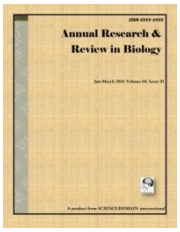

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Quantitative Real-Time PCR for Determination of Transgene in Callus of *Jatropha curcas*

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Authors' contributions

This work was carried out in collaboration between all authors. Author WTLY designed the study and wrote the protocol. Author SEJ performed the practical work and data acquisition. Authors KFR and JAG supervised the work in all its aspects and performed manuscript editing and review. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Jatropha curcas is an important plant belonging to the family Euphorbiaceae which is a potential candidate for biofuel production. Genetic transformation protocol for *J. curcas* callus mediated by *Agrobacterium tumefaciens* were optimized using a pCAMBIA1303 plasmid which carries green fluorescent protein (GFP) gene as a reporter. Results obtained were based on the highest percentage of GFP expression which was observed three days post-transformation. Immersion of callus into 1×10^5 cfu ml^{-1} (OD_{600nm} 0.6) of *A. tumefaciens* LBA4404 with addition of 300 µM of acetosyringone for 45 min, two days of pre-culture and three days of co-cultivation periods were determined to be ideal for *J. curcas* callus transformation. Putative transformants were selected in the presence of 25 mg/l hygromycin. Surviving calli were transferred into proliferation media (MS with 1 mg/l NAA and 1 mg/l BAP) to proliferate the callus for further molecular analyses and to confirm the presence of the target GFP transgene in the putative transformants. Polymerase chain reaction (PCR) was carried out using a 35S specific primer pair confirmed the presence of the 454 bp of 35S promoter region from the transformed callus. Quantitative real-time PCR (qRT-PCR) was carried out to demonstrate the integration and copy number of the 35S

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promoter in the putative tranformants. The 35S promoter gene (178 bp) as a target gene and *J. curcas* actin gene (179 bp) which functions as reference gene was designed to detect the positive transformants and control sample in real-time PCR reaction analysis. The results indicated that the actin specific PCR product was present in both the control and transformed calli, however the 35S PCR product was found only in the positive transformants. The similarity in C_T values confirmed that both the genes were present as single copy thus confirming a single integration event.

Keywords: Agrobacterium tumefaciens; genetic transformation; green fluorescent protein; putative transformants.

1. INTRODUCTION

Jatropha curcas, from the genus *Jatropha* classified as a shady woody plants belonging to the division Spermatophyla and family Euphorbiaceae. This type of fast growing plants, can survive in dry, semi dry and mining areas, especially in tropical and subtropical areas such as Southeast Asia, Central and South America, India and Africa. Although *J. curcas* trees can live in dry climates, these plants also required an adequate amount of water and nutrients for optimum growth [1]. *J. curcas* is considered one of the most highly promoted oilseed crops at present [2]. Other than oilseed producing plants, *J. curcas* also has medicinal properties and recognized as a multipurpose tree of significant economic importance [3].

To meet the global demand for biodiesel fuel, the effort to increase the cultivation and seed production can be done by collecting species in crude gathering centres, to encourage plantations and micropropagation of plants using *in vitro* culture techniques. Since 30 years ago, the method of tissue culture has been widely used in ornamental plant industry and the conservation of plant genetic resources, particularly for species that have many advantages and in demand. A poor seed germination and scanty rooting in vegetative cuttings has promoted the necessity of micropropagation of *J. curcas* through embryo derived explants cultures for conservation of elite germplasm [4]. Tissue culture is a method used to protect and propagate the plant cell and organ in the nutrient media under sterile environment that is free of microbial contamination [5].

