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Factors affecting gene transformation in embryogenic callus of oil palm by bombardment technique

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The study on chemical and physical factors affecting gene transformation in embryogenic callus (EC) of oil palm was conducted using bombardment technique. EC was bombarded with gold particles (1.0 µm of particle size) coated with plasmid pCAMBIA 1301 (1.5 µg/µl) which harboring *gus* and *hpt* gene used as screenable and selectable marker genes, respectively. The results revealed that an optimal conditions of physical factors for gene transformation in EC were bombardment at 5 kg/cm^2, 10 cm working distance and -0.1 MPa vacuum pressure. Those factors gave the highest transient expression of *gus* gene at 65.74%, 62.33% and 66.22%, respectively. The effect of chemical factors affecting to gene transformation in EC revealed that pre-cultured EC on MS medium supplemented with 0.3 M mannitol and 0.3 M sorbitol under dark condition for 16 h gave transient expression of *gus* gene at 68.75%. Post-cultured bombarded EC on the same medium and condition for 24 h gave the highest transient expression of *gus* gene at 38.63%. These expressions were stable until one month of culture. Polymerase chain reaction (PCR) revealed *gus* gene at 441 bp and *hpt* gene at 800 bp in genome of transformed plants.

Key words: oil palm, bombardment, embryogene cullus, gene transformation

Introduction

Oil palm *(Elaeis guineensis* Jacq.) is one of the most economically important crop and the highest oil yielding crop in the world. Interest in palm oil as a biofuel could eventually cause constraints on worldwide supply of edible palm oil and increase the pressure for higher yield and/or cultivatable areas (Biofuel, 2007). However, its improvement by conventional methods are slow due to the long generation time (approximately 7-10 years) and open pollinated behavior of the crop (Parveez *et al.*, 1998). Genetic engineering for resistance to fungus (*Ganoderma boninense*), the major oil palm disease, insects and pests would be beneficial (Abdullah, 2005). The biolistic

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technology of delivering DNA into plant cell or tissues has simplified plant transformation including monocotyledonous plants. The success of this method in a particular crop depends on the optimization of many parameters. In oil palm, physical and biological parameters were optimized for transient expression of *gus* and *gfp* reporter genes through particle bombardment (Parveez *et al*., 1997, 1998; Majid and Parveez, 2007). The objective of this study is to optimise parameters using *gus* transient gene expression and *hpt* selectable marker gene in embryogenic callus of oil palm using particle bombardment technique.

Materials and methods

Plant Material

Embryogenic callus (EC) used in this study was derived from young leaf of elite clone of oil palm cultivar tenera (DxP) from Thepa Research Station, Faculty of Natural Resources, Prince of Songkla University (Figure 2a). The calli were maintained on agar solidified embryogenic proliferation media (PM) which was Murashige and Skoog (MS) macro and micronutrients supplemented with 0.3 mg/l dicamba, 200 mg/l ascorbic acid, 3% sucrose and solidified with 7.5 g/l agar. The medium was adjusted to pH 5.7 with KOH prior to autoclaving at 121^{o}C for 15 min. Embryogenic calli were incubated at 28±2°C under light condition at 14 h photoperiod and subcultured onto fresh medium every 4 weeks.

Bacterial plasmid

The pCAMBIA 1301 plasmid carrying *gus* as the reporter gene and *hpt* gene as the selectable marker was used. Both genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter (Figure 1). The plasmid DNA was extracted using the protocols according to Bimboim and Doly (1979). The concentration of the plasmid DNA was qualitatively measured by 0.7% agarose gel electrophoresis, compared with standard lamda DNA (20 and 40 ng/µl) and stained with ethidium bromide for 20 min, finally visualized by gel documentation at 260 nm of UV.

