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Efficient Biolistic Transformation and Regeneration Capacity of an *EgTCTP* Transgene in Protocorm-like Bodies of *Phalaenopsis* Orchid

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Abstract

An efficient genetic transformation system using the biolistic method and protocorm-like bodies (PLBs) of *Phalaenopsis* orchid was established for introducing the *EgTCTP* gene obtained from oil palm leaves. A pCAMBIA 1302 vector containing the green fluorescent protein (*mgfp5*) as reporter gene and the selectable marker hygromycin phosphotransferase (*hpt*) gene under the cauliflower mosaic virus (CaMV) 35S promoter were used in this experiment. The transformed PLBs were cultured on MS medium containing 20 mg/L hygromycin for 2 months. The surviving PLBs with green fluorescence spots were used to calculate a transformation frequency (93.34%). PLBs containing the transformed *EgTCTP* gene had the highest percentage of regeneration frequency (95.66%) and numbers of regenerated shoots per explant (3.78±1.89 shoots) compared to the control. The time required for initiation of primordial shoots in the transformed PLBs (55.22±26.56 days) was much shorter than for the control. Evaluation of the regeneration efficiency, determined that the status of the *EgTCTP* transformants was above average: score=4.04±0.88. The *EgTCTP* gene was detected in the PLBs over a period of at least 6 months with subculturing every 4 weeks. The stability of the transgenes within the PLBs was confirmed by PCR and this indicated that the transgenes had been integrated into the genome of the transformants. This is the first successful report to introduce *EgTCTP* gene into PLBs of *Phalaenopsis* orchid.

Keywords: hygromycin, *mgfp5* gene, pCAMBIA 1302, PCR, transgenic plant, translationally controlled tumor protein

Introduction

Phalaenopsis, a member in the family Orchidaceae, is one of the most popular epiphytic orchids with high economic value in the orchid trade all over the world. It is noted for its good form, beauty, long-lasting flowers and long inflorescence which make it eminently suitable for ornamental plants. Nowadays genetically transformed plants of a *Phalaenopsis* orchid have been developed from both *Agrobacterium*-mediated gene transfer (Belarmino and Mii, 2000; Mishiba *et al.,* 2005; Sreeramanan *et al.,* 2008) and particle bombardment transformation (Anzai *et al.,* 1996; Fumika, 2007). In addition, biolistic transformation is more normally used in orchid gene transformation because of its higher transformation efficiency than *Agrobacterium*-mediated gene transfer (Chai and Yu, 2007).

The regeneration pathway through culture of inflorescence stem nodes of *Phalaenopsis* is either via protocorm-like bodies (PLBs) or via the proliferation of adventitious shoots. PLBs of several orchid genera such as *Dendrobium* (Chia *et al.,* 1994; Men *et al.,* 2003; Yu *et al.,* 1999), *Phalaenopsis* (Anzai *et al.,* 1996), *Cymbidium* (Yang *et al.,* 1999) and *Oncidium* (Yee *et al.,* 2008) have been widely used as target tissue for particle bombardment to recover transgenic orchids. However, an *Agrobacterium*-mediated transformation method was also used to produce orchid PLBs (Chai *et al.,* 2002; Chan *et al.,* 2005; Liau *et al.,* 2003) since the origin of the orchid PLB is from a single somatic cell, that has a high capability of regenerating into a new plant, is easy to root, presumed to be genetically uniform and can be induced efficiently from various somatic tissues including young leaves and stem segments (Chen and Chen, 2007; Yee *et al.,* 2008).

Genes of interest from other orchid genomes or plant species could be introduced for improving commercial traits such as enhancement of fragrance. However in this experiment, it was used the translationally controlled tumour protein (TCTP), as the transgene, because it is a highly conserved protein in all species studied and is a growth related protein believed to play an important role in cell growth and cell division (Bommer and Thiele, 2004). About 20 years ago the *TCTP* gene was found by three groups interested in translationally regulated genes. They named this protein P21, Q23 and P23, respectively (Gachet *et al.,* 1999). The TCTP name was coined (Gross *et al.,* 1989), based on the fact that the original cDNA was cloned from a human tumour and was regulated at the

translational level. Later, TCTP was found to be expressed in healthy animal tissues and controlled at both the transcriptional and post-transcriptional levels (Sanchez *et al.,* 1997; Xu *et al.,* 1999).

