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Full Length Research Paper

A highly efficient method for *Agrobacterium* mediated transformation in elite rice varieties (*Oryza sativa* L. spp. indica)

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An *Agrobacterium* mediated transformation method was developed for the Thai rice variety, Pathumthani 1 (PT1), and the Indian rice variety, Pokkali (PKL). Various aspects of the transformation method, including callus induction, callus age, *Agrobacterium* concentration and co-cultivation period were examined, in order to improve transformation efficiency. Optimized transformation conditions were established using *Agrobacterium* strain EHA105, which carries a virulent plasmid, pCAMBIA1301. A modified Murashige and Skoog (MS) medium supplemented with 1 mg/l 2, 4-D and 0.5 mg/l picloram was optimized for callus induction. Three week old calli were used to co-cultivate with 0.8 -1 OD_{600} *Agrobacterium* for 30 min and the culture was continued on agar medium without antibiotics for 2 days. This method can be used to induce high quality calli within three weeks. Based on GUS determination, it was demonstrated that the transformation method was improved significantly, with a high level of transformation efficiency.

Key words: *Agrobacterium tumefaciens*, indica rice, mature seed-derived callus, rice transformation, transgenic rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is an economically very important crop, with more than half of the world's population depending on it as a primary staple food (Lu, 1999). Its cultivation is concentrated particularly in Asian countries, which together make up approximately 90% of the rice cultivation area in the world. Around 80% of world rice production is based on indica varieties, which are grown in subtropical and tropical conditions as long grain rice, therefore securing a unique position and significance in the global agricultural economy (Khush, 1997). The genetic improvement of rice is of pivotal importance to agriculture, with emphasis on high and stable yields, resistance to insects, diseases, abiotic stresses and improving the grain quality to the level of consumer satisfaction. The potential of biotechnology in rice improvement has been recognized in recent years. Several gene transfer methods have been developed in order to introduce target DNA into the rice cell, of which, *Agrobacterium tumefaciens* mediated transformation has been well established, on account of its simplicity, low cost, and low copy number of transgene integration. There are several reports on *Agrobacterium* mediated transformation of rice varieties (Datta et al., 1999, 2000, 2001; Tu et al., 1998; Zhang et al., 1998; Mohanty et al., 1999; Labra et al., 2001). However, the transformation

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Abbreviations: PT1, Pathumthani 1; **PKL,** Pokkali; **MS,** Murashige and Skoog; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **PPF,** photosynthetic photon flux, **YEP,** yeast extract peptone.

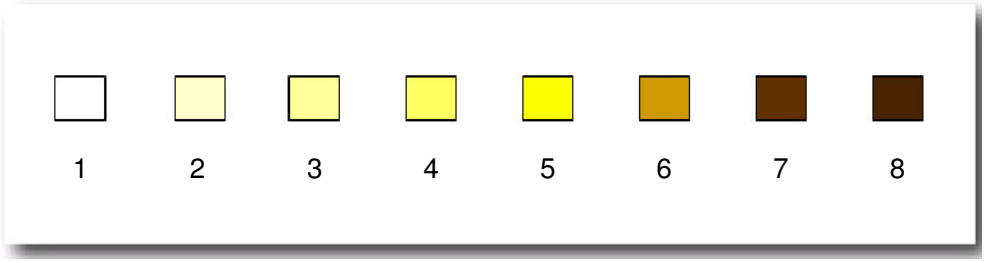

Figure 1. The color chart with different scores (1 to 8), used for determination of callus color.

efficiency in the indica rice varieties is very low. Most indica rice varieties also show a low rate of callus growth or low regeneration frequency in conventional culture (Nishimura et al., 2007).

