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Transient and Stable Transformation of Rice (*Oryza sativa* L.) Calli through Tissue Electroporation

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Abbreviations: NPT II - neomycin phosphotransferase, GUS -β-glucuronidase, PPT - phosphinotricin, MATAB- mixed alkil trimethyl ammonium bromide, MES- (N-morpholino) ethanesulfonic acid.

Abstract

Electroporation of microcalli and embryonic axes of a Brazilian Indica rice cultivar was performed. Some parameters influencing the recovery of transformed callus have been defined through transient *npt II* expression. Such parameters included the presence of light during incubation of microcalli used as target for electroporation, heat shock at 45℃, macerozyme pre-digestion of target tissues and the number of pulses during electroporation. Transgenic calli were obtained from embryonic axes after electroporation with plasmid pDM302, which encodes the gene phosphinotricin acetyl transferase (*bar*) under the control of *Act-1* promoter. Integration of the introduced gene into the genome was demonstrated by Southern blot hybridization.

Introduction

Progress in tissue culture and molecular biology techniques brought new alternatives for the genetic improvement of a variety of plants species. Transgenic cereals have been obtained through biolistics methods (Li et al., 1993; Zhan et al., 1996), electroporation of intact cells or tissues (D'Halluin et al., 1992; Sabri et al., 1996; Pádua et al., 2001), protoplasts transformation (Terada et al., 1993; Hosoyama et al., 1995) and *Agrobaterium tumefaciens* based systems (Hiei et al., 1994; Rashid et al., 1996). The two last methods suffer some drawbacks. Protoplast culture result in increased probability of genetic modifications (Yamagishi et al., 1997) and *A. tumefaciens* systems is limited by the host range (Wordragen and Dons, 1992). Since electroporation of intact tissues is among some of the less genotype dependent technique, which make use of regenerable explants, we have evaluated the use of wounded embryonic axes and microcalli as targets. The purpose of this research project was to develop a fast and feasible system for further studies on gene expression as well as on the effect of altered expression of particular genes, during the interaction of Brazlian rice cultivars with microorganisms. Using the optimized conditions, transient *gus* expression on microcalli and embryonic axes was observed. Calli resistant to phosphinotricin (PPT) were obtained upon electrotransformation with plasmid pDM302. The transgenic nature of selected calli was proved by Southern blot hybridization analysis.

Materials and Methods

Plant material and culture conditions

Mature seeds of an *Indica* rice subspecies (*Oryza sativa*, cv. IRGA 409), kindly supplied by CPACT (Centro de

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Pesquisa Agropecuária de Terras Baixas de Clima Temperado - EMBRAPA) were surface sterilized with 0.2% $HgCl_2$ for 5 minutes. Mature seeds and isolated embryonic axes were cultured on Matsuno et al. (1990) modified medium comprised of MS (Murashige and Skoog, 1962) medium supplemented with 12 mM proline, 9 μM 2,4-D, 10 g/L sucrose, 30 g/L sorbitol, 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) and solidified with 1% agar. Embryonic axes were excised from seeds soaked for 24 hours in MS liquid medium. Media pH (5.8) was adjusted before adding agar and autoclaving (15 min at 120°C). Cultures were maintained at 28±2°C, in the dark. With the purpose to study the effect of the light condition during incubation of the target microcalli, some of the cultures were maintained under a 16 h photoperiod regime provided by cool-white fluorescent lamps, with a photon fluency of 36 $\mu F.m^{-2}s^{-1}$.

Plasmids

Plasmids pDE108 (Denecke et al., 1989), pDM302 (Cao et al., 1992) and pAct1-D (McElroy et al., 1990) were used for electroporation experiments. pDE108 contains the *npt II* gene under the control of CaMV 35S promoter; pDM302 and pAct1-D contain the *bar* and *gus* genes respectively, under the control of rice actin promoter (Act-1). CsCl purified plasmid DNA used in the electroporation experiments was ressuspended in water at the concentration of 1 mg/mL. Plasmid pDE108 was linearized at the *Hin*dIII site and precipitated with ethanol prior to electroporation.

