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Strians of *Agrobacterium* affecting gene transformation through embryogenic cell suspension of hybrid tenera oil palm

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Abstract Embryogenic cell suspension was inoculated with 25 ml of *Agrobacterium* suspension strain EHA101 containing pIG121Hm plasmid in darkness at 28 ^{o}C for 6 hours. The cells were washed by liquid MS medium containing 200 mg/l cefotaxim and cultured in darkness at the above conditions for 3 days. After that, embryogenic cells were transferred to fresh medium of the same component supplemented with 20 mg/l hygromycin under light condition for 1 month. The result showed that proliferation rate of the cells in suspension was nearly 3 folds at 6 ml PCV after subculture for 4 times. Gus expressions of embryogenic cell suspension as analyze by GUS histochemical assay were 65.71%. Viability of the embryo, embryogenic cells suspensions was recorded to be 100 %. Assessment of transient expression of *gus* and *hpt*II gene by PCR technique from 15 samples of transformed tissues showed positive bands of the both *gus* gene and *hpt*II gene while control samples did not show these profiles of DNA.

Key words: Embryogenic cell suspension, oil palm, gene transformation, *Agrobacterium*

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a very important commercial crop in southern Thailand. It is an economically important source of vegetable oil, the most traded vegetable oil in the international market, and increasingly used in the food industry (Corley and Tinker, 2003). The development of oil palm varieties with higher yields, modified to marginal zones or expressing other imperative agronomic characters are important for the improvement of oil palm sustainability in Thailand. Oil palm has a long generation period of about 20 years, so propagation through tissue culture is widely used as commercial scale. Plantlets obtained from tissue culture processes could be induced directly or

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indirectly by the use of dicamba (Te-chato *et al*., 2002). The propagation of oil palm through cell suspension culture has been reported by several researchers (De Touchet *et al*., 1991; Aberlenc-Bertossi *et al*., 1999; Kramut and Te-chato, 2010; Inpuay and Te-chato, 2012). Embryogenic cell suspensions (ECSs) are the most ideal explants for genetic transformation because a single cell origin of somatic embryogenic cultures would avoid chimerism in regenerated plantlets (Huang *et al*., 2007).

Many researchers used ECS for transformation by *Agrobacterium*. For instance, Ganapathi *et al.* (2001) reported transformation of ECSs of banana cultivar Rasthali (AAB) using *A. tumefaciens* strain EHA105 containing the binary vector pVGSUN with the *als* gene as a selectable marker and *gusA* gene as a reporter gene. Expression of GUS activity could be detected after 6 days of inoculation. Moreover, Chong-Pe ŕez *et al.* (2012) used ECS of the 'Dwarf Cavendish' banana cultivar as explants for transformation by *Agrobacterium* strain EHA101 harboring vector pFAJ3000. Masli *et al.* (2009) reported a higher transformation rate in monocotyledonous plants by optimizing several other factors such as bacterial density and type of strain, pH, temperature, antibiotics, physical injuries, and the selectable marker. Different strains of *Agrobacterium* can be used as biological vector to transform DNA into plant cells, but there are species specific. Han *et al.* (2000) reported the superiority of *Agrobacterium* strain EHA105 over C58 and LBA4404 for T-DNA transfer based on transient assays with a reporter gene in *A. tumefaciens*-mediated transformation of hybrid cottonwoods (*Populus* sections Tacamahaca Spach. and Aigeiros Duby). Wang *et al.* (2006) reported transformation of *Actinidia eriantha* genotype E2 using leaf strips with the two *Agrobacterium* strains, EHA105 and A281. Over 80% of leaf strips inoculated by either strain formed calli on selection medium, M9 containing kanamycin and timentin after 4 weeks of inoculation. The explants inoculated with strain A281 produced slightly higher rate of callus induction than that inoculated with EHA105. In addition, phenolic inducers such as acetosyringone appear to act alongside specific temperature requirements and an acidic environment to promote the expression of *Agrobacterium vir* genes, the products of which mediate T-DNA delivery (Wu *et al.*, 2003). However, acetosyringone is not important factor in many monocotyledons gene transformation via *Agrobacterium*, such as wheat (Cheng *et al.*, 1997), rice (Goto *et al.*, 1999) and maize (Kim *et al.*, 2009).

The objective of this study is to develop and optimise gene transformation system through cell suspension of oil palm for transferring some important genes in order to improve desirable traits in the future.

