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Full Length Article

Agrobacterium-mediated Transformation of *Jatropha curcas* Young Leaf Explants with Lateral Shoot-Inducing Factor (*LIF*)

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ABSTRACT

Limited branching is considered as one of the major factors limiting oil-bearing seed yield in *Jatropha curcas* L. Seeking for promoting branches, Lateral shoot-Inducing Factor (*LIF*) was going to be transferred into the *J. curcas* genome. For this transgenic approach to be successful, we developed a stable and efficient transformation protocol using *Agrobacterium tumefaciens* strain LBA4404 for young leaf explants of *J. curcas*. Parameters optimized were bacterial strain, bacterial concentration, co-cultivation period, immersion time and concentration of cefotaxime and kanamycin. Transformation with *LIF* gene was achieved subsequently. PCR amplification confirmed that the transformation efficiency was 23.91±5.78%. Southern blot hybridization was performed to reveal that single and multiple copy events and further confirm that *LIF* was stably integrated into the *J. curcas* genome. © 2010 Friends Science Publishers

Key Words: *Jatropha curcas*; Lateral shoot-inducing factor; Young leaf; *Agrobacterium tumefaciens*

INTRODUCTION

From the family Euphorbiaceae, *Jatropha curcas* L., which produces seeds with high oil contents, is native from the south-American tropics. The oil produced by this crop can be easily converted to liquid bio-diesel, which meets the American and European standards (Tiwari *et al*., 2007). Moreover, *Jatropha* oil finds application in the detergent, soap, cosmetics, biopesticide and fertilizer industry (Kandpal & Mira, 1995; Adebowale & Adedire, 2006; Mazumdar *et al*., 2010). However, the popularization and application of *Jatropha* oil is limited by low oil-bearing seed yield. To date, *J. curcas* seed yield is still a difficult issue, which depends on various factors, such as environment (Openshaw, 2000), branching architecture (Achten *et al*., 2009), genetics (Ginwal *et al*., 2004) and management (Gour, 2006). Limited branching is considered as one of the major factors limiting seed yield in *J. curcas* among them. Therefore, it was suggested that the seed yield can be improved if the number of seedbearing branches could be increased (Gour, 2006; Abdelgadir *et al*., 2009). Manual pruning (Gour, 2006) and application of plant growth regulators (PGRs) under field conditions (Lovat & García, 2006; Abdelgadir *et al*., 2009) are traditional methods available for promoting *J. curcas* branching with healthy inflorescences. However, the stability and efficiency of these methods still remain a major concern.

In recent years, researches showed the possibility of optimizing the extent of branching by genetic modification in several plant species including Petunia (Bennett & Leyser, 2006; Lewis *et al*., 2008), tobacco (Bereterbide *et al*., 2001) and *Arabidopsis* (McSteen & Leyser, 2005). Nakagawa *et al*. (2005) found that overexpression of a zinc-finger protein of petunia, designated Lateral shoot-Inducing Factor (*LIF*), in transgenic petunia, tobacco and *Arabidopsis* resulted in a dramatic increase in lateral shoots and reduced plants height. Moreover, they suggested plant responses to *LIF* overexpression seem to be widely conserved, at least among dicots (Nakagawa *et al*., 2005). Thus, *LIF* could serve as a tool for genetic manipulation of branching patterns of plants.

For successful introduction of desirable genes, an efficient and stable *Agrobacterium*-mediated transformation protocol for *J. curcas* needs to be developed. There are few reports on transformation in *J. curcas*. Li *et al*. (2008) obtained *uidA* gene expression from regenerated plants arising from *J. curcas* cotyledons after *Agrobacterium*-mediated transformation, with a low transformation frequency (13%). Effects of age and orientation of the cotyledon explants on *Agrobacterium*-mediated transformation of *J. curcas* were studied by Mazumdar *et al*. (2010).