Plant genetic engineering is progressing very rapidly since the first success of introducing a foreign gene into a plant via *Agrobacterium tumefaciens* [6]. Since then the number of transgenic plants produced has increased exponentially. The International Service for Acquisition of Agri-biotech Applications (ISAAA) has summarized that the area commercially planted with transgenic plants worldwide has increased almost 53 fold, from 1.7 million hectares in 1996 to 90 million in 2005 [7]. Through genetic engineering, agronomic traits of a particular plant can be improved and furthermore, production of value added products and nutrients can also be obtained. Genetic engineering reduces the time required for introducing a novel trait into plants as compared to conventional breeding. The genetic transformation protocols for *J. curcas* have been discussed in a range of publication [8-10]. The methods used to confirm the transformation event have focused on immunohistochemical staining [9] or reverse transcription of putative RNA transcripts [10]. However, none of these experimental designs have incorporated quantitative real time PCR (qRT-PCR) as part of their validation.

This study was carried out to optimize the *Agrobacterium*-mediated transformation of *J. curcas* callus using Green Fluorescence Protein (GFP) as a reporter. The presence and integration of transgene into plant genome were confirmed by molecular analyses such as PCR and qRT-PCR. This study has specifically focused on the quantification of transgene copy number solely on the evidence derived by utilizing a primer that specifically targets the 35S promoter. This improves the accuracy of transgenic detection. Data obtained from this study will be of utility for further improvement of this plant species via introduction of economically important genes (viz. higher seed oil yield and biotic stresses resistance) under the optimized transformation protocol. Determination of transgene copy number using qRT-PCR can be useful for transformant characterization and selection of the appropriate transgenic lines for future experiments [11].

2. MATERIALS AND METHODS

2.1 Plant Material

Young and healthy green leaves of *J. curcas* were used as plant materials. The leaves were washed and soaked in running water for 20 min, surface sterilized using 30% (v/v) commercial bleach (containing 5.25% of sodium hypochlorite) added with 0.1% of Tween-20 for 15 min and rinsed with sterile distilled water several times before cultured on MS medium [12] supplemented with 1mg/L naphthalene acetic acid (NAA) and 1mg/L 6-Benzyl Amino Purine (BAP) for callus induction. The explants were cultured under 16 h light and 8 h dark photoperiod according to previous reported protocols [10,13] at 25 ± 2ºC. After 4 weeks, the induced calli were used for transformation study.

2.2 Plasmid DNA

The binary vector pCAMBIA1303 (CSIRO, Australia) harbouring the *mgfp*5 (green fluorescent protein) and *gus*A (histochemical GUS assay) genes was used to transform *J. curcas* callus using *Agrobacterium*-mediated method. The reporter genes are transcribed by the constitutive CaMV 35S promoter. The plasmid also contains *npt*II (coding region of neomycin phosphotransferase II gene for kanamycin resistance in bacterial culture) and *hpt*II (coding region of hygromycin phosphotransferase II gene for hygromycin resistance in plant system) as selectable markers. The plasmid is approximately 12.4 kb in size.

2.3 *Agrobacterium-mediated* Transformation

Calli were excised and pre-cultured on MS basal medium prior to transfer into *Agrobacterium* suspension. The bacterial suspension and explants were then mixed and gently shaken to ensure all the explants were fully submerged [14]. After immersion for an appropriate incubation time, the explants were blotted dry on sterile filter paper and transferred to the co-cultivation medium. For the control, the explants were directly placed on co-cultivation medium without immersion in *Agrobacterium* suspension. The cultures were incubated at 25 ± 2ºC under 16 hours light/8 hours dark photoperiod. After the co-cultivation, the explants were transferred to bacterial elimination medium containing 100 mg/L cefotaxime. In this study, the effects of the following parameters known to influence the transformation efficiency were assessed: bacterial concentration (0.2, 0.4, 0.6, 0.8 and 1.0 at OD_{600nm}), pre-culture period (1, 2, 3, 4 and 5 days), co-cultivation period (1, 2, 3, 4 and 5 days), immersion time (15, 30, 45, 60, 75 and 90 min) and acetosyringone concentration (100, 200, 300, 400 and 500 µM). All the parameters were optimized by screening for transient GFP expression

using a fluorescence microscope. All experiments were carried out with 10 samples and repeated thrice. Histochemical GUS assay was carried out to screen for β-glucuronidase activity in putative transformants according to [15].