Electroporation

Electroporation experiments were performed using microcalli (2-4 mm) or embryonic axes derived from mature seeds soaked for 24 hours in MS medium and longitudinally sectioned on the coleoptile side. DNA delivery was achieved using a homemade electroporation device constructed according to Dekeyser et al. (1990). The buffer used for electroporation consisted of EPRm, modified from EPR described by Dekeyser et al. (1990) and containing 5 mM calcium gluconate instead of 4 mM calcium chloride. Approximately 15-20 fragments of the target material were incubated during two hours in Petri dishes containing the electroporation buffer and washed three times with fresh buffer. Next, plant material was incubated for one hour with 150 μL of electroporation buffer in sterile cuvettes with 0.4 cm path length plus 20 μg of plasmid DNA previously complexed with 3 μL Lipofectin™. EPRm was supplemented with 8,5 μL 3 M NaCl before electropulsing. Electropulses were performed with different electric fields discharged from a 900 μF capacitor. Embryonic axes were electroporated with 1-5 pulses of different intensities and microcalli were electroporated with one pulse of 625 V/cm. Immediately after electropulse, 300 μL of MS medium was added to the electroporation buffer and the cuvettes were incubated on ice for 10 minutes before transfer to the culture media.

Optimization conditions

Following the basic procedure described above, different parameters related to the electroporation efficiency were analyzed. Using microcalli as target tissue, we analyzed the effect of DNA conformation (linearized or supercoiled), light condition during calli incubation on tissue culture media before electroporation, heat shock (15 min. at 45°C) and macerozime (previously dissolved in MS medium) predigestion of microcalli and embryonic axes without shaking for 1, 3 and 5 minutes. For embryonic axes, the macerozime predigestion was tested as described above and the effect of the number of pulses was monitored based upon PPT resistance.

NPT II and GUS assays

Neomycin phosphotransferase (NPT II) activity was detected by *in situ* gel assay according to the method of Reiss et al. (1994) on tissues electroporated with pDE108 plasmid. β-glucuronidase histological assays were performed according to Jefferson et al. (1987) after electroporation with pAct1-D plasmid. In the experiments conducted, NPT II and GUS activity were reproducibly detected 4-5 days after electroporation.

Selection of transformed callus

After electroporation with plasmid pDM302 microcalli and embryonic axes were transferred to callus induction medium supplemented with the selective agent PPT (3-4 mg/L). Subsequent experiments were as described for transient expression, using the optimized conditions.

Southern blot analysis

Genomic DNA was isolated using a modified procedure of Murray and Thomas (1980). Extraction buffer used was comprised by 0,1M Tris, HCl pH 8.0, 1.25 M NaCl, 0.02M EDTA, 2% MATAB and 14.26 mM β-mercaptoetanol. DNA was digested with *Sal* I which cleaves

pDM302 once. Uncut and digested genomic DNA was electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane (Hybond N-Filter, Armesham) and hybridized to a labelled *bar Eco* RI fragment from pDM302 plasmid.

Results and Discussion

Effect of plasmid conformation

NPT II activity was not influenced by using linearized or supercoiled DNA on the electroporation of microcalli (Figure 1A). Comparable results were found by Dekeyser et al. (1990) and D'Halluin et al. (1992) after electroporation of rice leaf bases and maize immature zygotic embryos, respectively. However, the results reported about this subject are contradictories as linearized DNA was associated to enhanced transient expression levels in electroporated seedling tissues of common bean (Dillen et al., 1995) and isolated scutela of barley (Hänsch et al., 1996).