This study had two objectives. The first was to establish an effective transformation protocol using young

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leaves of *J. curcas*. Although leaf disc regeneration system for *J. curcas* has been reported previously (Sujatha & Mukta, 1996; Lu *et al*., 2003; Deore & Johnson, 2008), details of successful plant transformation using leaf are still lacking. The second objective was to apply this protocol to transfer the *LIF* gene into the *J. curcas* genome.

MATERIALS AND METHODS

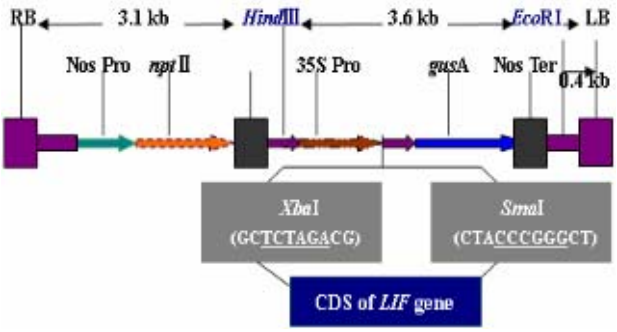
Plant materials: Mature seeds of *J. cur*cas were obtained from Panzhihua City, Sichuan Province, China. Seedlings were germinated and grown at 28°C during a 16 h light period and 8 h dark period. Young Leaves from the apex of seedlings were excised and sterilized by immersion in 75% (v/v) ethanol for 1 min followed by 15 min in 0.5% (w/v) sodium hypochlorite. Sterile young leaves of *J. curcas* were cut into 1 cm^2 sections and then pre-cultured on callus induction medium for 2 days. Seedlings of *Petunia hybrida* Vilm were grown in a controlled environment room at 22°C under a 14:10 h ratio of light to dark.

PCR amplification of *LIF* coding sequence (CDS) from Petunia: Cloning of the gene encoding *LIF* was achieved by Polymerase chain reaction (PCR) using cDNA of *Petunia hybrida* Vilm as the template. Based on the sequence in GenBank (accession number AB035093), two primers were designed for detecting the CDS of *LIF*. Their sequences are as follows: C1: 5'-GCTCTAGAATGGAAACTAGTAAAAATCAGCCGTC-3' and C2, 5'-CTACCCGGGTCATAAGAACTTTCTTGTGCCTAAACC-3'. An *Xba*I site (underlined) was added to the C1 primer and a *Sma*I site (underlined) to the C2 primer. PCR conditions were as follows: 95°C for 4 min, then 34 cycles of 94°C for 30s, 60°C for 40 s, 72°C for 1 min and ending with 10 min at72°C.

Plasmid construction and *A. tumefaciens* strain: The CDS of *LIF* was cloned into the plant transformation vector pBI121 (Hatanaka *et al*., 1999) at its *Xba*I/*Sma*I site. In the recombinant plasmid pBI121-*LIF*, which containing the GUS gene and the selectable marker neomycin phosphotransferase II (*npt*II), *LIF* was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1). Subsequently, the recombinant plasmid was mobilized into competent cells of *A. tumefaciens* LBA4404 (Hoekema *et al*., 1983) and EHA105 (Hood *et al*., 1986) by the freeze-thaw method (Holsters *et al*., 1978). The recombinant *A. tumefaciens* LBA4404 and EHA105 were grown overnight in YEB medium supplemented with 50 mg/L of streptomycin, 100 mg/L of rifampicin and 100 mg/L of kanamycin at 28°C. When the turbidity reached about OD_{600}=1.0, the bacteria were centrifuged and resuspended in hormone free MS liquid medium to a final OD_{600} ranging between 0.1 and 0.5.