2.4 DNA Extraction

The genomic DNA of the positively transformed and non-transformed *J. curcas* callus was extracted according to [16]. A total of 200 µl of 3% CTAB buffer (3% of CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl and 20 mM EDTA) was added to 100 mg of leaf powder and homogenized with plastic homogenizer before another addition of 300 µl of 3% CTAB to sample followed by incubation at 60ºC for 30 min. Then, 500 µl of chloroform was added to the mixture and centrifuged at 5000 rpm for 5 min before transferring the supernatant to a new 1.5 ml Eppendorf tube. The tube was added with 0.7 volume of isopropanol and centrifuged one more time in the same speed and the supernatant was discarded. The pellet was washed with 1 ml of 70% ethanol by centrifuged for 3 min in 5000 rpm. The supernatant was discarded and vacuum dried for 10 min prior to the addition of 30 µl of TE buffer. The genomic DNA product was confirmed by analyzing the DNA in 1.5% agarose gel at 80 V for 60 min.

2.5 Primer Design

Specific primer pairs were designed using Primer 3 software to amplify the 35S promoter in T-DNA region. In addition, another set of primers were designed for qRT-PCR assay. The primers to amplify 35S promoter region were designed as forward primer: 5'-GAA CTC GCC GTA AAG ACT GG-3' and reverse primer: 5'-GGT CTT GCG AAG GAT AGT GG-3' with a product size of 454 bp. For qRT-PCR assay, the primers used for the amplification of the 179 bp fragment of 35S promoter region were designed as 35SF (forward): 5'-AAA CCT CCT CGG ATT CCA TT-3' and 35SR (reverse): 5'-CTT TTT CCA CGA TGC TCC TC-3'. The β-actin primers for the 178 bp fragment size of the actin gene were β-ACTINF (forward): 5'-GAG CAG AGA GAT TCC GAT GC-3' and β-ACTINR (reverse) 5'-GCA ATG CCA GGG AAC ATA GT-3'.

2.6 Polymerase Chain Reaction

PCR was carried out in a total volume of 20 µl containing 5 µl of 5× GoTaq Buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.4 µl of 5 U/µl GoTaq DNA polymerase, 2 µl of 10 µM forward primer and reverse primer respectively, 2 µl of genomic DNA (DNA concentration= 50 ng/µl) and 6.6 µl of sterilized distilled water and the reactions were carried out under the following conditions: initial denaturation at 95ºC for 5 min, followed by 29 cycles of amplification with denaturation at 95ºC for 1 min, annealing at 54ºC for 40 sec and extension at 72ºC for 1 min, and an additional of final extension at 72ºC for 5 min. The product was confirmed by analyzing in 1.5% agarose gel at 100 V for 55 min. Putative transformants were analysed for the presence of transgenes.

2.7 Quantitative Real-Time PCR

The integration of 35S promoter region of T-DNA into the plant genome was determined by qRT-PCR using SYBR Green (SsoFast EvaGreen supermix) in an iQ5 Real-Time PCR detection system (Bio-Rad). This assay detects a specific sequence from the 35S promoter region gene, and an endogenous genomic DNA control sequence from the *J. curcas* actin

gene which is present in both untransformed and transformed plant sample. The qRT-PCR was carried out in 20 µl reaction volume which consist 10 µl Sso Fast Eva Green supermix, 2 µl of 0.5 µM of each forward and reverse primers, 2 µl of DNA template and 4 µl of RNase-free water. PCR reactions were performed under the following thermal cycling conditions: 1 min at 95ºC, 4 min at 95ºC, 35 cycles of 10 sec at 95ºC and 40 sec at 58ºC, 1 min at 95ºC, 1 min at 60ºC and finally 80 cycles of 10 sec at 55ºC to determine the specificity of the PCR. Results were confirmed by re-solving the product on a 1.5% agarose gel.

