



***Agrobacterium*-mediated Genetic Transformation of *Phalaenopsis bellina* Using GFP and GUS Reporter Genes**

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ABSTRACT

Genetic transformation protocols of *Phalaenopsis bellina* protocorm-like bodies (PLBs) were established using *gfp* (green fluorescent protein) and *gus* (β -glucuronidase) genes as the reporter system. *Agrobacterium tumefaciens* strain, LBA 4404 containing the binary vector, pCAMBIA 1304, with the *hptII* gene as the selectable marker and *gfp* and *gus*-intron genes as the reporter genes. Horizontally dissected PLBs were immersed in *A. tumefaciens* suspensions with 200 μ M acetosyringone (AS) for 45 minutes. This was followed by co-cultivation until the growth of *A. tumefaciens* was observed surrounding the PLBs on the co-cultivation medium. GFP detection and GUS histochemical assay were carried out to investigate the transient expression of both GFP and GUS reporter genes. The selection of proliferating PLBs was carried out on 4 mg/L hygromycin and 100 mg/L cefotaxime. GFP could be used as the reporter system as it is an effective, rapid and non-destructive system to monitor the transformed tissues.

Keywords: *Agrobacterium tumefaciens*, genetic transformation, *gfp*, *gus*, *Phalaenopsis bellina*

INTRODUCTION

Phalaenopsis bellina (Fig. 1) is a commercially important fragrant orchid species endemic to Borneo and Peninsular Malaysia. The attractive features of this orchid are that they flower freely all year round and produce strong sweet-flora fragrance. Genetic improvement of *P. bellina* through sexual hybridization is, however, restricted by a long growth period and limited genetic pool within the germplasm. Genetic engineering offers a promising approach in improving the orchid quality.

A protocol was developed to obtain transient expression of *P. bellina* via *Agrobacterium tumefaciens* (Strain LBA 4404 harboring vector pCAMBIA 1304). Prior to the genetic transformation study, hygromycin sensitivity of the PLBs was investigated to determine the minimal concentration required to sufficiently inhibit the growth of PLBs. Transient expression of *gfp* gene in PLBs was observed under the fluorescence microscope after co-cultivation period. The effect of inoculation time on transient GFP expression was also evaluated in this study. The putative transformants displayed distinguishingly strong fluorescence when observed under a GFP stereomicroscope using GFP2 filter.

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Fig. 1: The *P. bellina* flower

MATERIALS AND METHODS

Plant Materials

Induction and Establishment of PLBs

PLBs were induced using young leaf segments of approximately 1 x 1 cm², excised from three-month old *in vitro* seedlings. The leaf segments were placed adaxial side up in orientation inside the culture tubes containing 1/2 strength MS medium (Murashige and Skoog) supplemented with 100 mg/L myo-inositol, 0.5 mg/L niacin, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, and 2.0 mg/L glycine (Tokura and Mii, 2003 and Cheng and Chang, 2004). Twenty g/L sucrose and 3 g/L gelrite was adjusted to pH 5.6 (Islam *et al.*, 2003) before autoclaving for 15 min at 121°C. Different auxins at different concentrations were investigated. The auxins used were 2,4-D, NAA, picloram and dicamba and the range of concentrations was 0.2, 0.4, 0.6, 0.8 and 1.0 µM. The number of explants forming PLBs was recorded after 12 weeks of culture. In addition, the size and quantity of the PLBs induced were also observed. The proliferated masses of PLBs were subcultured every four weeks for six months to obtain large quantities of PLBs for *Agrobacterium*-mediated transformation.

Determination of Minimal Inhibitory Concentration of Hygromycin

Single PLBs of 3 - 4 mm in size (measured from shoot tip to root tip) were aseptically excised using scalpel and subjected to different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, and 35 mg/L) of hygromycin treatments to determine their minimal hygromycin killing level. Hygromycin was added into the selection medium consisting 1/2 strength MS basal medium. The hygromycin-free 1/2 strength MS medium served as a control medium. Observations were conducted on a weekly basis and the percentages of surviving PLBs per replicate were recorded. Changes in the physical appearance of the cultured PLBs, from healthy greenish to black, whitish or brownish colour, were used as indicators for scoring the PLBs. Meanwhile unaffected PLBs should remain green and continue to proliferate. Each treatment consisted of three replications with 16 PLBs per replicate, culturing on 20 ml of solidified medium in a 50 mm diameter petri dish. This

experiment was repeated three times. Eventually, the specific concentration that was sufficient to completely inhibit growth of PLBs or kill the PLBs would be determined and later applied for screening of putative transformed PLBs in the genetic transformation study.