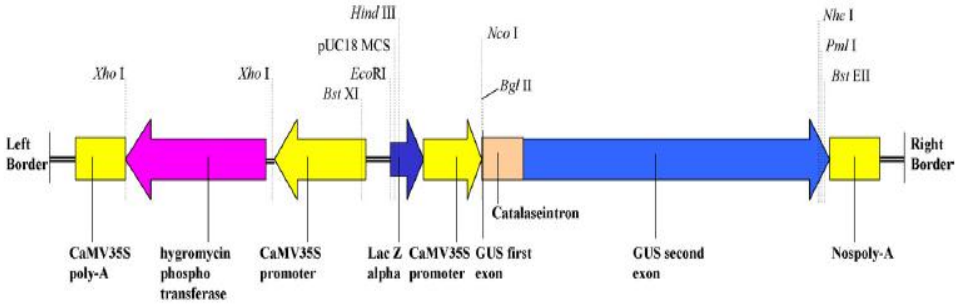

Fig. 1. Schematic map of T-DNA region of pCAMBIA 1301.

Bombardment with a particle in flow gun

The particle bombardment apparatus (Figure 2b) was the same as that used in a previous report (Akashi *et al.,* 2002). Sixteen hours prior to bombardment, EC was transferred to osmoticum medium (OM) containing MS medium supplemented with 0.3 M sorbitol and mannitol, then leaved on this medium for 24 h after bombardment for osmotic post-treatment. The plasmid DNA was precipitated onto gold particles (1µm diameter) following the protocol described by Parveez *et al.* (1997). Briefly, gold particles were sterile twice by sonication, each for 3 min in absolute ethanol. The particles at 0.3 mg in 50 µl sterile water were coated with coating mixture containing 5 µl plasmid DNA (1.5 ng/µl), 50 µl 2.5 M calcium chloride and 20 µl 0.1 M spermidine. After 5 min of incubation on vortex, the supernatant was removed, and the particles were vortexed in absolute ethanol. Coated particles were collected and re-suspended in 60 µl absolute ethanol, sonicated for 1-2 min, and 20 µl of the suspension were loaded on the screen holder of the inflow gun. Bombardment was carried out at various levels of reduced air pressure (-0.08, -0.09 and -0.1 MPa), target distances (3.4, 6.7, 10 and 13.4 cm) of 9.6 cm, helium pressures (2, 5 and 7 kgf/cm^2). For chemical factors; types of osmoticum (sorbitol or/and mannitol) and its concentrations (0.1 0.3 and 0.5 M), preculture treatment prior to bombardment (0, 4, 16, 24 and 48 h), post-bombardment incubation time (0, 12, 24, 72 and 168 h); and time delay (0, 3, 7, 15 and 24 days) were investigated. For each treatment, three replicates were performed. Each replicate contains 150 pieces of ECs at approximately 2 cm in size (Figure 2c).

Histochemical GUS assay and transient assessment

GUS assays were carried out using protocols described by Jefferson *et al.* (1987). GUS expression was observed by immersing transformed ECs in X-gluc buffer, 2 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM

potassium ferricyanide, and 0.5 mM potassium ferrocyanide. Transformed ECs were incubated overnight at 37^{o}C in the dark, and washed with absolute methanol on the following day. The number of the blue spots on ECs were scored, and recorded under stereomicroscope.

Selection of putative transformants

Hygromycin was used for selection of putative transformants. Selection medium (SM) was RM medium supplemented with 30 mg/l hygromycin. The fatal hygromycin concentration for calluses determined was used to select the putative transformed calli.

PCR analysis

Genomic DNA from the EC was isolated using the protocols according to Te-chato (2000). The *gusA* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3' and reverse primer sequence R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3'. The forward and reverse primer sequences for the *hpt* gene amplification were 5'-CCTGAACTCACCGCGACG-3' and 5'-AGACCAATGCGGAGCATATA-3' respectively. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2 µl ml of PCR buffer, 0.1 µl of Taq DNA polymerase (1 U/ml) and the volume was adjusted to 20 µl with sterile distilled water. The PCR conditions included hot start at 96^{o}C for 2 m, followed by 30 cycles of denaturation (96^{o}C, 20 s), annealing (55^{o}C, 1 min) and extension (72^{o}C, 2 min), with a final extension of 5 min at 72^{o}C. PCR amplified products were resolved in 1.5 % agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

Statistical analysis

Data were analysed using completely randomized design (CRD) and the differences among means were separated using Duncan's multiple range test (DMRT). All statistical analyses were performed at the level 5% using statistically analysis system (SAS).