3. RESULTS AND DISCUSSION

3.1 *Agrobacterium*-mediated Transformation of *J. curcas* Callus

The efficiency of *J. curcas* transformation was influenced by several factors such as bacterial concentration, pre-culture period, co-cultivation period, immersion time and acetosyringone concentration. The optimized condition for *J. curcas* callus transformation is illustrated in Table 1. The results obtained are based on the percentage of GFP positive transformants and confirmed by histochemical GUS assay (Fig. 1). *A. tumefaciens* at concentration of $1x10^5$ cfu/ml (OD_{600nm} 0.6) showed the highest virulence on *J. curcas* callus with 80.0±10.0% of GFP positive transformants. Two days of pre-culture and three days of co-cultivation were optimum for *J. curcas* callus transformation with 83.3±5.8% and 76.7±5.8% of positive transformants respectively. Results also showed that 45 min of immersion (80.0±0%) and addition of 300 µM acetosyringone (76.7±12.5%) gave the highest percentage of positive transformants for *J. curcas* callus.

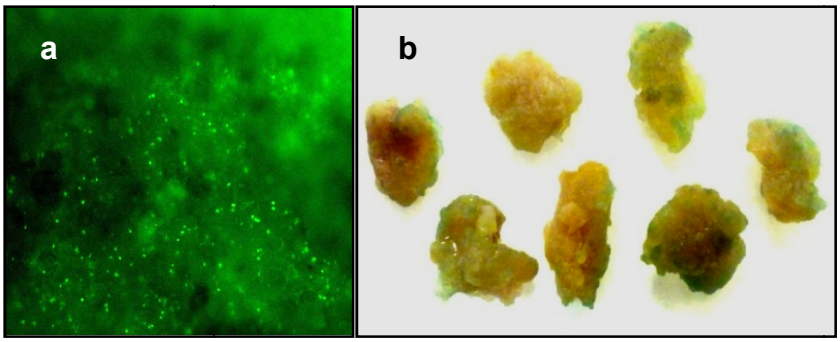

Fig. 1. GFP analysis (a) and histochemical GUS assay (b) of putatively transformed *J. curcas* callus

Transformation efficiency can be further increased by enhancing the competency of plant tissue for plant cell infection and the expression of *vir* gene to increase the virulence of bacteria [17-20]. Reporter genes have been used as convenient markers to visualize gene expression and protein localization *in vivo* in a wide spectrum of prokaryotes and eukaryotes [14]. The GFP gene has substantial advantages over other reporter and selectable genes because the detection of GFP is non-invasive, non-destructive and cell autonomous [21]. Histochemical GUS assay is used as a rapid way to detect the presence of β-glucuronidase gene in putative transformants. The compound, 5-bromo-4-chloro-3-indolyl- β-D-glucuronic acid (X-gluc) is used as a substrate. The β-glucuronidase enzyme catalyzes the cleavage of the colourless glucuronide substrate resulting in the release of an oxidized indolyl derivative that gives the characteristic blue precipitate. The main disadvantage of GUS assay is that it

requires destructive protocol that prohibited further proliferation and regeneration of identified transformed tissues [22].

