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Optimization of Certain Parameters for Transformation of *indica* Rice *Hom Kra Dang Ngah* Variety via *Agrobacterium*-Mediated Transformation

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ABSTRACT

A transformation experiment was carried out by optimizing three important parameters: the age of calli, infection time and the optimum density of *Agrobacterium* suspension. A high efficiency *Agrobacterium*-mediated transformation system was established for the *indica* rice *Hom Kra Dang Ngah* variety. Embryogenic calli with high regeneration capability were incubated under different conditions with *Agrobacterium tumefaciens* strain EHA105 harboring plasmid pCAMBIA1304-*EPSPs*. The plasmid contains the enzyme gene 5-Enolpyruvylshikimate 3-phosphate synthase (*epsps*) as a selection marker gene and *β-glucuronidase* (*gus*) as a reporter gene. After 2 wk of *Agrobacterium* elimination and 4 wk of culturing on a selection medium containing 0.5 mM glyphosate (subcultured at 2-weekly intervals) the results showed that the six-week-old calli infected with bacterial suspension at OD_{600} 0.6 for 20 min gave the highest frequency of *gus* gene expression of 83.5%. The target gene was confirmed using polymerase chain reaction.

Keywords: *Agrobacterium*-mediated, transformation system, local *indica* rice *Kra Dang Ngah*, embryogenic calli, *glyphosate* selection

INTRODUCTION

Rice transformation has become an important part of technology in the current research into molecular biology and breeding and has been reported by several laboratories. For example, rice improvement has been carried out by sexual hybridization (Repellin *et al*., 2001), electroporation and *Agrobacterium* (Marchand *et al*., 2007), particle bombardment (Baisakh *et al*., 2001) and protoplast and *Agrobacterium*-mediated gene transformation (Martino *et al*., 2007). Rice genetic transformation has developed rapidly since the first transgenic rice plant was produced a few years ago (Raineri *et al*., 1990). During the last two decades, an important breakthrough occurred with the genetic transformation of *indica* rice, such as elite *indica*, Pusa Basmatic I, R_2 and others (Azhakanandam *et al.*, 2000; Kumria *et al.*, 2001; Kumria *et al.,* 2002; Hoque *et al.,* 2005). To date, the focus of the study has moved to establish genetic transformation with the genotype-independent and recalcitrant cultivar (Hiei and Komari*,* 2008).

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Agrobacterium tumefaciens-mediation is a routine method and considered preferable due to the simple procedure and low cost, high transformation efficiency, the capacity to transfer relatively large segments of DNA with defined ends, the low copy number of transgenes inserted into the host genome and the expected Mendelian heredity of transgenes (Hiei *et al*., 1994; 1997; Shibata and Liu, 2000). Therefore, it is very important to establish an efficient and widely used protocol for varieties of rice and extend it to other recalcitrant species. It can also complement traditional breeding. However, there are many factors affecting the efficiency of transformation, such as the type and age of explants, the strain of *Agrobacterium*, the expression vector, the selectable marker genes and selection agents, as well as various conditions of tissue culture (Hiei *et al*., 1997; Cheng *et al*., 2004). Among these factors, the genotype of the explants is considered crucial and which is difficult to overcome or to complement through optimizing other external factors (Tie *et al*., 2012).

According to Wanichananan *et al*. (2010), *indica* rice is a kind of recalcitrant genotype and the efficiency of transformation is very low. There is no published data regarding the improvement of *indica* rice varieties using genetic transformation and there is still a lack of commonly used genetic transformation technologies for *indica* rice. Thus, the objectives of the present study were to examine some key factors affecting gene transformation and to establish the *Agrobacterium*-mediated transformation system of the local *indica* rice cultivar *Hom Kra Dang Ngah*.

MATERIALS AND METHODS

Plant material

Indica rice (*Oryza sativa* L.) landrace *Hom Kra Dang Ngah* was used for callus induction and transformation. Mature, healthy seeds were selected, manually dehusked and surface sterilized following the protocol described by Zhang and Te-chato (2012).

