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ORIGINAL PAPER

# Establishment of an *Agrobacteriuim*-mediated cotyledon disc transformation method for *Jatropha curcas*

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**Abstract** *Jatropha curcas* contains high amounts of oil in its seed and has been considered for bio-diesel production. A transformation procedure for *J. curcas* has been established for the first time *via Agrobacterium tumefaciens* infection of cotyledon disc explants. The results indicated that the efficiency of transformation using the strain LBA4404 and phosphinothricin for selection was an improvement over that with the strain EHA105 and hygromycin. About 55% of the cotyledon explants produced phosphinothricin-resistant calluses on Murashige and Skoog (MS) medium supplemented with 1.5 mg $l^{-1}$ benzyladenine (BA), 0.05 mg $l^{-1}$ 3–indolebutyric acid (IBA), 1 mg $l^{-1}$ phosphinothricin and 500 mg $l^{-1}$ cefotaxime after 4 weeks. Shoots were regenerated following transfer of the resistant calli to shoot induction medium containing 1.5 mg $l^{-1}$ BA, 0.05 mg $l^{-1}$ IBA, 0.5 mg $l^{-1}$ gibberellic acid (GA3), 1 mg $l^{-1}$ phosphinothricin and 250 mg $l^{-1}$ cefotaxime, and about 33% of the resistant calli differentiated into shoots. Finally, the resistant shoots were rooted on 1/2 MS media supplemented with 0.3 mg $l^{-1}$ IBA at a rate of 78%. The transgenic nature of the transformants was demonstrated by the detection of *β*-glucuronidase activity in the primary transformants and by PCR and Southern hybridization analysis. 13% of the total inoculated explants produced transgenic plants after approximately 4 months. The procedure described will be useful for both, the introduction of desired genes into *J. curcas* and the molecular analysis of gene function.

**Keywords** *Jatropha curcas* · Callus · Shoot · Plant regeneration · Transformation · Biofuel

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## Introduction

*Jatropha curcas* L. grows as a large shrub or small tree and belongs to the *Euphorbiaceae* family. The plant is found in the tropics and subtropics where is used to reclaim land and produce feedstuff, soap, candle, pesticide and anti-cancer medicine (Gupta 1985; Staubmann et al. 1999; Openshaw 2000; Li et al. 2001; Lin et al. 2004; Mampane et al. 2006). The seed oil 'Jatropha oil' can be easily processed to partially or fully replace petroleum based diesel fuel (Forson 2004). Thus, the use of this plant for large-scale bio-diesel production is of great interest with regards to solving the energy shortage, reducing carbon emission and increasing the income of farmers (Banerji et al. 1985; Martin and Mayeux 1985; Gubitz et al. 1999; Keith 2000; Zhou et al.

2006). Recently, the high yield of seed from the tree (~5 tons /ha/year) and the high oil content of its seeds (~66.4%) attracted global attention for the development of *J. curcas* as a source for bio-fuel (Openshaw 2000; Chen et al. 2006; Adebowale and Adedire 2006; Li et al. 2007). Governmental and non-governmental organizations in China have issued very ambitious plans for promoting *J. curcas* production on degraded lands and provided technical supports to farmers to harness the full benefit from this valuable plant (Li et al. 2007). The plant can be propagated on massive scale by direct seeding, planting stem cuttings, stumps, and root cuttings. Hot and humid weather is preferred for good germination of seed and plant growth. A seedling starts yielding seed at the end of the first year in South China. *J. curcas* is adapted well to semiarid marginal sites and can grow on gravelly or sandy soils with low nutrient content, and can also grow well in arid areas, where irrigation is provided. It prefers direct sunlight, however it can only survive a short mild frost, and it is slightly tolerant of salt spray. Its yield is still limited by some abiotic stresses, especially cold and drought (http://www.jatropha.org/; Adebowale and Adedire 2006). Therefore, additional genetic tools are required to further exploit the potential resources of *J. curcas* and provide additional genetic gain.