Preparation of *A. tumefaciens* Strain and Plasmid

A. tumefaciens strain LBA 4404 (pCambia 1304) was used for the transformation study. The T-DNA region of the binary vector pCambia 1304 contains the selectable marker *hptII* gene, encoding hygromycin phosphotransferase, the reporter *gfp*, and intron-*gus* genes. The β -glucuronidase gene was disrupted by an intron. This intron-*gus* reporter gene expresses GUS activity in plant cells but not in the cell of *A. tumefaciens*. Expression of *gfp*, *gus* and *hptII* genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

The Effect of Inoculation Time

The *A. tumefaciens* strain LBA 4404 was grown overnight at 28°C in liquid LB broth medium containing 100 mg/L kanamycin. The following day, 500 μ l of the bacterial suspension was spread over the surface of LB agar solid medium, and incubated at 28°C for 2 days. *A. tumefaciens* cells were collected with a aseptic flame inoculums and suspended in 30 ml LB liquid medium containing 100 mg/L kanamycin and 200 μ M AS to and OD600 at 0.7 - 1.0 and agitated (100 rpm) in a shaker, at 25°C for 30 min before inoculating. PLBs of *P. bellina* were cut into pieces, 3-4 mm in diameter. The PLBs were then immersed in the *A. tumefaciens* suspension for 15, 30, 45, 60, 75 and 90 min respectively, with 20 PLBs per treatment. These PLBs were blotted dry on sterile filter paper, and co-cultured on 1/2 MS medium containing 200 μ M AS at 25°C in the dark for 3 days until *A. tumefaciens* growth was observed. This experiment was repeated 3 times.

GFP Monitoring

GFP-expressing cells were detected using a fluorescence microscope (Leica MZFL III) equipped with GFP2 filter (Excitation filter: 480/40 nm) to mask the red fluorescence of chlorophyll, thereby permitting the visualization of the green fluorescence GFP-expressing cells. The PLBs observed with green fluorescent sections (using magnification 25X) were considered GFP positive. An imaging system (Laica DC 200) was attached to the fluorescence microscope to capture the image in real time using the Leica DC Viewer software.

Selection of Putative Transformants

After 2-3 days of co-cultivation, the cultures were transferred to selective medium (1/2 MS medium containing 100 mg/L cefotaxime, 4 mg/L hygromycin) and incubated at 25°C under 16 photoperiod. Previous research indicated that 4 mg/L hygromycin was sufficient to inhibit the growth of non-transformed tissues of *P. bellina*. Tissues were subcultured in a new selective medium every month. Cefotaxime were omitted after two months of selection. After three months of being cultured on the hygromycin-containing medium, the surviving and growing cell clusters were picked out and cultured on 1/2 MS medium supplemented with 4 mg/L hygromycin but without phytohormones for plant regeneration.

GUS Histochemical Assay

GUS activity assays were performed on PLBs, hygromycin-resistant PLBs and then on leaves and roots of the regenerated transformants using the method of Jefferson et al., (1986). Tissues were immersed in X-gluc solution, (1 mM EDTA, 50 mM NaH₂PO₄ (pH 7.0), 10 mM β -mercaptoethanol and 0.1% Triton X-100) and incubated overnight at 37°C. After staining, the materials were treated with 70% ethanol to remove chlorophyll before observation. Transient GUS expression of PLBs was examined after 2 days of co-cultivation.

RESULTS AND DISCUSSION

Protocorm-like Bodies (PLBs) Induction

There has been limited information on the utilization of leaf segments as explant source for PLBs induction. The appearance of nodular masses protruding from the wounded surfaces and epidermal layers of leaf explants could be observed as early as eight weeks after culture. The production of PLBs was greatest at week twelve. The highest frequency of PLB formation was 53% in media containing 0.8 μ M 2,4-D (Figs. 2 and 3). This was followed by 0.6 μ M 2,4-D (37%), 1.0 μ M 2,4-D (23%), and 0.2 μ M 2,4-D (10%). The frequency of PLBs production is low (5 - 12%) in treatments containing 0.6 μ M NAA and 0.4 μ M NAA, respectively (Fig. 2). Moreover, the NAA induced PLBs were small in size, low in quantity and did not proliferate readily (Table 1). No PLBs were formed in other treatments particularly in those containing picloram and dicamba, although the explants remain green for many months of culture (Fig. 4). The use of leaf segments to induce PLBs has recently been reported by Park *et al.* (2002) in different *Phalaenopsis* hybrids and Chen and Chang (2004) in *Oncidium* Gower Ramsey.