Callus induction and plantlet regeneration

Sterile seeds were placed on callus induction medium (CIM) of MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose, 2 $mg.L^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D), 1 $mg.L^{-1}$ α-naphthalene acetic acid (NAA), 1 $mg.L^{-1}$ 6-benzyladenine (6-BA), 0.5 $mg.L^{-1}$ kinetin (Kn) and 1 $g.L^{-1}$ casein hydrolysate (CH). The medium was solidified with 0.75% agar. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121 °C, 1.07 $kg.cm^{-2}$ for 15 min. All cultures were placed in the culture room at 27 ± 2 °C, under 16 h photoperiod. After 1 month of culture, the embryogenic calli were transferred to subculture medium (SCM) of MS medium containing 1 $mg.L^{-1}$ 2,4-D, 0.5 $mg.L^{-1}$ NAA, 0.5 $mg.L^{-1}$ 6-BA and 0.25 $mg.L^{-1}$ Kn. The other supplements in the subculture medium were the same as in CIM and this was cultured for a further 1 month. Embryogenic calli were then transferred to the regeneration medium (RM) as reported by Zhang and Te-chato (2012). The medium was oil palm culture medium (OPCM) which was modified for rice and oil palm. This medium was supplemented with 0.5 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ 6-BA and 2 $mg.L^{-1}$ Kn, plus 82 mM sorbitol in combination with 1 $g.L^{-1}$ CH. The culture medium was solidified with 0.3% phytagel.

Testing the sensitivity of the callus to glyphosate

Embryogenic calli were inoculated on SCM with four different concentrations of glyphosate (0, 0.15, 0.3 and 0.5 mM) and cultured at 27 ± 2 °C, under 16 h photoperiod for 2 weeks. The sensitivity was determined according to the growth status of the calli.

Strain of *Agrobacterium* and its preparation

Agrobacterium strain EHA 105 harboring the plasmid pCAMBIA1304 with β-glucuronidase as a reporter gene and glyphosate resistant gene (*epsps*) as a selectable marker gene was used for

the transformation experiment (Figure 1).

One loopful of bacterial stock was streaked onto an Luria-Bertani (LB) solid medium plate with 50 mg.L^{-1} kanamycin for correct transformed colony selection, incubated at 28 °C in the dark and cultured for 2 d. From this culture, one single colony of bacterium was taken and incubated in 20 mL of LB liquid medium containing 50 mg.L^{-1} kanamycin and 200 µM acetosyringone on a rotary shaker at 75–100 rpm at 28 °C for 16–18 hr. After that, the optical density (OD_{600}) of the bacterial cell suspension was adjusted to 0.3, 0.6 and 0.8 with liquid callus induction medium containing 200 µM acetosyringone as *Agrobacterium* inoculum and was directly used for infection.

Infection and co-cultivation period

The 6 wk-old and 8 wk-old embryogenic calli obtained on SCM were cut into approximately 0.5 cm pieces and inoculated by submerging them in the prepared 20 mL of the *Agrobacterium* inoculum for 10, 15 and 20 min (Table 1). Then, the calli were blotted dry on sterile filter paper to remove remnant *Agrobacterium* and transferred to the co-culture medium (CIM medium containing 200 µM acetosyringone). The co-cultivation was maintained in the dark at 26 °C for 3 d, followed by thoroughly washing in sterile water 3–5 times, then washing with liquid CIM medium containing 500 mg.L^{-1} cefotaxime. The washed calli were blotted dry on sterile filter paper to remove excess moisture and transferred to the *Agrobacterium* elimination medium (SCM medium containing 300 mg.L^{-1} cefotaxime) for 2 wk. After that, the incubated calli were transferred to the selection medium (SCM medium supplemented with 0.5 mM glyphosate). The cultures were placed in a culture room at 28 °C under 16 h photoperiod and subcultured at two-week intervals for 4 weeks. After 4 weeks of selection, the small transgenic glyphosate resistant embryogenic calli were recorded.

***GUS* histochemical assay**

After 3 d of co-cultivation, the rice embryogenic calli were washed with sterile distilled water and immersed in *GUS* assay buffer containing 50 mM phosphate buffer (pH 6.8), Triton X-100 and 1 mM histochemical substrate 5-bromo-4-chloro-3-indolyl-β -D-glucuronide (X-Gluc-Duchefa Biochemie B.V.; Haarlem, the Netherlands) according to the method described by Jefferson (1987). The reaction mixture was incubated overnight at 37 °C. After X-gluc treatment, calli were washed twice in 70% methanol. The characteristic of the blue color as the expression of *GUS* (β-glucuronidase) was recorded. The frequency of transient transformation was expressed as the ratio between the number of calli showing GUS expression and the total number of calli kept for staining. Proper control for GUS histochemical assay was done with the calli having no *Agrobacterium* infection.

