Evaluation of Green Fluorescence Protein (GFP) as a Selectable Marker for Oil Palm Transformation via Transient Expression

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Abstract. Green fluorescent protein (GFP) can be easily visualized under UV/blue light without any additional substrate or co-factor. Its assay is also non-destructive. Therefore, it has been widely used to monitor transgene expression and protein localization in variety of cells and organisms. To evaluate the effectiveness and practicality of GFP as a reporter and selectable marker for oil palm transformation, transient expression of GFP genes in oil palm embryogenic calli and immature embryos were analyzed after bombardment with different types (version) of GFP genes and driven by different constitutive promoters. Some of the GFP genes used were targeted to specific organelles within the cell, namely mitochondria, plastid and endoplasmic reticulum (ER). For transformation using non-targeted GFP genes, plasmid p35S.TYG.GFP showed the highest number of green fluorescent spots, followed by pHBT-sGFPS65T and pGEM.Ubi-sGFPS65T. The results demonstrated that the sGFPS65T gene version is the most active in oil palm. When organelle targeted GFP genes were transformed, localization of the genes into specific organelle was clearly demonstrated using laser scanning confocal microscopy. Using the organelle targeted GFP genes; the sGFPS65T gene version was again demonstrated to be the most active in oil palm cells. Long term transient expression of the GFP genes was also evaluated and is discussed.

Keywords. Transformation, green fluorescence protein (GFP), selectable marker, transient expression, oil palm

INTRODUCTION

Plant genetic engineering is progressing very rapidly since the first success of introducing a foreign gene into a plant via Agrobacterium tumefaciens (Fraley, et al., 1983). Since then the number of transgenic plants produced has increased exponentially. The International Service for Acquisition of Agri-biotech Applications (ISAAA) has recently summarized that the area commercially planted with transgenic plants worldwide has increased almost 53 fold, from 1.7 million hectares in 1996 to 90 million in 2005 (James, 2005). Through genetic engineering, agronomic traits of a particular plant can be improved and furthermore, production of value added products and nutrients can also be obtained. Genetic engineering reduces the time required for introducing a novel trait into plants as compared to conventional breeding. It was postulated for oil palm, up to 80% reduction in time could be achieved for introducing a novel trait through genetic engineering. Furthermore, transgenic plants can be used as bioreactors or bio-factories for producing novel products, such as pharmaceuticals, continuously.

Oil palm is one of the world’s main sources of vegetable oils and fats. Its production per planted area is 3 times and 10 times higher than coconut and soybean, respectively. In Malaysia, crude palm oil production is increasing annually; from 14 million tonnes in 2004 it has increased to 15 million tonnes in 2005, an increase of 7.1% (Basri, 2006). Similarly, the total export volume of oil palm products, such as palm oil, palm kernel oil and oleochemicals has increased by 7.3%, to 18.62 million tonnes in 2005 as compared to 17.36 million tonnes in 2004 (Basri, 2006). It is expected that the rate of production and export of oil palm products will continue to progress in the years to come and it will remain as one of the major sources of vegetable oils and fats to feed the...
world. However, the oil palm industry may face challenges such as the increase of demand over supply, due to the increase in world population, and the competition from other oil producing crops that are far more advanced in the application of genetic manipulations (Parveez, 1998). Due to the long regeneration time, narrow gene pool and open pollination behavior of oil palm (Rajanaidu and Jalani, 1995), improvement of the crop through conventional breeding approach alone is limited. Therefore, genetic engineering is earmarked to face these challenges.

A method for genetic manipulation of oil palm, via microprojectile bombardment, has been established with the production of transgenic oil palms that are resistant to the herbicide Basta (Parveez, 2000). To date, development of transgenic oil palms with novel traits such as high oleic acid, high stearic acid and synthesizing biodegradable plastics are progressing well (Parveez et al, 1999; Parveez, 2003). Nevertheless, further improvement of the genetic transformation method has been given a priority to ensure successful manipulation of oil palm in years to come.