Using MS (Murashige and Skoog, 1962) medium for *in vitro* culture of rice, 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used for callus induction. Several different levels of 2,4-D were used to induce callus in different rice cultivars (Venkata et al., 2007; Nishimura et al., 2007; Hiei and Komari, 2008). It has been found that 2,4-D produces a large quantity of callus, however, the quality is low. The callus induced by 2,4-D was light yellow or white in color and was compact in character (Islam et al., 2005). Frequently, albino plantlets resulted from the regeneration system. Since an embryogenic callus was used to improve the transformation efficiency of recalcitrant rice varieties (Nishimura et al., 2007), picloram (4-amino-3,5,6-trichloropicolinic acid), a synthetic growth regulator, acting as a potent auxin in a variety of test systems, could be of interest in order to induce embryogenic callus. There are several reports on callus induction in plants using combinations of 2,4-D and picloram, stating that they produce calli which are globular in shape, yellow in color and of the friable type (Can et al., 2008; Kiong et al., 2008; Sener et al., 2008). However, their effect on the quality of callus in rice is still restricted. Immature embryos of indica rice have been used as the starting material for *Agrobacterium* mediated transformation with only slight success. In comparison to mature seeds, the preparation of immature embryos is more difficult and laborious. There are also a few reports describing *Agrobacterium* mediated transformation using mature indica rice seeds, but success is limited to very specific varieties (Aldemita and Hodges, 1996; Rashid et al., 1996). An amelioration of the *Agrobacterium* mediated transformation method using mature rice seeds of an elite rice variety, Pathumthani 1 (PT1), and a salt tolerant rice variety, Pokkali (PKL) is reported here. Four steps of the transformation process, namely, callus induction, callus age, *Agrobacterium* concentration and co-cultivation period were examined, in order to improve the transformation efficiency, using an *Agrobacterium* strain EHA105, which carries a virulent plasmid pCAMBIA1301.

MATERIALS AND METHODS

Plant materials

Matured seeds of PT1 and PKL rice varieties were sterilized by dipping in 70% (v/v) ethanol for 5 min, soaking in 5% (v/v) Clorox (5.25% w/v sodium hypochlorite, Clorox Co., Ltd., USA) with 0.1% Tween 20 (Merck, Germany) for 1 h, soaking in 30% Clorox with 0.1% Tween 20 for 30 min and rinsing with sterile-distilled water three times. The rice seeds were germinated on MS medium (1962) for 3 days, at 25±2°C and 16 h photoperiod of 100±5 µmol/m^2/s photosynthetic photon flux (PPF) density provided by Cool-White fluorescent lamps (TLD 36 W/84, Philips, Thailand). Scutellum from the rice seeds were cultured on callus induction medium, containing MS salts with B5 vitamins, 30 g/l sucrose, 2.0 mg/l 2, 4-D, 0.3 mg/l Kinetin, 4 g/l Phytagel and 2 g/l agar for three weeks at 25±2°C under the condition of darkness (Kumar et al., 2005). The growth of embryogenic calli at zero, one, two and three weeks was measured, and the calli were then immediately infected with *A. tumefaciens*.

Optimization of callus induction

Callus induction in PT1 and PKL rice was optimized using the comparison of growth on five media formulae supplemented with 0.5 mg/l picloram + 1.0 mg/l 2, 4-D, 0.5 mg/l picloram + 2.0 mg/l 2, 4-D, 0.5 mg/l picloram + 3.0 mg/l 2, 4-D, 0.2 mg/l picloram + 2.0 mg/l 2, 4-D or 1.0 mg/l picloram + 2.0 mg/l 2, 4-D. Calli were cultured under light or dark conditions, 100 or 0 µmol/m^2/s. The growth of the callus was determined by its diameter size in millimeters, and the character was determined by the color and type of callus. The color score was determined by comparison with a color chart (Figure 1). The character of the callus was separated into two types with different scores, namely, compact type callus which was scored as three and friable type callus which was scored as one.

Optimization of *Agrobacterium* mediated transformation methods

A virulent *Agrobacterium* strain, EHA105, harboring pCAMBIA1301, which is widely used for rice transformation (Veluthambi et al., 2003), was cultured in yeast extract peptone (YEP) medium containing 10 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, pH 7.0 and supplemented with 100 mg/l Kanamycin to 0.6, 0.8, 1.0 or 1.2 OD_{600}. Subsequently, the culture medium was centrifuged at 500 xg for five minutes, and the pellet was re-suspended in an equal volume of the YEP medium. The calli were immersed in the bacterial suspension for 30 min, and the infected calli were

Table 1. Effects of light and callus induction media on size, color and callus type of P**a**thumthani1 (PT1) calli.

