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FACTORS AFFECTING GREEN FLUORESCENCE PROTEIN (GFP) GENE EXPRESSION IN OIL PALM AFTER MICROPROJECTILE BOMBARDMENT

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ABSTRACT

Expression of green fluorescence protein (GFP) gene can be visualized under ultraviolet or blue light without any substrate or co-factor addition. It has been used to monitor transient and stable transgene expression in many plant varieties. The effectiveness of gfp gene as a selectable marker for oil palm transformation was evaluated through transient expression of gfp genes in bombarded oil palm embryogenic calli and immature embryos. Different types (version) of gfp genes which are driven by different constitutive promoters were used to transform oil palm target tissues. Some of the gfp genes used were targeted to specific organelle: namely plastid, endoplasmic reticulum and mitochondria. Transient expression of the gfp genes could be detected in oil palm tissues as early as 16 hr after bombardment. It was observed that the number of gfp expressing cells and duration of the gfp gene expression differs from one construct to another. The differences in the gfp constructs performance in oil palm tissues were evaluated based on the following factors: version of the gfp genes, promoter used to drive the gfp gene, backbone vector and the size of the whole plasmid. The CaMV35S promoter was found to be the most effective promoter for driving gfp gene in oil palm tissues followed by HBT and maize ubiquitin promoter. The sGFPS65T was the most effective version of gfp gene for oil palm tissues followed by sGFP and mGFP5. It was also demonstrated that the pUC18 backbone vectors was the most effective vector backbone in expressing the gfp gene in oil palm. Finally, it was observed that the smaller the gfp vector, the higher the number of gfp expressing cells obtained. Possible reasons for these observations were elaborated and discussed.

Keywords: green fluorescence protein, transient expression, factors affecting gene expression, oil palm.

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INTRODUCTION

Palm oil is the largest source of edible oil in the world and is mainly produced in Malaysia and Indonesia (Anon, 2007). Malaysian palm oil industry is facing problems associated with labour shortage, limited arable land resources and also fluctuation in the price of the commodity. In order to remain competitive, the palm oil industry has to increase its yield as well as to improve the palm oil quality and produce novel fatty acids (Parveez, 1998). However, conventional improvement of oil palm suffers from a number of limitations, such as long generation time and open pollination behaviour. The success in oil palm tissue culture and the ability to transfer foreign genes into oil palm have made genetic engineering a promising tool for improving palm oil quality and making the plant synthesize novel products. It is estimated that only four to five years are required to produce

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valuable transgenic plantlets from the initial tissue culture stage (Parveez, 2000).

Increasing the oleic acid content is the main goal in oil palm genetic engineering today (Cheah *et al.*, 1995). However, producing other useful products, such as palmitoleic acid, ricinoleic acid, stearic acid and biodegradable plastics, are also being targeted to further increase the value of palm oil (Parveez *et al.*, 1999). These products are targeted as industrial feedstocks and for health food supplements and nutraceuticals. Transgenic plants are contained and evaluated in biosafety greenhouses.

Plant genetic engineering was first achieved almost a quarter century ago by the transfer of a bacterial gene into tobacco, mediated by a soil bacterium, *Agrobacterium tumefaciens* (Fraley *et al.*, 1983). The number of transgenic plants produced has since increased exponentially. According to a recent report by the International Service for Acquisition of Agri-biotech Applications (ISAAA), the areas that are commercially planted with transgenic plants worldwide has increased almost 60-fold, from 1.7 million hectares in 1996 to 102 million in 2006 (James, 2006).

A method to genetically manipulate oil palm, using the Biolistics™ approach, was successfully developed with the production of transgenic oil palm resistant to the herbicide Basta (Parveez, 1998). Subsequently, transgenic oil palm producing unique products, such as high oleic, high stearic and biodegradable plastics, have been produced (Parveez, 2003). Meanwhile, improvement of the transformation method, particularly to increase the transformation efficiency and production of transgenic lines, has been given priority to ensure successful manipulation of oil palm in the years to come. One of the approaches taken is to use a novel selectable marker system.

There are about 50 selectable marker genes being used or developed in transgenic plant research (Miki and McHugh, 2004). Generally, they are of two types *i.e.*, positive and negative. The negative gene works by killing the non-transformed cells in the presence of an antibiotic or herbicide in the plant regeneration medium thereby allowing only the transformed cells to grow and proliferate. Such genes commonly used in plant transformation are those that confer resistance to antibiotics, such as kanamycin (Bevan *et al.*, 1983) and hygromycin (Gritz and Davies 1983), and to herbicides, such as Basta (DeBlock *et al*., 1987). On the other hand, the positive gene allows proliferation of the transformed tissue by suppressing growth of the untransformed cells. An example of such is the *Phosphomannose isomerase* (pmi) gene, which converts mannose-6-phosphate to the easily metabolized fructose-6-phosphate that can be used by the plant cells as a carbon source (Joersbo *et al.*, 1998). The non-transformed cells will starve in a medium containing mannose, thus allowing only the transformed cells to proliferate and produce transgenic plants. Another example of positive selectable marker that allows visual isolation of transformed cells is the green fluorescent protein (GFP), gene isolated from a jellyfish, *Aequorea Victoria* (Chalfie *et al.*, 1994; Heim *et al.*, 1994). Compared to other selectable markers, GFP is stable, does not require any substrate or co-factor (Prasher, 1995) and can be easily detected under a fluorescence microscope (Cubitt *et al.*, 1995). The expression of GFP is also non-destructive and species independent, so it can be used to monitor transgene expression *in vivo*, *in situ* and in real time.

