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Full Length Research Paper

Agrobacterium tumefaciens-mediated transformation of biofuel plant *Jatropha curcas* using kanamycin selection

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Establishment of an efficient transformation system is a prerequisite for genetic improvement of *Jatropha curcas,* a promising biodiesel feedstock plant, by transgenic approach. In this study an efficient *Agrobacterium*-mediated transformation protocol using cotyledon explants from *J. curcas* seeds was developed. The integration and expression of the transgenes in the putatively transformed *J. curcas* plants was confirmed by polymerase chain reaction (PCR), Southern blot analysis and β-glucuronidase (GUS) activity staining. In the protocol used, the critical step for successful transformation is that the selective agent kanamycin (20 mg/L) is not included in the callus-inducing medium within the first 4 weeks after co-cultivation with *Agrobacterium*.

Keywords: *Agrobacterium*-mediated transformation, biofuel, *Jatropha curcas*, kanamycin, physic nut, plant regeneration, transgenic plants.

INTRODUCTION

Jatropha curcas is a perennial deciduous shrub belonging to the family Euphorbiaceae, which originated in Central America and is widely distributed in the tropics and subtropics (Carels, 2009). The oil content of *J. curcas* seeds is high and could be up to 40% (Kandpal and Madan, 1995). Biodiesel made from *J. curcas* seed oil has similar characteristics to those of fossil diesel and can be used as a substitute for fossil diesel (Makkar and Becker, 2009). Furthermore, *J. curcas* has strong adaptability to drought and impoverished soil, and may also be used to produce soap, green fertilizers, pesticides and intermediate products of medicines (Kumar and Sharma, 2008). At present, however seed yield of *J. curcas* is low and unstable (Sanderson, 2009). Transgenic approach has the potential to significantly improve seed productivity of *J. curcas* (Gressel, 2008; Sujatha et al., 2008), which requires an efficient genetic transformation and regeneration system. Although *in vitro* regenerated *J. curcas* plants have been obtained from various explants such as leaves (Sujatha and Mukta, 1996; Lu et al., 2003; Sujatha et al., 2005; Thepsamran et al., 2008), hypocotyls (Sujatha and Mukta, 1996; Lu et al., 2003), petioles (Sujatha and Mukta, 1996; Lu et al., 2003; Thepsamran et al., 2008), and nodes (Sujatha et al., 2005; Datta et al., 2007; Shrivastava and Banerjee, 2008), these regeneration systems have not translated into efficient genetic transformation protocols.

Using the herbicide phosphinothricin as a selective agent, Li et al. (2008) reported the first transgenic plants regenerated from cotyledonary leaf explants by *Agrobacterium*-mediated transformation which has been shown to be either inefficient or difficult to reproduce (He

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Abbreviations: BA, 6-Benzylaminopurine; **CaMV,** cauliflower mosaic virus; **GUS,** β-glucuronidase; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog; ***NPT II***, neomycin phosphotransferase II; **PCR,** polymerase chain reaction; **YEP,** yeast extract peptone; **CIM,** callus-inducing medium; **SIM,** shoot-inducing medium; **RM,** rooting medium; **CTAB,** cetyl trimethylammonium bromide.

et al., 2009; Mazumdar et al., 2010). Transformation attempts with antibiotic kanamycin (He et al., 2009; Mazumdar et al., 2010) or hygromycin (Trivedi et al., 2009) selection have produced transgenic calli, but failed to obtain regenerated transgenic plants of *J. curcas.* Although Purkayastha et al. (2010) have recently developed a method for generation of transgenic *J. curcas* plants by particle bombardment, *Agrobacterium*-mediated transformation method is preferred because of its high efficiency as well as the potential for delivery of a well-defined piece of transgenes into the plant cell chromosome (Newell, 2000).

In this report, we described an efficient *Agrobacterium*-mediated transformation system of *J. curcas* cotyledon explants from mature seeds by using kanamycin selection. Regenerated *J. curcas* plants with kanamycin-resistance were successfully obtained. Molecular characterization by polymerase chain reaction (PCR), Southern blot analysis and β-glucuronidase (GUS) activity staining revealed that a 30.8% of the kanamycin-resistant regenerated *J. curcas* plants was successfully transformed using this method.

