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RESEARCH ARTICLE

Transformation of Antisense *Chalcone Synthase* (*CHS*) Gene into Lotus (*Nelumbo Nucifera* Gaertn.) by Particle Bombardment

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Abstract: *Chalcone synthase* (*CHS*) is a key enzyme in the flavonoid biosynthesis pathway. *CHS* genes were cloned from genomic DNA and cDNA from the petals of '*Buntharik*' white lotus and '*Sattabangkacha*' pink lotus by the PCR technique using a specific primer of the *CHS* gene designed from the GenBank database. Semi-quantitative RT-PCR analysis revealed that the highest *CHS* gene expression was found in the early budding stage of the pink lotus and was reduced in later stages. Shoot tips from embryos of Buntharik and Rachinee lotus were used to induce shoot clusters by cultivation on a MS medium supplemented with 40 µM NAA and 0.5 µM TDZ for 8 weeks and a MS medium supplemented with 50 µM BA for 8 weeks. An antisense *CHS* gene (450 bp) from the cDNA of Buntharik lotus was used to construct a plant transformation vector; pCAMBIA1302CHSA. The vector construct was transformed into Buntharik and Rachinee shoot clusters by particle bombardment. After transformant selection and regeneration, two transformants of Buntharik shoot clusters showed *GFP* green spots and existence of the *GFP* gene and *hpt*II gene in the genomic DNA amplified by the PCR technique. In the Rachinee transformants, 3 of 5 showed the *GFP* green spots and the *GFP* and *hpt*II genes were identified in amplification by PCR. After *CHS* gene expression analyses by semi-quantitative RT-PCR, two transformed Rachinee shoot clusters had a reduction in *CHS* gene expression.

Keywords: Nelumbo, Gene Transfer, Particle Bombardment, *CHS* genes, Transformation, Lotus.

INTRODUCTION

Lotus (*Nelumbo nucifera* Gaertn.) is an aquatic plant of ornamental importance in Thailand. Its flowers are closely associated with Buddhism, in which it is used as a religious symbol and for decorative purposes. There are only four commercial varieties of lotus in Thailand and even though some variants have been produced through mutation induction using X-rays, gamma rays, and chemical mutagens, this approach has been unable to target specific characteristics [1]. In particular, we are interested in obtaining a variation in the flower color, which is limited to white and pink in Thai varieties.

The development of an embrygenesis protocol for lotus has been reported in bud explant culture with the bset results obtained on MS medium containing 4 µM 2,4-D and 1 µM BA. In this case, when calli were transferred to MS medium with 2 µM 2,4-D and 0.5 µM BA, somatic embryos were produced [2] and an apical bud from an embryo formed an embryogenic callus when cultured on MS medium supplemented with 40 µM NAA and 0.5 µM TDZ for 8 weeks. The highest number of shoots was achieved in a medium supplemented with 50 µM BA after 8 weeks [1].

Flower pigmentation is caused by the accumulation of pigments within the epidermal cells, including flavonoids, carotenoids and betacyanins [3]. The chalcone synthase (*CHS*) gene is required for biosynthesis of anthocyanin pigments that give color to various plant tissues, such as the flower and seed coat [4]. It is the key enzyme in flavonoid biosynthesis and catalyzes the condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-coA

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to form naringenin chalcone, which is the essential intermediate in biosynthesis of flavonols, flavones, isoflavonoids and anthocyanins [5]. The manipulation of this enzyme opens many possibilities for metabolic engineering of this pathway, which may be of value in the generation of some useful variations in the lotus plant [6].

Most previous plant transformation systems have used a herbicide or an antibiotic as a resistance selectable marker. However, visual markers for selection have also been used as these can increase transformation efficiency by reducing the time and the number of materials used at the screening step [7, 8]. Transformants expressing reporter genes have been generated for variety of studies. The histochemical *GUS* (β-glucuronidase) assay is widely used but is destructive for tissue and therefore not suitable for direct visual selection of transformed plants. The green fluorescent protein (*GFP*) from the jellyfish was reported to function as sensitive reporter. This gene emits bright fluorescence upon excitation with ultraviolet light. This gene was evaluated as a screening marker during cotton transforming and plant regeneration and was found to be very effective [9] The formation of the fluorescent chromophore requires no exogenous substrates or cofactors and is easily detected [10].