To determine either the function of a specific gene or that of the complete genome, many fundamental questions such as the gene expression pattern, localization of specific proteins, phenotypes of the plants when a gene is over-expressed or knocked down need to be answered. Most of these can be researched using an efficient genetic transformation system. *A. tumefaciens*-mediated genetic transformation has become a method of choice for basic plant research, as well as a principal technology for generating transgenic plants for the agricultural biotechnology industry (Stafford 2000; Gelvin 2003). This procedure has the advantage to result in stable integration of defined DNA sequences into the plant genome and it often results in a lower transgene copy number, fewer DNA rearrangements and higher long-term stability of expression as compared to direct DNA delivery methods (Dai et al. 2001; Travella et al. 2005). To apply this approach to *J. curcas* improvement, one prerequisite is to establish an efficient genetic transformation and plant regeneration system. Previously, shoot regeneration of *J. curcas* had been successfully obtained from cotyledon, petiole, hypocotyl, epicotyl, and leaf tissue (Sujatha and Mukta 1996; Lin et al. 2002; Lu et al. 2003; Wei et al. 2004). We have studied the factors influencing the *Agrobacterium*-mediated transformation of *J. curcas* based on transient GUS assay in a previous report (Li et al. 2006). However, successful genetic transformation of *J. curcas* has not been reported.

In this study, we developed an efficient and reproducible plant regeneration procedure for *J. curcas* by optimizing the culture conditions. Based on this procedure, transgenic *J. curcas* plants were successfully produced for the first time via *Agrobacterium*-mediated cotyledon disc transformation.

## Materials and methods

### Explant source

Seeds of *Jatropha curcas* were collected from Guizhou province, P. R. China. The seeds were surface sterilized for 30 s with 70% (v/v) ethanol after removing the outer seed coat, immersed in 0.1% (w/v) $HgCl_2$ for 30 min, and finally rinsed with sterile water for five times. The embryos were removed from the seed and incubated on hormone-free half-strength Murashige and Skoog salt (1/2 MS) medium (Murashige and Skoog 1962) containing 100 mg $l^{-1}$ myo-inositol, 10 mg $l^{-1}$ thiamine-HCl, 3% (w/v) sucrose, 0.7% (w/v) agar (Sigma), with the radicles in contact with the medium, and cultured in a growth chamber, at 24 ± 1°C in a 12 h light (35 µmol $m^{-2}$ $s^{-1}$)/12 h dark cycle.

### Media and sterilization conditions

MS basal medium containing 100 mg $l^{-1}$ myo-inositol, 10 mg $l^{-1}$ thiamine-HCl, and 3% (w/v) sucrose (MS medium) in combination with plant growth regulators was used. All media were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl, solidified with 0.7% (w/v) agar, and autoclaved at 121°C for 20 min.

### Plant regeneration from cotyledon explants

Cotyledons were excised from two-week-old seedlings, cut into small pieces (5 × 5 mm), and placed onto callus induction medium (CI medium: MS medium supplemented with 1.5 mg $l^{-1}$ BA, and 0.05 mg $l^{-1}$ IBA) for 3 weeks at 25°C in darkness. When still attached to their explants, developing calli were transferred onto shoot regeneration medium (SR medium: MS medium supplemented with 1.5 mg $l^{-1}$ BA, 0.05 mg $l^{-1}$ IBA, and 0.5 mg $l^{-1}$ GA3) and propagated at 25°C with a 14-h-photoperiod of 35 μmol $m^{-2}$ $s^{-1}$ provided by fluorescent light. After 4 weeks, the regenerated shoots were transferred onto shoot elongation medium (SE medium: MS supplemented with 0.3 mg $l^{-1}$ BA) for shoot elongation and bud multiplication. The elongated shoots (at a length of about 2.5 cm) were rooted on root induction medium (RI: 1/2 MS medium supplemented with 0.3 mg $l^{-1}$ IBA) within 2 weeks.