In plant research, a plant calcium binding *TCTP* gene has been successfully transformed into a tobacco plant. This transgenic tobacco encoding the TCTP gene grew about 30% faster than the parental plants during the juvenile growth stage (Kang *et al.,* 2005). Nowadays TCTP has attracted the attention of an increasing number of research workers interested in various biologically and medically relevant processes due to the importance of TCTP for cell cycle progression and malignant transformation. The ultimate goal of this research was to produce a stable transformation of the *EgTCTP (Elaeis guineensis TCTP)* gene into the PLBs of *Phalaenopsis* using a biolistic method and to validate its ability to play a role in the regeneration of transgenic orchids.

Materials and methods

Plasmids

The pCAMBIA 1302 vector (CAMBIA, Australia), containing an *EgTCTP* gene as the transgene together with the green fluorescent protein (*mgfp5*) reporter gene and the selectable marker hygromycin phosphotransferase (*hpt*) gene driven by the cauliflower mosaic virus (CaMV) 35S constitutive promoter was used in this experiment. This plasmid is approximately 10,549 bp in size.

Plant materials

PLBs of the white-flowered clones *Phalaenopsis* Blume were used as the target tissues. These PLBs were multiplied and maintained through four-week sub-culturing intervals in MS basal salt liquid medium (Murashige and Skoog, 1962) supplemented with 15% coconut water, at pH 5.7 and 25°C on a rotary shaker at 100 rpm with a 16 h photoperiod. Light was provided by fluorescent tubes with a photon flux density of 15 µmol m^{-2} s^{-1}. After four weeks, secondary PLBs were separated using a scalpel into individual PLBs and sub-cultured onto MS basal salt, solid medium. A total of 30 individual PLBs were plated at the centre of a 90-mm diameter Petri dish prior to bombardment. Each Petri dish was bombarded once but each parameter was performed in triplicate.

Biolistic bombardment experiments

Bombardment experiments were carried out using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). All the gold microcarriers, macrocarriers, stopping discs, and rupture discs were purchased from Bio-Rad (Hercules), California, USA. Precipitation of the DNA onto gold microcarriers was performed according to the manufacturer's instructions and all the bombardments were performed according to the standard procedures. Bombardments were carried out at the following constant conditions: 1100 psi rupture disc pressure, 11 mm rupture disc to macrocarrier distance, 9 cm stopping plate to target tissue distance, 11 mm macrocarrier to stopping plate distance, 27 mm Hg vacuum pressure and using 1 µm gold particles as the microcarrier.

Three controls were included, consisting of unbombarded PLBs, PLBs bombarded with microcarriers only (not coated with DNA) and PLBs bombarded with an empty vector. However, the PLBs bombarded with the empty vector might be classified into the same vector group. For the *EgTCTP* transformed PLBs, they would be the gene group. The bombarded tissues were then incubated at 25°C under 16 h of illumination provided by fluorescent tubes with a photon flux density of 15 µmol m^{-2} s^{-1}.

Post transformation and selection of putative transformants

Phalaenopsis PLBs were cultured on fresh MS medium for one week to recover from their bombardment injuries. Hygromycin (HYG) was used as a selective agent in this transformation. All transformed explants were transferred into selective medium containing 20 µg/mL HYG (Mishiba *et al.,* 2005) for selection of transformants. The newly formed secondary PLBs that survived were further subcultured at four-week intervals on fresh MS solid medium containing the same concentration of HYG as previously for at least 2 months.

Four parameters were assessed to evaluate the regeneration of the *Phalaenopsis* PLBs, the regeneration frequency was recorded as a mean percentage of explants with shoots (number of regenerated explants/total number of explants *100), the time for initiation of primordial shoots in days, the number of regenerated shoots per explant, and this scoring system was used to evaluate the regeneration efficiency as follows: Negative result = 1; Below average = 2; Average = 3; Above average = 4 and Excellent = 5.