The aim of this study was to establish the *CHS* gene expression in lotus flowers (cv. Buntharik and cv. Sattabangkacha) at five developmental stages and to analyse the suitability a method for transformation of the antisense *CHS* gene into these cultivars through particle bombardment. Visual selection of *GFP* expression was used to identify genetic modification.

MATERIALS AND METHODS

CHS Gene Expression in Lotus

Petals were collected from five developmental stages of lotus flowers cv. Buntharik and cv. Sattabangkacha: stage 1 - flower diameter was less than 3 cm (B1, S1), stage 2 - flower diameter 3-5 cm. (B2, S2), stage 3 - flower diameter 5-7 cm. (B3, S3), stage 4 - flower diameter 7-10 cm. (B4, S4) and stage 5 - open flower (B5, S5). Total RNA was extracted using an Invitrop spin RNA mini kit (Stratec Molecular, Germany) for each of the five petal stages. First-strand cDNA synthesis was performed using SuperScript™ III First–a strand Synthesis System for RT-PCR (Invitrogen) with an oligo(dt) according to manufacturer's instructions. Expression of *CHS* genes was determined by amplification of a 458 bp using the following primers5'AAGAGCTCCCGTCAAGAGACTCA3' and 5'AAGGATCCCAGAAAATTGAG TTC3'. The specific primer of CHS gene was designed from the GenBank (accession no. FJ999632) [11]. The PCR condition for the amplification of the *CHS* gene included 94°C for 5 min, 35 cycles of denaturation (94°C, 45 sec), annealing (58°C, 45 sec) and extension (72°C, 45 sec), and a final extension at 72°C for 10 min. The PCR products were electrophoresised on a 1% TAE agarose gel to allow visible amplification of gene on ethidium bromide.

Explants and Plant Regeneration

Seeds of lotus (*N. nucifera* Gaertn.) cv. Buntharik and cv. Rachinee were washed thoroughly under running water for 60 min, rinsed in 70% ethanol for 1 min, surface sterilized in 3% (v/v) NaOCl (50% Clorox plus two drops of Tween 20) for 20 min and rinsed three times in sterile distilled water [1]. Callus was initiated by culturing apical buds from embryos on Murashige and Skoog (MS) [12] medium containing 40 μM NAA and 0.5 μM TDZ for 8 weeks and callus was cultured on MS medium supplemented with 50 μM BA for 12 weeks to induce shoot clusters.

Plant and Plasmid Transformation

Shoot clusters were transferred to an osmotic medium (MS medium containing 2 M mannital and 2 sorbital) and subsequently transferred to Petri dishes (9 cm) and placed in the centre of a sterile, round Whatman filter paper (2.5 cm diameter). The plasmid pCAMBIA1302CHSA (Fig. **1**) was used in this experiment. This plasmid contains the *CHS* gene cloned from lotus petals, with a selectable hygromycin phosphotransferase II (*HPT*II) gene encoding resistance to hygromycin and a green fluorescent protein (*GFP*) gene as a reporter gene. The shoot clusters were bombarded with plasmid DNA-coated gold particles 1100 psi at 9 cm target distances. Four hours after bombardment, the shoot clusters were transferred from the osmotic medium to a regeneration medium (MS medium with 50 μM BA) containing 15 mg/l hygromycin for 8 weeks and subcultured every two weeks to fresh medium containing the selection agent.

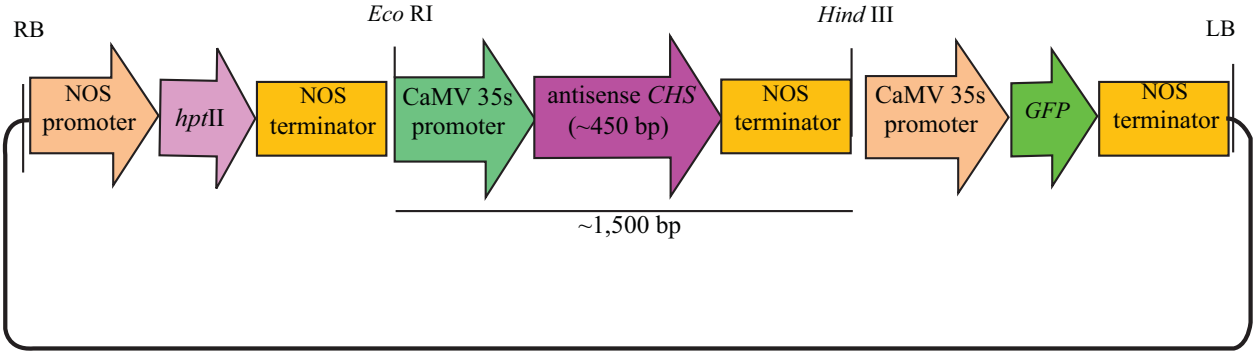

Fig. (1). pCAMBIA1302CHSA plasmid containing *anti-CHS*, *hpt*II and *GFP* gene.