One of the approaches taken to improve the efficiency of oil palm genetic transformation is through the application of a novel selectable marker system. One of the commonly used reporter genes is green fluorescent protein (GFP) (Heim et al, 1994). GFP is derived from jellyfish (Chalfie et al, 1994) and has the potential to be used as a universal reporter gene in living cells. Compared to other reporter genes, it is stable, does not require any substrate/co-factor or any protein assay to produce the green fluorescence (Heim et al, 1994). It is also species independent, which thereby can be used to monitor transgene expression non-destructively in vivo, in situ and in real time.

GFP has been successfully used as a selectable marker to produce transgenic plants such as sugarcane (Elliot et al, 1999), barley (Ahlandsberg et al, 1999), rice (Vain et al, 2000), oat (Kaeppler et al, 2002) and wheat (Jordan, 2000). Vain et al, (2000) also reported that in rice transformation, GFP decreases the amount of tissues handled by a factor of 4 and the time involved by a factor of 2. The above success clearly suggests that the utilization of GFP in other monocots, such as oil palm, may be useful and may subsequently enhance monitoring of transformation efficiency. Evaluation of GFP as a potential selectable marker gene for oil palm transformation was initiated. This paper reports on the transient expression studies of various GFP gene constructs in oil palm cultures.

**MATERIALS AND METHODS**

**Plant materials.** Oil palm embryogenic calli were derived either from calli originating from leaf or cabbage on medium containing MS salts (Murashige and Skoog, 1962) + Y3 vitamins (Eeuwans, 1976) + 0.1 g/l myo-Inositol and L-glutamin + 3% sucrose + 5 x 10^{-3}M, 2, 4-D + 0.25% activated charcoal + 0.7% agar and incubated at 28°C in the dark (Parveez, 1998). Any callus formed was subcultured every four weeks onto the same medium until embryogenic calli were formed.

Oil palm immature embryos were obtained from a Tenera variety at 11-12 weeks after anthesis. Callus and embryogenic calli were produced from the immature embryos on the medium containing Y_{macro}, micro nutrient and vitamin, + 0.05% (w/v) cystein + 0.5% (w/v) polyvinyl pyridolone (PVP) + 0.3% (w/v) activated charcoal + 5 X 10^{-3}M 2,4-D + 0.22% (w/v) gelrite (Gibco-BRL) and incubated at 28°C in the dark. Any callus formed was subcultured every four weeks onto the same medium until embryogenic calli were formed.

**GFP Gene Constructs.** The following GFP gene constructs were used in this study: p35S|CaMV-Ω-mt-sgfpS65T, p35S|CaMV-Ω-pt-sgfpS65T (Niwa et al, 1999), pB|IN|Ubi-mgfp5-ER, pB|IN|35S-mgfp5-ER, pGEM|Ubi-sgfpS65T, pTO134 (Elliot et al. 1998 & 1999), pHBT-sgfp, pHBT-sgfpS65T, p35S|CaMV-sgfpS65T (Sheen et al. 1995), p35S-mgfp (Clontech, US) and pCAMBIA 1302 (Dr. Richard Jefferson, CAMBIA, Australia). The first four gene constructs carry GFP gene which are targeted to a specific organelle. p35S|CaMV-Ω-mt-sgfpS65T has the GFP gene targeted to mitochondria (mt), p35S|CaMV-Ω-pt-sgfpS65T is targeted to plastid (pt) and finally pB|IN|Ubi-mgfp5-ER and pB|IN|35S-mgfp5-ER were targeted to endoplasmic reticulum (ER). The last seven GFP gene constructs were not targeted to any organelle. Detailed schematic representations of the constructs are given in Figure 1.