Media composition and culture conditions: The basal MS media (Murashige & Skoog, 1962) containing 3% sucrose

Fig. 1: Linear map of the T-DNA region of the recombinant pBI121-*LIF* showing the site of the inserted CDS of *LIF* gene. Also highlighted are the positions of *Hind*III and *Eco*RI restriction sites



(w/v) and 0.8% agar was used in all experiments. Callus induction (CI) medium consisted of the basal MS medium supplemented with 8.89 µM 6-benzylaminopurine (6-BA) and 0.45 µM 2, 4-Dichlorophenoxyacetic acid (2, 4-D). Plant regeneration (PR) medium was the basal medium containing 2.22 µM 6-BA, 2.32 µM kinetin (KIN), 0.45 µM Indole butyric acid (IBA) and 0.29 µM Gibberellic acid (GA_3). The pH of all the media was adjusted to 5.8 and all cultures were maintained at 26-28°C with 16/8-h light photoperiod. These media had been tested to have good shoot regeneration potential in our laboratory. *A. tumefaciens* were grown at 28°C in Yeast Extract Broth (YEB) medium supplemented with 50 mg/L of streptomycin and 100 mg/L of rifampicin with shaking (220 rpm).

Plant transformation and selection: Immediately after pre-cultured, the leaf explants were immersed in a 30 mL suspension of pre-induced *A. tumefaciens* for 10, 20 and 30 min with gentle shaking at 28°C and blotted dry with sterilized paper to remove excess bacterial suspension. These were co-cultivated on CI medium for 2-7 days in darkness at 28°C. Following co-cultivation, the explants were transferred to fresh CI medium containing cefotaxime (0, 100, 300, 400 & 500 mg/L) for 3 week to prevent bacteria overgrowth. Once calli were obtained, they were transferred to PR medium supplemented with different concentrations of kanamycin (0, 10, 20, 30 & 40 mg/L), as selective agent and 150 mg/L cefotaxime. After 4 weeks, the kanamycin-resistant (Kan^R) shoots were placed for a further 3–4 weeks on a solid rooting medium, which consisted of MS medium with 0.98 µM IBA and 40 mg/L kanamycin. Regenerated plantlets were placed into pots containing perlite, sand and soil [1:1:1 (v/v/v)] and also kept in the chamber for 2 weeks.

Identification of Transgenic Shoots

Staining for GUS activity: Expression of GUS activity was performed to make a distinction between transformed and untransformed tissues. Briefly, leaf explants after co-cultivation, calli induced or individual Kan^R shoots were incubated in 5-bromo-4-chloro-3-indoxylbeta-D-glucuronide (X-gluc) solution (Biosynth AG, Staad, Switzerland)

containing 100 mM phosphate buffer, 100 µM ethylene diamine tetraacetic acid, 0.5 M potassium ferricyanide and 0.5 M potassium ferrocyanide overnight at 37°C (Dutta *et al*., 2005). All the blue products were studied and observed under the UOP microscope (China) attached to Cannon camera and photographed.

PCR analysis: Total genomic DNA was isolated from stems of transformed and untransformed plants using the cetyltrimethyl ammonium bromide (CTAB) method (Murray *et al*., 1980). The forward and reverse primers:

qc1 (5'- ACCGTCGGGCAAAGACAGATT-3') and qc2 (5'- GACCGCATCGAAACGCAGCAC-3') were designed as to amplify a 538 bp fragment of the recombinant pBI121-*LIF* containing 246 bp of the internal sequence of the *LIF* gene and 292 bp of the internal sequence of the GUS gene. The reaction mixture was subjected to the following reaction conditions: a 5 min denaturation step at 95°C, followed by 34 cycles of 94°C denaturing for 30s, 62°C annealing for 40s and 72°C for 1 min, and 10 min at 72°C for final extension.

Southern blot analysis: To confirm the integration of *LIF* into plant chromosomal DNA, genomic DNA (20 µg) from the PCR-positive (PCR^+) shoot was digested overnight with *Eco*RI and *Hind*III at 37°C. *Eco*RI and *Hind*III cut plasmid pBI121 at only one site respectively and unpredictably in the plant genomic DNA (Fig. 1), which had no site neither in the gene of interest (region that is probed). The probe was Dig-labelled (digoxigenin, Roche) by PCR using the primer pair (qc1 & qc2). Hybridization, incubation, washing and detection of signals were performed following the earlier report (Raffeiner *et al*., 2009). The blot was exposed to film for 3 days (Kodak 8-10 X-Omat AR, Rochester).