Table 1. Optimization of *Agrobacterium* concentration, pre-culture period, co-cultivation period, immersion time and acetosyringone concentration for transformation of *J. curcas* callus

Parameters	Optimization range for *Agrobacterium*-mediated transformation	Percentage of GFP positive transformants (%)	Histochemical GUS assay
Agrobacterium concentration (OD_{600nm})	0.2	50.0 ± 0^{d}	-
	0.4	66.7 ± 5.8^{b}	-
	0.6	80.0 ± 10.0^{a}	Positive
	0.8	56.7 ± 11.5^{c}	-
	1.0	43.3 ± 5.8^{e}	-
Pre-culture period (days)	1	56.7 ± 5.8^{c}	-
	2	83.3 ± 5.8^{a}	Positive
	3	66.7 ± 5.8^{b}	-
	4	$63.3\pm11.5^{b,c}$	-
	5	$53.3\pm5.8^{c,d}$	-
Co-cultivation period (days)	1	53.3 ± 5.8^{d}	-
	2	70.0 ± 0^{b}	-
	3	76.7 ± 5.8^{a}	Positive
	4	60.0 ± 0^{c}	-
	5	50.0 ± 10.0^{d}	-
Immersion time (min)	15	43.3 ± 5.8^{d}	-
	30	63.3 ± 5.8^{c}	-
	45	80.0 ± 0^{a}	Positive
	60	70.0 ± 0^{b}	-
	75	$53.3\pm5.8^{c,d}$	-
	90	46.7 ± 5.8^{d}	-
Acetosyringone concentration (µM)	100	56.7 ± 12.5^{c}	-
	200	66.7 ± 16.3^{b}	-
	300	76.7 ± 12.5^{a}	Positive
	400	63.3 ± 12.5^{b}	-
	500	43.3 ± 4.7^{d}	-

Values are mean±SD, n=3 (10 samples per replicate)
Different letters indicate values are significantly different ($p<0.05$)

3.2 DNA Extraction and PCR Amplification

Genomic DNA from the putative *J. curcas* callus transformants was extracted using CTAB method due to the characteristic of CTAB as cationic detergent which can precipitate DNA and remove polysaccharides from both bacterial or plant preparations [23]. *Jatropha* species contain high polysaccharides and polyphenolics compounds posing a major problem in the isolation of good quality DNA [4]. DNA isolation method using CTAB developed by [24] has addressed this issue and the method is reported suitable for isolation of good quality genomic DNA from *Jatropha* that can be stored for longer period and lasting for several PCR reactions.

PCR amplification is based on the detection of the control sequences flanking the newly introduced gene, such as the 35S promoter of CaMV from *A. tumefaciens* Ti plasmid [25,26] or the kanamycin-resistance *npt*II marker gene [27]. Generally, the 35S-PCR test allows the detection of GMO contents of foods and raw materials in the range of 0.01-0.1% as reported by [28]. Fig. 2. shows the ethidium bromide-stained 1.5% agarose gel of the PCR

amplification of 35S promoter. Results indicated that amplification of 35S promoter (454 bp) was only observed in the putative transformants but not in the non-transformed calli.

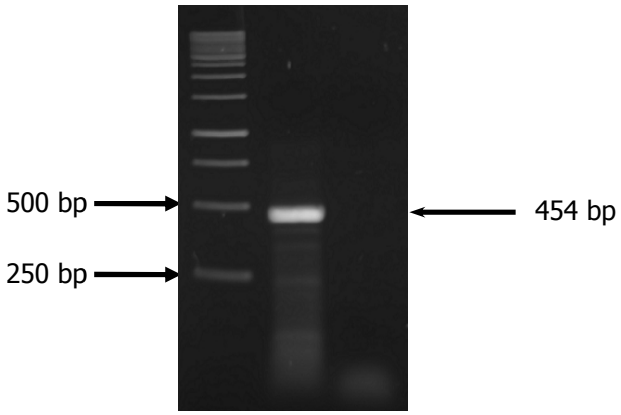

Fig. 2. PCR amplification of 35S promoter from putative transformant and non-transformed callus of *J. curcas*. lane M indicates 1 kb DNA ladder (Promega, Wisconsin). lane 1 indicates DNA sample from putative transformant and lane 2 indicates DNA sample from non-transformed callus as control