Since the first report on the suitability of GFP as selectable marker for plant (Niedz *et al.*, 1995), numerous applications of GFP on other plants have been carried out. However, low expression and quenching of the green fluorescence have been reported (Hu and Cheng, 1995). Initial sequence analysis of the original *gfp* gene revealed the presence of a cryptic intron which causes the aberrant slicing of 84 nucleotides and, consequently, the low expression. In order to keep the gene character similar to that of the wild type, the codon usage was altered and a new variant called mGFP4 produced (Haseloff *et al.*, 1997). Newer GFP variants were then produced through codon optimization to enhance protein fluorescence (reviewed by Stewart, 2001). The sGFP, for example, is a synthetic gene which has been human codon-optimized. Since human and corn have high similarity in codon usage, the human-codon optimized GFP has increased *gfp* gene expression up to 100-fold in maize plant cells (Chiu *et al.*, 1996). Subsequently, the sGFP S65T variant was produced by removing the cryptic intron in sGFP and altering the codon usage (according to Haseloff *et al*., 1997) by mutating the S65T chromophore (serine at position 65 to threonine). This variant was able to increase the detection limit by 19-fold (Reichel *et al.*, 1996) by inducing rapid chromophore formation and enhancing the fluorescent signal (Heim *et al*., 1995). With this advantage, the sGFP S65T gene has had broad applications in plants, such as to detect weak promoter activity, visualize the proteins targeted into nucleus or plastids, and in analysing of the signal transduction pathways in transgenic plants or other living cells (Chiu *et al*., 1996). Another GFP variant actively used in plant studies is eGFP (Clontech), it has the S65T, F64L and Y145F mutations and is also human-codon optimized (Yang *et al*., 1996).

GFP was successfully used as a selectable marker to produce transgenic plants, such as sugarcane (Elliot *et al.*, 1999), barley (Ahlandsberg *et al*., 1999; Holme *et al.*, 2006), wheat (Jordan, 2000), oat (Kaeppler *et al.*, 2002), brome grass (Nakamura and Ishikawa, 2006), American chestnut (Polin *et al*., 2006) and peach (Padilla *et al.*, 2006). In rice transformation, GFP could decrease the amount of tissues handled

by a factor of 4 and the time involved by a factor of 2 (Vain *et al.*, 2000). The use of GFP coupled with kanamycin selection resulted in a higher number of transformants produced for the conifer, *Chamaecyparis obtusa* and pear (Tanguchi *et al*., 2005; Yancheva *et al*., 2006). Interestingly, in carrot, 94% of the transformants selected using GFP also carried the *nptII* gene which was co-transformed (Baranski *et al*., 2006). This report suggests that GFP can easily replace antibiotics selection for co-transforming with useful genes. GFP has also been used to distinguish transgenics from chimeric plants and to easily screen for marker-free transgenic progenies (Chen *et al*., 2005). These findings clearly suggest that GFP can be used as an alternative selectable marker gene for plant transformation. Transient expression studies of various GFP gene constructs in oil palm cultures have recently been carried out (Na'imatulapidah and Parveez, 2007). In this article, the various biological factors affecting GFP gene expression in bombarded oil palm cultures, such as the versions of the GFP genes, promoters used to drive the GFP gene, backbone vectors and the size of the whole plasmid, will be discussed.

MATERIALS AND METHODS

Plant Materials

Oil palm embryogenic calli were produced from calli from either the young leaves or cabbage on OPEC medium [MS salts (Murashige and Skoog, 1962), 0.1 g litre^{-1} myo-Inositol and L-glutamin, 3% sucrose, 5 x 10^{-5} M 2, 4-D, 0.25% (w/v) activated charcoal, Y3 vitamins (Eeuwans, 1976) and 0.7% agar] incubated in the dark at 28°C (Parveez, 1998). The resultant calli were subcultured every four weeks on fresh OPEC medium until embryogenic calli were observed.

Oil palm immature embryos (11-12 weeks after anthesis, WAA) were obtained from the commercial oil palm variety (*tenera*). Calli from the immature embryos were produced on OPEC-IE medium containing Y_3 macro-, micronutrients and vitamins, 0.05% (w/v) cystein, 5 x 10^{-4} M 2,4-D, 0.5% (w/v) polyvinyl pyrolidone (PVP_{40}), 0.22% (w/v) gelrite and 0.3% (w/v) activated charcoal. The immature embryos were cultured in an incubator at 28°C without light. The calli formed were subcultured every four weeks on the fresh OPEC-IE medium until embryogenic calli were produced.

GFP Gene Constructs

In this study, 11 *gfp* gene constructs were used to evaluate their expression efficiency in oil palm embryogenic calli and immature embryos. They were p35SCaMVΩ-mt-sgfpS65T, p35SCaMVΩ-pt-sgfpS65T (Niwa *et al.*, 1999), pBIN.Ubi-mgfp5-ER, pBIN.35S-mgfp5-ER, pGEM.Ubi-sgfpS65T, pTO134 (Elliot *et al*., 1998; 1999), pHBT-sgfp, pHBT-sgfpS65T, p35SCaMV-sgfpS65T (Sheen *et al.*, 1995), pCAMBIA 1302 (Dr Richard Jefferson, CAMBIA, Australia) and p35S-egfp (Clontech, USA). The first four gene constructs carried the *gfp* gene and were targeted to specific organelles: mitochondria (mt), plastid (pt) and endoplasmic reticulum (ER). The last seven GFP gene constructs were not targeted to any organelle. Detailed information of each construct is given in *Table 1.*

Bombardment of Oil Palm Tissues Using PDS-1000/He Apparatus

Preparation of the DNA-microcarrier mixture for bombardment was carried out according to the instruction manual by the Biolistics PDS/He 1000 manufacturer (Bio-Rad). For each GFP plasmid, 5 µl DNA solution (1 µg µl^{-1}), 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M, free base form) were added in sequence to the 50 µl gold microcarrier suspension. The mixture was vortexed for 3 min and spun for 10 s in a microfuge. The pellet was washed twice with 250 µl absolute ethanol and the final pellet resuspended in 60 µl of absolute ethanol. Six microlitres of the DNA-microcarrier solution were loaded onto the centre of the macrocarrier, air-dried and used for bombardment.

For each GFP plasmid, bombardments were carried out on a minimum of five replicates for oil palm embryogenic calli and 10 replicates for oil palm immature embryos. The bombardments were carried out in the conditions optimized for oil palm embryogenic calli and immature embryos (Parveez *et al*, 1997; 1998). In this study, two controls were also incorporated, *i.e.*, tissues without bombardment and bombardment using plasmids without the *gfp* gene. After bombardment, the tissues were incubated at 28°C in light condition prior to GFP transient expression evaluation.