### *Agrobacterium* strains and binary vectors

The DNA fragment containing the cauliflower mosaic virus−35S (CaMV35S) promoter and the *gus*-int gene (*uidA*, encoding GUS) from the vector pBI121 (Jefferson et al. 1987) were inserted into the *Hin*d III and *Eco*R I sites of the vector of pCAMBIA 3301 (Cambia, Canberra, Australia), to result in p3301-BI121. Subsequently, the *gus* region in pBI121 was replaced by the full length cDNA (650 bp) of the gene *SaDREB1* (AY423713). This gene will be tested in transgenic plants subsequent experiments to evaluate its potential to protect from adverse environmental conditions. The resulting plasmid was designated p3301-BI121-SaDREB1. This vector also contains the herbicide resistance gene *bar* (de Block et al. 1987) controlled by the CaMV−35S promoter and encoding phosphinothricin acetyltransferase, and the *uidA* gene (Fig. 1). Its total size was 12.1 kb and it was transformed into *Agrobacterium tumefaciens* strains LBA4404 and EHA105 using the liquid nitrogen freeze-thaw method (Höfgen and Willmitzer 1988). The binary vector pCAMBIA 1301 (Cambia, Canberra, Australia) carried both the reporter gene *β*-glucuronidase (*uid*A) and the selectable marker gene for hygromycin phosphotransferase (*hpt*) and it was also transformed into *A. tumefaciens* strain LBA4404. A single colony of the transformed bacteria was used to inoculate liquid YEB medium supplemented with kanamycin (50 mg $l^{-1}$), streptomycin (25 mg $l^{-1}$) or rifampicin (50 mg $l^{-1}$). Bacterial cultures were grown overnight at 28°C. *Agrobacterium* cells were collected by centrifugation at 10, 000 × *g* for 30 s at 25°C. The precipitate was resuspended with 20 ml of liquid MS medium containing 20 mg $l^{-1}$ acetosyringone (AS) and adjusted to an $OD_{600}$ of 0.4–0.5, prior to transformation.

### Transformation of *J. curcas* cotyledon discs

Two-week-old cotyledons were cut into pieces of 5 × 5 mm and incubated with the *Agrobacterium* cells harboring p3301-BI121-SaDREB1 or pCAMBIA 1301 in 20 ml of CI liquid medium supplemented with 20 mg $l^{-1}$ AS for 10 min at 28°C. The explants were then transferred to the co-cultivation medium, (CI medium supplemented with 20 mg $l^{-1}$ AS) and co-cultured for 3 days at 25°C in the dark. Following co-cultivation, the explants were washed with sterile water containing 500 mg $l^{-1}$ cefotaxime to remove *Agrobacteria*. They were transferred to selection medium (CI medium

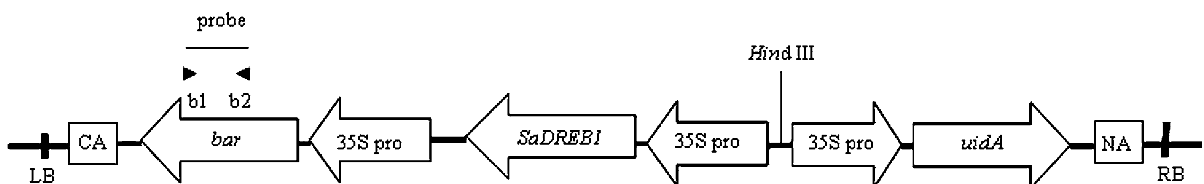


**Fig. 1** Expression cassette of the vector p3301-BI121-SaDREB1. RB, right border of the T-DNA; LB, left border of the T-DNA; 35S pro, 35S promoter from cauliflower mosaic virus; CA, CaMV35S polyA sequence; NA, nopaline synthase polyA sequence; *bar*, phosphinothricin acetyltransferase gene; *uid*A, *β*-glucuronidase gene; *SaDREB1*, DRE-binding protein (DREB1) gene from *Schedonorus arundinaceus*; PCR primers are indicated by b1 and b2, arrows indicate 5′–3′ direction of the gene

supplemented with 1 mg $l^{-1}$ phosphinothricin (glufosinate ammonium; AgrEvo USA) or 5 mg $l^{-1}$ hygromycin (A.G. Scientific, San Diego, Calif.) as selective agent for plant transformation and 500 mg $l^{-1}$ cefotaxime for elimination of *Agrobacteria*). After 3 weeks, resistant calli still attached to cotyledon explants were transferred to SR medium supplemented with 1 mg $l^{-1}$ phosphinothricin or 5 mg $l^{-1}$ hygromycin and 250 mg $l^{-1}$ cefotaxime for further selection. Finally, the resistant shoots were cut off the callus and rooted on RI medium supplemented with 1 mg $l^{-1}$ phosphinothricin or 5 mg $l^{-1}$ hygromycin and 250 mg $l^{-1}$ cefotaxime.