MATERIALS AND METHODS

Preparation of cotyledon explants

Jatropha curcas L. seeds were collected from Guizhou province, P. R. China. The decoated seeds were soaked in sterile distilled water for 1 - 2 h at room temperature. The seeds were surface sterilized with 70% (v/v) ethanol for 30 s, and rinsed 3 - 4 times in sterile distilled water. Seeds were then treated with a 20% (v/v) commercial bleach (containing 5.25% of sodium hypochlorite) supplemented with 0.1% of Tween-20 for 10 min with occasional agitation, followed by washing in sterile distilled water 4 - 5 times. Finally, embryos were removed from the seeds, and cotyledon explants were excised by cutting off the base of the cotyledons with a scalpel, as shown in Figure 1A, leaving approximate 3/4 of papery cotyledons for co-cultivation with *Agrobacterium*.

Agrobacterium tumefaciens strain and vector used for transformation

The *A. tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA2301 (CAMBIA, Canberra, Australia) was used for transformation experiments. A single colony of *A. tumefaciens*/ LBA4404 was inoculated into 5 ml of liquid yeast extract peptone (YEP) medium (An et al., 1988) containing 50 mg/L kanamycin and 25 mg/L streptomycin, and grown overnight at 28°C on a rotary shaker at 200 rpm. An aliquot (0.5 ml) of overnight culture was inoculated into 50 ml of liquid YEP medium containing the same antibiotics, and allowed to grow at 28°C with vigorous shaking until the OD_{600} reached about 0.8. *A. tumefaciens* cells were then collected by centrifugation at 4000 rpm for 5 min at 4°C, and resuspended at OD_{600} = 0.4 in about 100 ml of liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

Transformation and regeneration of *J. curcas*

Cotyledon explants were inoculated by soaking for 20 min in *Agrobacterium* suspension with agitation at 200 rpm on a rotary shaker at room temperature. Infected explants were blot-dried on sterile filter papers, and then transferred to MS-Jc1 medium (MS medium with 3 mg/L 6-benzylaminopurine (BA) and 0.01 mg/L indole-3-butyric acid (IBA)) which was modified from that of Sujatha and Mukta (1996) in Petri dishes for 3 days in the dark at 26 ± 2°C. The co-cultivated explants were rinsed several times with liquid callus-inducing medium (CIM, MS-Jc1 with 300 mg/L cefotaxime) to eliminate excess bacteria, blotted dry with sterile filter papers, and transferred to CIM for callus induction. Explants in Petri dishes were cultured under a 12 h light/12 h dark cycle at 26 ± 2°C. After 4 weeks, calli were subcultured onto the shoot-inducing medium (SIM, MS-Jc1 with 20 mg/L kanamycin and 100 mg/L cefotaxime). Regenerated shoots (1.5 - 2 cm) were detached and transferred to a rooting medium (RM, 1/2 MS with 0.3 mg/L IBA, 20 mg/L kanamycin and 100 mg/L cefotaxime). All media were adjusted to pH 5.8 using 1 N NaOH, solidified with 0.7% (w/v) agar, and autoclaved at 121°C for 20 min. Antibiotics were filter-sterilized prior to their addition to the autoclaved medium and cooled to 40 - 50°C.

Histochemical staining of β-glucuronidase (GUS) activity

Histochemical staining was performed according to the method of Jefferson et al. (1987). Leaves of kanamycin-resistant shoots were incubated in GUS staining solution (100 mM sodium phosphate pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.5% Triton X-100 and 2 mM X-gluc) at 37°C overnight. The leaves of the non-transgenic regenerated shoots were used as negative controls. After staining, the samples were washed with 70% ethanol several times to remove plant pigments.

Molecular characterization of transgenic plants

Genomic DNAs were isolated from leaves of GUS-staining positive transgenic plants and non-transgenic controls by the cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). The primer pair, the 35S CaMV promoter primer ZF56 (5′-TCCCACTATCCTTCGCAAGACCC-3′) and the GUS-specific primer ZF49 (5′-ATCCAGACTGAATGCCCACA-3′) were used to detect the *GUS* gene. The *NPT II* gene was amplified with the *NPT II*-specific primer pair, NPTDHL1 (5′-CCGCTTGGGTGGAGA-3′) and NPTDHL2 (5′-ATGTTTCGCTTGGTGGT -3′). PCR amplification reactions were performed in a 20-µl volume, and PCR reaction parameters were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C (for *GUS*)/ 58°C (for *NPT II*) for 30 s and 72°C for 1 min, and followed by a final extension of 10 min at 72°C. Plasmid pCAMBIA2301 DNA was used as a positive control, with non-transgenic plant DNA as a negative control. PCR amplification products were separated on a 1.0% (w/v) agarose gels and visualized by ethidium bromide staining under UV light.

