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ORIGINAL ARTICLE

Physical wounding-assisted *Agrobacterium*-mediated transformation of juvenile cotyledons of a biodiesel-producing plant, *Jatropha curcas* L.

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Abstract The non-edible plant *Jatropha curcas* L. is one of the most promising feedstock for sustainable biodiesel production as it is not a source of edible vegetable oils, produces high amounts of oil (approx. 30–60% in dry seeds) and does not require high-cost maintenance. However, as with other undomesticated crops, the cultivation of *J. curcas* presents several drawbacks, such as low productivity and susceptibility to pests. Hence, varietal improvement by genetic engineering is essential if *J. curcas* is to become a viable alternative source of biodiesel. There is to date no well-established and efficient transformation system for *J. curcas*. In this study, we tested various physical wounding treatments, such as sonication and sand-vortexing, with the aim of developing an efficient *Agrobacterium*-mediated transformation for *J. curcas*. The highest stable transformation rate (53%) was achieved when explants were subjected to 1 min of sonication followed by 9 min of shaking in *Agrobacterium* suspension. The transformation frequency achieved using this protocol is the highest yet reported for *J. curcas*.

Keywords *Jatropha curcas* L. · Cotyledon · *Agrobacterium*-mediated transformation · Physical wounding · Biodiesel

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Introduction

Within the framework of a constantly diminishing petroleum fuel supply and increasing evidence of deleterious environmental consequences from the use of petroleum fuel, alternative energy sources, such as plant oils, animal fats and waste oils, which are renewable and sustainable, have been become increasing attractive in recent years. The technology for producing biodiesel from plant oil has been available for many years. However, due to the high cost of refined vegetable oils, the production of biodiesel from these sources is less profitable than the production of petroleum diesel (Vasudevan and Briggs 2008). To reduce the production cost of producing biodiesel and make it competitive with petroleum diesel, researchers have been focusing on the use of low-cost feedstocks, such as non-edible oils, as promising sources of raw material for large-scale biodiesel production.

The oil-producing plant, *Jatropha curcas* L., commonly known as physic nut, belongs to the family Euphorbiaceae. It is a multipurpose plant which has been utilized for many years as a living fence to prevent and/or control soil erosion and reclaim land and in traditional medicines (Heller 1996). This plant has been attracting considerably attention as an alternative feedstock for biodiesel production for several reasons. *Jatropha*'s seeds contain high amounts of non-edible oil (approx. 30–60%) that can be used for biodiesel production. The plant is also highly adaptable and can grow in semi-arid, arid and marginal lands and thrive in areas with a wide range of rainfall (between 200 and 1500 mm per year). *J. curcas* grows relatively quickly and can bear fruits within a year (Kumar and Sharma 2008; Openshaw 2000). Given these characteristics, this plant has tremendous potential as a low-cost feedstock for sustainable biodiesel production. However, *J. curcas* remains

undomesticated, and thus its growth as well as its yield can be greatly affected by changes in climatic conditions. In effect, the cultivation of this plant provides relatively low economic returns. Moreover, *J. curcas* seeds contain many toxic substances, such as curcin, phorbol ester, trypsin inhibitor, lectin and phytate, which have adverse effects on human health and on nutrient utilization in animals. The toxicity of *J. curcas* subsequently raises concerns on the safety of using this plant as a biodiesel crop. Taken together, it is necessary to initiate a program of varietal improvement of *J. curcas* in order to enhance and stabilize its productivity in various land areas, including wastelands and non-arable lands, and to improve the quality of its oil and seed meal for diversified utilization (Sujatha et al. 2008). The successful improvement of this plant would make it a more promising and profitable plant source for the large-scale production of biodiesel to meet today's fuel needs.

Genetic engineering in combination with a conventional breeding program would be a potential tool for the improvement of *J. curcas.* However, information pertaining to the genetic transformation of *J. curcas* remains limited, with only a few reports on the use of cotyledons and leaf explants. Li et al. (2008) reported a basic cotyledon-disc transformation method using *Agrobacterium tumefaciens*, and Kumar et al. (2010) described the fundamental key parameters affecting the efficiency of *Agrobacterium*-mediated transformation of leaf explants. The results of these studies consistently demonstrate that stable genetic transformation can be achieved using the *Agrobacterium*-mediated method, but the transformation efficiency was low. A high transformation efficiency is essential in genetic transformation studies as it is important to be able to produce a large population of transgenic plants from which improved individuals can be selected and analyzed. Thus, a more efficient transformation system has to be established for *J. curcas*.