Statistical analysis: The mean values of triplicates were calculated and the standard deviations (±SD) were indicated. All data were statistically analyzed by the Duncan's multiple range test using the Statistical Package for the Social Sciences (SPSS) statistical software (ver. 11.0).

RESULTS AND DISCUSSION

Bacterial strain and concentration: *A. tumefaciens* strain plays an important role potentially influencing the efficiency of genetic transformation. In our study, transient GUS expression analysis was used as an indicator to monitor for T-DNA transfer to leaf explants of *J. curcas* from recombinant *A. tumefaciens*. After co-culture, transient blue GUS spots were observed in leaf explants inoculated with EHA105 or LBA4404. Generally, each particular strain of *A. tumefaciens* showed different level of virulence to the plant species (Yong *et al*., 2006). However, we detected no significant difference in number of blue spots per explant for the two bacterial strains (data not shown). These results indicated that LBA4404 and EHA105 had the same virulence on *J. curcas* young leaf, which was not in accordance with the reports where LBA4404 had the higher virulence than EHA105 on cotyledon explants of *J. curcas* (Li *et al*., 2008). Nevertheless, cefotaxime sensitivity study (see next part) indicated EHA105 was unsuitable for using in further *J. curcas* transformation protocols according to the tolerance of calli induced to cefotaxime.

Further analyses showed bacterial concentration had the significant effect on transformation efficiency as shown in Table I. At OD_{600}=0.1, LBA4404 showed the highest GUS^+ callus efficiency. At higher densities (OD_{600}>0.1), excrescent bacteria inhibited callus induction and changed the pH of the media, which led a corresponding reduction in the transformation efficiency. This was in conformity with other studies in which a higher concentration of *A. tumefaciens* resulted in low

Table I: Effect of bacterial strains and concentration on GUS expression efficiency of *J. curcas*

Bacterial strain	concentration (OD_{600nm})	Percentage of callus Induction (%)	Percentage of GUS^+ callus (%)
LBA4404	0.1	80.81±6.51[a]	68.31±8.82[a]
	0.2	63.23±6.27[b]	51.52±2.14[b]
	0.3	44.45±5.48[c]	38.48±2.76[c]
	0.4	19.05±1.47[d]	18.94±1.13[d]
	0.5	6.79±2.67[e]	6.79±2.67[e]

Table II: Influences of kanamycin on selection transformants of *J. curcas*

Concentration of kanamycin (mg/L)	Percentage of Kan^R shoots (%)	Percentage of GUS^+ shoots	Percentage of PCR^+ shoots
0	——	9.96±4.37[b]	4.87±3.85[bc]
10	48.61±4.61[a]	14.61±1.15[ab]	11.21±6.49[ab]
20	44.09±7.93[ab]	24.90±7.13[a]	21.17±1.90[a]
30	35.41±7.52[bc]	25.49±7.99[a]	25.49±7.99[a]
40	23.91±5.78[d]	23.91±5.78[ab]	23.91±5.78[a]

Results are means ± standard deviations. Values within a column followed by different letters are significantly different at P≤0.05 according to Duncan's tests

Fig. 2: Stages of development of transgenic plants by *A. tumefaciens* strain LBA 4404 mediated transformation of *J. curcas* harboring pBI121-*LIF*. A) Green-white calli induced from leaf disks on CI medium supplied 300mg/l cefotaxime. B) At 500 mg/l cefotaxime, calli turned brown and dead. C) Kanamycin-resistant (Kan^R) shoots regenerated after a 15-days culture on PR medium contain 40 mg/l kanamycin and 150 mg/l cefotaxime. D) Kan^R shoot was rooted on MS medium with 0.98µM IBA and 40 mg/l kanamycin. E) Hardened transgenic plantlet