GFP detection

A small section of any surviving orchid was observed for their expression of GFP using a confocal laser scanning microscope (CLSM) equipped with a GFP filter set for excitation between 455 and 490 nm and for an emission above 500 nm. The expression of the *gfp* gene was seen as a bright-green fluorescence caused by the presence of GFP in the cells. The difficulty of detecting GFP in the presence of the autofluorescence of the chlorophyll in the PLBs was overcome by using triple colour images acquired by sequentially scanning with settings optimal for the GFP (green), the autofluorescence of the chlorophyll (red) and the surface of organelles (blue). Filter sets tailored to the specific chromophores were used for the GFP (excitation by the 488 nm argon laser line and its emission collected from 500 to 543 nm, channel 1); the autofluorescence of the chlorophyll (excitation by the 543 nm argon laser line and its emission collected from 610 to 630 nm, channel

2) and the surface of the organelle (excitation by the 650 nm argon laser line and its emission collected from 667 to 750 nm, channel 3). Reflected light images were obtained by detection of light at the excitation wavelengths. Serial confocal optical sections were taken at different step sizes. The images of the individual channels were merged and stored as TIF files to facilitate visualization. Monitoring was carried out on random 3 days, in 1, 2, 3, 4 weeks, and 2, 3, and 4 months after bombardment.

Genomic DNA isolation from leaves of regenerated bombarded plantlets

After obtaining the viable *Phalaenopsis* PLBs, the PLBs were verified for the presence of the transgenes using the PCR technique. According to the manufacturer's instructions of the Genomic DNA Extraction Mini Kit (Plant) (RBC Bioscience Corp., Taiwan), total genomic DNA was extracted from the leaves of regenerated bombarded *Phalaenopsis* plantlets and used as the DNA template in the PCR reaction.

PCR analysis

Genomic DNA was extracted from about 100 mg of putative transgenic plants using a Genomic DNA Extraction Mini Kit (Plant). To confirm the presence of the *mgfp5* and *EgTCTP* genes in the transgenic plants, PCR experiments were performed using 2 pairs of specific primers for the GFP and EgTCTP:GFP. For the first PCR primer pair, it was designed to amplify an internal 362 bp *gfp* fragment: *gfp*F: 5'TCAGTGGAGAGGGTGAAGGTGATG3' and *gfp*R: 5'CGTTGTGGGAGTTGTAGTTGTATTC3'. The other PCR primer pair, resulting in a 887 bp fragment of *tctp-gfp* genes, *tctp-gfp*F: 5'ATGGTAATGTTGGTTTATCAG3' and *gfp*R: 5'CGTTGTGGGAGTTGTAGTTGTATTC3' was used in the PCR reaction.

In the PCR reactions, amplifications of the *gfp* and *tctp-gfp* genes were each carried out in a 12.5 µL reaction volume containing 1.25 µL of 10 x PCR buffer (10 mM Tris-Cl, pH 8.8, 50 mM KCl and 0.8% Nonidet P40), 0.75 µL of 1.5 mM $MgCl_2$, 0.25 µL of 5 mM dNTP mix (2.5 mM of each nucleotide dATP, dCTP, dGTP, and dTTP), 0.1 µL of 5 U Taq polymerase, 0.25 µL of 15 pmole primer each, 1 µL of template DNA at 100 ng/µL and 8.65 µL nuclease-free water.

The PCR run condition was set to an initial denaturation step of 5 minutes (min) at 94°C and subsequent 35 cycles of denaturation (94°C, 50 seconds), annealing (50°C, 1 min) and extending (72°C, 1 min) followed by a final extending step at 72°C for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel. The size of the amplified fragments were determined with reference to a 100 bp DNA ladder. Gels were stained with ethidium bromide to view fragments.

Statistical analysis

All experiments were conducted on three different days with at least 30 replicates per treatment. Population sizes varied between experiments, and these sizes are indicated together with the experiment results. Variance analysis (ANOVA) was applied to the results wherever indicated using the Sheffe's test at $p \leq 0.05$. The software used was SPSS 13.0 (SPSS Inc., USA) for Windows XP Professional.