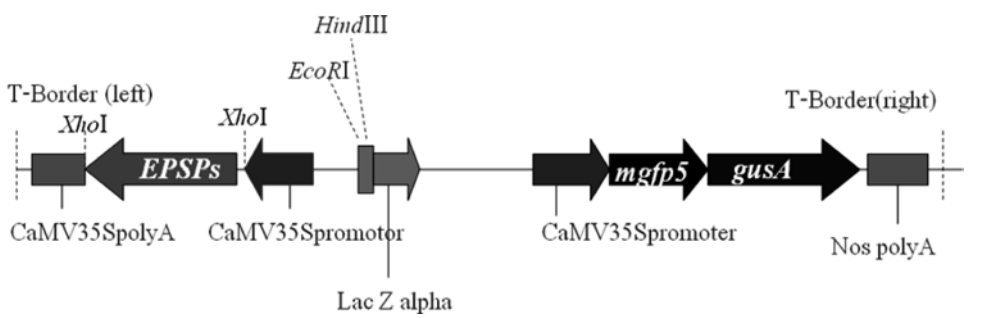

Figure 1 T-DNA regions of the cointegrate vector pCAMBIA1304 with β-glucuronidase (*gus*) and glyphosate resistant gene (*epsps*) used for rice transformation.

Polymerase chain reaction analysis

The genomic DNA of the putative transformed and non-transformed calli was isolated according to the method described by Techato (2000). The samples of DNA were subjected to polymerase chain reaction (PCR) amplification using *GUS* and *EPSPs* primers along with a positive control (plasmid DNA) and a negative control (non-transformed DNA) to confirm the presence or the absence of transgenes in the primary transformed calli. The primer sequences were: *GUS* gene fragment primer sequence F-primer 5′-CTGCGACGCTCACACCGATAC-3′and reverse primer sequence R-primer 5′-TCACCGAAGTTCATGCCAGTCCAG-3′ at expected size size of 441 bp; *EPSPs* gene primers forward 5′-CCATTCCGCTCGAGATGGCACAA ATTAACAACATGGC-3′ and reverse 5′-ATCC ACCGCTCGAGCGGTCATCAGGCAGCCTT CGTAT-3′ at expected size of 1,600 bp. PCR was performed for *GUS* and *EPSPs* in a 20 µL reaction volume containing 2 µL 10 × PCR buffer, 4 µL 1mM dNTP, 0.5 µL each of 10 mM primer, 1.0 U of Taq polymerase and 20 ng of DNA template. Amplification was carried out in a programmable Thermal cycler under the following conditions: predenature at 94 °C for 5 min, followed by 30 cycles of denature at 94 °C for 40 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. Amplification products were analyzed using electrophoresis at 100 V for 45 min on 1.5% agarose gel followed by staining with ethidium bromide and detection under ultraviolet illumination.

Statistical analysis

The experiment was arranged as a completely randomized design with three replicates per treatment. Analysis of variance was applied to indicate which treatment was the best for transformation. Data were analyzed using the SAS program Version 6.11 (SAS Institute, Cary, NC, USA). A probability level of 5% was chosen for all statistical inferences.

RESULTS

Embryogenic calli and regeneration

The embryogenic calli are the most amenable source of material for the genetic transformation of *indica* rice. In this study, two types of callus (embryogenic callus and non-embryogenic callus) were obtained on CIM after 4 wk. The embryogenic calli were creamy white with some being compact, friable and globular which were further developed into plantlets (Figure 2a).

The embryogenic calli were transferred to SCM for proliferation and the rate of the callus proliferation was 100%. After 3 wk of subculture, fast growing embryogenic calli and green spots were obtained (Figure 2b). Upon transferring the calli to RM for plantlet regeneration, a high frequency of 75% was obtained (Figure 2c).