Southern blot hybridization was performed with DIG Starter Pack for Southern Blots following the manufacture's instruction (Roche Diagnostics, Mannheim, Germany). 5 µg of genomic DNA was digested with the restriction enzyme *Eco*RI, separated on 0.8% (w/v) agarose gel, and transferred to Hybond N^+ nylon membranes. The *GUS* probe was labeled with digoxigenin (DIG) with a Roche PCR DIG Probe Synthesis Kit. Prehybridization, hybridization and chemiluminescent detection of the blots were performed following the manufacturer's instruction (Roche Diagnostics, Mannheim, Germany).

RESULTS AND DISCUSSION

In a previous study of the effects of antibiotics on *J. curcas* tissue culture (Deng et al., 2005), the kanamycin

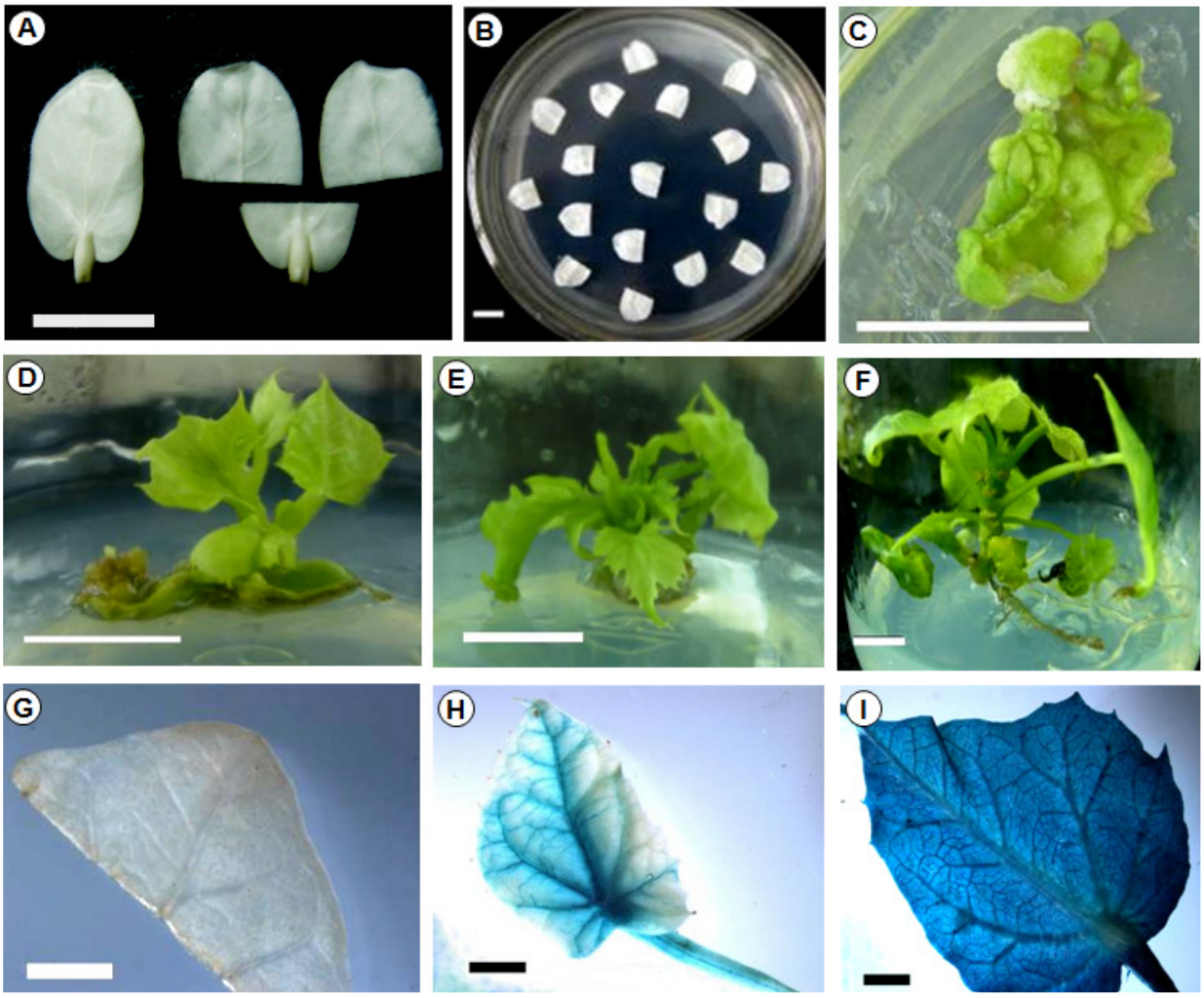

Figure 1. *Agrobacterium tumefaciens*-mediated transformation of *Jatropha curcas* using cotyledon explants of mature seeds. A, Cotyledon explants of mature seeds; B,cotyledon explants co-cultivated with *Agrobacterium* on MS-Jc1 medium; C, calli induced on callus-inducing medium containing 300 mg/L cefotaxime but no kanamycin; D and E, regenerated kanamycin-resistant shoots on shoot-inducing medium containing 20 mg/L kanamycin and 100 mg/L cefotaxime; F, rooted transgenic plantlet; G, GUS staining of non-transformed leaf; H and I, GUS staining of transgenic leaves. A-F, scale bar = 1 cm; G-I, scale bar = 200 μm.