Effect of presence of light during calli incubation

The procedure adopted here for *in vitro* regeneration includes calli proliferation in the dark and shoot induction in the presence of light. The effect of the presence of light was analyzed in order to determine the best phase to collect the target microcalli, before or after transfer to shoot induction medium. Figure 1B shows that calli grown

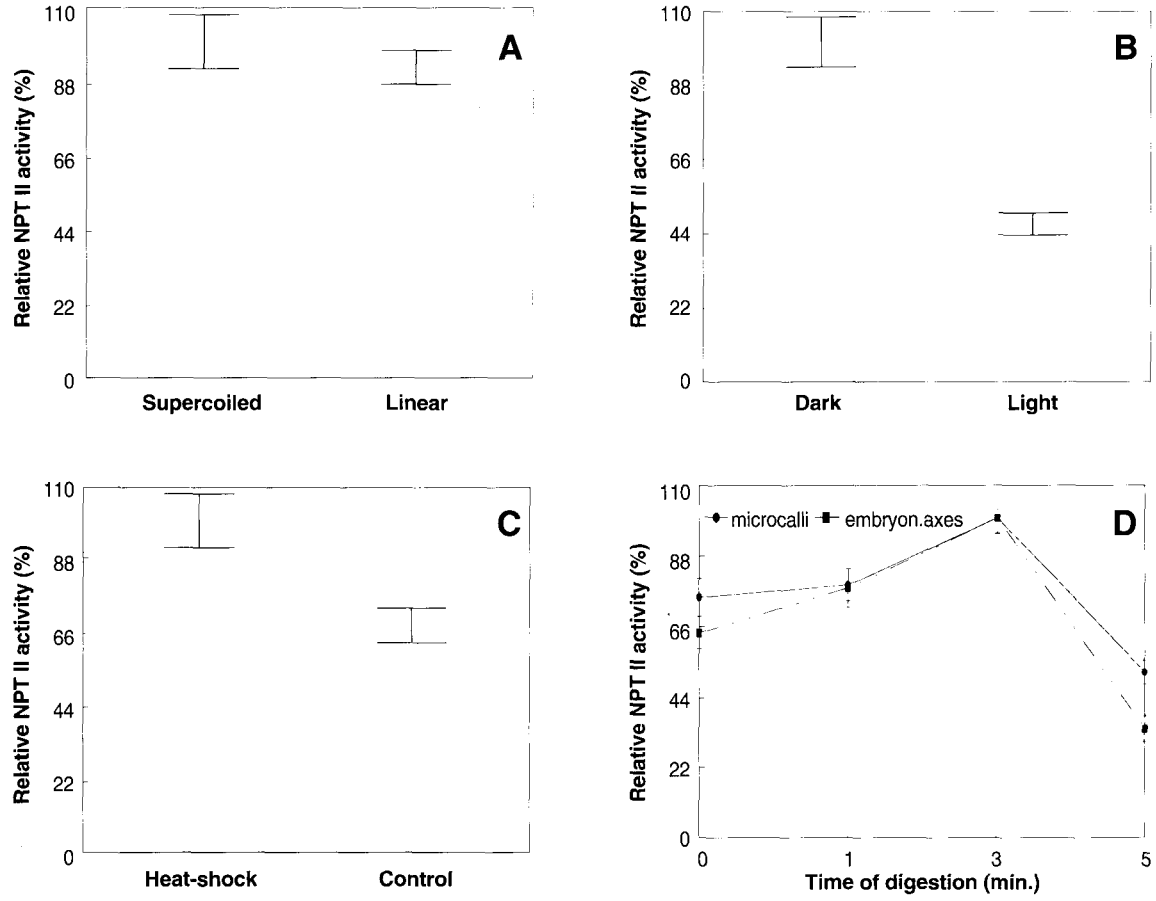

Figure 1. Relationship between the level of NPT II activity and parametres influencig the transient expression of the introduced foreign DNA in electroporated material. After measuring the NPT II activity for each treatment and calculating an average of at least three experiments, a relative transformation efficiency was calculated, representing the radioactive phosphorylated kanamycin detected relative to the maximum obtained within each set of experiments, A; Effect of plasmid conformation (linearized or supercoiled) in electroporated calli, B; Effect of dark/light cultivation condition of callus used a target for electroporation, C; Influence of 45°C heat shock pretreatment in electroporated calli, D; Effect of macerozime predigestion of calli and embryonic axes on the electrotransformation efficiency.

under dark condition presented highest transient gene expression level. It is not evident why those calli have increased competency for DNA uptake. One possible explanation is that the thickness of the cell wall of calli under light condition, is larger than in calli maintained in darkness. Also, it should be taken into account possible physiological differences between calli maintained in both circumstances, which could interfere in the *npt II* expression.