3.3 Quantitative Real-Time PCR

Results of the qRT-PCR assay indicated that the 35S promoter sequence was detected only in transformed calli. qRT-PCR assays are characterized by a wide dynamic range of quantification of 7-8 logarithmic decades, a high technical sensitivity (5 copies) and a high precision (2% standard deviation) method [29,30]. Another advantage of this method is that no post-PCR steps are required, thus avoiding the possibility of cross-contamination due to PCR products. This advantage is of special interest for diagnostic applications. Together with lower turn-around times and decreased costs it has revolutionized the field of molecular diagnostics [31]. New systems for field use, which can detect microorganisms in less than 10 min, have been developed [32]. In recent years, a powerful real-time fluorescence qRT-PCR method has been used to analyze gene copy number in transgenic corn, rapeseed, rice and cotton plants [33-36]. This method does require certain application conditions, such as the selection and copy number identification of the endogenous gene and accurate determination of DNA concentration. Detection of a GMO can be done by detecting a molecule (DNA, RNA or protein) that is specifically associated with or derived from the genetic modification of interest [37]. In qRT-PCR, it allows accumulation of amplified product to be detected and measured as the reaction progresses.

Fig. 3 shows the qRT-PCR amplification plot of DNA from six treatments. Sample A2, A4 and A5 were amplified by the β-actin primer, while A3, A6 and A7 were amplified by RT35S primer. A1 is the control sample with no template was performed. From the amplification profile shown, there is no amplification in sample A1 and A3. By using the β-actin primer as a housekeeping gene, amplification can be observed in both putative transformant and non-transformed callus sample, whereas only the putative transformant sample amplified by the RT35S primer indicated positive amplification of 35S promoter region. The reference gene was present in all the samples, however the 35S promoter region was only detected in transformed calli.

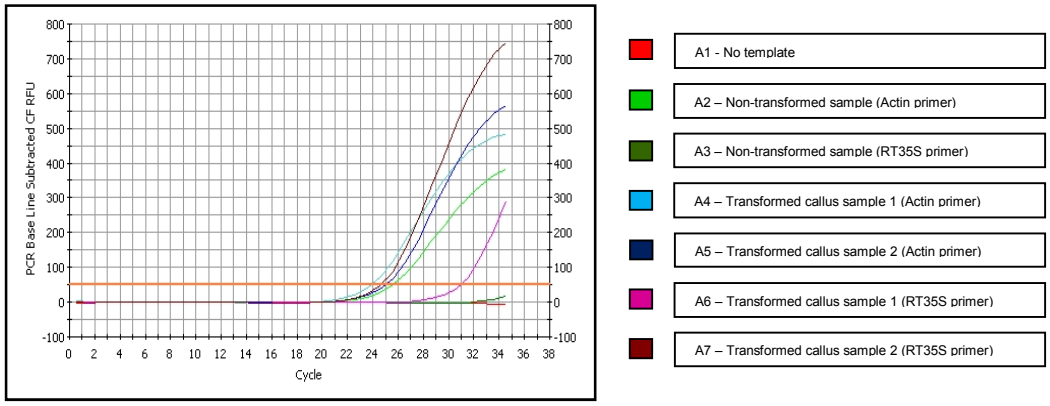

Fig. 3. qRT-PCR amplification plot of DNA from six treatments of J. curcas callus amplified by RT35S primer and β-actin primer using SYBR Green dye

Real-time PCR measures the amount of molecules produced during each stage of the PCR rather than just at the end [31]. The threshold cycle (C_T) is the PCR cycle at which fluorescence exceeds background and a significant increase in fluorescence is observed [38]. The higher the initial DNA amount, the lesser number of cycles are needed (low C_T values) to reach the threshold. C_T value corresponds to PCR product accumulation, thus it is correlated with the starting template amount. A lower C_T value implies a higher starting quantity of the nucleic acid target. Threshold is achieved during the exponential phase of PCR, where reaction components are not limiting, so C_T values are reproducible [38]. This leads to improved precision in DNA quantitation. It is important to confirm that the transgene and control gene amplify with approximately equal efficiencies because the internal control is used to normalize DNA concentration.

