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Sonication-Assisted *Agrobacterium*-mediated Gene Transformation of Oil Palm Secondary Somatic Embryo

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ABSTRACT

The influence was investigated of the hygromycin concentration on the survival rate of oil palm secondary somatic embryos (SSEs) and sonication-assisted *Agrobacterium*-mediated transformation (SAAT). The results showed that 20 $mg.L^{-1}$ hygromycin could completely inhibit growth of SSEs, suitable for selection of transformed SSEs. The transient *ß*-glucuronidase (*gus)* expression was affected by the different period of SAAT which was significantly enhanced by increasing the period of SAAT. However, the longer exposure to SAAT caused a decrement in the frequency of plantlet regeneration. The highest transformation efficiency was obtained when the SSEs were sonicated for 5 min. Polymerase chain reaction and Southern blot hybridization analysis confirmed the presence of the reporter genes in the transformed plantlet genome.

Keywords: sonication, *Agrobacterium*, oil palm, secondary somatic embryo, hygromycin

INTRODUCTION

Plant genetic engineering is a powerful tool to manipulate the genome of an organism for improving a new trait and the *Agrobacterium*-mediated transformation method has been used most widely for various plant species due to its easy protocol without the need for any special equipment (Gelvin, 2003).

A stable transformation procedure has been developed in various monocotyledonous plants such as lily (Ogaki *et al.*, 2008), rice (Rachamawati *et al*., 2004), maize (Sidorov *et al*., 2006), sugarcane (Manickavasagum *et al*., 2004) and sorghum (Zhao *et al*., 2000). One of the main disadvantages of using *Agrobacterium* for plant transformation is the organism's host specificity, resulting in a low frequency of transformation in certain plant species (Beranova *et al*., 2008). Although monocotyledonous plants, including oil palm, are not natural hosts of *Agrobacterium*, recent developments in technology have enabled the application of a protocol to increase the transformation efficiency in oil palm.

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is an efficient *Agrobacterium*-based transformation technology. The advantage of this method is that the cavitations caused by sonication result in thousands of micro wounds on and below the surface of the plant tissue and this wounding pattern permits *Agrobacterium* to penetrate deeper and more completely throughout the tissue than conventional microscopic wounding, increasing the probability of infecting plant cells (Liu *et al*., 2005). The SAAT method has been successfully employed to enhance the transformation efficiency in several plant species such as soybean (Trick and Finer,

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1998), squash (Ananthakrishnan, *et al*., 2007), flax (*Linux usitatissimum* L.) (Beranova *et al*., 2008) and cowpea (Bakshi, *et al*., 2011). In the present study, the concentration of hygromycin was determined for inhibiting the growth of secondary somatic embryos (SSEs) completely and that concentration was used for the selection of putative transformed plantlets focusing on the period of SAAT to enhance the efficiency of gene transformation in oil palm SSEs.

MATERIALS AND METHODS

Plant material

SSEs were induced from single haustorium embryos (HEs) of *E. guineensis* Jacq. *var* tenera according to the method described by Te-chato *et al.* (2002). Briefly, individual HEs were transferred to the SSE induction medium (SSEIM, a Murashige and Skoog (MS) medium) with 0.2 M sorbitol and 200 $mg.L^{-1}$ ascorbic acid and cultured for 3 mth. After this period of culture, a large number of SSEs were induced and ready to use for transformation.

Preparation of *Agrobacterium* cells

Agrobacterium tumefaciens strain AGL-1 containing the plasmid pCAMBIA1304 which harbored *ß*-glucuronidase (*gus*) and hygromycin phosphotransferase (*hpt*) genes was used in this study. A single colony of this bacteria was picked out and suspended in 30 mL liquid Luria-Bertani (LB) medium (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ bacto yeast extract, 5 $g.L^{-1}$ NaCl, pH 7.0) containing 50 $mg.L^{-1}$ kanamycin and incubated on a rotary shaker at 150 rpm in the dark at 28 °C. After proliferation in the LB medium overnight, the cells were collected and the density was determined using a spectrophotometer at optical density (OD) of 600 nm at 0.6.

Effect of hygromycin on survival rate of secondary somatic embryos

The SSEs were cultured on hormone-free MS medium containing 3% sucrose, 200 $mg.L^{-1}$ ascorbic acid and supplemented with hygromycin at different concentrations (0, 10, 15, 20 and 30 $mg.L^{-1}$). The cultures were maintained under 1,300 lux illumination, with a 14 hr photoperiod at 26 ± 2 °C and subcultured at 2 wk intervals for 6 wk. The number of survival SSEs was recorded every week for 6 wk.