Results

Transformation frequency in the bombardment of Phalaenopsis PLBs

The PLBs that survived with green fluorescence spots were calculated as the transformation frequency (%). No GFP green spots were found in the control group but 83.34% and 93.34% of GFP green spots were found in the vector and gene groups, respectively.

Evaluation of the regeneration of Phalaenopsis PLBs after bombardment

Germination and growth of the *Phalaenopsis* PLBs during the 1-6 months after bombardment at 1100 psi helium level were compared (Fig. 1). In the first month after bombardment, *Phalaenopsis* PLBs containing a transformed *EgTCTP* gene had swollen differentiated tissue while the others were not changed (Fig. 1a). One month later, the *EgTCTP* transformants regenerated and formed primordial shoots (Fig. 1b). However, in the third month after biolistic transformation, the control and vector alone transformants began to regenerate (Fig. 1c) and continued to grow (Fig. 1d).

To evaluate the regeneration of *Phalaenopsis* PLBs that was bombarded at 1100 psi helium pressure, four criteria were studied as follows: regeneration frequency, time for initiation of primordial shoots, number of regenerated shoots per explant and regeneration efficiency. Results are summarized in Tab. 1. For the first criteria, the regeneration frequency of the control, vector and gene groups in *Phalaenopsis* PLBs was 78.34, 80 and 95.66, respectively. During this time the gene transformants took fewer days to initiate primordial shoots than the control and vector transformants. The time for initiation of primordial shoots in the *EgTCTP* gene transformants took only 55.22 ± 26.56 days with 3.78 ± 1.89 regenerated shoots per explant. Moreover, the *EgTCTP* gene transformants achieved the highest score for regeneration efficiency (showing 4.04 ± 0.88 "above average" status). With regard to the time required to initiate primordial shoots, the control and vector transformant groups took 83.50 ± 33.89 and 61.87 ± 18.52 days, respectively. This time was significantly greater than for the gene transformed group. In addition, the numbers of regenerated shoots per explant in the control and vector transformant groups were 1.56 ± 0.51 and 1.14 ± 0.46, respectively. For the last criteria, on regeneration efficiency using the scoring system, the two groups of control and vector transformants could be classified into the same level. The score of the control and vector groups were 2.94 ± 0.52 and 3.16 ± 0.76, respectively which was evaluated as an "average" status.

Tab. 1 Evaluation of the regeneration parameters of the regenerated *Phalaenopsis* PLBs from the control, vector and gene groups

Evaluation criteria	Control	Vector	Gene
Regeneration frequency (%)	78.34	80	95.66
Time of shoot primordial initiation (days)	83.50 ± 33.89[a]	61.87 ± 18.52[b]	55.22 ± 26.56[b]
Number of regenerated shoots per explant	1.56 ± 0.51[a]	1.14 ± 0.46[a]	3.78 ± 1.89[b]
Regeneration efficiency*	2.94 ± 0.52	3.16 ± 0.76	4.04 ± 0.88

Each value represents the mean of thirty replicates. Means within a row followed by different letters show significant difference as analyzed by Sheffe's test at P≤0.05.
*Regeneration efficiency was expressed as a score from 1 to 5 (Negative result = 1; Below average = 2; Average = 3; Above average = 4 and Excellent = 5)

Visualization of GFP expression

The successful gene transformation and expression in transformants was examined with a CLSM fitted with a GFP filter set. GFP gene expression was seen as a bright-green fluorescence caused by the presence of GFP in the cells. In this research, monitoring was carried out at random on bombarded explants from 1 to 4 months after bombardment. All explants expressed bright-green fluorescence even though there was some in the control explants that was probably due to the auto-fluorescence from chloroplasts.

To solve this problem, three different fluorescent, green, red and blue signals were scanned at the same time and the three filters were overlain together. In Fig. 2, the GFP expression, 60 days after bombardment, in the PLBs of *Phalaenopsis* transformants is illustrated. When the control PLBs (Fig. 2a, b) were compared with the pCAMBIA 1302 transformants (Fig. 2c) and the *EgTCTP* orchid transformants (Fig. 2d), m*gfp*5 bright green spots were seen both in the vector alone transformants and the successful gene transformants but none in the controls.