The *Agrobacterium*-mediated transformation process relies on both the infectivity of bacterial cells involved in transgene delivery and the activity of host cells involved in transgene integration (Boyko et al. 2009; Citovsky et al. 2007). Serious research efforts have focused on manipulating various factors with the aim of improving T-DNA delivery into host cells, including the use of various wounding treatments. Zuker et al. (1999) wounded stem explants of carnation (*Dianthus caryophyllus* L.) by microprojectile bombardment prior to *Agrobacterium* infection, while Xue et al. (2006) observed that gentle stabbing by a multi-needle successfully improved the transformation of soybean cotyledonary nodes. The transformation of shoot apical meristems of *Vitis vinifera* L. was enhanced by treating the explants with a combination of nicking, abrasion and sonication (Dutt et al. 2007). Abrasion of the apical node by sand-vortexing was used in alfalfa (Weeks et al. 2008) and ultrasonic wounding has also been used to increase the transformation efficiency in many plant tissues (Pathak and Hamzah 2008; Santarem et al. 1998; Trick and Finer 1997).

In the study reported here, we attempted to enhance the transformation efficiency of *J. curcas* explants through physical wounding-assisted *Agrobacterium*-mediated transformation. Different means of physical wounding (i.e., sonication and sand-vortexing in addition to shaking) were employed to determine the method which would give the maximum transformation efficiency. The efficacy of each wounding treatment was evaluated in terms of both transient and stable transformation efficiency. Based on the stable transformation frequency of kanamycin (Kan) resistance, we demonstrate that physical wounding was able to efficiently ameliorate the transformation efficiency of juvenile cotyledons of *J. curcas*.

Materials and methods

Plant material and preparation of explants

The preparation of the plant material and the composition of all regeneration media used in this study have been described in Khemkladngoen et al. (2011). *Jatropha* seeds collected from the Philippines were husked and surface-sterilized with 30% (v/v) sodium hypochlorite and 0.01% Triton X-100 for 3 min and then rinsed three times with sterile distilled water. The sterilized seeds were then germinated on half-strength MS (Murashige and Skoog 1962) basal medium (1/2MS), which consisted of MS salts (Wako Pure Chemical Industries Ltd, Osaka, Japan) supplemented with 3% (w/v) sucrose, 10 mg L^{-1} thiamine and 100 mg L^{-1} myo-inositol (pH 5.8) and solidified with 0.7% (w/v) agar. Once the radicles emerged and the endosperm had opened slightly, juvenile cotyledons were incised into small pieces of 3 × 3 mm. The incised cotyledons were precultured on shoot regeneration medium consisting of MS basal medium supplemented with 3 mg L^{-1} benzyl adenine (BA) and 0.1 mg L^{-1} indole-3-butyric acid (IBA) for 1 week. All cultures were incubated at 25 ± 2°C under a 16/8-h (light/dark) photoperiod with light provided by cool-white fluorescent lamps at an intensity of 31–35 μmol m^{-2} s^{-1}. One week-precultured cotyledons were used as explants for genetic transformation.

Bacterial strain, plasmid and culture conditions

Agrobacterium tumefaciens, also known as *Rhizobium radiobacter*, strain LBA4404 containing the binary vector pBI121 was used for all transformation treatments. The

T-DNA region of pBI121 harbors the reporter β-glucuronidase (*GUS*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (*NOS*) terminator, and the neomycin phosphotransferase II (*npt*II) gene driven by the *NOS* promoter and terminator for Kan resistance. *Agrobacterium* cultures were grown overnight in 20 mL liquid YEB medium (5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$) containing 20 mg L^{-1} rifampicin, 25 mg L^{-1} streptomycin, 50 mg L^{-1} Kan and 20 mg L^{-1} acetosyringone at 30°C, 150 rpm. Overnight cultures of *Agrobacterium*-pBI121 were centrifuged at 3,000 *g* for 10 min, and the pellet was re-suspended in liquid MS basal medium containing 20 mg L^{-1} acetosyringone. The *Agrobacterium* suspension ($OD_{600} = 0.2$) was used for all transformation treatments.