Fluorescence Microscopy

Green fluorescence protein expression in shoot clusters of lotus was visualized under a fluorescence stereo microscope (Olympus, SZX12, USA) equipped with a 100 W mercury bulb light source and FITC/GFP excitation filter set with a 480/530 nm.

Analysis of Putative Transformed Plant

Shoot clusters of a lotus culture on MS medium with 50 µM BA and 15 mg/l hygromycin were putatively considered transformed. Genomic DNA was extracted from transformed and untransformed leaf tissue by using the CTAB method [13]. PCR analysis for detection of the *CHS* gene was done for a 458 bp fragment with primers: forward 5'AAGACTCCCGTCAAGAGACTCA3'; reverse 5'AAGGATCCCAGAAAATTGATTC3', the *HPT*II gene was examined for a 300 bp fragment with the primer: forward 5'ATTGACCGATTCCTTGCGGT3'; reward 5'GAGG-GCGTGGATATGTCCTG3' and the *GFP* gene was on a 400 bp fragment with the primer: forward 5'GGAGA-GGGTGAAGGTGATGC3'; reword 5'TGCCGTTCTTTTGCTTGTCG3'. The PCR reaction for amplification of the *CHS*, *HPT*II and *GFP* gene was done at 94°C for 5 min, 35 cycles of denaturation (94°C, 45 sec), annealing for *CHS* and *HPT*II (58°C, 45 sec); *GFP* (62°C, 45 sec) and extension (72°C, 45 sec), and a final extension at 72°C for 10 min. PCR products were electrophoresised on 1% TAE agarose gel and as before visible amplification of gene on ethidium bromide.

RESULTS AND DISCUSSION

Gene Expression for Each Stage of Flower Development

CHS gene expression at different stages of lotus flower development was compared by using semi-quantitative RT-PCR. 18S rRNA was used as an equal-amount control of a cDNA template for PCR reactions of the *CHS* gene. Flower development at different stages of petal flower of lotus cv. Buntharik (B) and cv. Sattabangkacha (S) were examined. The results show that expression of the *CHS* gene in petals of lotus cv. Sattabangkacha was higher than for lotus cv. Buntharik. The *CHS* gene in the petals of cv. Sattabangkacha was highly expressed at stages 1 to 3 and had lower expression in stages 4-5. In cv Buntharik, however, the *CHS* gene was expressed at the same level in each stage. The petal color of lotus cv. Buntharik was white, while the petals of lotus cv. Sattabangkacha had a pink color at early stages and changed to white at stage 5 (Fig. **2**). The expression of genes at each floral development stage can be used to predict the levels of protein and enzymes being translated by that gene. RNA was isolated from petals, with Buntharik having a white petal and Sattabangkacha having a pink petal lotus color. The pink color of Sattabangkacha was a dark pink during early flowering stages and changed to white in the last flowering stage. The results are similar to what has been reported for peony (*Paeonia lactiflora*) where the mechanism controlling flavonoid biosynthesis in different organs and different floral developmental stages was detected by expression levels by quantitative-PCR. For this species, nine genes (including the CHS gene) were investigated and their expression varied across the three cultivars examined [14]. Similarly, the expression pattern of *CHS* and *CHI* (the enzyme that catalyzes the early steps of flavonoid biosynthesis) in petals of *Gentiana triflora* cv. Maciry was high from stages 1 to 4 of flower development [15].