Fig. 3: Effect of immersion time on the rate of transient GUS expression of *J. curcas* leaf explants

Transient GUS expression efficiency was calculated as the percentage of number of survival explants showing transient GUS activity from the total number of explants. Different letters indicate significantly different at P≤0.05 according to Duncan's tests. The same below

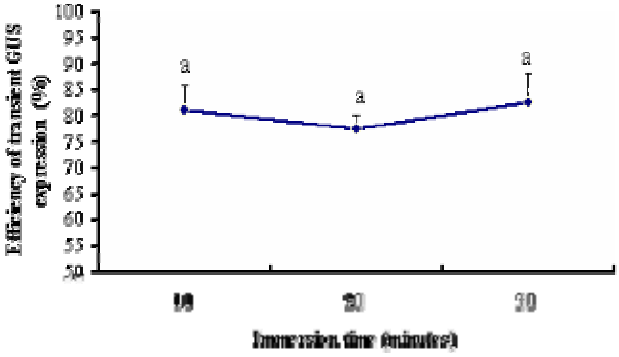
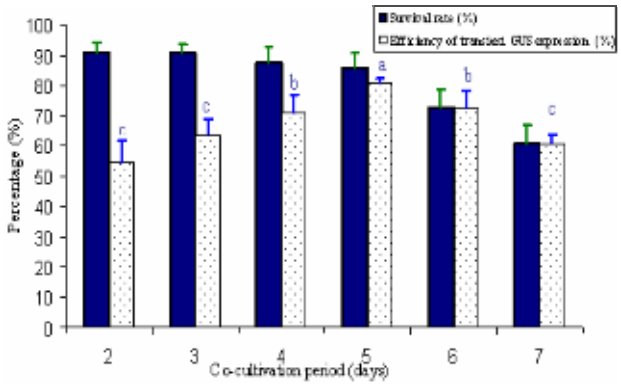


Fig. 4: Effect of co-cultivation period on the rate of leaf explants survival and transient GUS expression of *J. curcas.* Survival rate was calculated as the percentage of number of survival explants from the total number of explants



transformation efficiency (Yong *et al*., 2006; Xu *et al*., 2009). Therefore, OD_{600}=0.1 of LBA4404 was predominantly used in transformation protocols of leaf explants of *J. curcas*.

Cefotaxime sensitivity study: Cefotaxime was usually used for suppressing *A. tumefacien* (Valvekens *et al*., 1988). Sensitivity of both leaf explants and *A. tumefaciens* to various concentrations of cefotaxime (0, 100, 300, 400 & 500 mg/L) was tested. Results indicated 300 mg/L was the highest concentration of cefotaxime, which the leaf explants could produce regenerative calli (Fig. 2a). More than 40% of calli induced turned brown on the CI media containing 400 mg/L cefotaxime, while 70% of calli induced turned brown and lost differentiation capacity at 500 mg/L (Fig. 2b). This trend which cefotaxime was found to severely inhibit shoot regeneration from explants, was noticeable in transformation studies of other plants (Valvekens *et al*., 1988; Valobra & James, 1990; Li *et al*., 2007), but was contradictory to the previous reports, where 500 mg/L

Fig. 5: GUS histochemical assay confirms transgenic plants. (a) Transient GUS expression pattern from leaf explants after 5 d of co-cultivation with LBA4404. (b) Calli induced showed GUS activity. (c) Untransformed control calli. (d) Untransformed control shoot. (e) Node of transgenic plant showed GUS expression. (f-h) Petiole of transgenic plants showed blue spots