TABLE 1
Effect of different auxins (2,4-D and NAA) on quantity and size of induced
P. bellina PLBs after 12 weeks of culture

PGR (auxin)	Concentration (μ M)	^a Quantity of induced PLBs Sizes (< 0.3 mm)	^a Quantity of induced PLBs Sizes (> 0.3 mm)
2,4-D	0.0	0 a	0 a
	0.2	9.3 \pm 1.5 a	4.3 \pm 2.5 a
	0.4	12.3 \pm 3.5 a,b	7.7 \pm 3.8 a,b
	0.6	39.3 \pm 11.0 c	21.0 \pm 3.0 c
	0.8	82.3 \pm 6.8 d	43.7 \pm 4.0 d
	1.0	27.3 \pm 6.7 c,d	13.7 \pm 3.2 b,c
NAA	0.0	0 a	0 a
	0.2	0 a	0 a
	0.4	9.7 \pm 4.7 b	0 a
	0.6	1.7 \pm 1.5 a	1.0 \pm 1.0 a
	0.8	0 a	0 a
	1.0	0 a	0 a

Different auxins (2,4-D and NAA) and auxin concentrations were supplemented to half strength MS basal medium as described in Section 3.3.2.

^aThe quantity of induced PLBs was calculated as the average quantity of three independent experiments and represent mean (\pm SD). Three replicates were used in each treatment.

The data were analysed by one-way ANOVA in a completely randomised design using auxin concentration as factor. Mean values were compared by Tukey's multiple range test at 5 % ($p = 0.05$) significance level

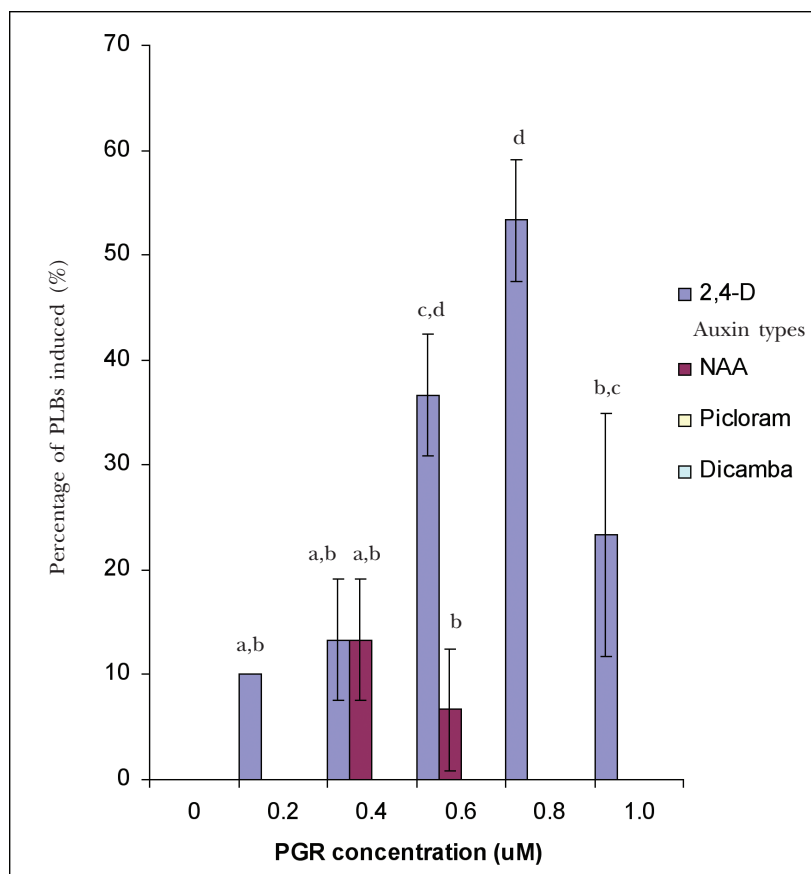


Fig. 2: Effect of different auxins concentrations on PLBs induction from leaf segments of *P. bellina*. All results were scored after 12 weeks of culture. The results indicate the mean standard error (\pm SE) of 3 independent experiments with 10 replicates for each treatment concentration. The experiment was repeated thrice. The data were analysed by one-way ANOVA in a completely randomised design using auxin concentration as factor. Mean values were compared by Tukey's multiple range test at 5 % ($p = 0.05$) significance level