Effect of sonication period on gene transformation

The SSEs were immersed in the bacterial suspension at an OD of 600 nm at 0.6. SAAT was applied by sonicating the SSEs immersed in 25 mL of bacteria in a 50 mL flask for 0, 1, 5, 10 and 15 min. The period of 0 min (without sonication) was used as a control. After sonication, SSEs were maintained in the bacterial suspension for a further 4 hr on a rotary shaker at 150 rpm. The excess of bacteria was removed from the SSEs by placing on sterile filter paper before transferring to the co-cultivation medium (solidified-hormone-free MS medium containing 200 μM acetosyringone) and kept in the dark at 28 °C for 3 d. After co-cultivation, the SSEs were washed with liquid MS medium containing 400 $mg.L^{-1}$ cefotaxime for 10 min to remove excess bacteria. Then, the SSEs were blotted dry on sterile paper and transferred for 2 wk to MS medium supplemented with 200 $mg.L^{-1}$ cefotaxime to eliminate bacteria. Then, the SSEs were transferred to a selective medium (hormone-free MS medium containing 200 $mg.L^{-1}$ ascorbic acid and 20 $mg.L^{-1}$ hygromycin) for early screening of transformed tissues. The cultures were placed under 1,300 lux illumination, with a 14 hr photoperiod at 26 ± 2 °C and subcultured at 2 wk intervals for 4 mth. The percentage of transgenic plantlet regeneration was recorded after 4 mth and subjected to confirmation by polymerase chain reaction (PCR) and Southern blot hybridization.

Histochemical assay for β-glucuronidase activity

Four weeks after culture on selective

medium, four pieces of SSE were collected from each treatment and subjected to transient histochemical *gus* assay by the protocol described by Jefferson *et al*. (1987). SSEs were incubated overnight at 37 °C in sodium phosphate buffer (50 mM) containing 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-Gluc) as the substrate. The stained SSEs were soaked in 70% methyl alcohol to remove the chlorophyll from the explant tissue. The transformation efficiency was evaluated using the percentage of SSE surface area expressing blue spots compared with the total area.

Molecular analysis of the transformed plantlets by polymerase chain reaction, Southern blot hybridization and Dot blot hybridization

Genomic DNA was isolated from young leaves (0.05 g) of non-transformed and transformed plantlets after 4 mth of culturing on selective medium using the CTAB method (Doyle and Doyle, 1990). To detect the presence of *gus* genes, genomic DNA was amplified using the *gus* primer (F-primer 5′-CTGCGACGCTCACACCGATAC-3′ and R-primer 5′-TCACCGAAGTTCATGC CAGTCCAG-3′). PCR products were then run on 1.0% agarose gel at 100 V and visualized using ethidium bromide staining.

For the Southern blot hybridization, the PCR products were separated using 1% agarose gel electrophoresis. The gel was treated with 0.25 N HCl to depurinate briefly the DNA and then denatured with an alkaline solution for 30 min and neutralized for 30 min. The denatured DNA was then transferred to a nylon membrane (Amersham Hybond-N; GE Healthcare Life Sciences; Little Chalfont, UK). The blotted membrane was dried by incubation at 80 °C for 1 hr and then prehybridized in hybridization solution (5× saline-sodium citrate (SDS) 0.1% N-lauroylsarcosine, 0.02 % sodium dodecyl sulfate and 1× blocking solution) for 1 hr at 65 °C. Hybridization was performed using a digoxigenin (DIG)-labeled DNA probe overnight at 65 °C, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science; Penzberg, Germany). The hybridized membrane was washed twice in low stringency buffer (2× SSC, 0.1 % SDS) for 15 min, twice in high stringency buffer (0.1× SSC, 0.1% SDS) for 15 min, once in washing buffer (1× Maleic acid buffer, 0.3% Tween 20) for 10 min. The membrane was blocked in blocking solution (diluted 10× blocking solution 1:10 with maleic acid buffer) for 30 min. After that, the anti-digoxigenin conjugate alkaline phosphate was added into the blocking solution and incubated for 30 min. Then, the membrane was transferred to detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star^{TM}; Roche; Indianapolis, IN, USA) and exposed to Biomax X-Omat film (Kodak; Rador, PA, US) for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

For Dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (Amersham Hybond-N; GE Healthcare Life Sciences; Little Chalfont, UK). The blotted membrane was dried by incubation at 80 °C for 1 hr. The blotted membrane was hybridized and detected using the same protocol according to the Southern blot hybridization described above.