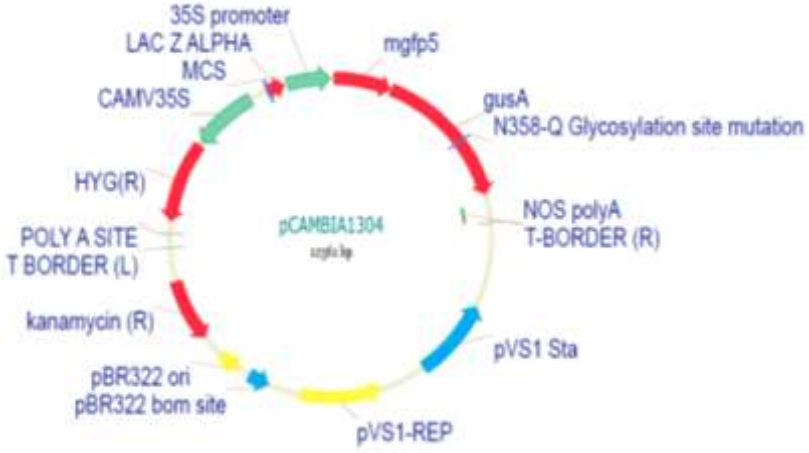
Materials and methods

Plant material, culture medium and environments

In this experiment embryogenic callus derived from young leave of DxP cross number 10 (Paorong company). Those calli were maintained on MS medium which was modified by supplementing with 3% sucrose, 200 mg/l ascorbic acid, 1 mg/l dicamba and 7.5 % agar. This medium is known as embryogenic callus induction medium. Subculture was carried out at monthly intervals for at least 10 years. The culture medium was adjusted to pH 5.7 before autoclaving at 1.07 kg/cm^2 at 121^{o}C for 15 min. The cultures were maintained under 10 µmol/m^2/sec illuminations, 14 h photoperiod at 27±1^{o}C.

Agrobacterium strains and plasmids

A. tumefaciens strain AGL-1 and EHA 101 containing a *gus* and *hpt*II gene in binary vector pCAMBIA 1304 and pIG121 Hm were used for transformation experiment. The details of constructed plasmid were shown in Figure 1. Fresh cultures of *Agrobacterium* strain AGL-1 were prepared by inoculating a single small colony into 25 ml LB medium containing 50 mg/l kanamycin. EHA 101 strain was prepared by inoculating a single small colony into 25 ml LB medium containing 50 mg/l kanamycin and 25 mg/l chloramphenicol.



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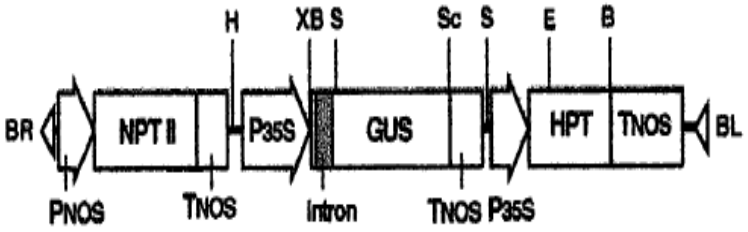

B

Fig. 1. The construct of plasmid pCAMBIA1304 containing in AGL-1 (CAMBIA,2006) (A) and pIG121 Hm containing in EHA101 (Belarmino and Mii,2000) (B)

Effect of strains of Agrobacterium and acetosyringone

A 10 ml of ECSs were incubated with 20 ml of two strains of *Agrobacterium,* AGL-1)pCAMBIA1304) and EHA101) pIG121Hm) in darkness at 28 ^{o}C for 6 hours in the absence or presence of 200 µM acetosyringone. The ECSs were washed by liquid medium supplemented 200 mg/l cefotaxim and cultured in darkness at the same conditions as mentioned earlier for 3 days. After that, ECSs were transferred to fresh medium supplemented with 20 mg/l hygromycin under light condition for 1 month. Packed cell volumes (PCV) were recorded after every 13 days of culture for 2 months. This experiment was arranged by completely randomized design (CRD) consisted of two strains of *Agrobacterium*. Each strain was four replicately done and significant different among treatments was carried out using analysis of variance (ANOVA) in SPSS version 16. Means among treatments were separated using T-test.