was found to be a suitable selective agent for *J. curcas*. Accordingly *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pCAMBIA2301 (CAMBIA, Canberra, Australia), which contains a neomycin phosphotransferase gene (*NPT II*), conferring kanamycin resistance, and a β-glucuronidase (*GUS*) reporter gene with an intron from the castor bean catalase gene inside the coding sequence to ensure that expression of GUS activity is derived from plant cells (Vancanneyt et al., 1990), was chosen for *J. curcas* transformation in this study. Cotyledon explants from mature seeds were used for *Agrobacterium*-mediated transformation (Figures 1 A and B), since cotyledons of *J. curcas* were found more susceptible to *Agrobacterium* infection than other explants such as hypocotyls, petioles, or leaves (Li et al., 2006).

Using a regeneration medium MS-Jc1 (MS medium with 3 mg/L BA and 0.01 mg/L IBA) based on that of Sujatha and Mukta (1996), we found cotyledon explants of *J. curcas* were very sensitive to kanamycin, and 5 mg/L kanamycin in the medium is sufficient to stop callus induction. Therefore, kanamycin was not included in the callus-inducing medium (CIM, MS-Jc1 with 300 mg/L cefotaxime) within the first 4 weeks after co-cultivation with *Agrobacterium* to induce calli (Figure 1C). Then a low concentration of kanamycin (20 mg/L) was used in the shoot-inducing medium (SIM, MS-Jc1 with 20 mg/L kanamycin and 100 mg/L cefotaxime) to select the transformants. Kanamycin-resistant regenerated shoots were obtained after 6 - 8 weeks of culture on the selective SIM (Figure 1D and E). Root induction, however, was very difficult and only one of the total 120 regenerated