Statistical analysis

Data of means were analyzed using a one-way analysis of variance. Significant differences among treatments were detected using Duncan's multiple range tests at the 0.01 or 0.05 levels of probability.

RESULTS AND DISCUSSION

Effect of hygromycin on survival rate of secondary somatic embryos

Hygromycin strongly reduced the regeneration capacity of SSEs after culture

for 6 wk. In the presence of hygromycin at 20 mg.L^{-1}, complete, inhibited regeneration of SSEs was obtained (Figure 1). Thus, this concentration of hygromycin was suitable for selection of transformed SSEs. Different concentrations of selective agents are needed to prevent the development of non-transformed SSEs. Hygromycin is an aminoglycoside antibiotic which causes plant cells death by inhibiting transcription and translation levels (Datta *et al*., 1999). A similar result was obtained by Abdullah *et al.* (2005), who reported the use of 20 mg.L^{-1} hygromycin for selecting a putative transformant of oil palm.

Effects of sonication-assisted *Agrobacterium*-mediated transformation on transformation efficiency and plantlet regeneration

Gene transformation into SSEs by immersion of the SSEs in bacterial solution and sonication for 15 min gave the highest percentage of transient *gus* expression at 63.33% which was significantly different from the other treatments. The percentage of transient *gus* expression increased with the period of sonication. However, the percentage of plantlet regeneration decreased with long periods of sonication. After 15 min of sonication, SSEs could not be regenerated into plantlets (Figure 2c). Therefore, the present study found that sonication for 5 min gave the best transformation efficiency (Table 1 and Figure 3). Similar results were reported in the transformation of kidney bean (*Phaseolus vulgaris* L.) according to Liu *et al*. (2005) who found that sonication for 5 min gave the best transformation efficiency. The micro wounds produced by sonication allow *Agrobacterium* to efficiently infect deep within the tissue and stably transform (Beranova *et al*., 2008). However, it was observed that the percentage of plantlet regeneration decreased as the period of SAAT increased because sonication over a long period usually damaged plant cells and resulted in lower cell recovery that ultimately reduced the transformation frequency. This means that the period of sonication should be very carefully evaluated and optimized for each species and type of tissue. The results indicated that higher transient *gus* expression frequencies did not necessarily result in higher stable transformation frequencies.

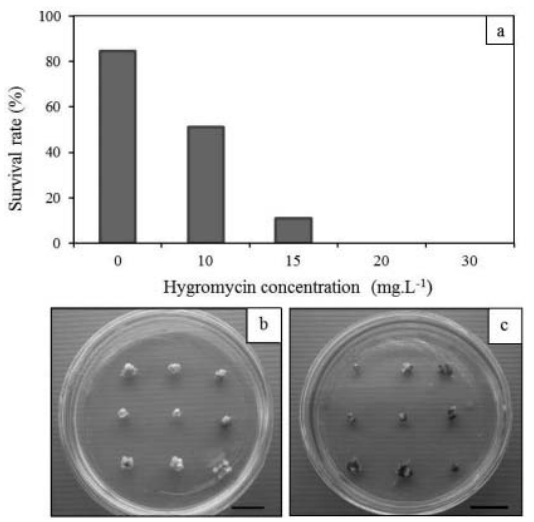

Figure 1 Effect of concentration of hygromycin on survival rate of oil palm secondary somatic embryos (SSEs): (a) After 6 wk culture; (b) Characteristics of SSEs cultured on Murashige and Skoog medium without hygromycin; (c) With 20 mg.L^{-1} hygomycin. (scale bar = 2 cm.)

Molecular analysis of the transformed plantlets by polymerase chain reaction, Southern blot hybridization and dot blot hybridization

Compared to the control plantlets, PCR and Southern blot hybridization confirmed the presence of the *gus* gene with a size of 919 bps from transformed plantlets (Figure 4). The transformed plantlets showed the positive results of PCR and Southern blot hybridization in 8 of the 14 samples (57.14%). Dot blot hybridization confirmed the positive signals of the *gus* gene in the genomic DNA of transformed plantlets in

five of the eight samples (62.5%). The positive transgenic plant samples developed dark black spots as well as the positive control sample, while the non-transformed plantlet samples did not show the dark spots (Figure 5). In this study, the transgenic oil palm was successfully produced after using the sonication-assisted *Agrobacterium*-mediated transformation method. The transformation efficiency obtained from this study was related to Abdullah *et al.* (2005), who reported the optimization of *Agrobacterium*-mediated gene transfer into oil palm immature embryos were easily carried out with a transient transformation frequency of 64.4%.