Agrobacterium-mediated transformation and plant regeneration systems

The first step in the transformation process consisted of immersing 13–15 excised explants in 10 mL of *Agrobacterium* suspension contained in 50-mL Falcon tubes. The explants in this *Agrobacterium* suspension were exposed to different physical wounding treatments. For shaking, the explants were subjected to shaking on an orbital shaker at 150 rpm, 30°C for 10 min. For the sonication treatment, the explants in the Falcon tube were placed in the middle of a bath-type sonicator set and then exposed to ultrasound for 30 s or 1 min, 2 min (2 × 1-min treatment, with a 15-s interval between treatments) or 3 min (3 × 1-min treatment, with a 15-s interval between treatments). Following the sonication treatment, the explants were maintained in *Agrobacterium* suspension and subjected to shaking at 150 rpm, 30°C for a further 10, 9, 8 or 7 min, respectively, to give a total infection time of 10 min. For the sand-vortexing treatment, 0.5 g of quartz sand was added to the *Agrobacterium* suspension. The explants in the *Agrobacterium* suspension were vortexed for 15 s, 30 s, 1 min or 3 min and further subjected to shaking at 150 rpm, 30°C to obtain a total infection time of 10 min. The excess *Agrobacterium* suspension was removed by blotting the explants on a sterile paper towel before the explants were cultured on co-cultivation medium (shoot regeneration medium supplemented with 20 mg L^{-1} acetosyringone) at 25 ± 2°C in the dark for 3 days. The explants were subsequently washed with sterile distilled water containing 200 mg L^{-1} cefotaxime to remove excess bacteria, and they were blotted onto a sterile paper towel. The transformed explants were cultured on selection medium (shoot regeneration medium supplemented with 200 mg L^{-1} cefotaxime and 20 mg L^{-1} Kan). The frequency of explants resistant to Kan selection was scored after 8 weeks of culture on shoot regeneration medium containing Kan. Shoot-generating explants were transferred to selection medium for shoot elongation (MS basal medium supplemented with 2 mg L^{-1} BA, 200 mg L^{-1} cefotaxime, 20 mg L^{-1} Kan). The well-developed shoots were then transferred to root induction medium containing 1/2MS basal medium and 0.2 mg L^{-1} IBA. Rooted plantlets were carefully transplanted into a jar containing autoclaved gardening soil and gradually acclimatized by opening the culture chamber. Plantlets were eventually transplanted to plastic pots and moved to the glasshouse. For each treatment, 25–30 explants were used, and the experiments were repeated three times.

Histochemical GUS assay

Transient GUS expression in cotyledon explants was monitored at 7 days post-*Agrobacterium* transformation, and stable GUS expression was examined in Kan-resistant leaves and shoots as described by Cervera (2005) with some modifications. Tissues were immersed in staining solution {0.1 M $NaHPO_4$ buffer (pH 7.0), 10 mM EDTA (pH 7.0), 2 mM $K_3[Fe(CN)_6]$, 2 mM $K_4[Fe(CN)_6] \cdot 3H_2O$, 0.1% (v/v) Triton X-100, 500 mg L^{-1} X-Glu} and subjected to vacuum for 20 min. The tissues were further incubated in staining solution overnight at 37°C, following which the chlorophyll was removed from the tissues using ethanol prior to visualization and photography. The GUS expression rate was calculated by comparing the number of explants/leaves that showed GUS expression to the total number of explants/leaves assayed. The relative GUS expression, which represents the number of transformants that showed both resistance to Kan and GUS expression from all samples/explants subjected to transformation, was evaluated by multiplying the GUS expression rate by the frequency of Kan-resistant explants. The formulas used for Kan-resistant explants (%), GUS expression (%) and relative GUS expression (%) are shown below.

$$\text{Kan-resistant explants } (\%) = \frac{\text{No. of explants with regenerated shoots}}{\text{No. of transformed explants}} \times 100$$

$$\text{GUS expression } (\%) = \frac{\text{No. of GUS-expressing leaves}}{\text{No. of kanamycin-resistant leaves}} \times 100$$

$$\text{Relative GUS expression} = \frac{\text{GUS expression} \times \text{kanamycin resistance frequency}}{100}.$$

Molecular analysis of transformed plantlets

Genomic DNA was isolated from young leaves using the DNeasy Plant Mini kit (QIAGEN, Tokyo, Japan) according

to the manufacturer's protocol. PCR analysis of the isolated genomic DNA was performed to check for the presence of transgenes in putative transformants using primers for both the *GUS* and *npt*II genes. The pair of primers used to amplify the *GUS* coding region was 5′-CACCATGTTA CGTCCTGTAGAAACCCCAACC-3′and 5′-TTGTTTGC CTCCCTGCTGCGG-3′, which amplifies a 1,800-bp fragment. The pair of primers used for *npt*II amplification was 5′-GGCTATGACTGGGCACACCA-3′ and 5′-GCGATAC CGTAAACCACGAG-3′, which amplifies a 680-bp fragment. DNA samples were amplified using Go Taq Green Master Mix (Promega, Madison, WI). The amplification reactions were performed according to standard protocols and consisted of an initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 58°C (for *npt*II gene) or 59°C (for *GUS* gene) for 30 s and 72°C for 1 min (for *npt*II gene) or 1 min 30 s (for *GUS* gene), with a final extension step of 5 min at 72°C. The PCR products were separated by gel electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized with a UV transilluminator.