Determination of Hygromycin Killing Curve

In this experiment, 16 two-month-old PLBs of *P. bellina* were placed on 1/2 strength MS basal medium containing various concentrations of hygromycin (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, and 35 mg/L) for five weeks, and the number of surviving PLBs was recorded weekly. Black, whitish or brownish colour was an indicator of dead PLBs. An early inhibitory effect of hygromycin at 10 mg/L on PLBs was initially observed during the first week of culture. Increased hygromycin concentration reduced the growth frequency from 100% at hygromycin 0 mg/L to 43% at hygromycin 1 mg/L, to 32% at hygromycin 2 mg/L, to 23% at hygromycin 3 mg/L and to 0% at hygromycin 4 - 35 mg/L (Fig. 5). All of the PLBs multiplied healthily and remained greenish on hygromycin-free medium, whereas, PLBs cultured on medium containing ≥ 4 mg/L hygromycin, for 5 weeks resulted in complete fatality, giving entirely black, brown or white necrotic PLBs

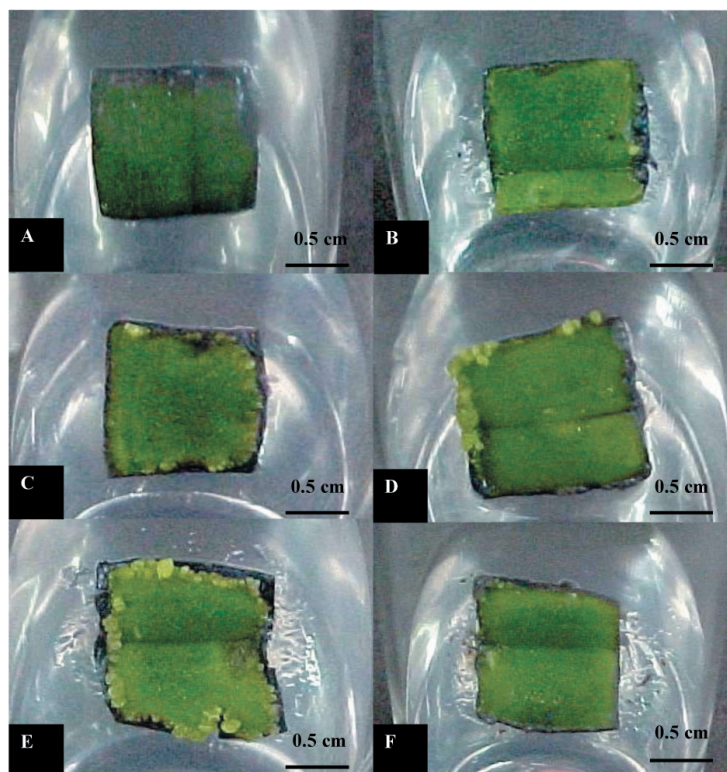


Fig. 3: Effect of different 2,4-D concentrations on *P. bellina* PLBs induction after 12 weeks of culture. A Control treatment B-F PLBs induction on 1/2 strength MS basal medium supplemented with 0.2, 0.4, 0.6, 0.8, and 1.0 μM 2,4-D respectively