Visualization of GFP and Fluorescence Microscopy

Evaluation of GFP spots on the bombarded oil palm embryogenic calli and immature embryos was carried out using a Leica MZ12.5 stereomicroscope with a fluorescence GFP Plus filter module (Leica Microscopy and Scientific Instruments, Switzerland). GFP spots were scored every hour for the first day, then everyday for the next two weeks and, finally, once a month. Chlorophyll interference was minimized using a narrow bandpass interference filter (S550/100 NP). Images of the GFP-expressing cells were captured and analysed using a Leica IM50 Image Manager. The 'green' level of the images was then analysed to quantify the green fluorescence

TABLE 1. PLASMIDS CONTAINING DIFFERENT *gfp* VARIANTS OR DIFFERENT PROMOTERS USED IN THIS STUDY

No.	Plasmid	Promoter	*gfp* version	Additional element	Backbone vector	Size (kb)
1	p35SCaMV-sgfpS65T	CaMV35S	*sgfpS65T*	-	pUC18	~4.09
2	pHBT-sgfpS65T	HBT	*sgfpS65T*	35S enhancer	pUC18	~4.09
3	pHBT-sgfp	HBT	*sgfp*	35S enhancer	pUC18	~4.09
4	pGEM.Ubi-gfpS65T	ubiquitin	*sgfpS65T*	-	pGEM	6.20
5	p35S-mgfpClontech	CaMV35S	*egfp*	-	pUC18	4.50
6	pCAMBIA 1302	CaMV35S	*mGFP5*	-	pCAMBIA	~4.32
7	p35SCaMVΩ-pt-sgfpS65T	HBT	*sgfpS65T*	transit peptide of rbcs-1a for plastid targeting & 35S enhancer	pUC18	4.29
8	p35SCaMVΩ-mt-sgfpS65T	HBT	*sgfpS65T*	pre-sequence of α subunit of F1 ATPase used for mitochondria targeting & 35S enhancer	pUC18	4.44
9	pTO134 sgfpS65T	CaMV35S	*sgfpS65T*	*bar* gene	pTO	12.65
10	pBIN.35S-mgfp5-ER	CaMV35S	*mgfp5*	ER targeting signal & HDEL	pBIN	~13.30
11	pBIN.Ubi-mgfp5-ER	ubiquitin	*mgfp5*	ER targeting signal & HDEL	pBIN	14.30

Note: No.1 - 6 and 7-11: plasmids listed in descending order of *gfp* fluorescent cells observed in the first two weeks after bombardment (refer to *Figures 3* and *4*).

intensity using a Leica QWin Pro software (Leica System, Germany).

RESULTS AND DISCUSSION

Visualization and Quantification of *gfp* Fluorescent Spots in Oil Palm Tissues

Oil palm embryogenic calli were bombarded with 11 GFP plasmids carrying different types (or versions) of the GFP gene and driven by different constitutive promoters. The backbone for the GFP plasmids used varied. Detailed information for the plasmids is given in *Table 1*. For each plasmid, bombardments of embryogenic calli were carried out with a minimum of five replications. For the oil palm immature embryos, bombardments were carried out with 10 replications for each plasmid. After bombardment, the embryogenic calli and immature embryos were incubated at 28°C, without light. Initially, the bombarded embryogenic calli and immature embryos were screened for GFP spot at every hour using a Leica MZ12.5 fluorescence microscope. The GFP spots were easily detectable at 16 hr post-bombardment even with a magnification at as low as 10X (*Figure 1a*). No GFP spot was detected in the controls (*Figure 1b*).

The GFP spots were randomly distributed on the surface of the bombarded oil palm embryogenic calli (*Figure 1a*). In contrast, the non-bombarded embryogenic calli or embryogenic calli bombarded with plasmids carrying no *gfp* gene only appeared as a faint green background. The random distribution of *gfp* expressing cells in the oil palm embryogenic calli was due to transformation using the microprojectile bombardment method. During the bombardment, the plasmid that carried the *gfp* gene, and which bounded to the gold particles (microcarrier) would have been randomly distributed in the oil palm cells following the helium gas flow. This resulted in non-specific insertion of the *gfp* genes into the cells throughout the surface of targeted oil palm cultures. Successful delivery of the transgene into the oil palm cells could be assessed as early as 16 hr after transformation. The intensity and size of the GFP detected on the embryogenic calli varied, some spots being brighter and bigger than others. The green fluorescence intensity was also quantitatively measured as the Grey level value using the Leica QuantaPro Software. The Grey level was set from 0 to 286, ranging from the undetectable level up to the highest intensity that could be detected using the software.

The highest Grey level for the *gfp* expressing spots found were 150-200 compared to the non-transformed cells, recording only the background green fluorescence Grey level value, which had only 25-80 (*Figures 1c* and *1d*). This clearly demonstrated that the *gfp* expressing cells can be quantitatively distinguished from the non-transformed cells using the available equipment and software. It is believed that when clumps of *gfp* expressing cells are obtained after proliferation, they can be easily distinguished, isolated and regenerated to produce *gfp* expressing transgenic oil palm plantlets.

In the oil palm embryogenic calli, GFP spots appeared on the immature embryos as early as 16 hr after bombardment, although the number and sizes of the spots varied from one to another. The