### PCR and Southern hybridization

Genomic DNA was prepared from leaves of transgenic plants using cetyltrimethyl ammonium bromide (CTAB), as described by Muhammad et al (1994). To verify the presence of the introduced *bar* gene in regenerated plantlets, PCR amplification of 441 bp of the *bar* coding region was carried out with the primers b1 (5′ -GTC TGC ACC ATC GTC AAC C- 3′) and b2 (5′ -GAA GTC CAG CTG CC AGA AAC- 3′). The reaction mixture for PCR was incubated in a DNA thermal cycler under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final 10 min extension at 72°C. PCR products were separated on a 0.8% agarose gel run at 100 Volt for 25 min and visualized under UV light. For Southern hybridization, 5 µg of genomic DNA was digested with the restriction enzyme *Hin*dIII, separated on 0.8% (w/v) agarose gel, and transferred to Hybond $N^+$ nylon membranes (Amersham-Pharmacia, USA). The *bar* probe was labeled with digoxygenin (DIG) by PCR using the b1 and b2 primers and using the same cycling conditions as described above. Prehybridization, hybridization and chemiluminescent detection of the blots were performed according to the manufacturer's instructions (Roche Diagnostics GmbH).

### Detection of *uidA* expression in transgenic plantlets

Histochemical staining of GUS activity was performed according to Jefferson (1987). Shoots of transgenic and wild type plants were submerged into the assay solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 3 h and then destained in 70% alcohol.

## Results and discussion

### Capacity of cotyledon explants to form calluses and shoots

Plant growth regulators (PGR) are important factors, which can selectively influence the genes triggering differentiation of cells in culture (Thorpe 1983). BA and IBA were shown to be effective growth regulators for the induction of callus and shoot regeneration from various explants of *J. curcas*, and their optimal concentrations ranged from 0.1 mg $l^{-1}$ to 1 mg $l^{-1}$ (Sujatha and Mukta 1996; Lin et al. 2002; Wei et al. 2004). Our studies demonstrated that IBA together with BA is necessary for callus induction and shoot regeneration, and higher concentrations of BA (1.5 mg $l^{-1}$) and lower concentrations IBA (0.05 mg $l^{-1}$) were superior in inducing compact and yellowish calli, which could subsequently be used for shoot regeneration. Other growth regulators such as 2, 4-dichlorophenoxyacetic acid (2, 4-D, 1 mg $l^{-1}$), α-napthaleneacetic acid (NAA, 1 mg $l^{-1}$), and 1, 2, 3-thiadiazol-5-yl (TDZ, 1 mg $l^{-1}$) were also efficient in callus induction, but no shoot regeneration could be obtained in these cases (data not shown). Many adventitious buds could be observed on the margins or inner regions of cotyledon explants after 3 weeks incubation on the CI medium (Fig. 2a). 33 to 38% of the total inoculated explants produced adventitious shoots.

The effect of GA3 on shoot regeneration from calli of *J. curcas* was evaluated. GA3 has commonly been used in shoot regeneration, proliferation, and elongation in some plants (de Winnar 1988; Mohamed et al. 1996; Mathur et al. 2001; Karim et al. 2003; Hussain et al. 2004). We showed that GA3 was very effective in improving shoot regeneration from calluses. When GA3 was included in the medium, more calli differentiated into shoots (Table 1, Fig. 1b). Multiple buds were further transferred to SE medium (MS medium containing BA at 0.3 mg $l^{-1}$) for shoot elongation. Repeated multiplication of buds by node cuttings was achieved through a biweekly transfer to