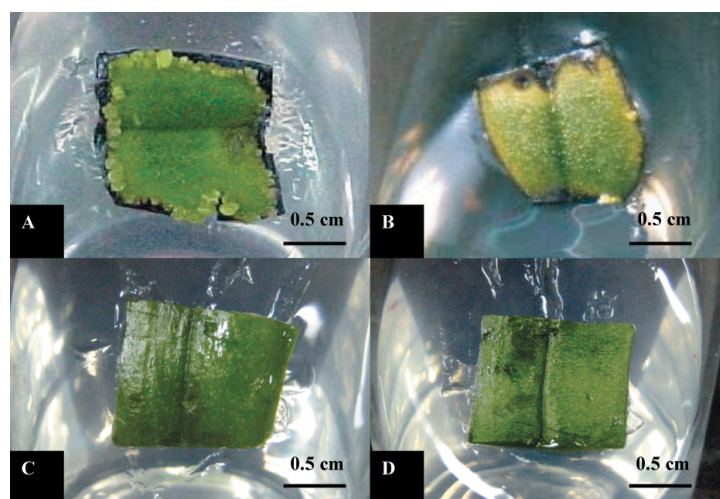


Fig. 4: Effect of different auxins on *P. bellina* PLBs induction after 12 weeks of culture. A PLBs induction on medium containing 2,4-D (0.8 μM) B PLBs induction on medium containing NAA (0.4 μM) C-D Leaf treated with picloram and dicamba respectively

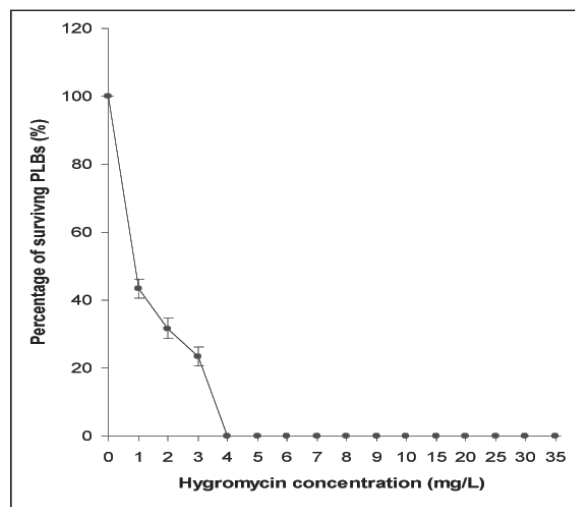


Fig. 5: The hygromycin killing curve for *P. bellina* PLBs. Data were recorded based on percentage of surviving PLBs after 5 weeks of culture in selection medium containing different concentrations of hygromycin (0 - 35 mg/L). Black, whitish or brownish colour was the indicator of the dead PLBs. Each point represents the mean \pm standard error (SE) for $n = 3$ replicates with 16 PLBs on each replicate. The experiment was repeated 3 times