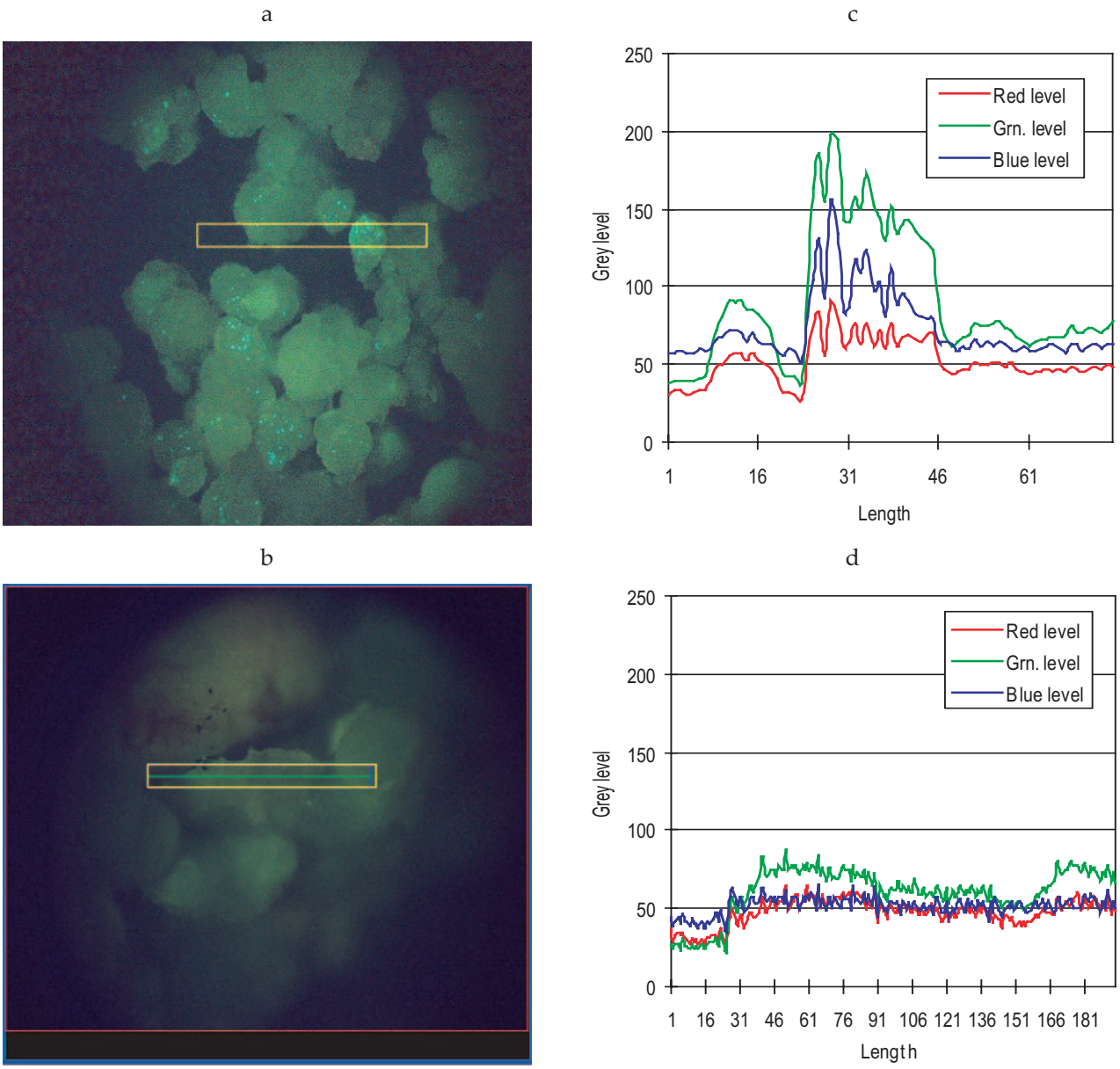

Figure 1. Visualization of green fluorescence in gfp*-bombarded oil palm embryogenic calli (a) 16 hr after bombardment as compared to non-bombarded embryogenic calli (b). Green level quantification on green fluorescent spots on the bombarded embryogenic calli (c) and non-bombarded embryogenic calli. (d) Magnification: X10.*

severity of spoting could be classified into three categories, *i.e.* <50, 50-80 and >80% of the surface area of the immature embryos covered (*Figure 2*). As with the embryogenic calli, the random distribution of GFP spots on the embryos appeared due to the transformation method used.

The expression patterns of the 11 *gfp* gene constructs were monitored and studied over 10 months. Six of thems were not targeted to any organelle. The mean (five bombardments) numbers of *gfp* expressing cells (GFP spots) were counted for each of the constructs at different interval and were plotted against time (*Figure 3*). Scoring of the numbers of GFP spots was started at 16 hr post-bombardment. The average number of GFP spots for the p35S-mgfp (Clontech), p35S-mgfp5 (CAMBIA-1302), pGEM-Ubi-sgfpS65T and pHBT-sgfp constructs were less than 200. Interestingly, embryogenic calli bombarded with constructs 35S-sgfpS65T and HBT-sgfpS65T showed the highest number of GFP spots, *i.e.* more than 800 and almost 600, respectively. The 35S-sgfpS65T construct also demonstrated the longest duration for which the GFP signals could be observed, *i.e.* up to eight months post-bombardment.

Figure 3 shows that within two weeks of bombardment, the number of GFP spots had begun to decline for all the plasmids tested on oil palm embryogenic calli. However, for the most efficient plasmids, 35S-sgfpS65T and HBT-sgfpS65T, the rates of GFP spots reduction were much slower. For most of the GFP plasmids tested, the highest number of