Sensitivity of calli to glyphosate

The present study aimed to optimize the glyphosate concentration for the selection procedure. After culturing the calli on SCM containing different concentrations of glyphosate for 2 wk, all calli survived without supplementation with glyphosate (Figure 3a). Glyphosate at a concentration higher than 0.3 mM caused browning and necrosis of the calli. Glyphosate at a concentration of 0.5 mM caused partial death of the calli after 2 wk of culture (Figure 3d). Among the different concentrations of glyphosate, 0.5 mM was found to be a suitable concentration for the selection of gene transfer in the *indica* rice cultivar *Hom Kra Dang Ngah* (Figure 3d). Consequently, 0.5 mM was concluded to be the minimum concentration which effectively prevented callus growth. This experiment provided the basis for the choice of glyphosate concentration in the selection media for the transformation experiments.

Effects of callus age on gene transformation

The age of explant plays an important role in transformation. In order to investigate

the effect of callus age on T-DNA delivery and transformation efficiency, 6 week-old and 8-week-old calli were separately immersed in *Agrobacterium* suspension at OD_{600} of 0.8 for 20 min. Through assaying the *gus* gene histochemical activity and observing the frequency of *gus* gene expression, the results indicated that 6-week-old calli showed more transient *gus* gene expression than did 8-week-old calli. The frequency of *gus* gene expression obtained from 6-week-old calli was 70.5% whereas 8-week-old calli gave only 51.3% (Table 1). Non-transgenic calli did not show a blue color which thus meant there was no *gus* activity (Figure 4a). In contrast, the 8-week-old infected calli showed a small zone and a light blue color (Figure 4b). The 6-week-old infected calli showed a blue zone on the entire surface of the transformed calli with a dark blue color (Figure 4c). In addition, on the transformed 8-week-old calli it was difficult to eliminate *Agrobacterium*, finally leading to contamination. Thus, 6-week-old calli were better and were used for the following experiment.

Density of *Agrobacterium* and infection period

The bacterial density and infection time are also important factors in transformation experiments mediated by *Agrobacterium*. In the present study, 6-week-old calli were used for genetic transformation. During the transformation process, different concentrations of *Agrobacterium* (OD_{600} = 0.3, 0.6, 0.8) and durations (10, 15, 20 min) of inoculation gave different results. After 3 d of co-culture, the histochemical activity and the frequency of *gus*-gene transient expression indicated that the *gus* gene response increased

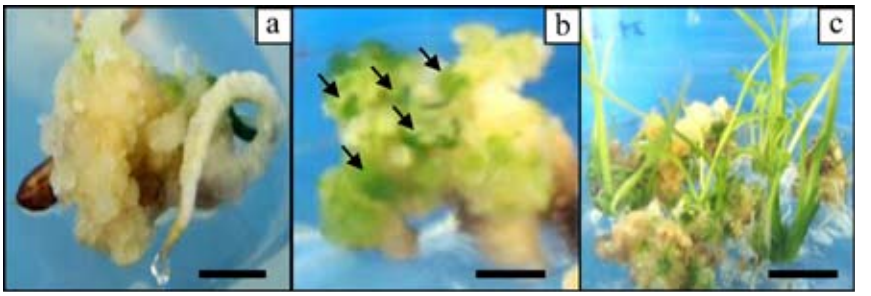

Figure 2 Embryogenic callus induction and regeneration of *indica* rice cv. *Hom Kra Dang Ngah:* (a) Callus with embryogenic structures derived from mature seed after 1 month on callus induction medium; (b) Proliferation of callus with green spots (arrows) on subculture medium after 3 weeks of subculture; (c) Plantlets regenerated from embryogenic callus after 2 months on regeneration medium. (Scale bar = 0.5 cm.)