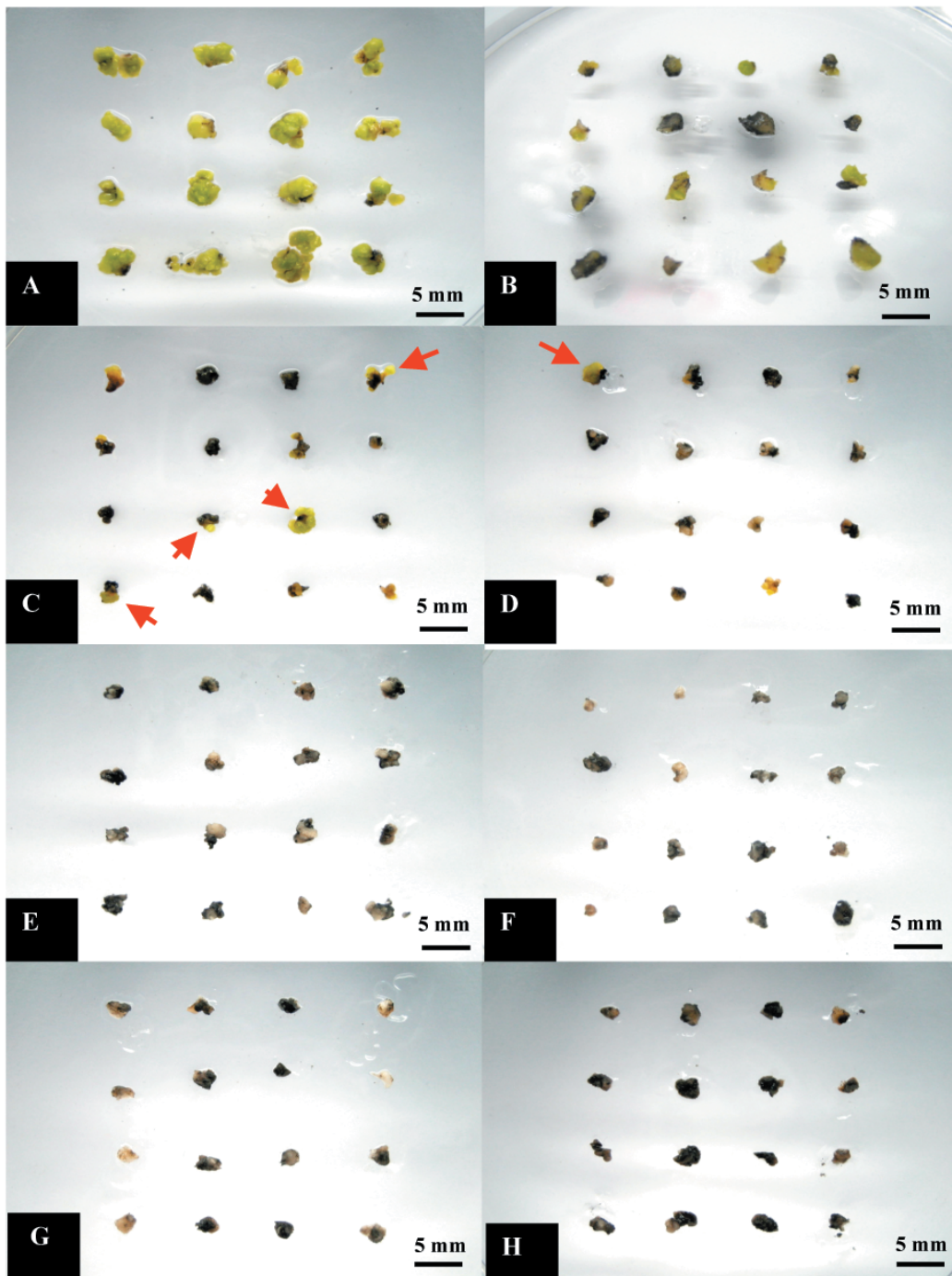
appearances (Fig. 6). It was clearly evident that *P. bellina* PLBs were very sensitive to hygromycin as most of the PLBs were killed at very low (4 mg/L) hygromycin concentration after five weeks. This experiment, therefore, revealed that 4 mg/L hygromycin was the lowest concentration required for *P. bellina* to discriminate between transformed and non-transformed PLBs. The hygromycin killing level observed from the present study was very similar to the findings reported by Chai *et al.* (2002), who also examined the hygromycin killing curves in four different *Phalaenopsis* lines, and found low hygromycin concentrations (1.5-3 mg/L) were suitable for putative transformed PLBs selection.

Effect of Inoculation Time

An inoculation period of 45 min to 90 min resulted in the highest percentage of transient expression compared to other inoculation periods (Table 2) as detected by the GFP reporter system (Fig. 7 a - d). The degree of transient expression is different with different hybrids (Tee *et al.*, 2004; Tee and Maziah, 2005). After eight weeks on selection medium, with hygromycin and cefotaxime antibiotics, green colour, hygromycin-resistant PLBs were observed. These hygromycin-resistant PLBs were then selected and subcultured in the cefotaxime-free medium. Putative transformed PLBs clumps were obtained after 2 months of culture.

Transient Expression Detection

Putative transformed PLBs can also be detected in GUS histochemical assay by showing GUS positive sections (sections that stained in blue colour) (Fig. 7 e and f). However,