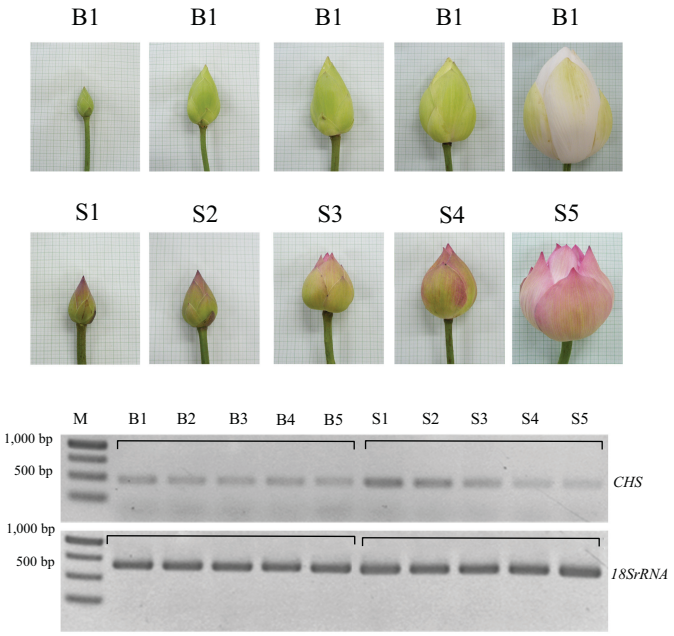

Fig. (2). Comparison of *CHS* gene expression in each stage of flower development by Semi-quantitative RT-PCR and *18S rRNA* gene is control. Lotus cv. Buntharik (B) and cv. Sattabangkacha (S), flower stage 1 diameter of flower lower 3 cm (B1, S1), stage 2 diameter of flower 3-5 cm (B2, S2), stage 3 diameter of flower 5-7 cm (B3, S3), stage 4 diameter of flower 7-10 cm (B4, S4) and stage 5 open flower (B5, S5).

Shoot Cluster Induction

When apical buds from embryos were cultured on MS medium containing 40 µM NAA and 0.5 µM TDZ, there was callus growth and good formation of embryogenic callus after 8 weeks. Shoot clusters were generated from the embryogenic callus when cultured on MS medium with 50 µM BA after 8 weeks (Fig. **3**). The shoot clusters from these cultures were used as the target tissue for transformation. Apical buds from embryos cultured on medium containing 40 µM NAA and 0.5 µM produced more embryogenic callus. Shoots were successfully produced from embryogenic callus explants using MS medium containing 50 µM of BA [1]. This result confirms earlier observations in other species (*e.g. Zantedeschia aethiopica*) [16].

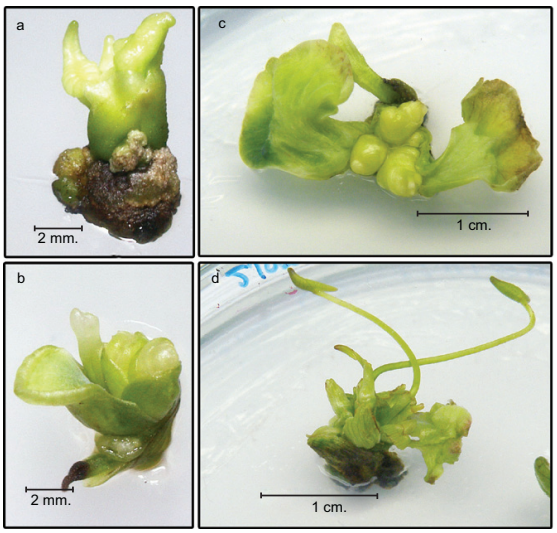

Fig. (3). Regenerating culture of *N. nucifera* Gaertn. from apical bud of embryo after 8 weeks of culture on MS medium with 40 µM NAA and 0.5 µM TDZ: (**a**) lotus cv. Buntharik; (**b**) lotus cv. Rachinee and change to culture on MS medium with 50 µM NAA until 16 weeks; (**c**) cv. Buntharik; (**d**) cv. Rachinee.

GFP-Fluorescence in Transgenic Lotus

The *GFP* gene as a reporter system was visually detected in organs and transient expression rate could be detected on shoot clusters after one week of culture. Transformed shoot clusters showed transient *GFP* activity in the shoot area (Fig. **4** arrow). The number of shoot clusters showing transient expression in lotus cv. Buntharik and cv. Rachinee were similar (72 and 75 pieces, respectively; Table **1**). The *GFP* gene was also reported as a reporter gene in transgenic *Phytothora palmivora* and provided better visualization and was superior to *GUS* [17]. *GFP* can partially replace antibiotic selection and is particularly important when organogenesis or conversion of transformation procedures is inefficient under antibiotic or herbicide selection. It is also helpful in isolating events during the early stages of transformation [18].