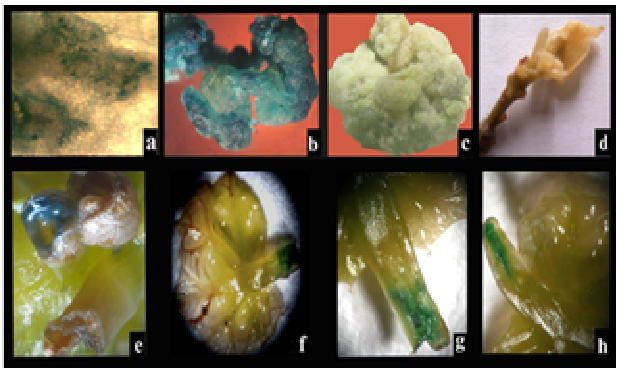
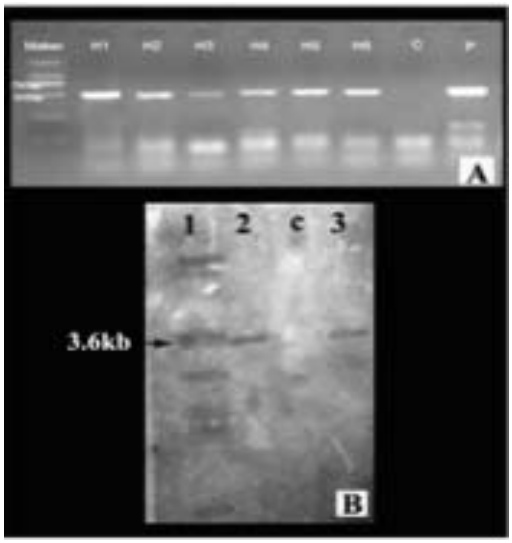


Fig. 6: Molecular analysis of transgenic plants obtained from independent transformation by PCR and Southern hybridization. A) PCR amplification of a 540 bp fragment expected of independent transgenic plants (H1–6), untransformed control shoot (C), recombinant plasmid pBI121-*LIF* (P). B) Southern blot analysis of *Eco*RI/ *Hind*III-digested DNA from PCR$^+$ shoots (lane 1-3) with a DIG-labelled probe, untransformed control shoot (C)



cefotaxime did not inhibit both calluses induction and shoots regeneration from cotyledon explants of *J. curcas* (Li *et al*., 2008).

The concentration of cefotaxime also varied between *A. tumefaciens* strain (Prakash & Gurumurthi, 2005). In our investigation, no residual LBA4404 was observed associated with the callus at 300 mg/L cefotaxime, while more than 500 mg/L cefotaxime could not eliminate

EHA105 completely. Thus, EHA105 was shown to be unsuitable for using in further transformation experiments, because eliminating it required a much higher concentration of cefotaxime than the tolerance of calli.

Immersion time and co-culture duration: Immersion of explants in *Agrobacterium* suspension enhanced the attachment of bacteria to the explants (Yong *et al*., 2006). However, there was no significant difference in transient GUS expression frequency between 10, 20 and 30 min of immersion for *J. curcas* leaf explants (Fig. 3). Thus, 10 min was suggested to be used in transformation due to economical purpose. Longer duration of co-cultivation (6-7 days) cause leaf explants death, while at shorter duration (2-3 days) the transient GUS expression was not obvious (Fig. 4). Normally 2–3 days of co-cultivation are standard for most transformation protocols indicating transient expression (Men *et al*., 2003; Weber *et al*., 2003; Yong *et al*., 2006). But, results indicated 5 days of co-cultivation was optimal, which gave the highest transient GUS expression efficiency for leaf explants of *J. curcas* (Fig. 5a).