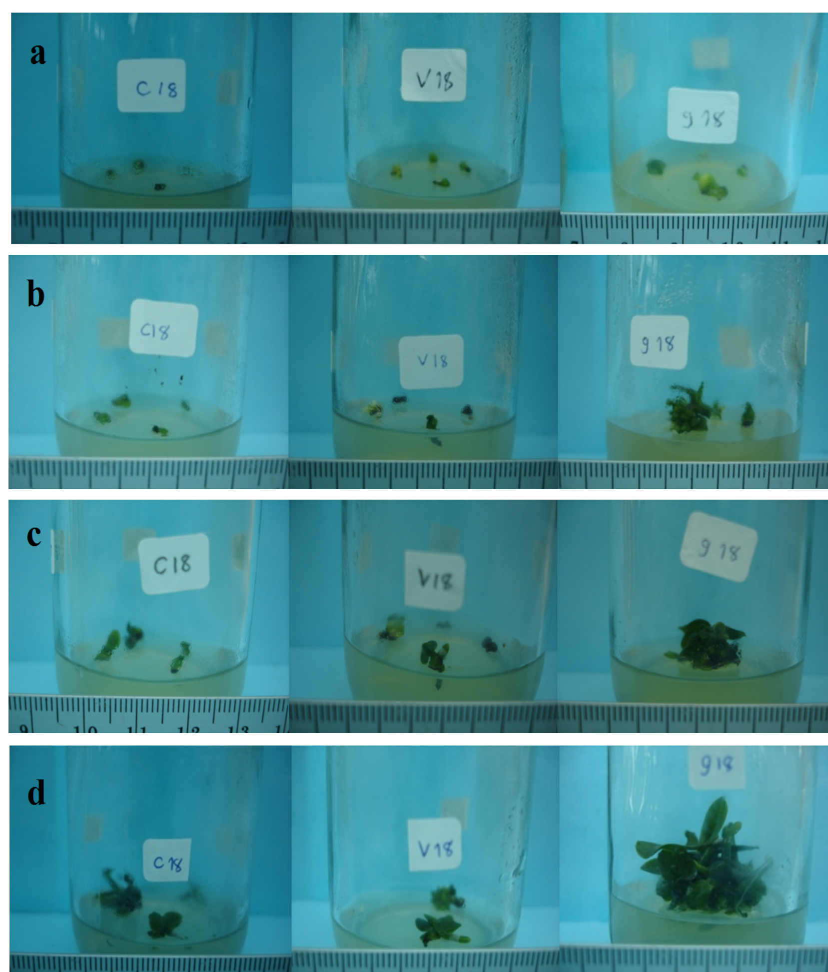

Fig. 1. Growth and development of *Phalaenopsis* PLBs after bombardment at 1100 psi helium level (from left to right is control, vector-transformed and the *EgTCTP* gene transformed PLBs, respectively). (a) One-month-old PLBs. (b) Two-month-old PLBs. (c) Three-month-old PLBs and (d) Six-month-old PLBs after transformation