Results

Helium pressure

Helium pressure at 5 kgf/ cm^2 gave the highest result in transient *gus* gene

expression in EC at 65.74% (Figure 2d) in comparison to 7 kgf/ cm^2 (62.74%) and 2 kgf/ cm^2 (36.35%) (Table 1, Figure 2f). Bombardment of the microcarries without DNA did not show transient GUS gene expression (Figure 2e). Similar to hygromycin resistance of the calli, a helium pressure at 5 kgf/ cm^2 resulted in the highest resistant to hygromycin. Non bombarded calli gradually turned brown (Figure 2g), whereas hygromycin-resistant calli formed a new calli or somatic embryos after bombardment (Figure 2h).

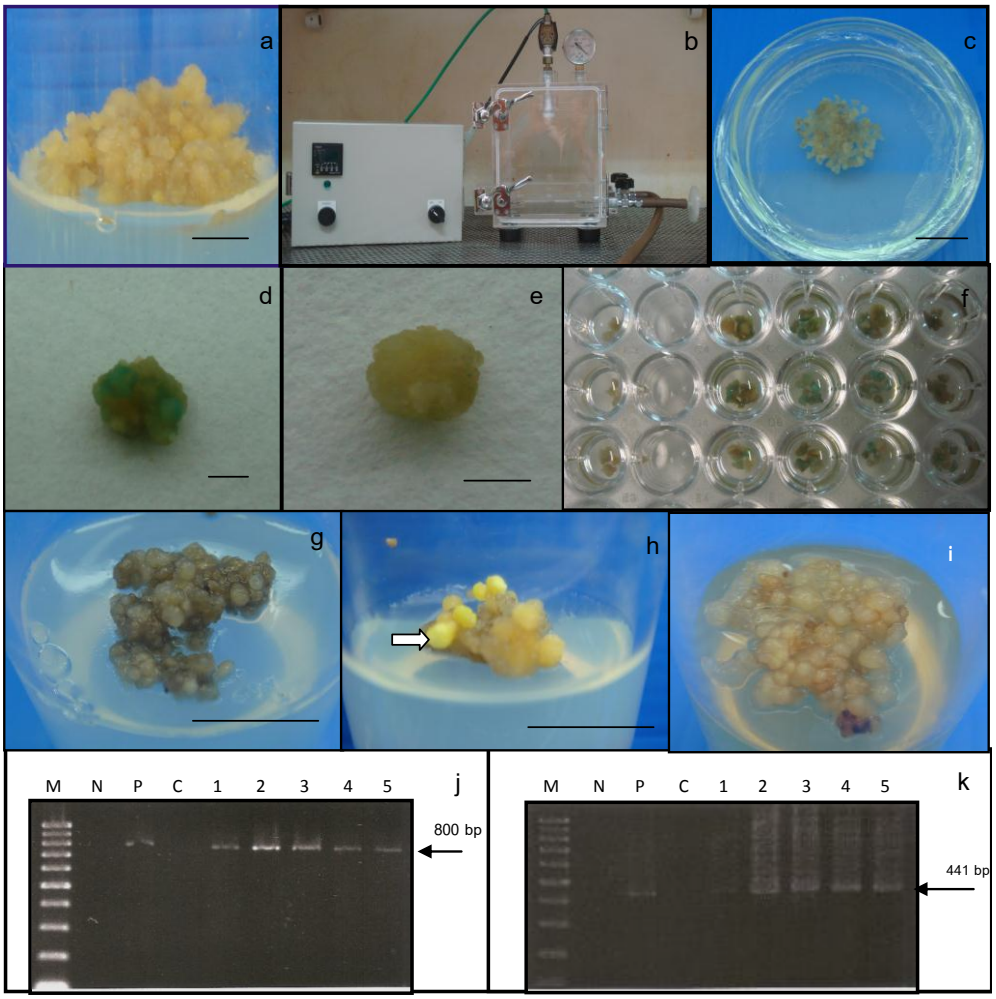

Fig. 2. Bombardment of embryogenic callus of oil palm and assessment of gene transformation: a young leaf-derived embryogenic callus used for bombardment (bar = 0.5 cm); b The particle bombardment apparatus; c target tissue ready to bombard (bar = 1 cm); d GUS assay in transgenic callus and (e) non- transgenic callus (bar = 1 mm); f histolochemical detection of GUS expression; g non- transgenic callus and (h) proliferation of transgenic callus on 30 mg/l hygromycin; *arrows* indicate somatic embryo on embryogenic callus (bar = 1 cm). i Turgor pressure of cells; j detection of *hpt* and (k) *gus* genes in transgenic embryogenic callus by PCR (M: marker, N: non transformed calli, C: negative control, P: positive DNA control, 1-5: transformed calli).

Distance from microcarrier to target tissue

In this study, the distance at 10 cm from microcarrier to target tissue gave significantly higher (62.33%) transient GUS gene expression as compared to 6.7 cm with 51.19% and 13.4 cm with 36.76% (Table 1). In addition, the highest hygromycin resistance calli was observed using 10 cm (92.22%) and followed by 13.4 cm (88.33%) distance from microcarrier to target tissue.

Vacuum pressure

It was observed that vacuum pressure at -0.1 MPa gave the highest result in *gus* gene expression. The lowest result in transient expression determined by using GUS expression in EC was observed at -0.08 MPa (Table 1). However, Overall pressures were not difference significant ($p<0.05$) in hygromycin resistance calli. In these study suggested that -0.1 MPa was more significant ($p<0.05$) vacuum pressure which could be compensated with high level of GUS expressions.