Table 1 Effect of sonication period on *Agrobacterium*-mediated gene transformation into oil palm secondary somatic embryos.

SAAT duration (min)	*gus* expression after 4 wk transformation (%)	Regeneration after 4 mth transformation (%)
Control	0.00^{d}	0.00^{c}
0	21.67^{c}	6.25^{bc}
1	28.33^{bc}	25.00^{ab}
5	38.33^{bc}	31.25^{a}
10	46.67^{ab}	18.75^{abc}
15	63.33^{a}	0.00^{c}
F–test	**	**
C.V. (%)	32.48	89.70

SAAT = Sonication-assisted *Agrobacterium*-mediated transformation; *gus* = β-glucuronidase.
Means not having the same lowercase superscript letters within a column are significantly different by Duncan's multiple range test at $P < 0.05$; ** Significant differences at $P \leq 0.01$.

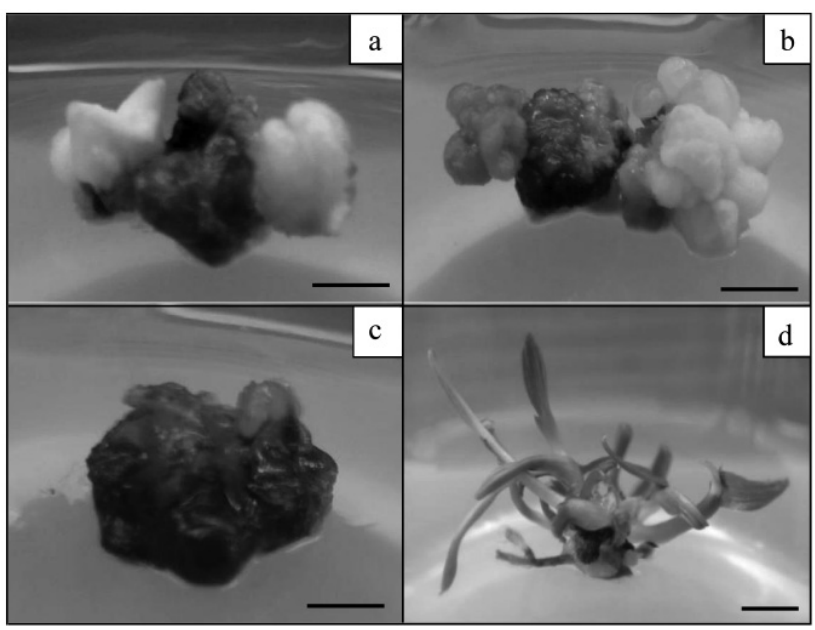

Figure 2 Hygromycin-resistant secondary somatic embryos obtained from the sonication treatment cultured on selective medium (scale bar = 5 mm). (a) control (without sonication); (b) sonication for 5 min; (c) sonication for 15 min; (d) transformed plantlets obtained from 5 min of sonication treatment.