Rice cultivar	Light condition	Media	Size (mm)	Color (1-8)	Callus type (1-3)
PT1	Dark	0.5 mg/l picloram + 1.0 mg/l 2,4-D	3.2 ± 0.4 [a]	5.2 ± 0.4	1.4 ± 0.5
		0.5 mg/l picloram + 2.0 mg/l 2,4-D	3.6 ± 0.9	2.4 ± 0.5	1.0 ± 0.0
		0.5 mg/l picloram + 3.0 mg/l 2,4-D	4.4 ± 0.5	2.0 ± 0.0	1.0 ± 0.0
		0.2 mg/l picloram + 2.0 mg/l 2,4-D	4.0 ± 0.0	4.2 ± 1.6	1.0 ± 0.0
		1.0 mg/l picloram + 2.0 mg/l 2,4-D	3.8 ± 0.4	2.0 ± 0.0	1.0 ± 0.0
	Light	0.5 mg/l picloram + 1.0 mg/l 2,4-D	4.8 ± 0.5	3.2 ± 0.4	1.4 ± 0.5
		0.5 mg/l picloram + 2.0 mg/l 2,4-D	3.8 ± 0.5	6.8 ± 0.4	2.2 ± 0.4
		0.5 mg/l picloram + 3.0 mg/l 2,4-D	2.2 ± 0.5	7.0 ± 1.0	2.2 ± 1.1
		0.2 mg/l picloram + 2.0 mg/l 2,4-D	2.6 ± 0.5	7.4 ± 0.5	2.2 ± 0.4
		1.0 mg/l picloram + 2.0 mg/l 2,4-D	3.4 ± 0.5	3.2 ± 0.4	2.2 ± 0.8
Light (L)			**	**	**
Media (M)			*	**	ns
L x M			**	**	ns

[a] Means ± standard deviation; *, ** and ns: significant differences at P ≤ 0.05, P ≤ 0.01 and not significantly different, respectively using Duncan's multiple range test (DMRT). The color of callus was separated into eight colors with different scores from white to brown (1 to 8 scored). The type of callus was separated into two types with different scores (compact callus = 3 or friable callus = 1).

transferred onto an MS co-cultivation medium (MS salts, B5 vitamin, 30 g/l sucrose, 100 mM acetosyringone, 2.5 g/l Phytagel [pH 5.7] without antibiotics), overlaid with Whatman no. 1 filter paper (Kumar et al., 2005). The optimum time for callus infection was determined by culturing infected calli for two, three, four, five or six days at 25±2°C and a 16 h photoperiod of 100±5 µmol/m^2s PPF.

GUS determination

Histochemical assay of β-glucuronidase and measurement of GUS activity by fluorimetry were performed by standard methods (Clark, 1997).

RESULTS AND DISCUSSION

Optimization of callus induction

In order to determine the optimum conditions for callus induction, five formulae of modified MS basal medium under both light and dark conditions were tested using seedlings of PT1 and PKL rice. Size, color and callus type of the PT1 calli were affected significantly by the condition of light at P ≤ 0.01 (Table 1). Under dark conditions, the PT1 calli had a larger average size (3.8±0.6 mm) than in light conditions (3.4±1.0 mm). The browning of calli (scored 2.0 - 5.2) in the dark was less than in light conditions (scored 3.2 - 7.4). The type of calli produced in the dark was mostly friable (Table 1), while calli growing in the light were compact (Table 1). The effects of the medium significantly influenced the size at P ≤ 0.05 and color at P ≤ 0.01 of the PT1 calli, but not their character. Under dark conditions, the callus size was not different in any medium. The brown color of the callus decreased with increasing 2,4-D and picloram concentration. The type of media had a highly significant effect on the color of the PT1 calli at P ≤ 0.01, and it also had a significant effect on the size of the PT1 calli at P ≤ 0.05, while it had no effect on callus type. The callus size decreased with increasing 2,4-D concentration. The brown color also increased with increasing 2,4-D concentration, while it decreased with increasing picloram concentration (Table 1). In addition, the medium containing picloram and 2,4-D induced PT1 calli which differed from those of the medium containing only 2,4-D (Kumar, 2005) from our preliminary experiment (2.6±0.1 mm and 1.6±0.2 scored, respectively), in being larger and in yellow color (3.6±0.9 mm and 4.3±2.1 score, respectively) (Figure 2). The interaction between the light conditions and the type of medium greatly affected the size and color, at P ≤ 0.01, of the PT1 calli, while it had no effect on callus type (Table 1). Modified MS medium supplemented with 0.5 mg/l picloram and 1.0 mg/l 2,4-D was found to be the optimum medium for callus induction in the PT1 rice cultivar under light conditions.