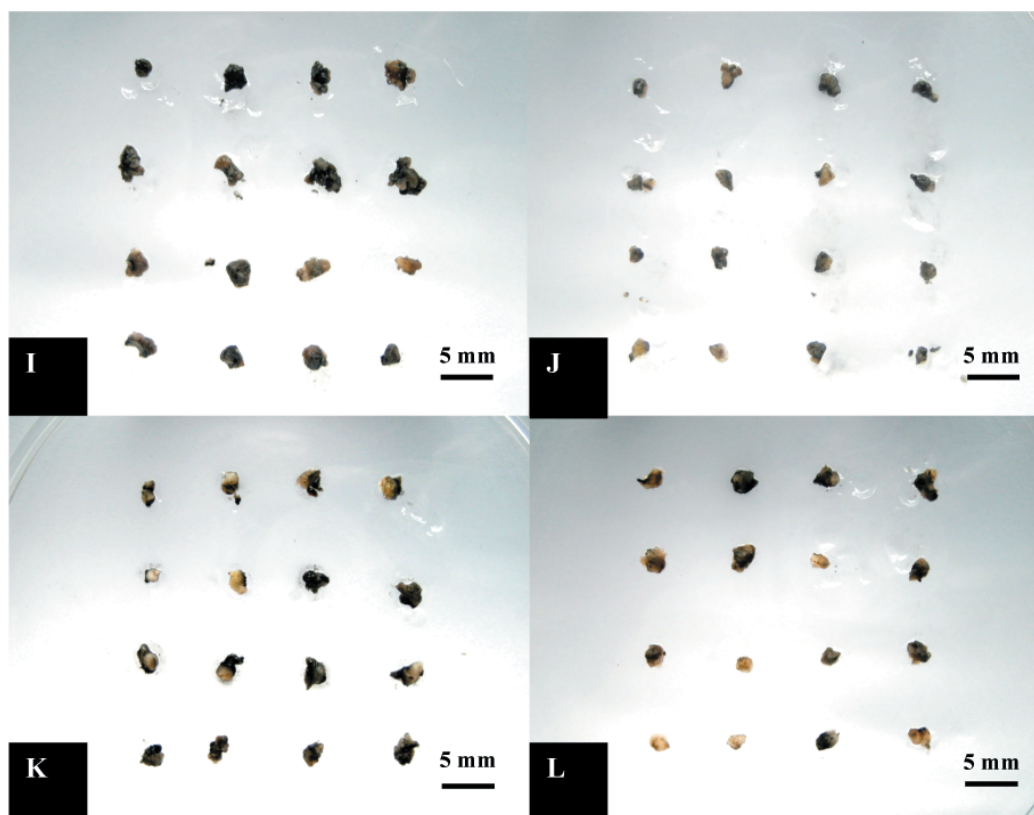


Fig. 6: Physical effects exhibited by PLBs after five weeks of culture on selection medium containing various concentrations of hygromycin. Black, whitish yellowish or brownish colour was the indicator of the dead PLBs. A All PLBs remained greenish and proliferating on hygromycin-free medium B 50% of the PLBs remained greenish and survived on media containing 1 mg/L hygromycin C-D Most of the PLBs were dead on media containing 2 mg/L and 3 mg/L hygromycin respectively E- L All PLBs were dead on medium containing 4, 5, 6, 7, 8, 9, 10 and 15 mg/L hygromycin respectively. Red arrows show the surviving PLBs cultured on hygromycin selection medium

TABLE 2
Effect of inoculation period on the percentage of GFP transient expression of *P. bellina*

Length of inoculation time (min)	Number of PLBs inoculated	Percentage of GFP transient expression (%)
15	40	2.8
30	40	2.8
45	40	5.6
60	40	5.6
75	40	5.6
90	40	5.6

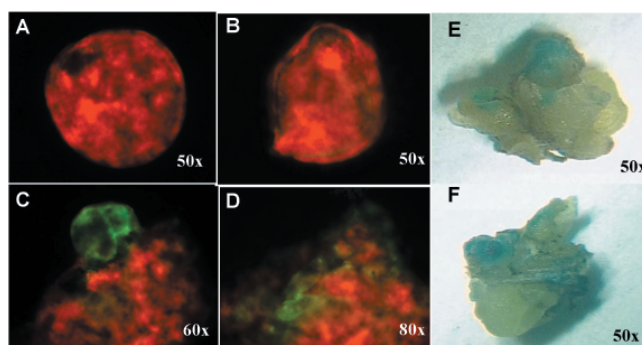


Fig. 7: GFP and GUS transient expression on *P. bellina* PLBs. A Negative control B Positive control C-D GFP transient expression in PLBs E-F GUS transient expression in PLBs

GUS assay for transient detection was not favorable throughout this study due to the irreversible destructive character to transient tissues or somehow, stable transformants (Schopke *et al.*, 1997).

CONCLUSIONS

In accessing the genetic transformation in this study, *GFP* was successfully used in *Agrobacterium*-mediated transformation of *P. bellina* PLBs. GFP has certainly proved its superior characteristics of being non-destructive, direct and rapid detection, non-cofactor or substrate required.

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