Fig. (4). Expression of *GFP* gene in shoot clusters lotus cv. Buntharik after transfromation 1 week (arrow). (**A, B**) non-transgenic shoot cluster. (**C-F**) transgenic shoot cluster. The upper photos use a fluorescent light and the lower photos use UV irrasiatin (7x). Shoot clusters lotus cv. Rachinee (**G, H**) non-transgenic shoot cluster. (**I-L**) transgenic shoot cluster.

Table 1. Efficiency of transformation anti-CHS gene by particle bombardment to shoot cluster of lotus cv. Buntharik and cv. Rachinee.

	Buntharik	**Rachinee**
Number of shoot clusters	100	100
Number of shoot cluster found *GFP*	72	75
Score of *GFP* gene expression (1-5)	0.97 ± 0.20	0.99 ± 0.17
Percentage of number survived shoot clusters	56.00 ± 13.87	61.00 ± 18.51
Number of shoot regeneration	2	5
Number of *GFP* gene expression	2	3

Genetic Transformation of Shoot Clusters

Shoot clusters were transformed with pCAMBIA1302CHSA plasmid containing anti-*CHS*, *GFP* and *HPT*II genes. Shoot clusters were selected on selection medium. Most shoot clusters become brownish and this was sufficient to kill the untransformed shoot cluster. The number of hygromycin-resistant shoot clusters was recovered from 10-week-old shoot clusters bombarded with the plasmid. When shoot clusters were cultured on MS medium containing 50 µM BA

and 15 mg/l hygromycin for 10 weeks, the percentage of surviving shoot clusters was 56.00% (Buntharik) and 61.00% (Rachinee). Subsequently, when transferred shoot clusters were cultured on MS medium containing 50 μM without hygromycin found shoots were regenerated on medium cv. Buntharik, which had only 2 shoots and cv. Rachinee, which had 5 shoots (Table **1**).

Putative transformants were analyzed by PCR with a specific primer of *CHS*, *HPT*II and *GFP* gene. In lotus cv. Buntharik, there were three genes from two parts, the leaf and petiole. A 450 bp fragment was identified as a *CHS* gene, a 300 bp. fragment was identified as a *HPT*II gene, and a 400 bp. fragment was identified as a *GFP* gene (Fig. **5**). The *CHS* gene was found in all samples. Because this primer was amplify *CHS* endogenous gene or transgene. The *GFP* gene was found in three samples from five respectively (Table **1** and Fig. **6**).

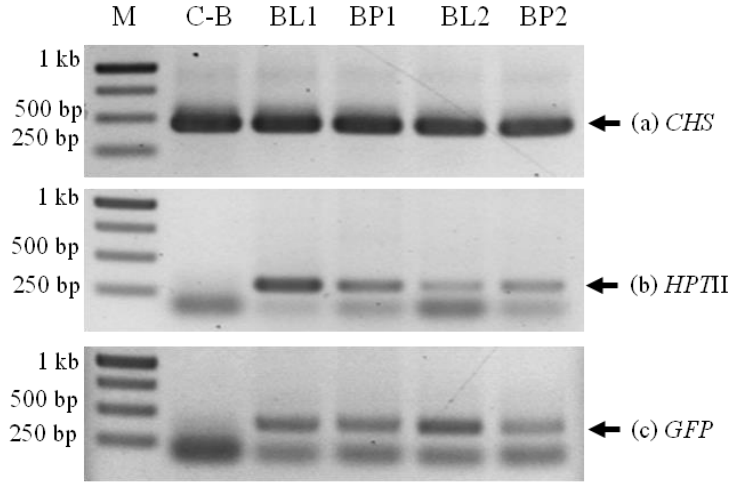

Fig. (5). PCR analysis of lotus cv. Buntharik transgenic (**a**) *CHS* gene (**b**) *HPT*II gene and (**c**) *GFP* gene. (C-B) non transgenic lotus, (BL1) leaf of transgenic, (BP1) petiole of transgenic no. 1, (BL2) leaf of transgenic no. 2, and (B5/12S) petiole of transgenic no. 2. M = 1 kb ladder.