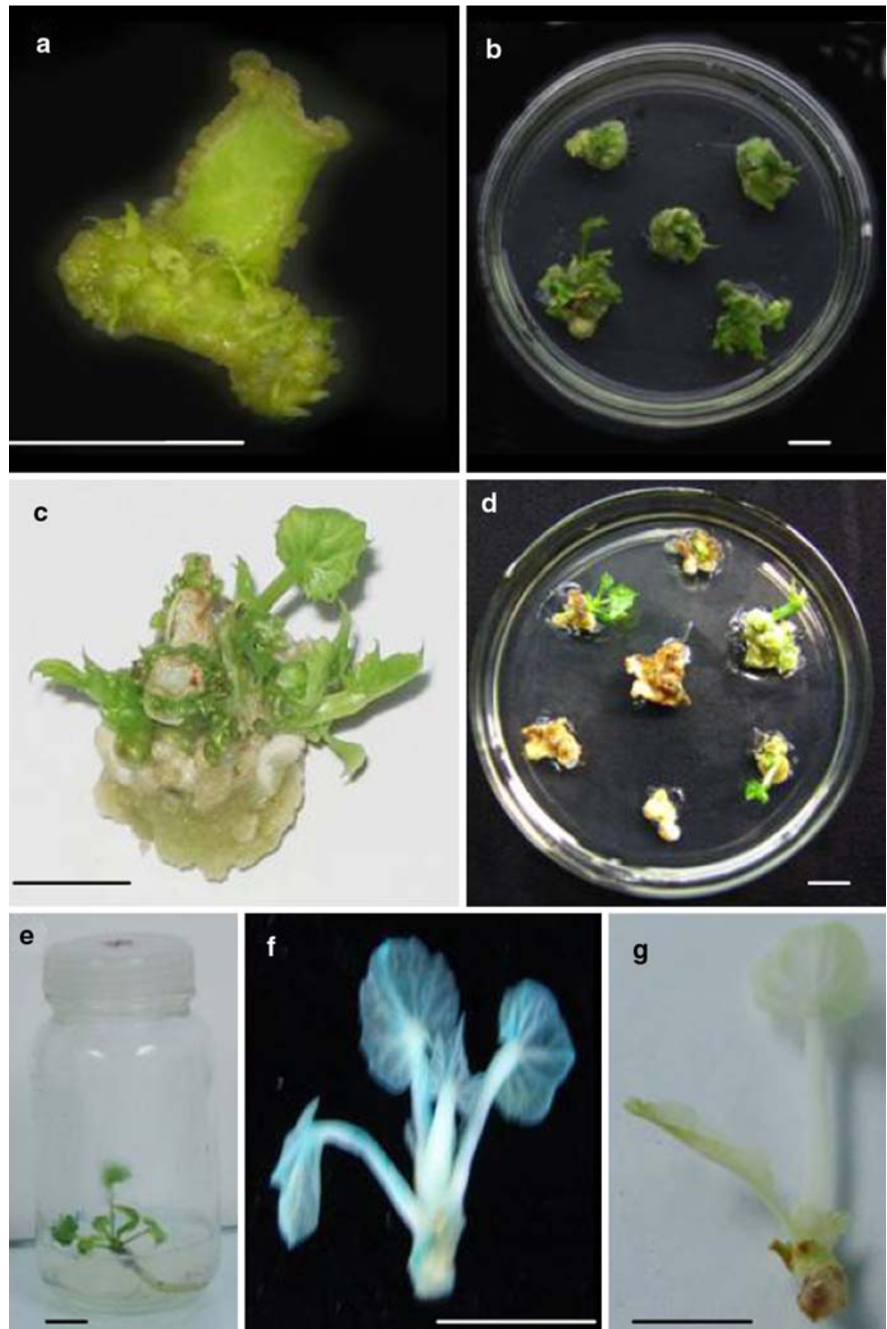


**Fig. 2** Regeneration and transformation of *J. curcas*. (**a**) Direct induction of adventitious shoots from cotyledons after a 21-day culture on CI medium containing BA (1.5 mg $l^{-1}$) and IBA (0.05 mg $l^{-1}$) in the dark. (**b**) Differentiation of multiple shoot/bud clumps after a 30-day culture on SR medium containing BA (1.5 mg $l^{-1}$), IBA (0.05 mg $l^{-1}$) and GA3 (0.5 mg $l^{-1}$). (**c**) Shoots/buds grew out from the cut end of a node after a 21-day culture on SE medium containing 0.3 mg $l^{-1}$ BA. (**d**) Resistant shoots regenerated after a 4-weeks culture on SR medium containing 1 mg $l^{-1}$ phosphinothricin and 250 mg $l^{-1}$ cefotaxime. (**e**) Putative transgenic plantlet on 1/2 MS medium containing 0.3 mg $l^{-1}$ IBA, 1 mg $l^{-1}$ phosphinothricin, 250 mg $l^{-1}$ cefotaxime after a 4-week culture. (**f**) and (**g**) GUS assay of the transformed plant (**f**) and non-transformed plant (**g**). Scale bar = 1 cm

fresh SE medium (Fig. 2c). After reaching a height of approximately 2.5 cm, the shoots were cut off and placed onto root induction media for rooting. About 86% of the shoots successfully produced roots on 1/2 MS medium supplemented with 0.3 mg $l^{-1}$ IBA.