The factor of light had a highly significant effect on the size, color and type of the PKL calli at P ≤ 0.01. Under the light, the PKL calli were smaller in size, brown and compact, when compared with those in the dark conditions (Table 2). The factor of the medium significantly affected the size at P ≤ 0.05, and was highly significant regarding the color of the PKL calli at P ≤ 0.01, but had no effect on callus character. The size of the PKL

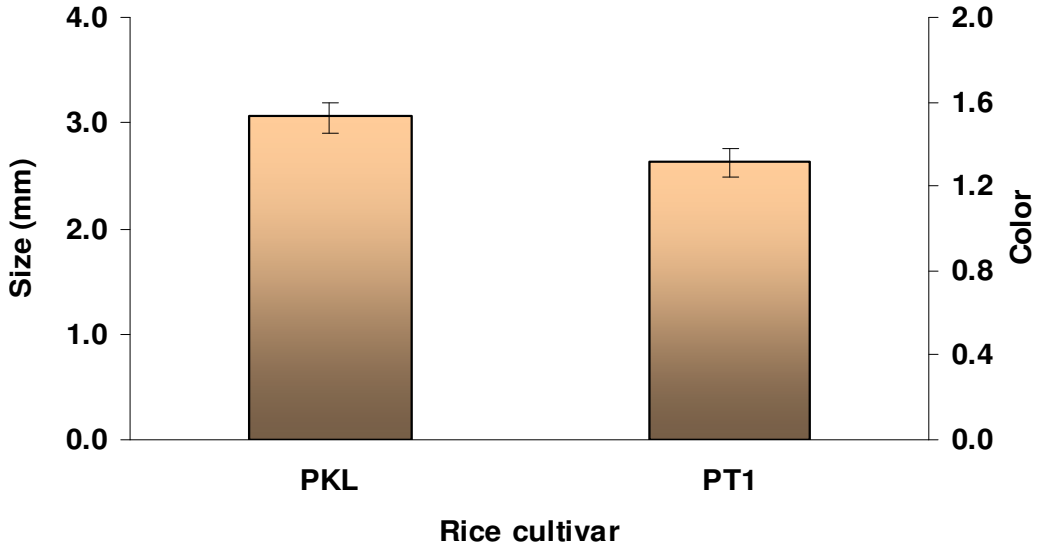

Figure 2. Results for callus induction medium containing 3.0 mg/l 2, 4-D. Size and color of Pokkali (PKL) and Pathumthani 1 (PT1) rice varieties.

Table 2. Effects of light and callus induction media on size, color and callus type of Pokkali (PKL) calli.

Rice cultivar	Light condition	Media	Size (mm)	Color (1-8)	Callus type (1-3)
PKL	Dark	0.5 mg/l picloram + 1.0 mg/l 2,4-D	4.2 ± 0.4[a]	3.8 ± 0.4	1.4 ± 0.5
		0.5 mg/l picloram + 2.0 mg/l 2,4-D	5.0 ± 0.7	2.2 ± 0.4	1.0 ± 0.0
		0.5 mg/l picloram + 3.0 mg/l 2,4-D	5.6 ± 0.5	2.2 ± 0.4	1.0 ± 0.0
		0.2 mg/l picloram + 2.0 mg/l 2,4-D	2.6 ± 1.1	3.8 ± 0.4	1.4 ± 0.5
		1.0 mg/l picloram + 2.0 mg/l 2,4-D	5.0 ± 0.7	2.2 ± 0.4	1.0 ± 0.0
	Light	0.5 mg/l picloram + 1.0 mg/l 2,4-D	2.8 ± 1.3	5.4 ± 0.5	3.0 ± 0.0
		0.5 mg/l picloram + 2.0 mg/l 2,4-D	3.6 ± 0.5	5.6 ± 1.1	2.6 ± 0.9
		0.5 mg/l picloram + 3.0 mg/l 2,4-D	4.2 ± 0.4	8.0 ± 0.0	3.0 ± 0.0
		0.2 mg/l picloram + 2.0 mg/l 2,4-D	3.8 ± 0.8	7.6 ± 0.5	3.0 ± 0.0
		1.0 mg/l picloram + 2.0 mg/l 2,4-D	3.6 ± 1.5	5.6 ± 0.5	2.6 ± 0.0
Light (L)			**	**	**
Media (M)			*	**	ns
L x M			**	**	ns