Southern hybridization was performed to check the integration of the T-DNA in the *J. curcas* genome. A 5-μg sample of genomic DNA was digested overnight at 37°C using *Hin*dIII (New England Biolabs, Ipswich, MA) to cleave a unique site in the T-DNA. The DNA was subsequently separated on a 1% agarose gel, then denatured and neutralized before being transferred to a positively charged nylon membrane (Roche Diagnostic Systems, Indianapolis, IN). The digoxigenin (DIG)-labeled probe was prepared using the PCR DIG Probe Synthesis kit (Roche Diagnostic Systems) and utilizing the *GUS* primers described above with pBI121 as the template. Hybridization was carried out in Hybri Easy Hyb (Roche Diagnostic Systems) at 48°C. The membrane was washed twice in low-stringency buffer [2× standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS)] at room temperature for 5 min each time, then twice in high stringency buffer (0.5× SSC, 0.1% SDS) at 68°C for 15 min each time. Detection of the hybridized probe was carried out according to the instructions in the manual supplied by Roche Diagnostic Systems using CSPD substrate.

Statistical analysis

In this study, 25–30 explants were used for each treatment and the experiments were repeated three times. To determine the frequency of Kan-resistant explants, we performed the *t* test to compare the data between the shaking and 2-min sonication treatments. The software program KaleidaGraph (http://www.synergy.com/) was used for all statistical analyses.

Results

Effect of physical wounding treatments on transient GUS expression

Different time-courses of wounding treatments were tested with the aim of obtaining the highest *Agrobacterium* infection together with the lowest cell damage. We found that all wounding treatments were able to injure plant tissues, and blue foci indicating GUS expression were detected in most of the explants in all treatments (Fig. 1). However, the size and distribution pattern of the blue foci were different among the three treatments. With shaking, expression was found mainly on the cut end of explants (Fig. 1b). In contrast, the blue foci were located both on the cut end of explants and inside or in the middle of explants when the explants were treated with sonication and sand-vortexing (Fig. 1c–j). Moreover, explants treated with sonication and sand-vortexing displayed a substantial number of blue foci on the explant surface, but only a very small number of blue foci were observed on the explant surface in the shaking treatment.

Effect of the physical wounding treatments on stable genetic transformation

To evaluate the effect of physical wounding on stable transformation, we selected transformed explants on shoot induction medium supplemented with cefotaxime and Kan to suppress the growth of *Agrobacterium* cells and non-transformed cells, respectively. The frequencies of Kan resistance in explants subjected to the shaking, sonication and sand-vortexing treatments were approximately 54, 52–72 and 50–56%, respectively (Table 1). The highest Kan resistance frequency (approx. 72%) was obtained from the 2-min sonication treatment followed by 8 min shaking. The addition of the 2-min sonication treatment during *Agrobacterium* infection significantly increased the transformation efficiency of *J. curcas* based on Kan resistance frequency as compared to the shaking treatment at $\alpha = 0.1$ (*t* test, $P = 0.089$).

After 8 weeks of culture in selection medium for shoot induction, explants were transferred to shoot elongation medium containing antibiotics (Fig. 2a–c). To investigate whether physical wounding has an effect on stable GUS expression, we collected leaves from well-developed shoots (Fig. 2d) for the histochemical GUS assay (Fig. 3b–d). The number of leaves showing GUS expression was compared to the total number of leaves showing resistance to Kan and the product of this comparison was set as the GUS expression rate. To obtain a representative stable transformation frequency for all samples/explants subjected to