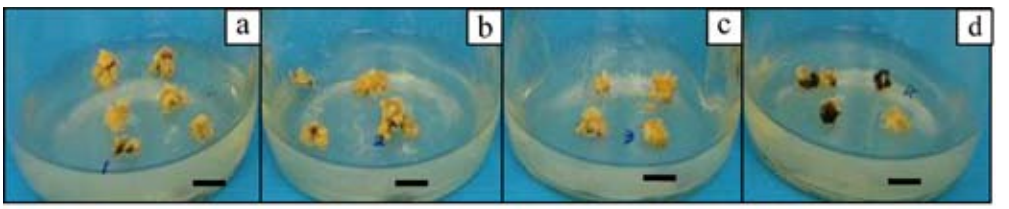

Figure 3 Growth and proliferation of embryogenic calli of *indica* rice cv. *Hom Kra Dang Ngah* cultured on medium containing various concentrations of glyphosate after 2 weeks: (a) without glyphosate; (b) 0.15 mM glyphosate; (c) 0.3 mM glyphosate; (d) 0.5 mM glyphosate. (Scale bar = 1 cm.)

with the increasing time of infection (Figure 5). A lower bacterial density (OD_{600} = 0.3) and shorter time of infection (10 min) decreased the frequency of transformation (Table 1). The highest frequency of gene transformation according to blue spot detection was obtained at 83.5% from a 20-minute infection time and the OD_{600} of bacterial density at 0.6 (Figure 5c).

Polymerase chain reaction analysis

The surviving samples of transgenic calli after the selection processes were subjected to PCR analysis. The genomic DNA was isolated from the putative transgenic calli and the non-transgenic calli as the control. The DNA samples were amplified using *gus* primers and *epsps* primers separately for PCR analysis. Nine of the 10 putative transgenic calli were found to contain *gus* transgene with the expected band size at 441bp, while non-transformed callus could not amplify that band (Figure 6). These results preliminarily confirmed that *A. tumefaciens* could transfer the T-DNA into the *indica* rice. However, the *epsps* gene was the only one from the 10 DNA samples that showed amplification of this sequence as expected at the size of 1600 bp though these transformants showed a positive result for the *gus* gene amplification (Figure 7).

DISCUSSION

The foreign gene transformation mediated by *A. tumefaciens* is the result of bacterium strains and plant cell interaction. Several factors were found to have an impact on the efficiency of *A. tumefaciens*-mediated transformation, including

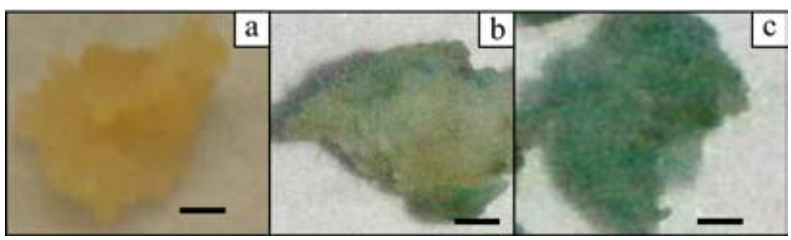

Figure 4 Histochemical assay of *gus* expression in transformed calli of rice cultivar *Hom Kra Dang Nga* at different ages after inoculation with *Agrobacterium* at OD_{600} of 0.8 for 20 min: (a) Nontransformed calli (negative control); (b) Transformed at 8-weeks-old transgenic calli with a small amount of light blue color; (c) transformed at 6-weeks-old transgenic calli with a blue zone on the entire surface of infected calli. (Scale bar = 0.5 cm.)

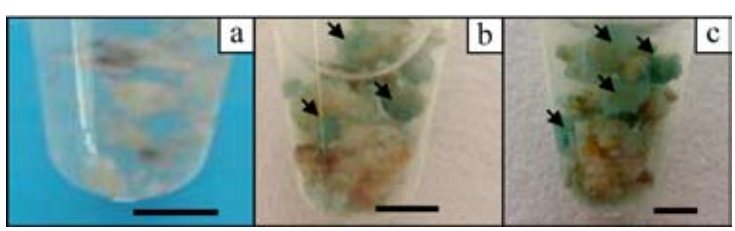

Figure 5 Histochemical assay of transient gene expression in infected calli of rice, *Hom Kra Dang Ngah* at different densities of *Agrobacterium* for different times of infection (Scale bar = 0.5 cm): Callus excised from non-transformants (control); (b) Calli inoculated in OD_{600} = 0.3 and infected for 10 min; (c) Calli inoculated in OD_{600} = 0.6 and infected for 20 min. (arrows showed blue staining calli)

Table 1 Ages of calli, density of *Agrobacterium* and infection time affecting gene transformation in term of transient *gus* expression.