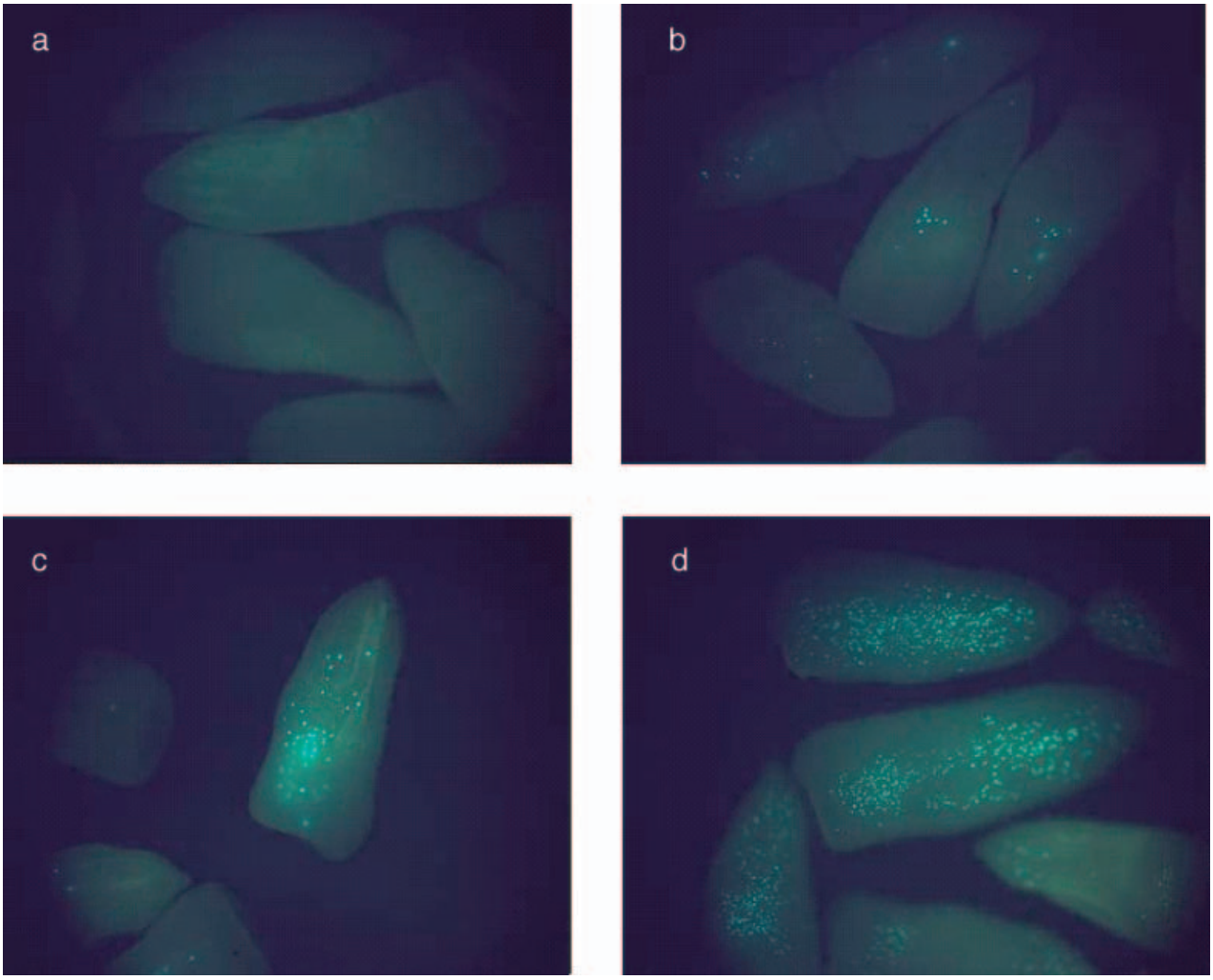

Figure 2. GFP spots appearing in bombarded immature embryos: (a) non-bombarded and bombarded with (b) <50%, (c) < 80% and (d) > 80% of the immature embryo surface area covered with GFP spots.

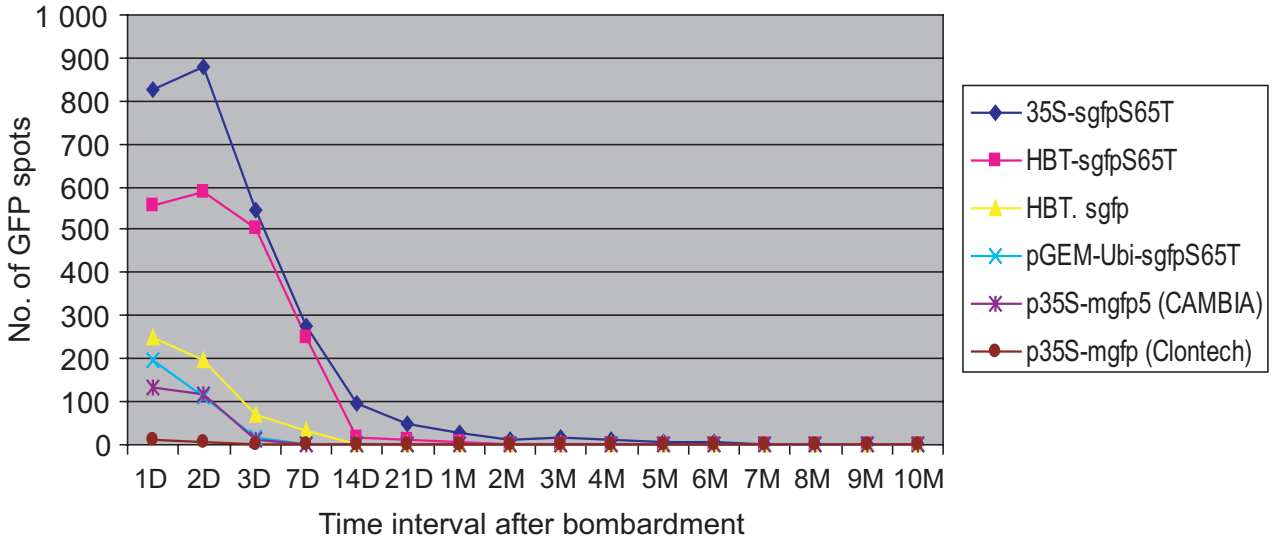

Figure 3. GFP expression pattern of non-targeted GFP plasmids in oil palm embryogenic calli. The expression pattern was measured based on the GFP spots counted at different times post-bombardment. D: day and M: month. Note: The highest peak of green fluorescent spot occurred in the second day after bombardment for 35S.sGFPS65T.

GFP spots was obtained in the second day post-bombardment. This suggests that the highest transient expression of the *gfp* gene occurs in the second day in oil palm embryogenic calli. This result agrees with findings in other plants such as orchid (Tee *et al.*, 2003) and barley (Carlson *et al*., 2001), which the highest *gfp* gene expression also occurred in the second day after transformation. Similarly, it was reported previously that the highest *gus* gene expression in oil palm embryogenic calli was obtained on the second day post-bombardment (Parveez, 1998). It has also been demonstrated that the GFP spots were usually only visible a few hours after transformation and started to decline within a few days, indicating transient expression of the *gfp* gene (Mercuri *et al*., 2001; Jeoung *et al*., 2002; Pishak *et al*., 2003). A similar observation was made in oil palm cultures.