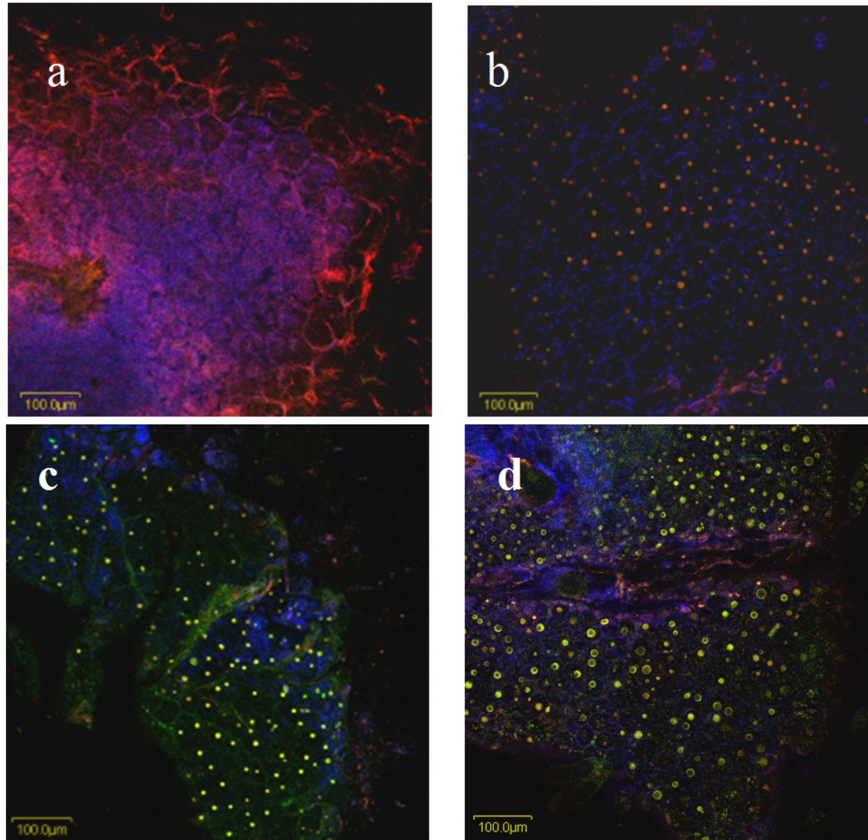

Fig. 2. Localization of GFP in *Phalaenopsis* PLBs 60 days after bombardment as shown by confocal laser scanning microscope images. 35S CaMV-GFP expressed from pCAMBIA1302 vector. (a) Control: umbombarded (b) Control: bombarded without vector and gene (c) Bombarded with vector and (d) Bombarded with transgene plasmid

1 2 3 4 5 6 7 8 9 10 11 12 13

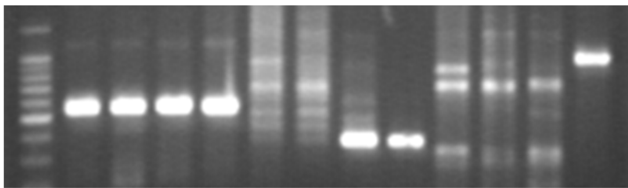

Fig. 3. PCR analysis of transformed *Phalaenopsis* orchid. Chromosomal DNA was amplified with the specific primer to 18s rRNA (Lane 2-5), *gfp* (Lane 6-9) and *gfp-EgTCTP* (Lane 10-13) genes. Lane 1 molecular marker DNA, lane 6, 10 negative control (untreated orchid), lane 7, 11 negative control transformed with microcarrier only, lane 8, 12 transformed with vector alone, lane 9 positive control transformed with the *gfp* gene and lane 13 positive control transformed with the *gfp-EgTCTP* genes

PCR analysis of transformants

All transformed explants were screened for the presence of the *mgfp5* and *EgTCTP* genes using PCR amplification of fragments within the transferred genes. The chromosomal DNA of *Phalaenopsis* explants was isolated and used as the template in the PCR reactions later. The result of the PCR reaction is shown in Fig. 3. In transformed *Phalaenopsis* PLBs, the presence of the *mgfp5* transgene produced the expected band at 362 bp whereas the fragment at 887 bp is specific to the recombinant *gfp-EgTCTP* gene. The positive bands of the *gfp* gene are seen in Fig. 3 (lane 8-9) while the positive band of the *gfp-EgTCTP* gene was detected in Fig. 3 (lane 13). No amplification was detected in the non-transformed orchids.

Discussion

Transformation frequency in the bombardment of Phalaenopsis PLBs

In this experiment the transformation frequency in the survived *Phalaenopsis* PLBs was much higher when compared to that obtained by the bombardment of the PLBs of *Phalaenopsis* ('Danse' × 'Happy Valentine'). In this previous work only 7 bialophos-resistant plantlets from 622 bombarded PLBs were obtained (Anzai *et al.,* 1996) and the transformation frequency in the bombarded *Dendrobium* protocorms was 19.87% (Suwanaketchanatit *et al.,* 2007). However the percentage transformation frequency in the present work with *Phalaenopsis* PLBs bombardment was nearly as high as that obtained by the bombardment of *Cymbidium* PLBs that produced about 85% of GUS positive shoots (Yang *et al.,* 1999). These results support

the transformation efficiency of this *Phalaenopsis* PLBs bombardment.