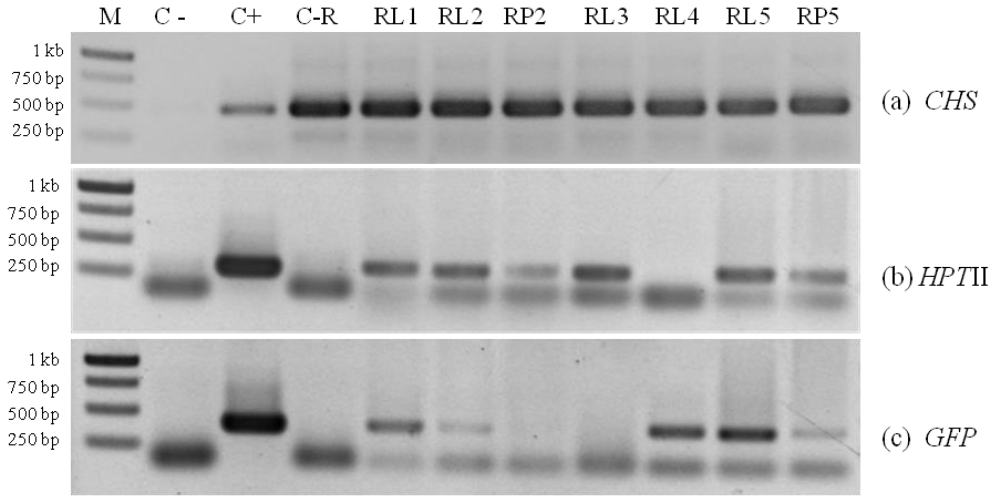

Fig. (6). PCR analysis of lotus cv. Rachinee transgenic (**a**) *CHS* gene (**b**) *HPT*II gene and (**c**) *GFP* gene. (C-R) leaf of non-transgenic, (RL1 leaf of transgenic no. 1, (RL2) leaf of transgenic no. 2, (RP2) petiole of transgenic no. 2, (RL3) leaf of transgenic no.3, (RL4) leaf of transgenic no. 4, (RL5) leaf of transgenic no. 5 and (RP5) petiole of transgenic no. 5., C dH_2O, C+ pCAMBIA1302CHSA plasmid and M = 1 kb ladder.

To confirm expression, semi-quantitative RT-PCR was done using a *CHS* gene and *18S rRNA* as control. *CHS* gene expression could be found in some samples, with decreased *CHS* gene expression in lotus cv. Buntharik and Rachinee (Fig. **7**). However, it has reported *CHS* gene was expressed in various tissues of *N. nucifera*, with the highest expression in red flower and lowest level in the leaves [19].

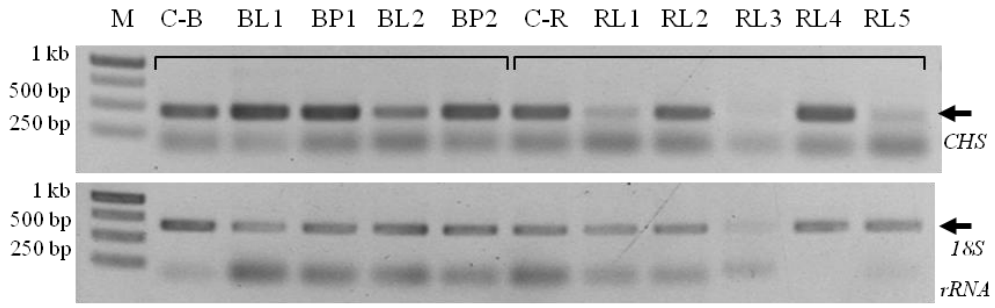

Fig. (7). Expression of *CHS* (458 bp) in transgenic lotus by semi-quantitative RT-PCR using *18S rRNA* primer (600 bp); leaf of non-trasgenic cv. Buntharik (C-B), leaf of transgenic (BL1) and petiole (BP1), leaf (BL2) and petiole of transgenic (BP2), leaf of non-trasgenic cv. Rachinee (C-R)), leaf of transgenic cv. Rachinee 1-5 (RL1-5) M = 1 kb DNA marker.

In this paper, we found a high *GFP* transient expression rate The *GFP* gene was found in three samples from five respectively one-week after bombardment. Shoot clusters were selected by hygromycin. Shoots could be regenerated for only 2 and 5 shoots in Buntharik and Rachinee, respectively. Transient *GFP* expression was used to evaluate factors affecting transformation efficiency. With *GFP* as a reporter marker, the growth and proliferation of an individual transgenic event could be readily tracked visually without disturbing the tissue in any way [10]. Different numbers of PCR-positive explants were achieved in the shoots of the transgenic plants. Some samples exhibited negative results for some of the genes. Some of the putative transformations were non-transgenic. Some explants also mostly likely escaped the selective medium, as observed by Kuvshinov *et al*. [20] and Saetiew *et al*. [21].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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