[a] Means ± standard deviation; *, ** and ns: significant differences at P ≤ 0.05, P ≤ 0.01 and not significantly different, respectively using Duncan's multiple range test (DMRT). The color of callus was separated into eight colors with different scores from white to brown (1 to 8 scored). The type of callus was separated into two types with different scores (compact callus = 3 or friable callus = 1).

calli increased with increasing 2,4-D concentration. The color of the PKL calli changed from yellow to brown with increasing 2,4-D concentration, or a decrease in the picloram concentration. In addition, the medium containing picloram and 2,4-D induced larger, yellow colored (4.0±1.2 mm and 4.6±2.1 scored, respectively) calli when compared to those of the medium containing only 2,4-D (Kumar, 2005) from our preliminary experiment on PKL rice cultivars (3.1±0.1 mm and 1.9±0.1 scored, respectively) (Figure 2). The interaction between the light conditions and the type of media greatly affected the size and color, at P ≤ 0.01, of the PKL calli, while it had no effect on callus type (Table 2). In the case of the PKL rice cultivar, It was found that the optimum conditions to increase growth, with a high quality of friable callus, yellow in color (Can et al., 2008; Kiong et al., 2008; Sener et al., 2008) were a modified MS medium supplemented with 0.5 mg L^{-1} picloram and 1.0 mg/l 2,4-D under dark

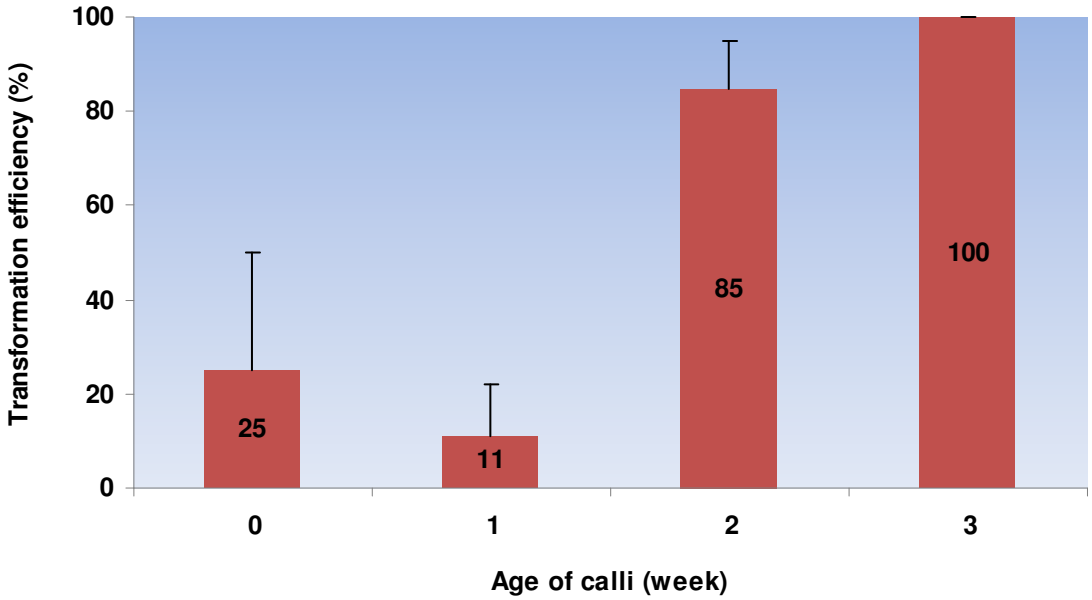

Figure 3. Transformation efficiency (%) of Pathumthani 1 (PT1) calli at 0, 1, 2 or 3 weeks old as determined by GUS expression. Transformation efficiency was calculated by the number of GUS.

conditions. Although there are many reports on genetic transformation through callus in rice cultivars in both the *japonica* and indica subspecies, callus induction was done under light conditions (Ozawa and Kawahigashi, 2006; Ali et al., 2007; Nishimura et al., 2007), and dark conditions (He et al., 2006; Luo et al., 2006; Rao and Rao, 2007; Hiei and Komari, 2008). However, there is still a lack of knowledge about effects of light on the induction of friable-embryogenic callus. Our results clearly show that large, friable calli, with no brown coloration were induced most significantly under dark conditions.