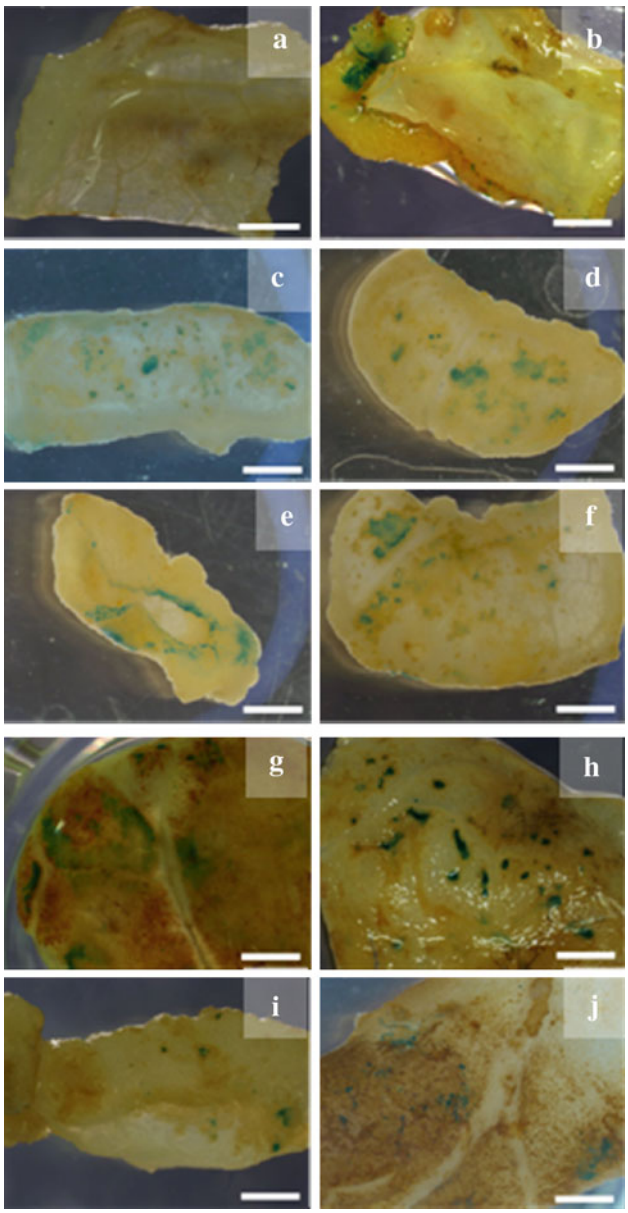

Fig. 1 Histochemical assay of β-glucuronidase (GUS) transient expression at 7-days post-transformation. The histochemical assay was performed to investigate the expression of the *GUS* gene and to locate GUS expression in the transformed explants subjected to each treatment. **a** Non-transformed explants, **b** explants treated with shaking, **c–f** explants treated with 30 s (**c**), 1 min (**d**), 2 min (**e**) and 3 min (**f**) sonication followed by shaking, **g–j** explants treated with 15 s (**g**), 30 s (**h**), 1 min (**I**) and 3 min (**j**) sand-vortexing followed by shaking. *Bars*: 2 mm

transformation, we computed the relative GUS expression rate by multiplying the GUS expression rate by the frequency of Kan resistance. The GUS expression rate and relative GUS expression rate are shown in Table 1. We found that Kan-resistant tissues obtained from the 1-min sonication and 15-s sand-vortexing treatments showed the highest GUS expression rate, namely, about 90%, of all

Table 1 Effect of wounding treatments on kanamycin resistance frequency, stable β-glucuronidase (GUS) expression rate and relative stable GUS expression rate in *Jatropha curcas*

Treatments	Kan-resistant explants[a] (%)	GUS expression (%)	Relative GUS expression (%)
Shaking			
10 min	54.2 ± 4.9	90.5	49.0
Sonication			
30 s	60.0 ± 10	75.0	45.0
1 min	60.7 ± 9.4	87.5	53.1
2 min	72.3 ± 6.4	59.3	42.8
3 min	52.4 ± 9.9	47.4	24.8
Sand-vortexing			
15 s	54.4 ± 21.4	91.7	49.8
30 s	50.1 ± 15.4	76.0	38.1
1 min	55.7 ± 9.1	61.5	34.3
3 min	54.1 ± 12.9	50.0	27.0

The statistical difference between the frequency of kanamycin (Kan)-resistant explants obtained from the 10-min shaking + 2-min sonication treatment was observed at a threshold α value of 0.1 (t test, $P = 0.089$)

[a] Values are presented as the mean ± standard error (SE) of three replicate treatments ($n = 3$)

sonication treatments and sand-vortexing treatments, respectively (Table 1). The relative GUS expression rates shown in Table 1 reveal that the duration of the wounding exposure times during the sonication and sand-vortexing treatments had an effect on stable GUS expression: the longer the exposure of explants to sonication and sand-vortexing, the lower the GUS expression rate. Moreover, the highest transformation frequency in terms of relative GUS expression (approx. 53%) was obtained from the 1-min sonication treatment followed by 9 min of shaking. In the histochemical GUS assay of Kan-resistant shoots, whole shoots from all treatments showed the blue coloration indicative of GUS activity, indicating stable non-chimeric GUS expression (Fig. 3f, g). Well-developed shoots 2–3 cm in height were transferred to root induction medium, and after 4–6 weeks, approximately 60–70% of the plantlets had developed roots (Fig. 2e). The rooting plants (approx. 90%) were successfully acclimatized and cultured in the glasshouse (Fig. 2f). The morphological appearance and growth rate of transgenic plants was normal when compared to those of wild-type plants germinated from seeds and cultured in vitro.