### The effect of antibiotics and *Agrobacterium* strains on regeneration from cotyledons

For transformation of *J. curcas*, cotyledon pieces were the preferred explants. Previously, results showed that cotyledons were more susceptible to

**Table 1** The effect of GA3 on shoot regeneration from cotyledon-derived callus of *J. curcas* in combination with different concentrations of BA and IBA

| Callus induction media (mg $l^{-1}$) | | | Shoot regeneration media (mg $l^{-1}$) | | | Frequency of shoot formation (%) |
|---|---|---|---|---|---|---|
| BA | IBA | GA3 | BA | IBA | GA3 | |
| 0.5 | 1 | 0 | 1.5 | 0.05 | 0 | 53 ± 4.9 |
| 0.5 | 1 | 0 | 1.5 | 0.05 | 0.5 | 81 ± 4.3 |
| 0.5 | 1 | 0 | 1.5 | 0.05 | 1 | 63 ± 5.3 |
| 1.5 | 0.05 | 0 | 1.5 | 0.05 | 0 | 10 ± 3.2 |
| 1.5 | 0.05 | 0 | 1.5 | 0.05 | 0.5 | 94 ± 1.8 |
| 1.5 | 0.05 | 0 | 1.5 | 0.05 | 1 | 71 ± 2.2 |
| 1.5 | 0.05 | 0.5 | 1.5 | 0.05 | 0.5 | 2 ± 0.6 |
| 1.5 | 0.05 | 0.5 | 1.5 | 0.05 | 1 | 0 |

Cotyledons were cultured on callus induction media for 3 weeks in darkness, and then the resulting calli were transferred onto shoot regeneration media in the light. After another 4-week of propagation, the number of calli differentiating into shoots was recorded. Sixty cotyledon disc explants were used in each experiment. The experiment was repeated three times. Values are the mean ± SD

*Agrobacterium* infection than other explants such as petioles, hypocotyls, epicotyls, or leafs (Li et al. 2006). In addition, cotyledons of *J. curcas* are easy to obtain from germinating seed and large enough to obtain many explants for transformation in a short time. The factors influencing *Agrobacterium*-mediated cotyledon disc transformation were investigated. The selectable marker genes used for different plant species may vary according to their efficiencies. Generally, the selection of transformants is achieved using the well-characterized selection systems based on antibiotics or herbicides (Brasileiro and Aragão 2001). To define a suitable selectable marker, we tested the sensitivity of cotyledons to some antibiotics that are currently used as selection agent in plant transformation (Li et al. 2006). The results showed that 50 mg of kanamycin, 0.5 mg of phosphinothricin, or 5 mg of hygromycin per liter completely blocked shoot regeneration from untransformed callus and 100 mg of kanamycin, 1 mg of phosphinothricin, or 7.5 mg of hygromycin per liter completely killed untransformed shoots. In addition, the growth of the calli inoculated on the media supplemented with either phosphinothricin (1 mg $l^{-1}$) or hygromycin (7.5 mg $l^{-1}$) was suppressed obviously, and the calli turned brown quickly, while the calli inoculated on the media supplemented with kanamycin (100 mg $l^{-1}$) did not show the same phenomena at the same time. Therefore, kanamycin (100 mg $l^{-1}$) was shown to be unsuitable while both phosphinothricin and hygromycin significantly reduced further growth of the callus, hygromycin and phosphinothricin were used for selection of transformed cells in *J. curcas*. In the initial transformation experiments, cotyledon explants infected with LBA4404 containing p3301-BI121-SaDREB1 or pCAMBIA1301 respectively produced similar numbers of resistant calli (data not shown). However, the phosphinothricin resistant calli showed a higher shoot regeneration capacity (Table 2). Phosphinothricin has been found to stimulate somatic embryogenesis in grape by Hébert-Soulé et al (1994). It is hypothesized that, phosphinothricin might also stimulate resistant shoot development in *J. curcas*. In addition, selection with hygromycin caused rapid browning and cell death in cotyledon explants at the beginning of selection, which might have adverse effects on the growth of transformed cells. Therefore, we have chosen phosphinothricin as selection reagent in *J. curcas* transformation experiments.