Regeneration evaluation of Phalaenopsis PLBs after bombardment

As far as the regeneration properties are concerned, the *EgTCTP* transformed *Phalaenopsis* PLBs using the optimal helium pressure at 1100 psi promoted the regeneration frequency and efficiency as well as a shorter time for initiation of primordial shoots and produced more regenerated shoots per PLBs. In an evaluation of the number of regenerated shoots per explant, after being bombarded the explants with the *EgTCTP* gene produced the highest shoot number compared to the control and vector groups. For the evaluation of regeneration efficiency, the status of the *EgTCTP* orchid transformants was in the 'above average' level. These results were in agreement with transgenic tobacco which had been transformed with the *ntTCTP* gene using the *Agrobacterium*-mediated method grew 30% higher than the parental plants during the juvenile growth stage (Kang *et al.,* 2005). The present results also clearly demonstrated the importance of the role of *EgTCTP* which also seems to assist with the transformation process and provides benefits for plant growth and regeneration.

Visualization of GFP expression

GFP monitoring was an effective tool for screening the transformed explants because the *gfp* gene offers advantages such as any transformants do not contain antibiotic resistance genes to use for selection and its presence also avoids the need to use a destructive *GUS* assay for identification. It was therefore relatively easy to establish if transformation had occurred by looking for the green fluorescence because GFP was part of the pCAMBIA 1302 plasmid that also contained the *EgTCTP* gene. The expression of GFP was in agreement with Tee and Maziah (2005) who reported that the GFP expression rate transformed into *Dendrobium* Sonia 17 was higher than that for the GUS under the control of the same promoter based on the GUS or GFP spot counts. Almost all bombarded PLBs of orchid showed many bright green spots from the GFP fluorescence inside the cells compared to the controls due to the GFP expression under control of the strong constitutive CaMV 35S promoter. Normally, the brightness of fluorescence was maintained at full intensity during the subculture. This indicated that explants and their plasmids were able to proliferate under HYG selective conditions. However, interference by plant chlorophyll with the GFP fluorescence was a complication for evaluation. By preventing autochlorophyll fluorescence in orchid PLBs, the sequential scanning of the triple color image method could easily detect the difference between GFP fluorescence and the autofluorescence of chlorophyll. In addition, the red autofluorescence of the chlorophyll interacted with the green fluorescence of the GFP to make the plants transformed with GFP appear yellow under UV light. Furthermore, there are other methods able to distinguish GFP from endogenous autofluorescence such as using optimized filter sets, dual-wavelength differential fluorescence correction, fluorescence polarization, use of image analysis software, autofluorescence quenching, the use of spectrally different GFP mutants, etc (Billington and Knight, 2001). Thus, the GFP-based visual selection has made it possible and simple to detect and select transgene-carrying tissues.

In this study, for the first time, the *mgfp5* gene was used in transformation of *Phalaenopsis* PLBs with a high efficiency. The ability of the above tissues to express GFP activities more than 4 months post-bombardment further indicated that the transgene had been successfully and stably integrated into the genome of the putative transformants. Nevertheless, the stable integration of the *EgTCTP* gene after integrating into the orchid cells received further proof via PCR analysis.

PCR analysis of transformants

In the past, several research studies in orchids (Anzai *et al.,* 1996; Chia *et al.,* 1994; Men *et al.,* 2003; Tee and Maziah, 2005; Suwanaketchanatit *et al.,* 2007; Yang *et al.,* 1999; Yu *et al.,* 1999; Yee *et al.,* 2008) have shown the stable integration of transgenes via biolistic transformation although Janna *et al.* (2006) reported only a transient expression of the transgenes. Eventually, the presence and integration of the *mgfp5* transgene via the particle bombardment method was established in other plants genome such as petunia (Garabagi and Strommer, 2000), papaya (Zhu *et al.,* 2004) and high oleic acid sunflower (Mohamed *et al.,* 2006). With regard to the PCR analysis in the *Phalaenopsis* explants in this study, the obtained PCR bands confirmed the presence of *mgfp5* and *EgTCTP* sequences. Therefore this observation proves the stable integration of the *EgTCTP* gene into the transformed orchids.

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