Four of the constructs used to transform the oil palm immature embryos contained the *gfp* gene targeted to organelles mitochondria (mt), plastid (pt) and endoplasmic reticulum (ER). In addition, two non-targeted GFP plasmids were also bombarded as reference. The mean (10 bombardments) numbers of GFP spots were counted for each of the constructs at different intervals and plotted against time (*Figure 4*). In this experiment, it was revealed that plasmid p35S-mt-sgfpS65T had the highest average GFP spots, followed by pHBT-pt-sgfpS65T and p35S-sgfpS65T (pTO134). Plasmids pGEM-Ubi-sgfpS65T, pBIN-Ubi-mgfp5-ER and pBIN-35S-mgfp5-ER showed the lowest number of GFP spots in the oil palm immature embryos.

Similar to observations made in embryogenic calli using non-targeted *gfp* gene constructs, the number of GFP spots in oil palm immature embryos bombarded with *gfp* gene constructs (both organelle-targeted and non-targeted) had reduced to almost zero within two weeks (*Figure 4*). However, long-term expression of the *gfp* gene, demonstrated in embryogenic calli, was not observed for the immature embryos. Irrespective of the type of *gfp* gene constructs used (organelle-targeted or not), almost all the GFP spots fully disappeared in the third week post-bombardment.

Factors Affecting *gfp* Gene Expression in Oil Palm Cultures

Previous studies revealed that *gfp* gene expression and regeneration of transgenic plant via GFP marker selection can be improved by modifying the wild-type *gfp* gene sequence (Chiu *et al*., 1996; Haseloff *et al*., 1997; Niwa *et al.*, 1999; Sheen *et al.*, 1995). The modifications have resulted in the development of new *gfp* variants, with some having altered chromophores, stability or solubility. Transgene expression and patterns can be optimized by using a promoter suitable for specific plants, plant tissues and transgenes (Potenza *et al.*, 2004; Kamate *et al.*, 2000). Therefore, the influence of the *gfp* gene variant, promoter driving the *gfp* gene, backbone vector, size of the whole construct and the organelle-targeted sequence on *gfp* gene expression or number of GFP spots, in oil palm culture was evaluated. The discussion in this section will be based on the number of GFP spots appearing plotted against time, for bombardment of both embryogenic calli and immature embryos, as demonstrated in *Figures 3* and *4*, respectively.

Promoter. Three GFP constructs containing the sGFPS65T gene version driven by three different promoters (CaMV35S, HBT and ubiquitin) were used in oil palm embryogenic calli transformation (*Figure 3)*. It was demonstrated that the CaMV35S promoter

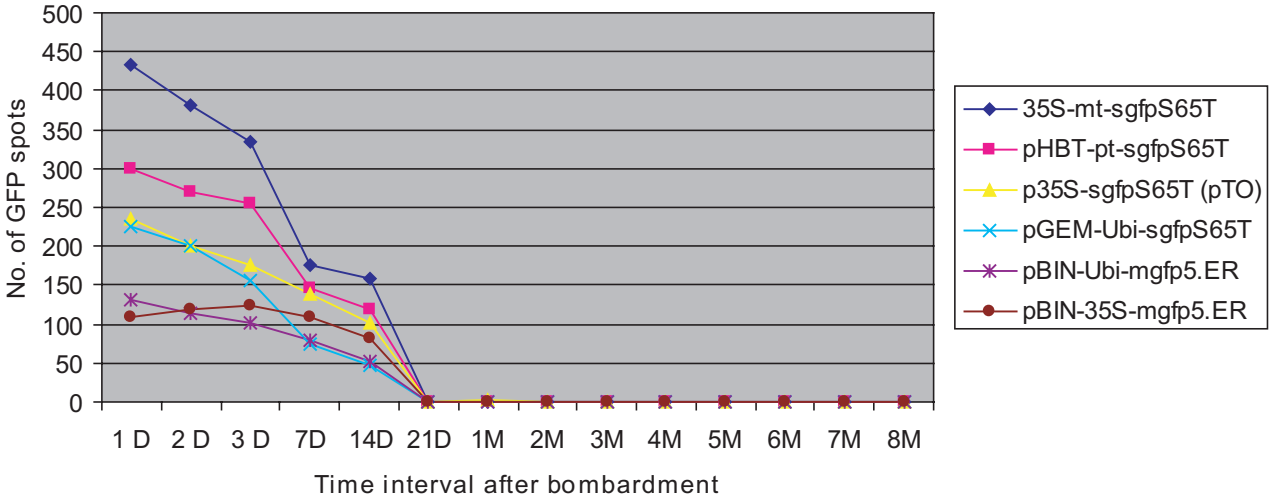

Figure 4. GFP expression pattern of organelle-targeted GFP plasmids in oil palm immature embryos. The expression was based on the GFP spots counted at different times post-bombardment. M: month. D: day. mt – mitochondria, pt – plastid and ER- endoplasmic reticulum.

(p35S-sGFPS65T) showed the highest number of GFP spots. The differences in number of GFP spots produced by the CaMV35S and HBT promoters were not significant as compared to the differences between CaMV35S and ubiquitin and also between HBT and ubiquitin. The HBT promoter is a maize C4PPDK gene promoter, enhanced by addition of a CaMV35S promoter (Sheen *et al.*, 1995). The construct driven by the CaMV35S promoter also demonstrated the longest *gfp* gene expression, of up to eight months. The constructs driven by the HBT promoter showed *gfp* gene expression for up to three months compared to ubiquitin which effect only lasted one week.

The effect of the promoter can also be evaluated by comparing the six constructs with the two *gfp* variants, namely the sGFPS65T and mgfp5 genes. Three constructs, p35S-mt-sGFPS65T, p35S-pt-sGFPS65T and p35S-sGFPS65T, all driven by the CaMV 35S promoter, showed a higher number of GFP spots than pGEM.Ubi1-sGFPS65T, driven by the ubiquitin promoter (*Figure 4*). The CaMV35S promoter also showed longer *gfp* gene expression than the ubiquitin promoter. However, when using the mgfp5 gene variant, the difference between CaMV35S and ubiquitin was not significant. The number of GFP spots and the duration of the *gfp* gene expression were almost the same (*Figure 4*). This finding suggests that the CaMV35S promoter is more efficient than the maize ubiquitin promoter in directing *gfp* gene expression in oil palm tissues.