There are also a few reports describing *Agrobacterium*-mediated transformation using matured indica seeds, but successes were limited to very specific varieties. Most indica rice varieties show a low rate of callus growth when using matured seeds on MS medium (Nishimura et al., 2007). In order to induce the optimum embryogenic callus for transformation, they were induced from the matured PT1 seeds by varying the induction time from zero, one, two or three weeks (age of calli). They were infected by a virulent *Agrobacterium* strain EHA105 harbouring pCAMBIA1301 vector. *Agrobacterium* was grown in YEP medium containing 100 mg/l Kanamycin for 16 h or until the optical density at 600 nm was about 1.0. The calli were immersed in the bacterial suspension for 30 min, and the infected calli transferred onto an MS co-cultivation medium (MS salts, B5 vitamin, 30 g/l sucrose, 100 mM acetosyringone, 2.5 g/l Phytagel [pH 5.7] without antibiotics), and overlaid with Whatman no. 1 filter paper (Kumar et al., 2005) for three days at 25±2°C and a 16 h photoperiod of 100±5 µmol/m^2/s PPF. The GUS activity of the infected calli was determined by the standard methods of Clark (1997). Transformation efficiency was calculated from the proportion of GUS positive calli to the total number of rice calli. The transformation efficiency of the PT1 calli at two (85%) and three (100%) weeks old was significantly higher than those at zero and one week old (Figure 3). GUS positive calli are shown in Figure 4.

Optimization method for *Agrobacterium* mediated transformation

There are many reports describing *Agrobacterium*-mediated transformation using varying concentrations of *Agrobacterium*, from 0.5 to 1.5 OD_{600} (Ali et al., 2007; Nishimura et al., 2007; Hiei and Komari, 2008; Lin et al., 2009). The optimum concentration of *Agrobacterium* was determined by co-cultivation of the PT1 calli with different concentrations (0.6, 0.8, 1.0 or 1.2 OD_{600}) of *Agrobacterium* for three days. Transformation efficiency was determined by measuring the number of GUS positive calli per total calli (Clark, 1997). The transformation efficiencies of the PT1 calli at 0.8 and 1.0 OD_{600} were significantly higher than those at 1.2 and 0.6 OD_{600}, respectively (Figure 5).

The optimum time for callus infection was determined by the co-cultivation of PT1 calli with *Agrobacterium* strain EHA105 harbouring pCAMBIA1301 plasmid, for two, three, four, five or six days at 25±2°C and a 16 h photoperiod of 100±5 µmol/m^2/s PPF. Transformation efficiency was determined by GUS positive calli per total