Selection of transgenic plantlets: The significant effect of kanamycin on selection of transformed shoots has been reported in several wood species (Fillatti *et al*., 1987; McGranahan *et al*., 1988; James *et al*., 1993; Prakash & Gurumurthi, 2009). Kanamycin sensitivity depends on the explant and species (Klimaszewska *et al*., 2001). Our tests showed that 20, 30 and 40 mg/L kanamycin led the highest rate of PCR^+ shoots and there was no statistically significant difference ($p \leq 0.05$) between them (Table II). However, only 40 mg/L kanamycin completely restrained untransformed shoots production. A concentration reduction than this level for selection resulted in many shoots, which were not transformed "escapes". Thus, 40 mg/L kanamycin was suggested to be used in selection of transformation. Here, we demonstrated kanamycin produced efficient selection, but previous studies reported kanamycin was not a suitable selectable marker for *J. curcas* (Li *et al*., 2008). Four weeks later, kanamycin–resistant (Kan^R) shoots (2-3 cm) were transferred to rooting medium with 40 mg/L kanamycin for keeping selection (Fig. 2d). Continuous selection allowed progressive elimination of untransformed plants (Athmaram *et al*., 2005). After 1 month, rooting plantlets were placed into pots and also kept in the chamber for 2 weeks (Fig. 2e).

GUS histochemical assay: In order to determine the production of stable transformants, calli induced and kan^R individual shoot were assayed by histochemical GUS staining. We found approximately 68% of calli induced showed GUS-positive (GUS^+) (Table I). Four weeks later, about 35% of the GUS^+ calli differentiated into Kan^R shoots (Fig. 2c). Moreover, all of the independent shoots selected by 40 mg/L kanamycin were GUS-positive (Table II). The position of GUS blue spot could be used as a signal for locating the expression of *LIF*. The blue spots were localized all along the node of the stem and the petiole in Kan^R plants (Fig. 5e-h), while blue spots showed an intense distribution in induced calli (Fig. 5b). The phenomena were in accord with *LIF*-overexpressed transgenic petunia plants where the GUS activity was localized all along the junction of the stem and the petiole. Nakagawa presumed *LIF* might be involved in the regulation of local cytokinin metabolism near the axillary buds of transgenic petunia plants (Nakagawa *et al*., 2005). Activity of GUS enzyme was not detected in control tissues (Fig. 5c, d).

Molecular verification: All the GUS^+ shoots were further confirmed by PCR analysis. This was a verification that the observed bands were not due to residual bacteria in the tissue but rather to stable transformation. The transformation frequency was calculated by dividing the number of the PCR^+ independent shoots by the number of infected explants. PCR products amplified from transgenic shoots DNA had the expected size of 0.54 Kb (Fig. 6a). The DNA from untransgenic plants did not show any bands. Finally, a total of 48 independent transgenic shoots were obtained from 201 leaf disks of *J. curcas*, representing transformation efficiency of 23.91±5.78% (Table II). The transformation efficiency was relatively higher than the previous reports using cotyledon explants (13%, Li *et al*., 2008). Subsequently, integration of T-DNA into genome of all the PCR^+ shoots was confirmed by Southern blot analysis. The fragments were expected to be over 3.6 kb (Fig. 1). Fig. 6b revealed the presence of insertions in 3 independent PCR^+ shoots. Lane 1 contained seven hybridization signals, suggesting that there were multiple copies of the T-DNA inserted in the plant genome. In addition to two bands larger than 3.6 kb, which suggested it contained an intact T-DNA, it also possessed four smaller hybridizing bands, presumably due to a deletion or re-arrangement resulting in a partial T-DNA. The partial integration of T-DNA can be frequently observed in many transgenic plants (Sanikhani *et al*., 2008). Lane 2 and Lane 3, respectively demonstrated a single copy T-DNA insertion which was approximately 3.6 kb. As expected, no hybridization signal was detected in non-transformed control plants.

CONCLUSION

A stable and efficient *Agrobacterium*-mediated young leaf transformation method for *J. curcas* has been demonstrated in this study. Based on this procedure, the lateral shoot-inducing Factor (*LIF*) from petunia has been introduced successfully into *J. curcas*. In future, we will work on field testing of these transgenic plants for the ability of branching and yield. Furthermore, this stable transformation protocol is expected to be applicable for introduction of relevant genes into *J. curcas.*

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