The suggestion that CaMV35S is a more efficient promoter in oil palm, is in agreement with the findings in *Dendrodium* Sonia 17 orchid (Tee *et al.*, 2003). *Dendrodium* callus bombarded with the sGFP(S65T) gene driven by a CaMV35S, HBT or ubiquitin promoter revealed that the CaMV35S promoter produced the highest number of GFP spots followed by HBT and ubiquitin. In contrast, CaMV35S was a weak promoter in driving the sGFPS65T gene in *Chamaecyparis obsuta* (a conifer) embryogenic calli (Tauguchi *et al*., 2005). In another conifer, *Picea abies*, the ubiquitin promoter was reported as 12-16-fold more active than the CaMV35S promoter in expressing the *gusA* gene in embryogenic calli (Clapham *et al*., 2000). Similarly, when oil palm embryogenic calli were bombarded using the *gusA* gene, the ubiquitin promoter gave a higher number of blue spots than the CaMV35S promoter (Chowdhury *et al*., 1997). The differences in effect by the two promoters in oil palm embryogenic calli were probably due to the differences in the transgenes used, *gfp* versus *gus*A. The two transgenes (*gfp* and *gusA*) may require different transcription factors in oil palm embryogenic calli.

GFP gene variant. Many gfp gene variants have been produced for plant transformation studies. The *gfp* gene can be varied by altering its codon usage and removing its cryptic intron (Stewart, 2001). In this study, four *gfp* variants, namely sGFP(S65T) (Chiu *et al*., 1996), sGFP (Haas *et al*., 1996), mGFP5 (Haseloff *et al*., 1997) and eGFP (Yang *et al*., 1996), were evaluated. Using the CaMV35S promoter, sGFP(S65T) produced a higher number of green spots than mGFP5 and eGFP (*Figure 3*). The sGFP(S65T) also produced more GFP spots compared to sGFP when the genes were driven by the HBT promoter in embryogenic calli. These *sGFP(S65T)*-containing plasmids consistently showed a higher GFP expression than other plasmids, from day one to up to more than five months after bombardment.

In immature embryos, CaMV35S drove sGFP(S65T) (for both organelle-targeted and non-targeted) to produce more GFP spots as compared to mGFP5 (*Figure 4*). Similarly, sGFP(S65T) were superior to mGFP5 when the genes were bombarded into immature embryos under the control of the ubiquitin promoter. It was also shown that the four constructs carrying the sGFP(S65T) gene showed better results compared to the two carrying the mGFP gene. Furthermore, in both experiments, regardless of the promoter and vector used, the sGFP(S65T) variant consistently produce more GFP spots than the sGFP, mGFP5 and eGFP variants. These observations clearly suggest the sGFP(S65T) variant to be the most effective and reliable GFP for transforming oil palm cultures.

The results above are in agreement with findings in sugarcane in which a higher number of spots and brighter fluorescence were observed with sGFP(S65T) than mGFP5 (Elliot *et al*., 1999). It was also suggested that the sGFP(S65T) variant and its derivatives are more suitable for use in monocots, mGFP5 and the wild type gfp gene for dicots (Stewart, 2001). Both oil palm and sugarcane are monocots. However, in canola, eGFP was more efficient than mGFP5 (Cardoza and Stewart, 2003), with the transformation rate 50% higher using the former than the latter.

Surprisingly, in the conifer, *Chamecyparis obutsa*, sGFP(S65T) turned out to be a good reporter gene despite its low expression in *Agrobacterium*-mediated transformation (Tauguchi *et al*., 2005). Similarly, when *Pinus strobus* was transformed using *Agrobacterium* with mGFP5 variant, low expression of the mGFP5 gene was observed (Levee *et al*., 1999). However, when the gene was transformed into other conifers using the biolistic approach, a higher level expression and more GFP spots were obtained (Tauguchi *et al*., 2004). It is postulated that the particle bombardment had introduced higher copies of the transgene and, as a result, a higher intensity of green fluorescence was obtained. This may also be the case for oil palm cultures in which the

sGFP(S65T) gene was demonstrated to be the most efficient GFP variant.

Backbone vector. Comparisons on the effect of the vector backbone on *gfp* gene expression were carried out separately for two target tissues, - embryogenic calli and immature embryos. Six constructs containing the non-organelle-targeted *gfp* gene were used in the bombardment of oil palm embryogenic calli. Two of the constructs, - p35S-sGFP(S65T) and pHBT-sGFP(S65T) produced the highest number of GFP spots (*Figure 3*). Both constructs were based on pUC18 as the backbone vector (*Table 1*). The other four plasmids, namely pHBT-sGFP, pGEM-Ubi-sGFP(S65T), p35S-mGFP (CAMBIA1302) and p35S-eGFP (Clontech) - were constructed using pUC18, pGEM, pCAMBIA and pUC18 as the backbone vector, respectively. Within the sGFP(S65T) variant, the pUC18-based vectors consistently produced higher numbers of GFP spots than those using pGEM as the backbone. However, for the other GFP variants, no comparison could be made because two *gfp* gene constructs were cloned using the same pUC18 backbone. Only one of the constructs carried mGFP5 gene which was cloned within the pCAMBIA backbone.

Bombardment of oil palm immature embryos with the organelle targeted *gfp* gene revealed that the p35S-mt-sGFP(S65T) and HBT-pt-GFP (S65T) constructs produced the highest number of GFP spots (*Figure 3*). *Table 1* again shows that both plasmids were constructed using the plasmid pUC18 as the backbone vector. The next four had plasmids p-sGFP(S65T)(pTO), pGEM-Ubi-sGFP(S65T), pBIN.35S-mgfp5-ER and pBIN.Ubi-mgfp5-ER, incorporated into pTO, pGEM and pBIN backbones, respectively. It was demonstrated again that within the sGFP(S65T) gene, the pUC18-based constructs produced higher numbers of GFP spots than those with pTO and pGEM backbones. However, for the other gene, mGFP5, no comparison could be made since both constructs were cloned using the same backbone, pBIN.