Molecular analysis of transgenic plantlets

In total, 113 GUS-expressing transformants were obtained from all transformation treatments. A number of representative transformants were subjected to PCR analysis to

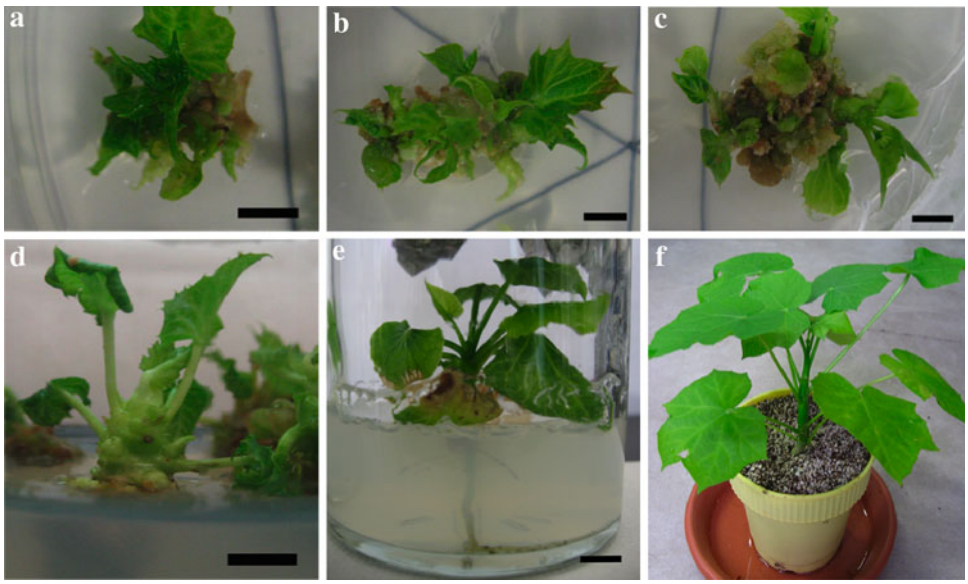

Fig. 2 Regeneration of transformed *J. curcas* plants. **a–c** Transformed explants treated with 15 s of vortexing (**a**), 1 min of sonication (**b**) or 2 min of sonication (**c**) in shoot elongation medium with antibiotics, **d** well-developed shoots in shoot elongation medium with antibiotics, **e** transformed rooting plantlets, **f** transformed plant cultured in gardening soil. *Bars*: 5 mm

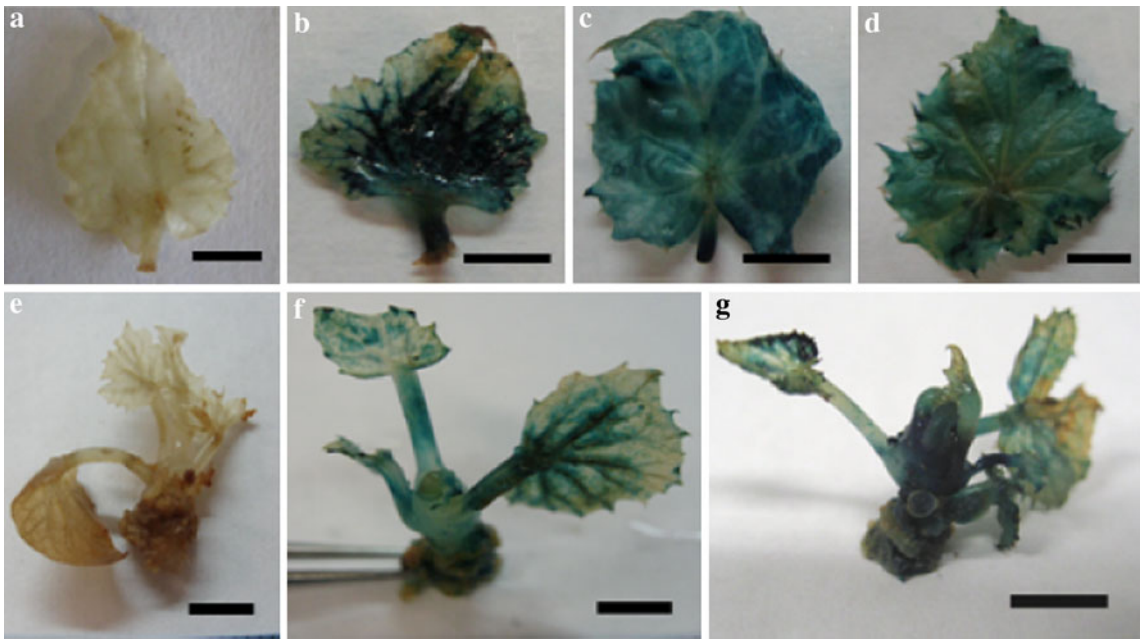

Fig. 3 Histochemical assay of GUS stable expression in a non-transformed leaf (**a**), a non-transformed shoot (**e**), kanamycin (Kan)-resistant leaves (**b–d**) and Kan-resistant shoots (**f**, **g**). GUS-expressing leaves were obtained from explants treated with shaking (**b**), 1 min of sonication (**c**) and 3 min of vortexing (**d**). GUS-expressing shoots were regenerated from explants treated with 1 min of sonication (**f**) and 30 s of vortexing (**g**). *Bars* (**a–d**) 2 mm, (**e–g**) 5 mm