Effect of heat shock (45℃)

Microcalli were submitted to 45℃ heat shock during 15 minutes before addition of DNA. This treatment resulted in a 31% increase in the relative level of NPT II activity (Figure 1C). Similar observations related to the improvement of electroporation efficiency due to preheating were described by Shillito et al. (1985), Rathus and Birch (1992) and Luong (1995). Any kind of treatment which could improve cell viability can potentially enhance the recovery of transgenic cells. Since heat shock increases the viability of rice protoplasts (Thompson et al., 1987), we could assume that the positive effect of heat shock resulted from increased cell viability. Other mechanism which could increase the transformation efficiency due to preheating treatment is possibly related to alterations on the membrane permeability (Gervais and Maranon, 1995; Morozov et al., 1997). However, the fact that the stimulation of gene expression is observed when the material is heated before the electric pulse might indicate that some process in the early events in the control of gene expression is affected (Zakai et al., 1993).

Effect of macerozime pre-digestion

Pre-digestion with 0.3% macerozime resulted in a increase of NPT II activity by 25 and 36% for respectively electroporated calli and embryonic axes (Figure 1D). Accordingly, D'Halluin et al. (1992) and Hänsch et al. (1996) obtained improved transformation efficiency of maize embryos and isolated scutellum respectively, by electroporation after enzymatic wounding. In contrast, Klöti et al. (1993) and Rao (1995) reported no differential response on the number of transformed cells, after enzymatic pretreatment of scutellum of wheat embryos and immature embryos of rice, respectively.

Influence of the number of pulses

Embryonic axes were electroporated with one pulse of 625 V/cm, three of 375 V/cm and five pulses of 225 V/cm in order to determine the influence of the number of pulses on the efficiency of transformation events as settled by the number of explants resistant to PPT following electroporation with pDM302 plasmid. Whereas the frequency of explants resistant to PPT was increased after 3 electrical pulses, after five 225V/cm pulses, the viability of the explants was drastically reduced (Figure 2A).

In addition, the competence for calli proliferation of the explants was observed as a result of the number of pulses applied. Embryonic axes were submitted to the complete procedure of electroporation with exception of the incubation with the DNA. After an average of 30 days, the explants were monitored for the competency for calli proliferation. As shown in the Figure 2B, electric pulses decreased the potential for callusing. In spite of that, 3 electropulses were chosen for the subsequent electroporation experiments.

In an attempt to further improve the efficiency of

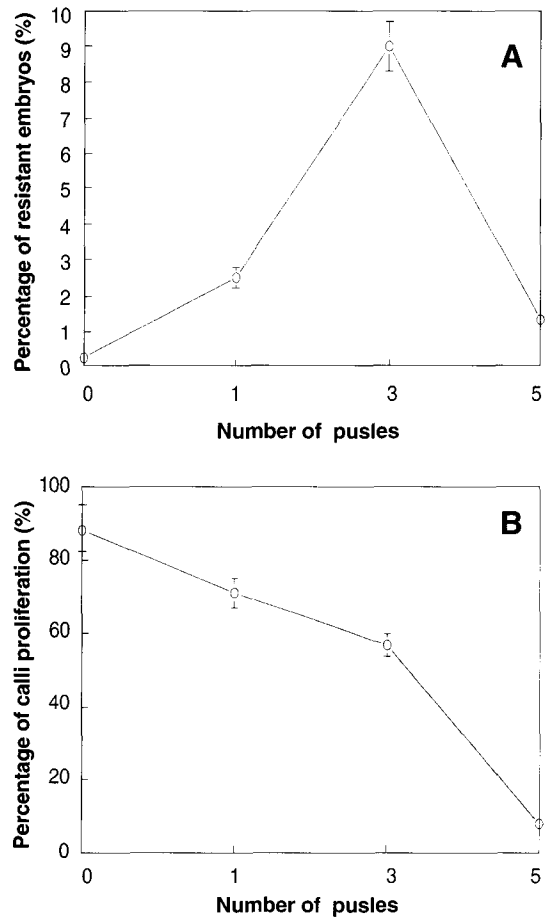

Figure 2. Effect of number of pulses in the frequency of resistant electroporated embryonic axes (A) and in the potential for calli proliferation (B).