Preculture treatment prior to bombardment

Transient *gusA* gene expression was monitored from 48 h after bombardment. Explants (EC) without preculture period before bombardment were used as control. The highest GUS expressions were observed from preculture treatment for 16 h (68.73%). However, significant difference ($p<0.05$) in *gus* gene expression was not observed among preculture time. Similar result was obtained in hygromycin resistance calli. Preculture treatment for 16 h (95.85%) gave the highest results (Table 1).

Post-bombardment incubation time

In this experiment, the exposure period for post-bombardment was studied based on GUS expressions for 0-168 h. Bombarded EC were cultured on OM medium for 24 h gave significantly higher transient expressions as compared to other treatments tested. The results implies that it take at least 12 h to 24 h for the cells or tissues to recover from the injuries.

Table 1. Transient expression of the *gus* gene and hygromycin resistance calli in oil palm embryogenic calli after bombarment

	Treatment	**GUS expression (%) 48 h after bombardment**	**Hygromycin resistant calli (%) 4 weeks after bombardment**
Helium gas pressure	0(kg/cm^2)	0.00d	36.67c
	2(kg/cm^2)	36.35c	82.22b
	5(kg/cm^2)	65.74a	91.67a
	7(kg/cm^2)	62.74b	82.22b
	F-test/ C.V. (%)	*/ 5.13	*/ 6.20
Target tissue distance	3.4 cm	30.36c	68.56c
	6.7 cm	51.19b	80.56b
	10 cm	62.33a	92.22a
	13.4 cm	36.76c	88.33ab
	F-test/ C.V. (%)	*/ 12.83	*/ 5.34
Vacuum pressure	-0.08 MPa	48.91b	81.67a
	-0.09 MPa	51.02b	84.17a
	-0.1 MPa	66.22a	91.67a
	F-test/ C.V. (%)	*/ 21.46	ns/ 4.03
Pre-culture duration	0 h	51.06a	84.17b
	4 h	65.28a	91.67a
	16 h	68.73a	95.83a
	24 h	66.30a	90.83ab
	F-test/ C.V. (%)	ns/ 21.46	*/ 4.02
Post-bombardment duration	0 h	25.29ab	71.67a
	12 h	27.87a	78.33a
	24 h	38.63a	70.00a
	72 h	11.99ac	63.33a
	168 h	0c	0b
	F-test/ C.V. (%)	*/ 37.7	*/ 20.15
Time delay	0 d	15.14b	30.00c
	3 d	25.82ab	65.00b
	7 d	27.55a	92.50a
	15 d	28.06a	90.83a
	24 d	29.68a	90.83a
	F-test/ C.V. (%)	*/ 23.75	*/ 8.11