Histochemical assay for GUS activity

GUS expression using histochemical analysis for ß-glucuronidase was performed according to the method described by Jefferson *et al.* (1987). Briefly, after transformation for 48 hours, ESCs were brought to microtubes and incubated with X-gluc solution [10% X-Gluc (5-bromo-4-chloro-3-indolyl-glucuronide) dissolved in DMSO (dimethyl sulfoxide) and 50 mM $NaPO_4$]. The solutions were added in microtubes until explants were flood. Microtubes containing mixture of suspension cells and X-gluc solution were incubated at 37 ^{o}C for 12 hours or overnight. Then, the solution was removed from microtubes and 70% methanol was added periodically until blue spots appeared.

DNA analysis

DNA extraction

After 8 weeks of transformation, 0.05 g fresh weights of ECSs were subjected to extract DNA following the method described by Te-chato (2000). In brief, explants were put in 1.5 µl microtubes and filled with 150 µl TE buffer (20 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). A 20 µl of 10 %SDS (sodium dodecyl sulfate) was added, crushed well with pestle and incubated at 70 ^{o}C for 15 min. After that, 110 µl of 5M NH_4OAc was added in microtubes, mixed well, incubated at 0 ^{o}C for 30 minutes and centrifuged at 15,000 rpm for 10 min. After centrifugation, supernatant was removed and transferred to a new microtubes, A 500 µl of Isopropanol was added and gently inverted the tube until DNA precipitate appeared. Then a mixture of DNA solution was centrifuged at 15,000 rpm for 10 minutes and discarded the supernatant. The pellet of DNA was washed by chill 70 %ethanol twice and dried at room temperature. The DNA pellet was finally dissolved by 20 µl TE buffer and kept in freezer at -30 ^{o}C until further analysis.

DNA quantification

DNA were electrophoresed in 0.8 % agarose gel in comparison with λ standard DNA in TAE buffer)Tris base, glacial acetic acid and EDTA 0.5 M, pH 8.0 (at 100 V of 15 A for 20 minutes. The gel were then dyed in ethidium bromide and observed under 260 nm UV of gel document.

Detection of gus and hptll gene by PCR technique

DNA obtained from ECSs was amplified by PCR technique using specific primers of *gus* and *hpt*II. The primer for *gus* gene is F-primer 5'-CCTGAACTCACCGCGACG-3' and R-primer 5'-AAGACCAATGCG GAGCATATA-3'. The fragment size of the gene is 441 bp. The primer for *hpt*II gene is F-primer 5'-CTGCGACGCTCACACC GATAC-3' and R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3' and the fragment size of this gene is 800 bp. The PCR components or mixtures were shown in Table 1 and the PCR reaction was shown in Table 2. The PCR reaction of 10 ng DNA from transformed ECSs, 10 ng of plasmid DNA from pCAMBIA 1304 and pIG121Hm were separated on 1.5% agarose under 100 V of 15 A.

Table 1. Components of solution in PCR reaction used for detection of gene transformation in oil palm ECSs

Components of solution in Reaction	Concentration of the components	Volume	Final concentration
PCR buffer(+50 mM $MgCl_2$)	10 X	2.0 µl	1 X
dNTP mix	1 mM	4.0 µl	200 µM
Primer GUS (or HPT)-F	50 µM	0.5 µl	0.1 µM
Primer GUS (or HPT)-RV	50 µM	0.5 µl	0.1 µM
Taq polymerase	2U/50 µl	0.1 µl	0.5 U
DNA template		1 µl	
dH_2O		11.9 µl	

Table 2. The PCR reaction for gene amplification in transformed ECSs of oil palm

Steps	Temperature (^oC)	time	cycles
	96	2 min	1 cycle
Denaturing	96	20 sec	
Annealing	55	1 min	30 cycles
Extension	72	2 min	
	72	5 min	1 cycle

Results

Effect of strains of Agrobacterium and acetosyringone

After 1 month of transformation contamination of *Agrobacterium* was not observed. Viability of somatic embryos in ECSs as stained by FDA was high. The highest proliferation rate of embryogenic cells in ECS in term of PCV was obtained from strain EHA 101 haboring pIG121Hm while strain AGL-1 haboring pCAMBIA1304 gave the lowest result. PCV obtained from inoculation ECS with EHA101 (pIG121Hm) was nearly 3 folds of origin after being subcultured for 4 times (Figure 2). Adverse effect was found when 200µM acetosyringon was added to inoculation medium. Both strains of *Agrobacterium* gave far lower proliferation of PCV in the presence of acetosyringone (Figure 2).