Cefotaxime, which was used for suppressing *Agrobacterium*, was found to severely inhibit shoot regeneration from root explants of *Arabidopsis* (Valvekens et al. 1988). In the case of *J. curcas*, cefotaxime did not inhibit both of the callus induction and shoot regeneration capacity of its cotyledon explants and, therefore, cefotaxime was used to inhibit *Agrobacterium* after co-cultivation with cotyledon explants (Li et al. 2006). The *Agrobacterium* strain is another important factor potentially influencing the efficiency of genetic transformation. Generally, the *Agrobacterium* strains available for

**Table 2** The effect of phosphinothricin and hygromycin on the rate of cotyledon explants forming resistant shoots in *J. curcas*

| Experiment | | Number of explants | Number of explants forming resistant shoots (%) |
|---|---|---|---|
| Phosphinothricin ($1\ mg\ l^{-1}$) | 1 | 50 | 10 (20) |
| | 2 | 60 | 9 (15) |
| | 3 | 80 | 11 (13) |
| Hygromycin ($5\ mg\ l^{-1}$) | 1 | 50 | 3 (6) |
| | 2 | 60 | 5 (8) |
| | 3 | 80 | 6 (7) |

Cotyledons were infected with *Agrobacterium tumefaciens* LBA4404 (containing p3301-BI121-SaDREB1 or pCAMBIA1301 respectively), then cultured on callus induction media containing phosphinothricin or hygromycin respectively, for 3 weeks in darkness. Subsequently, the resistant calli were transferred onto resistant shoot selection media in the light. After another 4-week of propagation, the number of explants forming resistant shoots was recorded

different plant species may vary according to the susceptibility of plant species to the strains. Both of LBA4404 and EHA105 harboring the same binary vector pCAMBIA 1301, which contains the *hpt* gene and the *gus*-int gene produced different transient blue GUS spots. We detected more than 20 blue spots per explant for LBA4404 (Li et al. 2006), but only about 10 blue spots per explants for EHA105. Our results indicated that LBA4404 was superior to EHA105 in producing both the resistant callus and allowing shoot formation (Table 3). Therefore, *Agrobacterium* strain LBA4404 was selected for further *J. curcas* transformation experiments.

## Production of transgenic plants of *J. curcas*

Cotyledon explants were incubated with the *Agrobacterium* strain LBA4404 harboring pCAMBIA 1301 for different time intervals (5, 10, 15, 20, 25, and 30 min). We observed that the transient expression of GUS increased with the incubation time (Li et al. 2006). Considering the post infection process and the need to remove *Agrobacterium* cells during the selection process, an incubation time of 10 min was selected in subsequent transformation experiments. After 3 days of co-cultivation, the explants were washed with sterile water and transferred onto selection media, (CI media supplemented with $1\ mg\ l^{-1}$ phosphinothricin and $500\ mg\ l^{-1}$ cefotaxime). 14 days later, nodular calli grew out from the cut end or the surface of the yellow-brown explants; about 55% of the cotyledon explants produced resistant calli within 3 weeks. Subsequently, resistant calli were transferred to selection media (SR media supplemented with $1\ mg\ l^{-1}$ phosphinothricin and $250\ mg\ l^{-1}$ cefotaxime). Approximately 33% of the resistant calli differentiated into shoots (Table 3; Fig. 1d). Root formation efficiency from resistant shoots occurred at a rate of 78% on RI/phosphinothricin selective media (Fig. 2e; Table 4). Histochemical GUS assays showed that *uidA* expressed in resistant shoots (Fig. 2f), whereas no blue GUS signal was observed in non-transformed shoots (Fig. 2g).

The insertion of T-DNA into the *J. curcas* genome was confirmed by PCR and Southern hybridization. A DNA band was detected at a size of 441 bp in the

**Table 3** The effect of *Agrobacterium* strains LBA4404 and EHA105 on the rate of cotyledon explants forming phosphinothricin - resistant shoot in *J. curcas*

| Experiment | | Number of explants | Number of explants forming resistant calli | Number of explant forming resistant shoots (%) |
|---|---|---|---|---|
| LBA4404 | 1 | 80 | 40 | 12 (15) |
| | 2 | 100 | 52 | 19 (19) |
| | 3 | 50 | 32 | 10 (20) |
| EHA105 | 1 | 80 | 17 | 3 (3) |
| | 2 | 100 | 23 | 5 (5) |
| | 3 | 50 | 12 | 1 (2) |

