970) with pCaMVNEO (Fromm et al. 1986) carrying a chimeric gene consisting of the CaMV 35S promoter, the neomycin phosphotransferase (NPTII) gene from Tn5 and the nos polyadenylation region. pH2P3 was provided in the same E. coli strain. This plasmid was constructed by fusing the 35S promoter of CaMV to the 5' end of the EcoR1 fragment of pA2DI. The EcoR1 fragment carried the NPTII gene and the 19S promoter from gene VI of CaMV (Paszkowski et al. 1986). After construct verification (Holmes and Quigley 1981), large scale plasmid isolation from E. coli was performed using the alkaline lysis method (Birnboim and Doly 1979). Plasmid was sterilised by ethanol precipitation and dissolved in sterile TE buffer (Maniatis et al. 1982) at 1.0 mg/ml.

Electroporation of protoplasts

Protoplasts were resuspended at 2.5 x 10^6/ml in electroporation medium which was modified from a published formulation (Fromm et al. 1986) and contained 0.8 g/l NaCl, 0.02 g/l KCl, 0.02 g/l KH_2PO_4 and 100 g/l glucose, pH 7.1. One ml volumes of protoplast suspension were mixed with 20 μg of pCaMVNEO in 20 μl of TE buffer. Four hundred μl samples of protoplast/plasmid mixture were transferred to the chamber of an electroporator (DIA-LOG, G.m.b.H., 4 Düsseldorf 13, West Germany). The plexiglass chamber, which had two parallel
stained and maintained under the same conditions to
hormone-free Murashige and Skoog (1962) based
compartment. Four hundred colonies were
medium (Chu et al. 1975) containing 8% w/v sucrose
onto the surface of hormone-free N6 regeneration
kanamycin, and the same number to N6 medium lacking
0.4% w/v agarose (Sigma, type I) in 100 mm
transferred to agar-solidified (0.8% w/v; Sigma)
cultures, the liquid medium in each dish was
replaced by 3.0 ml of KPR medium containing 100
mg/ml of kanamycin. The kanamycin supplemented
medium was itself replaced every 7 days over a
period of 28 days with medium containing the same
concentration of antibiotic. The number of colonies which
reached a diameter of 1.0 mm or
more in antibiotic-free KPR medium and in the
kanamycin selection plates was recorded 42 days
after protoplast plating.

Samples of protoplasts which had been heat shocked
and electroporated in the absence of plasmid were
also cultured on kanamycin selection to ensure a supply of non-transformed
cellus for biochemical analysis.

Protoplast regeneration from kanamycin resistant tissues

Individual cell colonies from the kanamycin
selection plates were placed, using fine forceps,
onto the surface of hormone-free N6 regeneration
medium (Chu et al. 1975) containing 8% w/v sucrose
and 0.4% w/v agarose (Sigma, type I) in 100 mm
square 5 x 5 compartment dishes (Sterilin Ltd.,
Feltham, Middlesex, UK). Four colonies were
cultured on 2.0 ml aliquots of medium in each
compartment. Four hundred colonies were
transferred to N6 medium with 50 µg/ml of
kanamycin, and the same number to N6 medium lacking
antibiotic. Cultures were maintained at 27°C in
the dark for 2 to 3 weeks before transfer to the
light (continuous daylight fluorescent
illumination; 2500 lux). Plantlets were
transferred to agar-solidified (0.8% w/v; Sigma)
hormone-free N6 medium and Skoog (1962) had
medium and maintained under the same conditions to
stimulate shoot and root development.

Fifty randomly selected protoplast-derived colonies
which failed to regenerate on N6 medium were
transferred to AA medium (Abdullah et al. 1986)
(27°C; dark) and this material used for biochemical
analysis.

Regenerated plants were potted, 10-15 cm tall, in
mixture of equal volumes of John Innes No. 3
potting compost and Levington M3 compost (Pisces
Horticultural Division, Ipswich, UK). Plants were
sprayed with distilled water after potting and
covered with a plastic bag. They were maintained for
6 weeks in a growth room (day and night temperatures
of 28°C and 25°C respectively; 10 h day/14 h night
cycles) during which time they were hardened off by
gradual removal of the plastic bags. Subsequently,
they were transferred to the greenhouse and grown
under natural daylight with day and night
temperature maxima of 30°C and 18°C respectively.

A similar plant regeneration procedure was used for
tissues derived from heat shocked protoplasts which
had been electroporated in the presence of plasmid.
Some colonies from such protoplasts were also grown
on AA medium in the absence of kanamycin to provide
non-transformed callus for analysis.

DNA isolation and hybridisation

Total genomic DNA was isolated from rice callus and
leaves of regenerated plants using a published
technique (Dellaporta et al. 1983). Ten µg of DNA
each of sample were digested with BamHI as described
by the manufacturer (Northumbria Biochemicals Ltd.,
Cramlington, UK) and the fragments electrophoresed
(25V; 16h) in a 0.8% w/v agarose gel. One µg of
pCaMVNEO DNA restricted with BamHI was also run on the
gel. DNA fragments were blotted to nylon
membrane (Hybond-N; Amersham International,
Amersham, UK) and hybridised to the 1.0 Kb BamHI
fragment of pCaMVNEO containing the structural
sequence of the NPTII gene. The 1.0 Kb BamHI
fragment was labelled using a Multiprime DNA
Labelling System (Kpn.16011; Amersham
International). After hybridisation, the membrane
was washed twice with a solution of 2 x SSPE
(Maniatis et al. 1982) and 0.1% w/v SDS (10 min
each wash) at 22°C, followed by 2 washes (30 min
each) at 65°C. The membrane was given 2 additional
washes (30 min each) in 0.3 x SSPE with 0.1% SDS at
65°C, dried, and exposed to X-ray film (Fuji RX)
using an intensifying screen.

Neomycin phosphotransferase (NPTII) assay

Two hundred µg of leaf material or 0.5g of callus
were ground with 50 µl of extraction buffer
containing 62.5 mM Tris-HCl (pH 6.8), 40 mM
dithiothreitol, 1.0 mM 1,10-phenanthroline and 5.5
mM phenylmethyl-sulphonyl fluoride. Aprotinin
(SIGMA) was added to the final concentration of 100
µg/ml. Extracts were centrifuged (12000 rpm; 5
min). Protein concentrations were measured
(Bradford 1976). Fifty µg of protein of each
sample were electrophoresed in a 10% w/v
polyacrylamide gel for 18 h (80V) at 4°C. Subsequent
stages of the procedure were as reported (Schreier
et al. 1985).

RESULTS

Protoplasts electroporated with pCaMVNEO entered
division within 4 days of plating. Heat shock
treatment of freshly isolated protoplasts prior to
electroporation increased the percentage of
protoplasts dividing by day 14. This stimulatory
effect of heat shock on protoplast division was