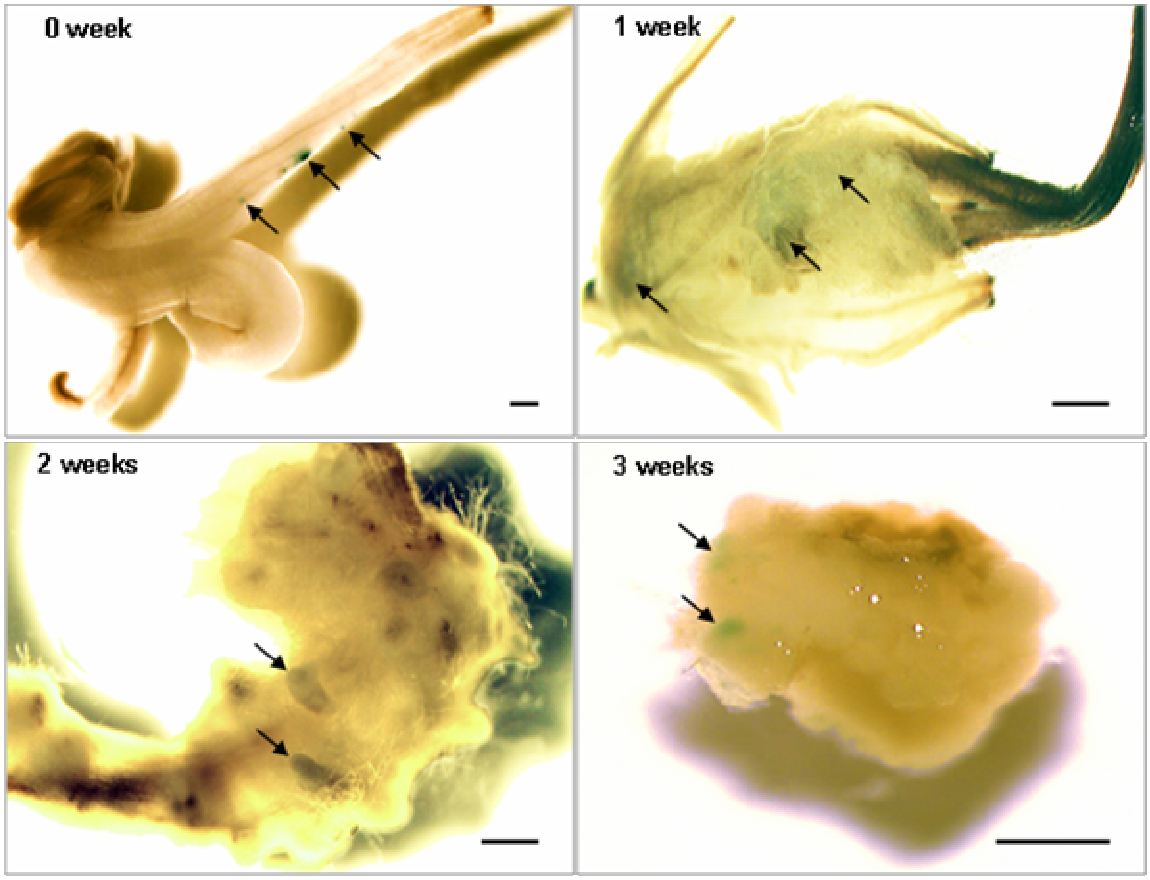

Figure 4. GUS expression of Pathumthani 1 (PT1) calli at 0, 1, 2 or 3 weeks old. Arrows indicate the blue spot of the GUS positive signal. Bar = 1 mm.

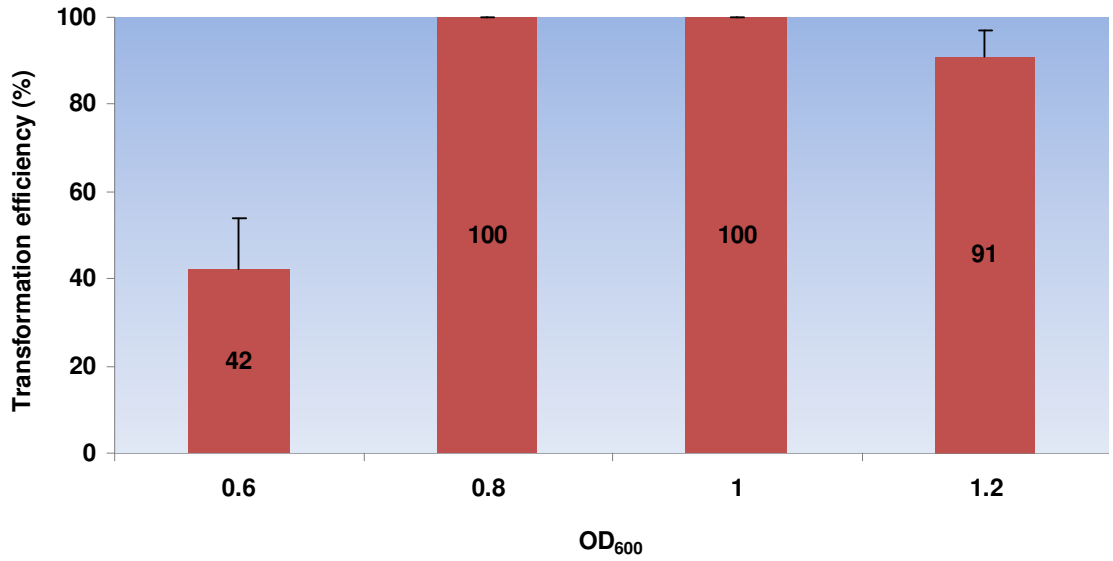

Figure 5. Transformation efficiency (%) of the 3 week old calli of Pathumthani 1 (PT1) co-cultivated with *Agrobacterium* strain EHA105 harbouring pCAMBIA1301 plasmid at 0.6, 0.8, 1.0, or 1.2 OD_{600} determined by GUS expression. The transformation efficiency was calculated by the number of GUS positive calli divided by the total number of calli.