confirm the presence of the transferred genes. The expected *npt*II and *GUS* fragments of 680 and 1,800 bp, respectively, were amplified from DNA samples of all representative transgenic plantlets (Fig. 4a, b), indicating that the transgenic plantlets contained both transgenes, namely, the *npt*II and *GUS* genes. The integration of the *GUS* gene in the transgenic *Jatropha* plants was confirmed by Southern hybridization analysis (Fig. 4c) performed on two well-developed transgenic plantlets. No hybridization band was detected in the wild-type plant. However, one band (approx. 7 kb) was obtained for transgenic plant #1, while two bands (at 10 and 12 kb) were detected for transgenic plant #2 (Fig. 4c). These results indicate that one copy of the *GUS* gene was integrated into transgenic plant #1 and more than two copies of the *GUS* gene were integrated into transgenic

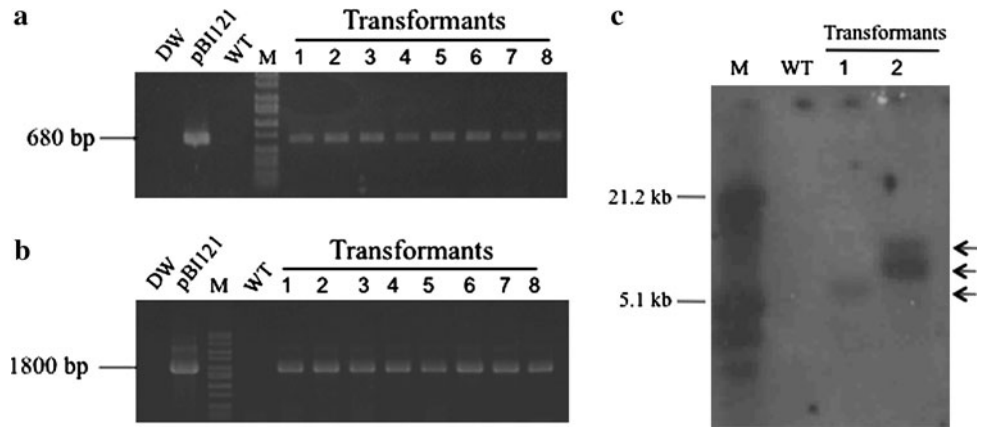

Fig. 4 Molecular analysis of transformants. PCR amplification indicated the presence of the neomycin phosphotransferase II (*npt*II) gene (**a**; 680 bp) and *GUS* gene (**b**; 1,800 bp) in the genome of transgenic plantlets. **a**, **b** Transformant *#1* was obtained from the shaking treatment, transformants *#2–5* were obtained from the sonication treatments and transformants *#6–8* were obtained from the sand-vortexing treatments. **c** Southern hybridization of genomic DNA isolated from the wild type (*WT*) and from transformants *#1* (shaking treatment) *#2* (1 min of sonication). The digoxigenin-labeled *GUS* fragment was used as probe. *M* DNA marker, *DW* distilled water

plant #2. They also confirm that the regenerated plants were stably transformed.

Discussion

The ultimate aim of our study was to enhance the transformation efficiency of *J. curcas* and to produce large populations of transformants. To this end, we attempted to enhance the transfer of DNA from *Agrobacterium* to plant cells by increasing the contact between *Agrobacterium* and the plant cells. A wounding treatment is crucial for efficient transformation and has been successfully employed to improve transformation efficiency in many plants (Du and Pijut 2009; Dutt et al. 2007; de Oliveira et al. 2009; Pathak and Hamzah 2008; Santarem et al. 1998; Trick and Finer 1997; Weeks et al. 2008). During wounding by sonication, the explosion of small bubbles from ultrasonic cavitation causes mechanical damage and creates micro-wounds on plant cells. The wounds on the surface of tissues are large enough to allow *Agrobacterium* to invade and infect the wounded cells and neighboring cells. Trick and Finer (1997) reported that sonication causes very localized and deep micro-wounding without collateral damage to the neighboring cells. In a similar manner, sand-vortexing was found to wound explants by acting as an abrasive on the epidermal cells of explants. We hypothesized that increasing the number of wounding sites on the plant tissue would promote *Agrobacterium* infection and DNA transfer from *Agrobacterium* to plant cells and subsequently improve the transformation efficiency. Various phenolic compounds are produced at the wounding site that can activate the expression of *vir* genes in *Agrobacterium* and lead to the recognition of *Agrobacterium* by plant cells and subsequent infection (Citovsky et al. 2007).