Cotyledons were infected with *Agrobacterium tumefaciens* LBA4404 or EHA105 (each containing p3301-BI121-SaDREB1), cultured on callus induction media containing phosphinothricin for 3 weeks in darkness, then the number of explants forming resistant calli was recorded. Subsequently, the resistant calli were transferred onto shoot selection media in the light. After a further 4-weeks of culture, the number of explants forming resistant shoots was recorded

**Table 4** Summary of *Agrobacteriuim*-mediated cotyledon disc transformation results in *J. curcas*

| Experiment | Number of explants | Number of explants forming resistant shoots | [a]Number of resistant plants | [b]PCR+ (%) |
|---|---|---|---|---|
| 1 | 50 | 10 | 8 | 8 (16) |
| 2 | 60 | 9 | 7 | 7 (11) |
| 3 | 80 | 11 | 8 | 8 (10) |
| 4 | 80 | 12 | 9 | 9 (11) |
| 5 | 100 | 19 | 14 | 14 (14) |
| 6 | 50 | 10 | 8 | 8 (16) |

[a]Only one shoot from one individual resistant callus and rooted on 1/2 MS containing 1 mg $l^{-1}$ phosphinothricin, was scored

[b]Resistant plants were checked by *bar*-PCR, *bar*-PCR positive result are indicated as "+"

transgenic lines by genomic PCR with primers specific for *bar*, while this band was not detected in non-transformed plants (Fig. 3a). The presence of the *bar* gene was also confirmed by Southern hybridization using part of the *bar* sequence as the DIG-labeled probe. Multiple copies of the T-DNA insertion were detected in the transgenic lines tested (Fig. 3b). As there is only one *Hin*dIII site in the T-DNA region, the number of hybridization bands represents the number of copies of T-DNA integrated, while the intensity of the bands indicated that tandem or multimer arrangements of the T-DNA region occurred during the transformation process. A total of 54 independent transgenic plants, each derived from an independently infected cotyledon disc, were obtained from 420 cotyledon discs within 4 months following infection with *Agrobacterium* (Table 4). On average, 13 transgenic lines were obtained from 100 cotyledon explants of *J. curcas*, representing an overall transformation efficiency of 13%.

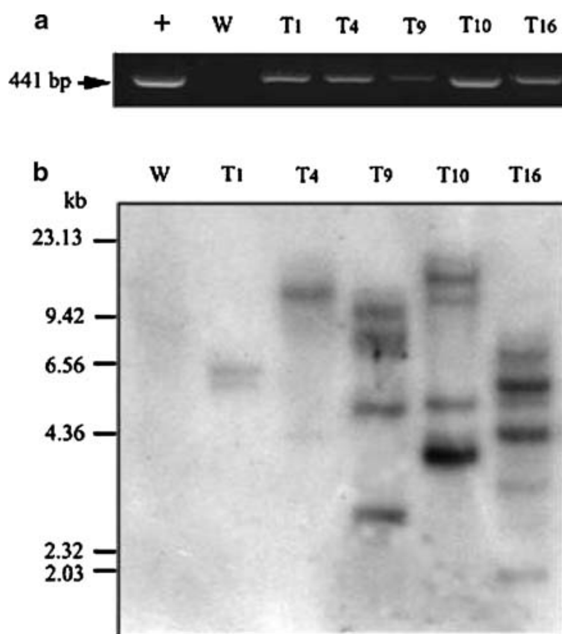


**Fig. 3** Molecular analysis of transgenic plants. (**a**) PCR amplification of the *bar* gene in transgenic lines. +, Plasmid control (p3301-BI121-SaDREB1). W, Non-transformed plant. T1 to T16, Putative transgenic lines. (**b**) Southern hybridization using the *bar* gene as probe in transgenic lines. Genomic DNA was digested with *Hin*dIII. W, Non-transformed plant. T1, T4, T9, T10 and T16 are different transgenic lines. λ/*Hin*d III size marker is indicated on the left

In summary, highly efficient genetic transformation and plant regeneration methods were established for *J. curcas* using cotyledon explants. These methods have the potential to facilitate the genetic modification and subsequent in vitro multiplication of *J. curcas* cultivars for various uses, for example the bio-diesel industry. Further, the technology can lead to a better understanding and subsequent improvement of the oil biosynthesis pathway in this species, which could have positive implications on reducing the worlds dependence on fossil reserves.

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