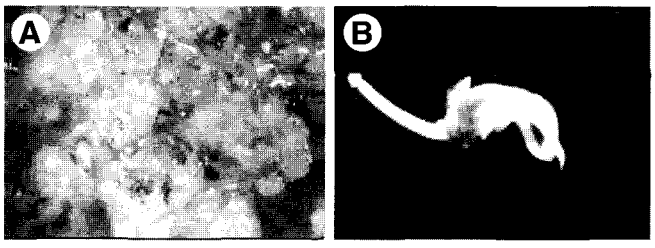

Figure 3. Histochemical GUS assay after gene transfer with pAct1-D plasmid after 4-5 days culture in callus (A) and embryonic axe (B).

DNA uptake, we tested 30 minutes of vacuum at low pressure (2.5×10^{-4} mbar) during the co-incubation period of target material with DNA. Efficiency of transfer of DNA concerning the latter variable has not been systematically investigated by transient gene expression but under that condition there was a increase of approximately 48% of the electroporated material capable of initiating callus.

Using the optimized conditions, transient transformation through electroporation was showed after histochemical GUS assay (Figure 3). Klöti et al. (1993), obtained rice immature embryos with transient expression only on the coleoptile side after electroporation with chimeric anthocyanin regulatory genes. In contrast, transient GUS activity was observed in electroporated rice immature embryos without any tissue specificity (Rao, 1995). In another instance, Chaudhury et al. (1995) used as target seed-derived embryos 48 hours post-germination and showed that plantlets derived from germination of these electroporated embryos showed transient *gus* expression predominantly in the shoot and also in the root and remainder of endosperm. In our experiments, after 4 days in MS medium, embryonic axes expressed the gus transgene mostly at the exposed areas during the pulse delivery, scutelum and coleoptile side, indicating that transgenic plants can be obtained through callus regeneration but not following germination of the embryos (Jones and Rost, 1989). For the obtaining of transgenic plants using the germination approach, shoot and root apex inside embryo should be reachable and responsive to DNA penetration. In fact, electroporated embryos germinated in selective medium for at maximum 10 days while the control embryos, electroporated without DNA, did not developed plantlets (data not shown).

Selection of stably transformed calli

Around 8% electroporated tissues callused in the presence of PPT after electroporation with pDM302. Non-transformed calli turned yellow to brown and became soft (Figure 4). Transformed calli were white and friable in spite of non-regenerable, a phenomenon already observed by Zhou et al. (1993), Sabri et al. (1996) and Rhodora and Hodges (1996) after gene transfer procedures. Six of the potentially transgenic material from electroporated microcalli were confirmed for the presence of *bar* gene after Southern blot hybridization (Figure 5A). There was no detectable hybridization of the radioactively *bar* fragment in control calli electroporated without DNA. Analysis of resistant calli undigested samples hybridized to a *bar*