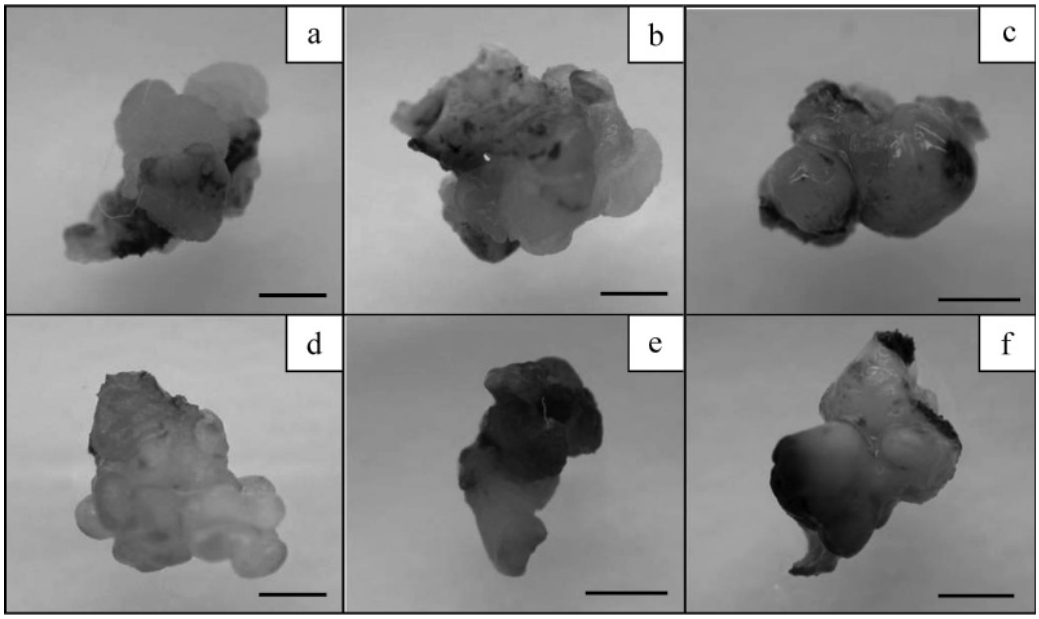

Figure 3 Transient β-glucuronidase expression in secondary somatic embryos (SSEs) after transformation by sonication treatment and culture for 4 wk for selection (scale bar = 2 mm): (a) Non-transformed SSEs; (b) Without sonication, (c–f) Sonication for 1, 5, 10 and 15 min, respectively.

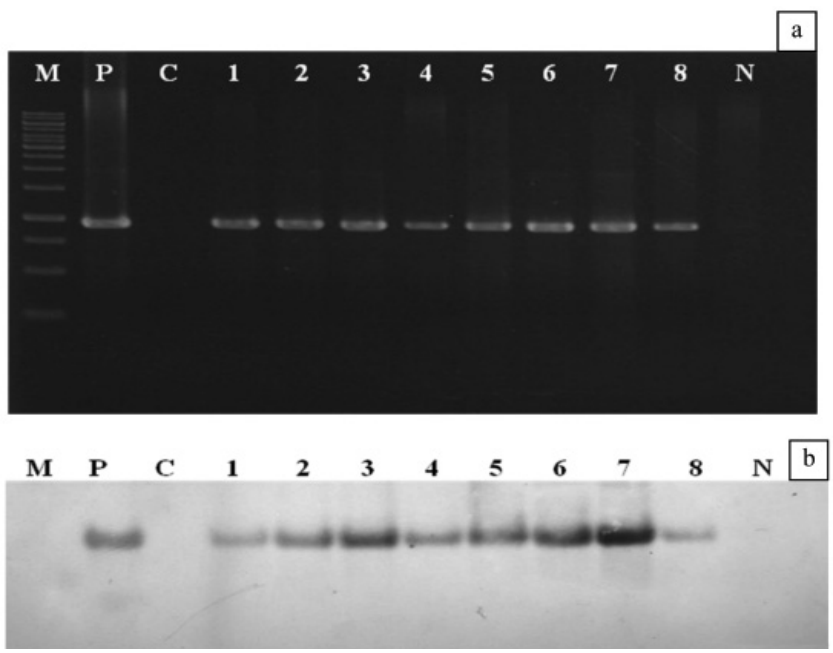

Figure 4 Detection of β-glucuronidase gene in oil palm transformed plantlets after 4 mth of selection: (a) By polymerase chain reaction (PCR); (b) By Southern blot hybridization. (M = DNA marker; P = Positive control; C = Non-transformed; N = Negative control; 1–8 = Transformed plantlets.)

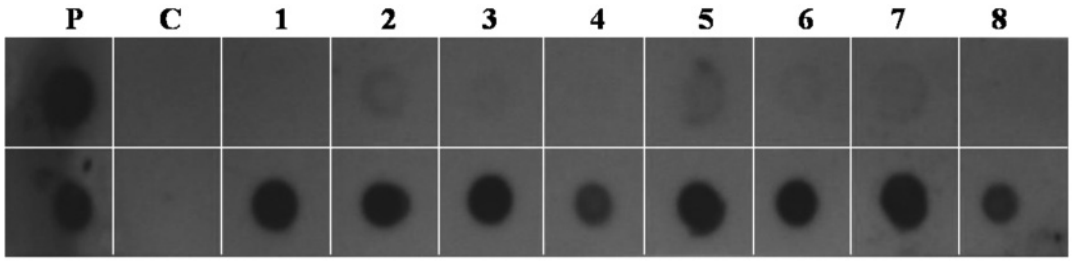

Figure 5 Detection of β-glucuronidase gene in genomic DNA (top row) and polymerase chain reaction products (bottom row) of oil palm transformed plantlets after 4 mth of selection using dot blot hybridization. (P = Positive control; N = Non-transformed; 1–8 = Transformed plantlets.)

CONCLUSION

The suitable concentration of hygromycin for selection of transformed SSEs was 20 mg.L^{-1}. The highest regeneration efficiency of transformed SSEs was obtained when they were sonicated for 5 min. Polymerase chain reaction (PCR) and Southern blot hybridization analysis confirmed the presence of the transgene in the genome of the transformed plantlets.

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