Callus age	OD_{600}	Infection time (min)	Expression of *gus* (%)
6-week-old	0.3	10	5.3
		15	33.5
		20	56.7
	0.6	10	40
		15	68.6
		20	83.5
	0.8	20	70.5
8-week-old	0.3	10	0
		15	14.6
		20	20.5
	0.6	10	32
		15	36
		20	46.3
	0.8	20	51.3

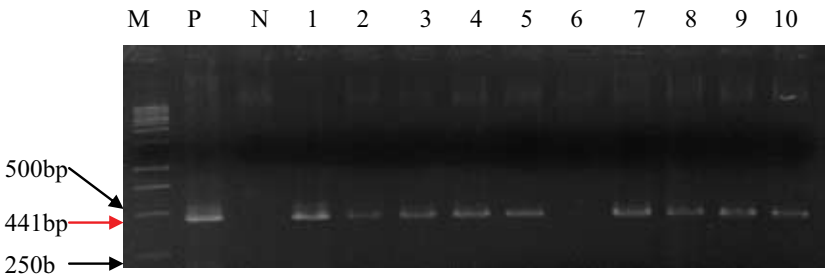

Figure 6 Polymerase chain reaction (PCR) analysis of genomic DNA of putative transgenic calli to detect the presence of the *gus* gene. M: 1kb size marker (Promega). P: positive control. N: negative control. lane 1–10 were putative transformants. The 441 bp arrow indicates the expected PCR product of the *gus* gene.

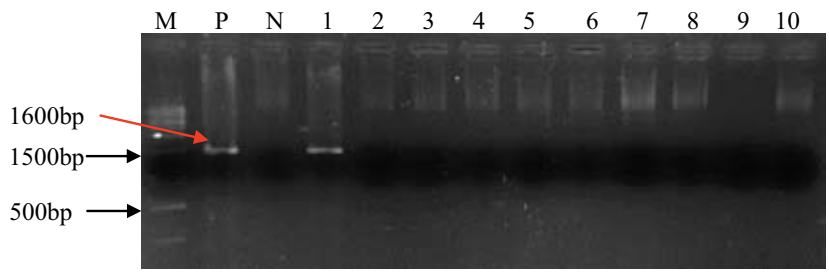

Figure 7 Polymerase chain reaction (PCR) analysis of genomic DNA of putative transgenic calli to detect the presence of the *epsps* gene. M: 1kb size marker (Promega). P: positive control. N: negative control. 1–10 lines were putative transformants. The 1600 bp arrow indicates the expected PCR product of the *epsps* gene.

the Ti plasmid, plant cell response capability, the co-cultivation condition with *Agrobacterium* and the regeneration ability of the transformants (Gao and Hang, 1999). *A. tumefaciens*-mediated transformation included steps such as attachment, *vir* gene induction, T-DNA processing, transporting and integrating, which were completed during the co-cultivation period (Shibata and Liu, 2000). Thus, the key factor affecting successful transformation was the time of co-cultivation.

Embryogenic callus and regeneration

Embryogenic callus and the regeneration system are prerequisites for genetic transformation mediated by *Agrobacterium* in *indica* rice. Plant regeneration from embryogenic callus was achieved initially in *japonica* rice varieties (Nishimura *et al*., 2007). Successful regeneration of fertile plants has been limited in *indica* rice varieties (Rueb *et al*., 1994). The transformation of *indica* rice is still difficult due to the low induction rate of embryogenic callus and plantlet regeneration. Many *indica* rice varieties are particular genotype-dependent (Hiei *et al*., 2006; Hiei *et al.,* 2008). Although a few successful cases of genetic transformation in *indica* rice have been reported (Lin *et al*., 2009; Wang and Tian, 2009), it has a strong recalcitrant nature and is genotype-independent, even with the same types. In the present study, the high efficiency of the regeneration system was established using a mature embryo as the explant and by culturing on MS medium supplemented with 2 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ 6-BA and 0.5 $mg.L^{-1}$ Kn containing 3% sucrose and 1 $g.L^{-1}$ CH. After 1 mth of culture, the embryogenic callus was transferred to the proliferation medium (ARDA medium supplemented with 0.5 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ 6-BA and 2 $mg.L^{-1}$ Kn) and cultured for a further 1 mth. Finally, the calli were transferred to the plantlet regeneration medium (MS solidified with phytagel at concentration of 0.3%, plus 82 mM sorbitol combined with 1 $g.L^{-1}$ CH). The highest frequency of plantlet regeneration obtained was 75%. This culture system in callus-based *indica* rice was used for the transformation system.