In our experiments, wounding of the tissues by sonication and sand-vortexing in addition to shaking were employed to facilitate efficient *Agrobacterium* infection. The results, based on the frequency of stable Kan resistance transformation, demonstrate that physical wounding by the 2-min sonication treatment followed by 8 min of shaking obtained the highest transformation efficiency, compared to shaking, at $\alpha = 0.1$ (t test, $P = 0.089$). The stable transformation frequency based on Kan resistance obtained from our method is higher than those reported previously (Li et al. 2008; Kumar et al. 2010). Li et al. (2008) used mature cotyledons as plant material; however, these explants have a lower regeneration ability and are less susceptibility to *Agrobacterium* infection than the young/juvenile cotyledons (Mazumdara et al. 2010) used in our study.

Kumar et al. (2010) studied the key factors affecting *Agrobacterium*-mediated transformation with the aim of improving transformation in *J. curcas*. These authors pointed out that wounding treatments by glass beads and hand pricking with needle were unnecessary for transformation and deleterious to plant regeneration in *J. curcas*. However, they did not describe their wounding protocol in detail and did not mention the optimization of conditions for wounding. Establishing the optimum conditions for physical wounding is essential, since too much or too little wounding can affect the transformation and regeneration efficiencies of plant tissues. In contrast, our results demonstrate that certain conditions of physical wounding (2-min sonication) were able to significantly improve the transformation efficiency in *J. curcas*, based on Kan

resistance frequency, as compared to the shaking treatment. As a result, the transformation efficiency obtained in our study was higher than that reported by Kumar et al. (2010). Moreover, to further increase transformation efficiency in *J. curcas*, the combination of sonication or sand-vortexing with other physical treatments, such as vacuum, is recommended.

We utilized the *GUS* reporter gene as an indicator of successful transformation and expression of the transgenes. The expression of the *GUS* gene among the Kan-resistant leaves/shoots was examined and set as the GUS expression rate. To evaluate GUS expression based on the initial explants used, the relative GUS expression was calculated (Table 1). We found that an approximately 53% stable transformation rate was obtained in *J. curcas*, based on relative GUS expression, following physical wounding of the tissues by a 1-min sonication during *Agrobacterium*-mediated transformation. Moreover, a longer exposure of the tissues to the wounding treatment was observed to adversely affect the stable transformation rate compared to shorter wounding times. It is highly probable that long periods of sonication and sand-vortexing increased the death of *Agrobacterium* cells, resulting in a low DNA transfer efficiency from *Agrobacterium* to plant cells. Results from previous studies also suggest that relatively longer sonication treatments often cause damage to plant tissues, as reflected in a low transformation efficiency (Santarem et al. 1998; Trick and Finer 1997). Different levels of wounding have different effects on plant cells; for example, a high level of wounding results in immediate cell lysis, sublethal levels cause temporary suppression of RNA and protein synthesis and moderate levels induce rupture of the cell walls (Joersbo and Brunstedt 1992). Based on our GUS expression rate (%), we noted that many Kan-resistant shoots did not show GUS expression. Non-transformed tissues are often encountered even after selection using antibiotics. One explanation is that transformed cells protect many non-transformed cells from exposure to the selection agents, although the mechanism by which they do this is not clear. However, it is likely that the *npt*II provided by transformed cells detoxifies the aminoglycosidic antibiotics, hence reducing their effective concentration in the medium (Dong and McHughen 1993). A second explanation for the occurrence of non-transformed plants is the long preculture period. Barik et al. (2005) and Kumar et al. (2010) investigated the effect of preculture period on the transformation efficiency of *Agrobacterium*-mediated transformation using epicotyls of grasspea and mature leaves of *J. curcas*, respectively. They found that a preculture period of longer than 4 days produced more non-transformed shoots. Our results showing that a 7-day preculture period resulted in the occurrence of many non-transformed shoots (data not shown) are consistent with these earlier findings. Therefore, shortening the preculture period would most likely reduce the number of non-transformed tissues.

Overall, the method established in this study, consisting of a 1-min sonication treatment of explants followed by 9 min shaking in *Agrobacterium* suspension, is an effective protocol for obtaining the transformation of *J. curcas* with a high stable transformation efficiency (approx. 53%). The transformation efficiency obtained using this method was fourfold and twofold higher than those reported using cotyledons (Li et al. 2008) and young leaves (Kumar et al. 2010), respectively. It is therefore a promising methodology for achieving genetic modification of *J. curcas*. The information reported here may also facilitate molecular analysis studies aimed at the genetic improvement of *J. curcas*, which in turn may result in the usage of this biodiesel crop as an energy source being more feasible and sustainable in the near future.

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