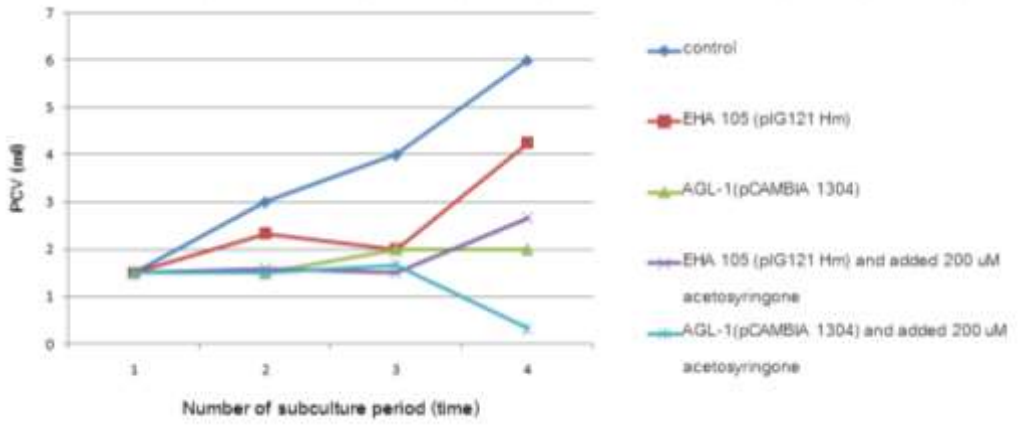

Fig. 2. Effect of *Agrobacterium* strains and acetosyringone. ECSs were cultured on MS medium supplemented with 3%sucrose, 200 mg/l ascorbic acid, and 1.0 mg/l Dicamba, and 200 mg/l cefotaxim. The suspensions were incubated with *Agrobacterium* suspension for 6 hours.

GUS histochemical assay

From the present study it was clear that strains of *Agrobacterium* and acetosyringone added to inoculation medium had an interaction together. Strain AGL-1 haboring pCAMBIA1304 gave a higher frequency of gene transformation in the presence of 200 µM acetosyringone while strain EHA101 haboring pIG121Hm gave higher result without that chemical (Table 3). The highest frequency of gene transformation was obtained from inoculation ECS with EHA101 haboring pIG121Hm at 65%. However, significant difference was not found from AGL-1 haboring pCAMBIA1304 together with 200 µM acetosyringone (Table 3, Figure 3).

Table 3. Effect of strains of *Agrobacterium* and acetosyringone on gene transformation expressed by GUS-histochemical assay in ECS of hybrid tenera oil palm

***Agrobacterium* strains and acetosyringone**	**Blue spot**	**% gus expression**	**% viability**
Control	+	27.3	100
AGL-1)pCAMBIA1304)	+++	35.56	50.00
AGL-1)pCAMBIA1304) + 200 µM Acetosyringone	++++	65.28	0
EHA101) pIG121Hm)	++++	65.71	100
EHA101) pIG121Hm)+ 200 µM Acetosyringone	+++	39.50	100

- No blue spot + 1-20 % blue spot ++ 21-40 % blue spot
+++ 41-60% blue spot ++++ 61-80 % blue spot +++++ 81-100 % blue spot

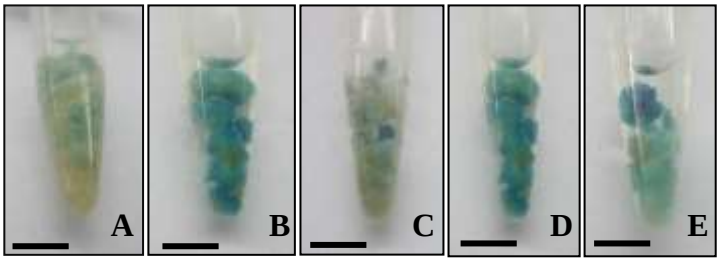

Fig. 3. Gus expression of ECSs in oil palm after transformed 3 weeks by *Agrobacterium* strain AGL-1 (pCAMBIA 1304) and strain EHA101 (pIG121Hm) (bar = 10 mm.)
A control, B. AGL-1 (pCAMBIA 1304), C. AGL-1 (pCAMBIA 1304) and added 200 µM acetosyringone, D. EHA101 (pIG121Hm), E. EHA101 (pIG121Hm) and added 200 µM acetosyringone

The expression of gus and hptll gene by PCR technique

Assessment of transient expression of *gus* and *hpt*II gene by PCR technique from 15 samples of transformed tissues showed positive bands of 441 bp for *gus* and 800 bp for *hpt*II while control samples did not show these profiles of DNA.