Effect of callus age on transformation efficiency

Callus is an excellent source of cells for the production of transgenic rice (Rashid *et al*., 1996; Hiei *et al*., 1997). The use of actively growing, embryogenic callus is one of the most important factors in efficient transformation. Short-term culture and long-term culture significantly affect the efficiency of transformation. Liu *et al.* (2005) studied the effect of callus age on transient *GUS* expression and showed that the 42-day-old calli performed better and exhibited the maximum numbers of blue zone samples in the variety *Pusa Basmati* L. A similar result was also found in the present study. Six-week-old calli gave better results in transformation than 8-week-old calli. This might have been due to the difference between the physiological status of the two callus ages. The 6-week-old calli had a strong capability of division due to the cells having mitogenetic status which benefited attachment by *Agrobacterium*. In addition, mitogenetic cells promote T-DNA to integrate into the genomic DNA of rice (Cheng *et al.,* 2004). Therefore, after 6 weeks of sub-culture, the calli were easier to transform than short-term and long-term cultured calli.

Effect of bacterial density and infection time on transformation efficiency

The efficiency of co-cultivation was affected by the bacterial cell density, infection methods, infection time, co-cultivation temperature among others. Bacterial density plays an important role in the transformation. According to study results, the OD_{600} of the bacterial density in a range from 0.3 to 1 was suitable for transformation (Aananthi *et al*., 2010; Baskaran and Dasgupta, 2012). In the present study, the OD_{600} of bacterial density at 0.6 showed the highest transformation efficiency at 83.5% as the result of assaying *gus* activity.

Infection time is also one of the key factors affecting the success of transformation experiments mediated by *Agrobacterium*. Research has shown that infection for 10–20 min produced the best results in transformation (Wang and Tian, 2009). A longer time of infection leads to contamination, while a shorter time of infection does not promote *Agrobacterium* attachment to calli completely and T-DNA processing. In the present study, calli were infected for 10, 15 and 20 min and gave incremental expression of a *GUS* response. An infection time of 20 min showed the highest response of *GUS* activity. Unfortunately, only one from ten DNA samples showed amplification of the *EPSPs* band or activity as expected at a size of 1600 bp (Figure 7). This might have been due to the transportation mechanism of the T-DNA from *Agrobacterium* to the plant genome. Enzyme restriction endonuclease might cut T-DNA at the right border (RB) from the Ti plasmid and may not have completely inserted all of the T-DNA into the plant genome through the left border. Normally, the RB is connected with the *gus* and *EPSPs* genes, respectively. In some cases, it is possible that incomplete transfer of T-DNA was performed resulting in only some of reporter genes at the first part being sent to plant genomes while the others were not. Thus, in the present study, all of the transgenic samples showed expression of the *gus* gene, but the *EPSPS* gene didn't show in some samples. To prove the integration of these two genes into *Hom Kra Dang Ngah* rice genome, Southern blot analysis must be performed in a future experiment.

CONCLUSION

The present study reported on a high-efficiency regeneration system and succeeded in establishing an efficient gene transfer system in the *indica* rice cultivar *Hom Kra Dang Ngah* by *Agrobacterium*-mediated method. The results of the study suggested that the most suitable conditions for transformation were using 6-week-old embryogenic callus derived from mature seeds infected by *Agrobacterium* at the OD of 0.6 for 20 min. The presence of the *gus* and *epsps* gene was confirmed by PCR. In summary, this report described the use of *A. tumefaciens* strain EHA 105 (pCAMBIA 1304- *EPSPs*) to transfer screenable and reporter genes into the *indica* rice cultivar *Hom Kra Dang Ngah* and the tissue culture standardization and optimization of transformation conditions in *Hom Kra Dang Ngah* might help in the transformation of other related genotypes for their genetic improvement.

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