From the above observations, it can be suggested that vectors having pUC18 as the backbone consistently drove higher *gfp* gene expression as that the vectors having other backbones. However, no conclusion could be drawn specifically for pCAMBIA and pBIN, as no comparison could be made due to the lack of vectors to be compared with. In soyabean, the *gfp* gene cloned on a pUC18 backbone showed a delay in losing the GFP spots that with the same gene cloned on a pBIN backbone, i.e. 320 hr for pUC18 and 168 hr for pBIN (Ponnapa *et al*., 1999). The numbers of GFP spots were not significantly different. It is proposed that the delay in loosing the GFP spots by pUC18 was due to the gene having been transformed using the biolistics method which would have occurred more with pUC18 that pBIN binary vector.

Size of construct. The size of the constructs may play an important role in successful DNA delivery and expression in plant cells via particle bombardment. Comparison on the effect of construct size on the number of GFP spots obtained in oil palm cultures was carried out separately for two target tissues, - embryogenic calli and immature embryos. The first experiment involved bombarding oil palm embryogenic calli with six *gfp* gene constructs. Three of the GFP constructs, p35S-sGFP(S65T), pHBT-sGFP(S65T) and pHBT-sGFP - showed the highest number of GFP spots after bombardment (*Figure 3*). From *Table 1*, these were the smallest of the constructs used, only 4.09 kb. The other three plasmids, - pGEM-Ubi-sGFP(S65T), pCAMBIA1302 and p35S-sGFP - were ~6.20, ~4.32 and ~4.50 kb, respectively, and showed fewer GFP spots (*Figure 3*). Generally, within the sGFP(S65T) variant, the smaller plasmids consistently gave a higher number of GFP spots that the larger plasmids.

An experiment involving some organelle-targeted and non-targeted *gfp* genes on oil palm immature embryos revealed that constructs pHBT-mt-sGFP(S65T) and pHBT-pt-GFP(S65T) gave the highest number of GFP spots (*Figure 4*). Both plasmids had the smallest constructs size, - ~4.44 and ~4.29 kb, respectively (*Table 1*). The other four plasmids, - p35S-sGFP(S65T) (pTO), pGEM-Ubi-sGFP(S65T), pBIN.35S-mgfp5-ER and pBIN.Ubi-mgfp5-ER - gave a lower number of GFP spots. They were ~12.65, ~6.20, ~13.20 and ~14.30 Kb, respectively. Within the sGFP(S65T) gene, the smaller plasmids showed higher expression except for the last two plasmids [p35S-sGFP(S65T) (pTO) and pGEM-Ubi-sGFP(S65T)], between which the larger (~12.65 kb) showed more GFP spots than the smaller (~6.20 kb). The difference may be due to the different promoter used. For mGFP5, the smaller plasmids showed higher expression than the larger plasmids.

From the above observations, in general, smaller plasmids produced more GFP spots than larger plasmids. However, in some cases the situation is reversed, possibly be due to a different promoter and vector backbone used. Large plasmids (>10 kb) may be more subject of fragmentation during DNA-gold preparation and transformation, resulting in a lower transient expression (Mendel *et al*., 1989; Fitch *et al*., 1990). Furthermore, in Biolistics transformation, the differences in size may result in a difference in the number of plasmid copies used since the same amount (˜g) of DNA is used in all the bombardments, regardless of the plasmid size. Consequently, more copies of smaller plasmids would have been added to the microcarrier compared to the larger plasmids per µg of the material used. The more copies of plasmids used in Biolistics transformation may also

increase the transgene expression as reported previously in oil palm (Parveez *et al*., 1997). However, to verify this postulation on the effect of plasmid size on the number of GFP spots, a proper set of experiments that using different size constructs on the same *gfp* variant, promoter and enhancers should be carried out.

Organelle targeted. In this study, three *gfp* genes were targeted to three organelles, - plastid (pt), mitochondria (mt) and endoplasmic reticulum (ER). In an earlier experiment, exact localization of the *gfp* genes to the targeted organelle was reported (Na'imatulapidah and Parveez, 2007). Plastid- and mitochondria-targeted sequences were constructed within the p35S-sGFP(S65T) variant construct, while the endoplasmic reticulum-targeted sequence was constructed within the pBIN-mGFP5 variant construct. The oil palm immature embryos bombarded with the sgfp.S65T variant-targeted to the mitochondria [p35S-mt-sGFP(S65T)] showed the highest number of GFP spots, followed by the same variant-targeted to the plastid (p35S-pt-sGFP(S65T) (*Figure 4*). The other two constructs with the same variant but not specifically targeted to any organelle (pGEM-Ubi-sGFPS6T and p35S-sGFPS6T) showed a lower number of GFP spots. This shows that targeting the sGFPS65T variant to a particular organelles increased the transient expression or number of GFP spots on the oil palm tissues used.

Up to now, no comparison between organelle-targeted constructs has been reported. However, targeting the *gfp* gene into plastid and mitochondrial has been done in tobacco, petunia and *Arabidopsis* (Köhler *et al*., 1997a, b; Niwa *et al*., 1999). In rice, plastid-targeted sGFP did not increase the number of GFP spots compared to non-targeting. However, the plastid-targeted GFP resulted in 20-fold more soluble protein compared to the non-targeted gene (Jang *et al*., 1999).

In soyabean the number of GFP spots increased slowly for the ER-targeted mGFP (peaking at 24 hr) compared to non-targeted mGFP which number of GFP spots peaked at around 4 hr post-bombardment (Ponappa *et al*., 1999). It was proposed that the expression of the gene variant in the organelle may require secretion and retention of the protein in the lumen of the ER for proper folding and maturation. Conversely, in sugarcane mGFP5, the variant-targeted to ER showed a lower GFP intensity than the non-targeted mGFP5 (Elliot *et al*., 1999). In oil palm, the ER-targeted seemed to produce fewer GFP spots.

CONCLUSION

Detection of GFP in bombarded oil palm tissues was simple and reliable, especially by using gfp gene constructs driven by the CaMV 35S promoter. However, the expression observed was mainly transient. Stable expression of the gfp gene could be monitored up to eight months after bombardment. This observation showed that gfp gene constructs can direct the transgene expression at least up to the transient expression level. Evaluation of the factors affecting the *gfp* gene expression in oil palm showed that the promoter driving the gene, *gfp* gene variants, backbone vectors, size of constructs and targeting the gene into a specific organelle can influence the *gfp* gene expression. This finding is very useful for future application of the technique for producing transgenic oil palm. An effort in this direction has been initiated.

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