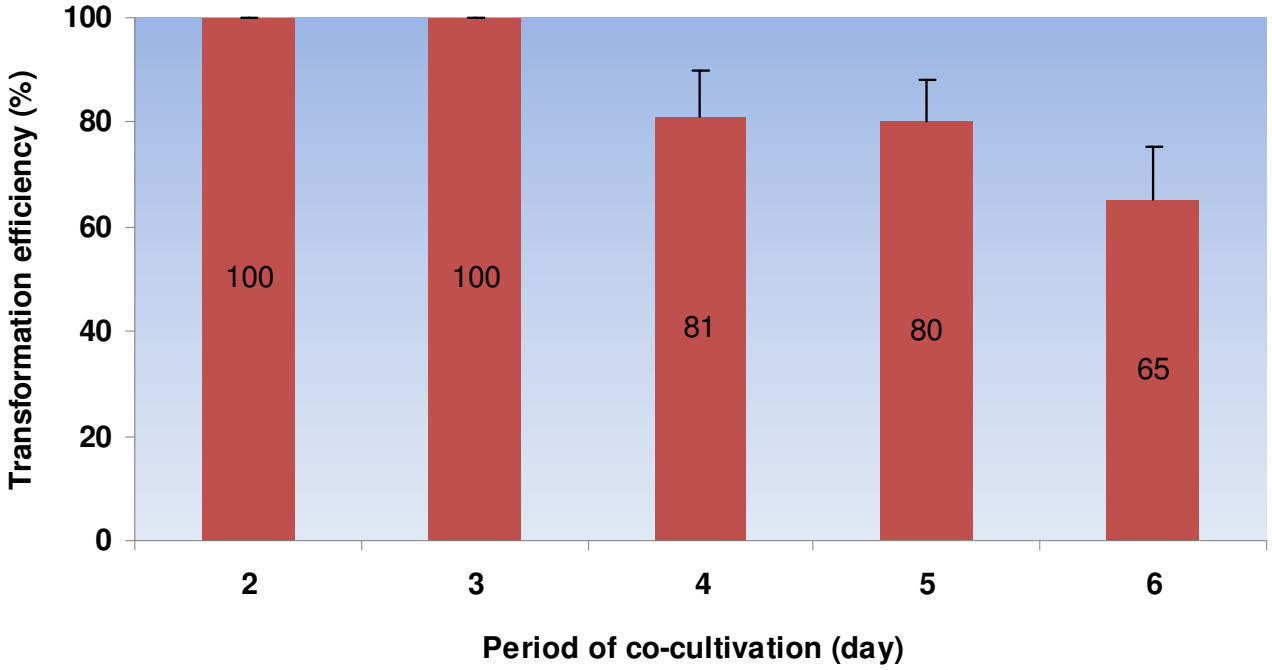

Figure 6. Transformation efficiency (%) of 3 week old Pathumthani 1 (PT1) calli co-cultivated with *Agrobacterium* strain EHA105 harbouring pCAMBIA1301 plasmid for 2, 3, 4, 5 or 6 days as determined by GUS expression. The transformation efficiency was calculated by the number of GUS positive calli divided by the total number of calli.

calli (Clark, 1997). The transformation efficiency of the PT1 calli co-cultivated for two and three days was significantly higher than that of calli infected at four, five and six days (Figure 6). The optimum time for infection corresponds closely with several reports in indica rice varieties such as Kasalath, IR8 and IR72 (Nishimura et al., 2007; Hiei and Komari, 2008), KDML105 (Yookongkaew et al., 2007) and Pusa Basmati1 (Rao and Rao, 2007). The results of our experiment clearly show that both the growth and character of PT1 and PKL calli were significantly affected by light and media factors. A modified MS medium supplemented with 0.5 mg/l picloram and 1.0 mg/l 2,4-D was the optimal medium for callus induction under light conditions in the PT1 rice cultivar, but under dark conditions in the PKL rice cultivar. Optimized transformation conditions were established using three week old calli, co-cultivated with 0.8-1 OD_{600} *Agrobacterium* for 30 min with the culture being continued on agar medium without antibiotics for two days.

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