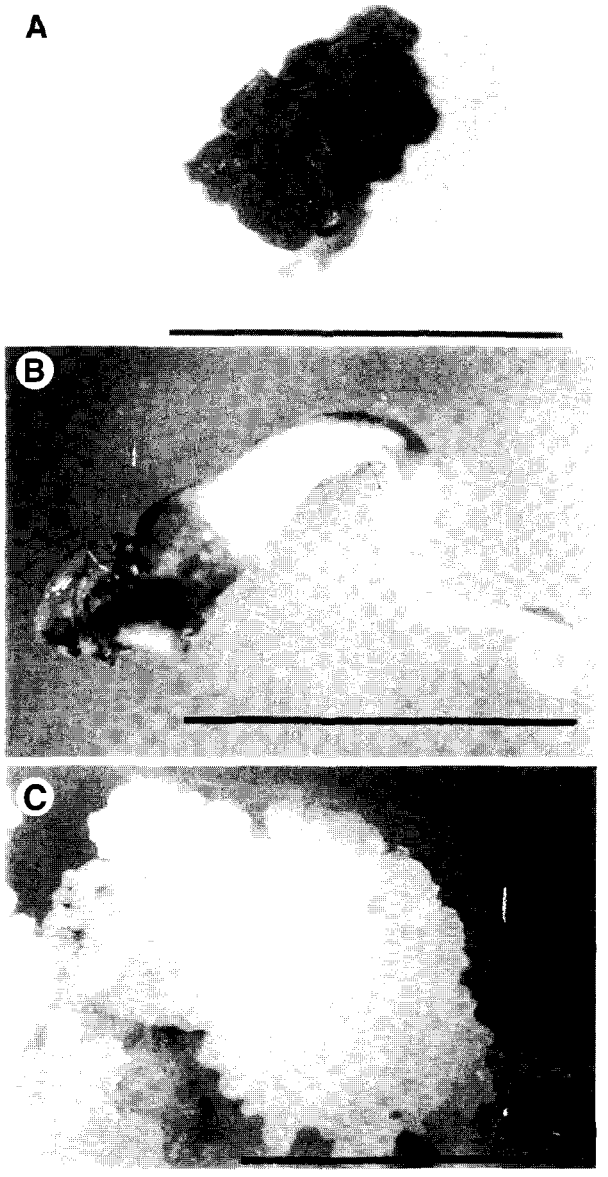

Figure 4. Phenotypically resistant calli in selective medium (PPT). Microcalli (A) and embryonic axes (B) were electroporated without exogenous DNA and in the presence of pDM302 plasmid (C).

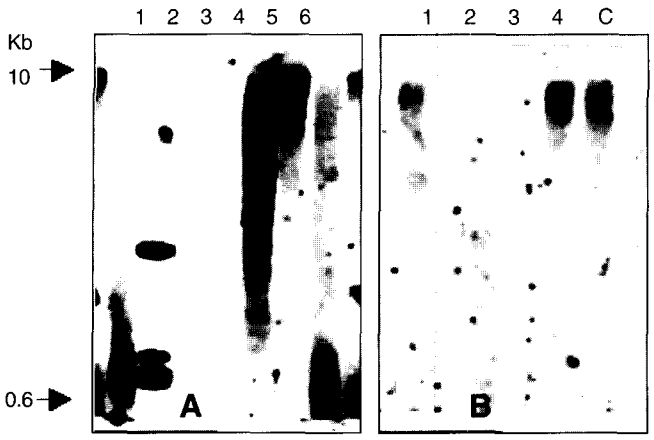

Figure 5. Southern blot analysis of DNA isolated from transgenic calli derived from electroporated embryonic axes. 20 μg *Sal* I digested (A) and uncut (B) DNA samples were hybrydized to the ^{32}P-labelled *bar* probe. The negative control contains undigested DNA isolated from nontransformed rice cells. The positive control is a plasmid DNA harboring *bar* gene.

probe showed a pattern equivalent to high molecular weight DNA, providing evidence that the transgene was integrated into the genomic DNA. Southern blot analysis of the *Sal* I-digested DNA revealed banding patterns characteristic of multiple insertions of *bar* gene (Figure 5B).

The main purpose of this research project was to generate a fast and feasible system for further studies on gene expression as well as on the effect of the altered expression of any particular gene during the interaction with microorganisms, through either transient and/or stable transformation approaches. The analysis of factors, which influence successful DNA delivery, is a time-saving approach aiming at the use of the settled conditions as a start point for gene incorporation and expression. The strategy of the experiments conducted here included the use of two recipient target materials (microcalli and embryonic axes) responsive to *in vitro* plant regeneration through embryogeneses or organogenesis (Willians and Maheswaram, 1986; Jones and Rost, 1989; Rueb et al., 1994), making possible further production of stable transgenic plants.

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