ns = Non significant difference * = Significant difference ($p<0.05$)
Mean with different letter within column indicates significant differences by DMRT.

Caused by bombardment (Table 1). After 72 h of post-bombardment, the GUS transient expression was reduced by 31% in comparison to 24 h. There were no blue spots observed in the absence of post-bombardment incubation (which act as turgor pressure of cells) after 168 h of culture (Figure 2i).

Types and concentrations of osmoticum

Embryogenic calli were transferred to RM medium supplemented with 2 types of osmotic agents; mannitol and sorbitol, each at 0-0.5 M. The *gus* gene expression showed a significant difference between the two different osmotic agents. Mannitol at 0.1 M gave the best percentage of *gus* gene expression at 43.42 % (Table 2). The highest hygromycin resistant calli were obtained in 0.1 M sorbitol containing RM medium (82.5%) (Table 2).

Table 2. Effect of osmoticum types and concentrations on transient expression of the *gus* gene (%) in oil palm after 48 h of bombardment

Osmoticum Concentrations (M)	**GUS expression (%)**			
	Osmoticum types			**Average concentration**
	Sorbitol	Mannitol	Sorbitol+ Mannitol	
0.1	25.25b	43.42a	38.78ab	35.81A
0.3	38.43ab	30.17ab	39.33a	35.97A
0.5	39.56a	34.51ab	35.65ab	36.57A
C.V. (%)	21.41			

Mean with different letters within column indicates significant differences at $p<0.05$

Table 3. Effect of types and concentrations of osmoticum on hygromycin resistance calli (%) of oil palm after 4 weeks of bombardment

Osmoticum Concentrations (M)	**Hygromycin resistance calli (%)**			
	Osmoticum types			Average concentration
	Sorbitol	Mannitol	Sorbitol+ Mannitol	
0.1	82.50a	75.00abc	68.33bc	75.28A
0.3	80.00ab	70.00abc	65.00c	71.67A
0.5	73.33abc	65.00c	51.67d	63.33B
Average^{type}	78.61A	70.00B	61.67C	
C.V. (%)	10.30			

Mean with different capital letter indicate significant differences among treatments ($p< 0.05$) and means with different small letter indicate significant differences among treatment combination ($p< 0.05$)

Time delay

Before transferring bombarded EC to SM medium they were cultured on PM medium for 0-24 days after post-bombardment. This time is so called time delay. Upon culturing EC on PM medium for 24 days, then transfer to SM

medium and cultured for 4 weeks gave the highest percentage of GUS transient expression (29.68%), followed by 15 days (28.06%) (Table 1). However, 7 days of time delay gave the significantly increased in level of hygromycin resistant calli (92.5%) in comparison with 15 days (90.83%).

PCR analysis of transgenic plant

PCR was used to detect the integration of foreign genomes into putative transgenic oil palm EC. Four weeks after bombardment, PCR analysis showed the presence of *hpt* gene at 800 bps (Figure 2j) and *gus* gene at 441 bps (Figure 2k) in the positive control and all five putative-transgenic-ECs which was amplified by the two different sets of *gus* and *hpt* primers. There was no amplification of DNA from negative control or non-transformed calli.