SYBR Green in qRT-PCR, the cycling program should always be followed by melting curve analysis. As illustrated in the melting curve in (Fig. 4.) small variations in the Tm indicated a different pattern of amplification. There were five peaks, each represented by callus of putative transformant sample and non-transformed sample. Two peaks represented by DNA amplified from putative transformants using RT35S primer with Tm of 85.5ºC for sample A7 and 86.0ºC for sample A6. Another three peaks represented by the DNA amplified using β-actin primer with Tm of 83.5ºC for samples A2, A4 and A5. Other than the significant single sharp peak, there was an extra single low peak produced. This low peak was suspected to be generated by non-specific amplification from β-actin primer. Primer-dimers will appear as a peak with a Tm that is less than the Tm of the specific product.

This assay detected a sequence from CaMV 35S promoter and an endogenous control sequence from the actin gene, which was presented in both putative transformants and non-transformed *J. curcas* calli. β-actin is a housekeeping gene, whose expression remains constant under a wide variety of physiological conditions. For this reason, β-actin is commonly used as a standard reference in qRT-PCR. In this study, it was proven that the putative transformed calli contain 35S promoter region. The C_T value was similar indicating that the gene was inserted as a single copy. According to [39], transgenic plants generated through *Agrobacterium*-mediated transformation may contain lower transgene copy numbers and simpler integration patterns as compared to direct DNA transfer such as particle bombardment.

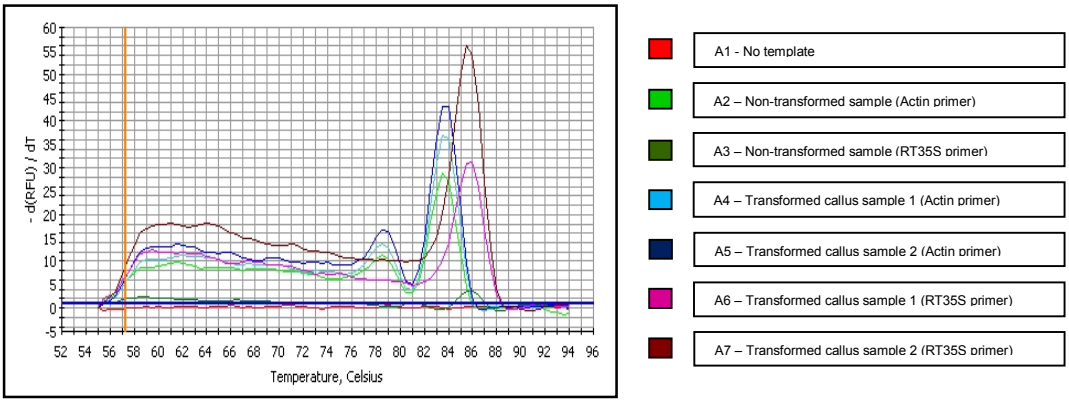

Fig. 4. Dissociation curves for the endogenous reference actin gene and 35S promoter gene in qRT-PCR assay. The x-axis and y-axis represent temperature and the negative first derivative of fluorescence intensity (-dFU/dT), respectively

4. CONCLUSION

Agrobacterium-mediated transformation was optimized using GFP as reporter for *J. curcas* callus and the integration of transgene into plant genome was verified using qRT-PCR. Recent years, plant genetic engineering has been utilized in many different ways to increase the qualitative and quantitative yield of crop plants, to enhance protection against pests and to produce sustainable raw materials for industry and pharmaceutical purposes. With the established and optimized transformation protocol for *J. curcas*, transformation of economically important genes is recommended to improve the quality and valuable trait of this plant, such as to reduce the toxic level of substances in seeds, increase resistance to biotic stresses, and modify the seed oil characteristics for higher engine efficiency.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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