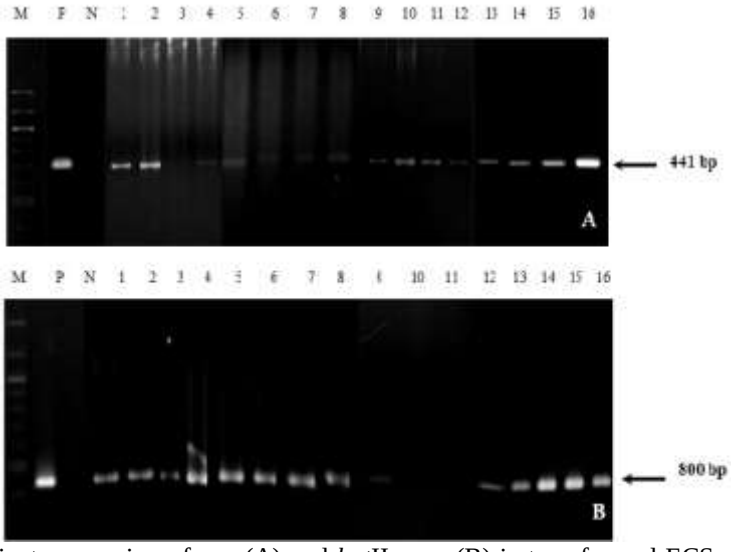

Fig. 4. Transient expression of *gus* (A) and *hpt*II gene (B) in transformed ECS as assessed by PCR technique. DNA was extracted from ECSs transformed by *Agrobacterium* strain AGL-1 (pCAMBIA 1304) and strain EHA 101 (pIG121 Hm) and dissolved in bacteria suspensions (OD_{600} = 0.8) for 6 hours and then co-cultured for 3 days.
M: Standard DNA P: positive control N: negative control
1-8: DNA from ECSs were transformed by *Agrobacterium* strain AGL-1 (pCAMBIA 1304)
9-16: DNA from ECSs were transformed by *Agrobacterium* strain EHA101 (pIG121Hm)

Discussion

Generally, ECSs are the most ideal explants for *Agrobacterium* transformation in oil palm because it is a single cell origin of somatic embryogenic cultures. By this system it would avoid chimerism in regenerated plants obtained from genetic transformation (Huang *et al*., 2007). Masli *et al.* (2009) reported that a higher transformation frequency can still be achieved by optimizing several other factors in monocots, such as strains and densities of *Agrobacterium*, pH, temperature, antibiotics, physical injuries and selectable marker. In this study, different of *Agrobacterium* strains provided nearly the same transformation frequency, however, there are specific to different plant types. In case of ECSs of hybrid tenera oil palm in the present study, *Agrobacterium* strain EHA101 gave better results than AGL-1. Contrary results was found when embryogenic callus of the same species was applied. Yenchon and Te-chato (2012) reported that strain AGL-1 haboring plasmid pCAMBIA1304 gave better transformation frequency than the other strains. Although, acetosyringone has been reported to be an important chemical in gene transformation in many plant species, but it was not improved transformation frequency in ECSs transformation in this present study.

However, transformed ECSs showed *gus* activity and viability in the presence of acetosyringone at higher frequency than those without acetosyringone in inoculation with *Agrobacterium* strain EHA 101. Obtaining results from the present study were the same as has been found in many monocotyledonous plants, such as wheat (Cheng *et al.*, 1997), rice)Goto *et al.*, 1999(and maize (Kim *et al.*, 2009). At present, ECSs of oil palm are difficult to regenerate into plantlets. Thus, we are now put attempt to regenerate transformed ECSs into plantlets and examined stable transformation of those transformed plants in order to set up gene transformation system through ECS of hybrid tenera oil palm.

Conclusion

ECSs inoculated with 25 ml of *Agrobacterium* suspension strain EHA101 containing pIG121Hm plasmid in darkness at 28 ^{o}C for 6 hours gave the highest transformation frequency of *gus* expressions at 65.71%. Proliferation of EC in the suspension under selective culture medium was 3 folds after subculture for 4 times. Viability of the embryo in ECSs was recorded to be 100 %. Assessment of transient expression of *gus* and *hpt*II gene by PCR technique from 15 samples of transformed tissues showed positive bands of the both *gus* gen and *hpt*II gene while control samples did not show these profiles of DNA.

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