Discussion

Optimization was done using transient GUS expression and hygromycin resistance calli as indicators for efficiency of the parameters studies. Helium pressure has been shown that the gas blast and the acoustic shock generated by the particle bombardment were the major causes of cell injury (Tadesse *et al.,* 2003). Increasing the pressure will not only cause the tissues to be dislodged but also significantly reduced the GUS expression, possibly due to tissue damage. Lower expression at reduced pressures could be correlated to the poor penetration capability of the microcarriers as they approach the recipient tissues. On the other hand, higher pressures cause the increase in penetration force of the particles which might injure the cells (Janna *et al.*, 2006). This result is in agreement with the published data on bahiagrass (*Paspalum notatum*) tissue (Himuro *et al.,* 2009). In addition, cell and tissue type also play an important role in determining the optimum pressure of helium gas. In *Dendrobium* Sonia 17, different types of calli gave different responses to helium pressure. High GUS expression was obtained from helium gas at pressure of 1,100 psi in types A calli whereas the pressure at 650 psi was suitable for type B calli (Tee and Maziah, 2005).

Distance from the stopping plate to target tissue is necessary to allow even spreading of the DNA microcarrier onto the target tissue without causing damage (Tadesse *et al.,* 2003).

In this study is in agreement with the published data on bahiagrass tissue (Himuro *et al.,* 2009). Helium pressure has been shown that the gas blast and the acoustic shock generated by the particle bombardment were the major causes of cell injury (Tadesse *et al.,* 2003). Increasing the pressure will not only cause the tissues to be dislodged but also significantly reduced the GUS

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Application of vacuum in bombardment chamber plays an important role in the acceleration of the microcarrier from the microcarrier to the target tissue (Parveez *et al*., 1997). Lower vacuum pressure did not allow particles to reach the target tissues (Walter *et al.*, 1998). Rapid deceleration occurs when microcarriers pass through gas. For microcarriers, which are very small and light, the deceleration will be very high. By removing the gas (vacuum) from bombardment chamber, the deceleration will be minimized resulting in better acceleration of the microcarrier achieved (Sanford *et al.*, 1993).

Effect of osmoticums (sorbitol and mannitol) on increasing efficiency of transient GUS expression. High velocity bombardment causes the penetration of microcarrier into cells to driven DNA. The penetration can disturb the intracellular lipid membrane structure causing cell destruction and ethylene accumulation (Imaseki, 1986). Osmoticum can stabilize cell membrane better than healing of the lesion caused by the microcarrier penetration and reduce turgor pressure of cells, thus reducing leakage and cell rupture (Perl *et al.*, 1992). In oil palm, manipulating the osmoticum treatment (0.4 M mannitol) resulted in a 2.5 fold increase in transient transformation (Parveez *et al.* 1998) and date palm (Mousavi *et al.*, 1998). However, the different results might be due to the different conditions in plant cell culture, and species specific. The results obtained in the present study suggested that 0.1 M mannitol was optimum to obtain a high efficiency of gene transformation in EC.

Timing of antibiotic selection (hygromycin) of the bombarded EC critically affected transformation efficiency. In *Hypericum perforatum*, time delay resulted in high proliferation of organogenic cell suspension but low transformation efficiency (Franklin *et al.*, 2007). The results in this experiment suggested that time delay for 15 days was optimum for obtaining high transformation efficiency in EC. PCR analysis also revealed that the *gus* gene and *hpt* gene had integrated into the oil palm genome.

Conclusion

Optimum conditions for bombardment of ECs using both *gus* reporter gene and *hpt* selecable gene systems were 5 kgf/cm^2 of He pressure, 10 cm distance from microcarrier to target tissue, -0.1 MPa vacuum pressure, 16 h preculture in osmoticum medium containing 0.1 M sorbitol prior to bombardment, 24 h postculture in osmoticum medium containing 0.1 M mannitol after bombardment and 15 days time delay. PCR revealed the presence of both *gus* reporter gene and *hpt* selectable gene in all conditions. Therefore, these conditions or systems are suitable for transformation of oil palm ECs.

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