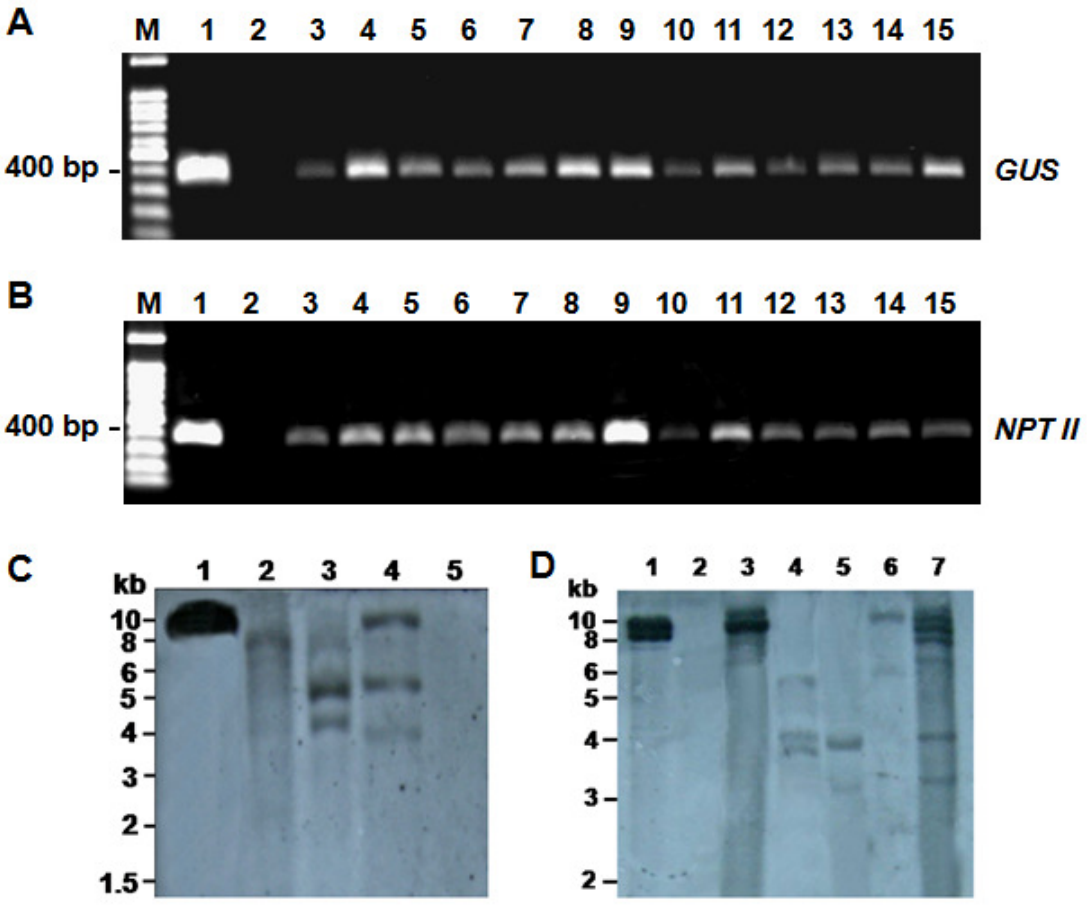

Figure 2. Molecular characterization of transgenic *Jatropha curcas* plants. A, PCR analysis for detection of the 355-bp *GUS* gene fragment; B, PCR analysis for detection of the 380-bp *NPT II* gene fragment. Lanes: M, 1 kb DNA marker; 1, positive control (plasmid pCAMBIA2301); 2, negative control (non-transformed plant); 3-15, transgenic plants (No. 3, 35, 50, 65, 67, 69, 73, 78, 83, 86, 98, 105 and 120); C and D, Southern blot analysis. Five µg of genomic DNA from transgenic plant No. 3, 35 and 105 (C, lane 2-4), 50, 67, 86, 98 and 120 (D, lane 3-7), and non-transgenic plants (C, lane 5; D, lane 2) was digested with *Eco*RI and fractionated on a 0.8% agarose gel. The DNA was blotted and hybridized to digoxigenin (DIG)-labeled *GUS* probe. Plasmid pCAMBIA2301 DNA (C and D, lane 1) was included as a positive control.

shoots was successfully rooted on a rooting medium (RM, 1/2 MS with 0.3 mg/L IBA, 20 mg/L kanamycin and 100 mg/L cefotaxime) for another 4 - 5 weeks (Figure 1F).

Reporter gene (GUS) expression was examined by histochemical staining of GUS activity in leaves of kanamycin-resistant regenerated shoots. Of the total 120 kanamycin-resistant shoots, 37 shoots showed GUS expression in leaves (blue staining, Figure 1H and I), whereas no blue staining was observed in leaf of the non-transformed shoot (Figure 1G).

To confirm the presence of the transgenes in the putatively transformed plants, genomic DNAs were extracted from GUS-staining positive regenerated plants and a non-transformed plant for PCR amplification (Figure 2A and B) and Southern blot analysis (Figure 2C) of the transgenes. A 355-bp fragment corresponding to the reporter gene *GUS* was detected in all GUS-staining positive plants (Figure 2A, lanes 3 - 15) and positive control (Figure 2A, lane 1), but no amplification product was found in non-transformed control plant (Figure 2A, lane 2). The presence of the selective marker gene *NPT II* was also confirmed in all GUS-staining positive shoots (Figure 2B, lanes 3 - 15). The integration of *GUS* gene into the *J. curcas* genome was further verified by Southern blot analysis of genomic DNAs from 8 putative transgenic plants showing GUS staining (Figure 2C, lanes 2 - 4; Figure 2D, lanes 3 - 7). The transgenic plants showed insertion of single (Figure 2C, lanes 2, Figure 2D, lanes 5 and 6) to multiple copies (Figure 2C, lanes 3 and 4; Figure 2D, lanes 3, 4 and 7) of the *GUS* gene.

In conclusion, the authors demonstrated that using cotyledons of mature seeds as explants *J. curcas* can be transformed with *Agrobacterium* with kanamycin selection

marker gene. The selective agent kanamycin, which completely inhibits callus formation, was not included in the callus-inducing medium is a key to successful transformation of *J. curcas*. The main remaining problem is to improve rooting ability of the kanamycin-resistant regenerated shoots of *J. curcas*. To further increase the transformation efficiency of *J. curcas*, the selectable marker-free method (de Vetten et al., 2003) is worthy of exploration and since the in planta transformation methods (Bent, 2000), which avoid tissue culture and regeneration, have been successfully developed for several plant species including *Arabidopsis* (Chang et al., 1994; Clough and Bent, 1998) and *Medicago truncatula* (Trieu et al., 2000), it is also possible to adapt these methods to the transformation of *J. curcas*.

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