**Large Scale Plasmid Isolation.** One millilitre of overnight *E. coli* culture containing the desired GFP gene construct was inoculated into 500ml of LB medium (5gm NaCl, 5gm tryptone and 2.5gm yeast extract) containing 75μg/ml of antibiotic (ampicillin). The overnight culture was transferred into large centrifuge bottles and the cells were pelleted by centrifugation (6,000g, 10min and 4°C). DNA isolation was carried out using the QIAGEN Maxiprep kit. The supernatant was removed and the cell pellets were resuspended in 10 ml of Buffer P1. The mixture was vortexed until no cell clumps remained. 10 ml of Buffer P2 was added and mixed gently by inverting 4-6 times and incubated for 5 minutes at room temperature. 10 ml of chilled Buffer P3 was added slowly, mixed and left on ice for 20 minutes. The mixture was centrifuged at 20,000g for 40 minutes at 4°C. While centrifuging, a QIAGEN-tip 500 was equilibrated by allowing 10 ml of QBT buffer to flow through the resin by gravity. The supernatant from the GSA bottle was loaded into the column promptly. The QIAGEN-tip was washed twice with 30 ml of Buffer QC. A 30 ml SS48 centrifuge tube was placed below the tip and the DNA was eluted using 15 ml of Buffer QF. A total of 10.5 ml of chilled isopropanol
was added and the mixture was incubated at 4°C for 30-60 minutes. The mixture was centrifuged at 20,000 g for 40
minutes and the pellet obtained was washed with 5 ml of
70% (v/v) ethanol. The tube was centrifuged at 20,000 g
for 10 minutes and the pellet DNA was air dried in laminar
flow. Finally, the DNA was dissolved in 1 ml of TE buffer
(10 mM Tris, 1 mM EDTA, pH 8).

The concentration and purity of the plasmid was
determined using a spectrophotometer. The DNA yield
ranged from 300 to 500 µg per 100 ml of overnight culture
with good purity A260/280 i.e. 1.8-2.0 was obtained. The
DNA quality was further verified by restriction digests
followed by electrophoresis on 1% agarose gels.

**DNA microcarrier Preparation and Bombardment for
PDS-1000/He Apparatus.** DNA precipitation onto gold
microcarriers was carried out according to manufacturer’s
instructions for the Biolistics PDS/He 1000 (Bio-Rad)
device. Five microlitre of DNA solution (1µg/µl), 50µl of
CaCl₂ (2.5M) and 20µl spermidine (0.1M, free base form)
were added sequentially to the 50µl gold microcarrier
suspension. The mixture was vortexed for 3 minutes, spun
for 10 second in a microfuge and the supernatant was
discarded. The pellet was washed with 250µl of absolute
ethanol. The final pellet was resuspended in 60µl of absolute
ethanol. Six microlitre of the solution was loaded onto the
centre of the macrocarrier and was air dried.

For each GFP plasmids, bombardments were carried on
a minimum of five replicates. For bombardment of oil palm
embryogenic calli, five replications were carried out and for
bombardment of immature embryos, 10 replicates were used.
Bombardment were carried out at the following conditions;
1100 Psi rupture disc pressure; 6mm rupture disc to
macrocarrier distance; 11mm macrocarrier to stopping plate
distance, 75mm stopping plate to target tissue distance and
67.5 mmHg vacuum pressure (Parveez, 1998). Two controls
were also incorporated i.e. tissues without bombardment
and bombardment using microcarriers without DNA. The
bombarded tissues were then incubated at 28°C in the dark
prior to GFP transient expression evaluation.

**Visualization of GFP and fluorescence microscopy.**
Visualization of GFP fluorescence on bombarded oil palm
calli was carried out using a Leica MZ12.5 stereomicroscope
with fluorescence GFP Plus filter module (Leica Microscopy
and Scientific Instruments, Switzerland), at several time
intervals i.e. everyday for the first 2 weeks and followed
by once a month. The number of GFP-fluorescent spots
was counted. Autofluorescence was differentiated and
eliminated by repeating the above on non-bombarded tissues.
A narrow bandpass interference filter (S550/100 NP) was
used to minimize chlorophyll interference. Images of GFP-
expressing cells were captured using a 4910 Series RS-170 &
CCIR monochrome CCD camera (COHU, San Diego, CA)
and analyzed using Leica IM50 Image Manager. The images were further processed and stored using Adobe Photoshop 3.0 software (Adobe System Incorporated, USA). The green level of the images was then analyzed to quantify the green fluorescent intensity using Leica QWin Pro (Leica System, Germany).

Bombarded calli were also imaged with a Bio-Rad MRC 1024ES Confocal Imaging System, Argon, Cripton, Laser (488/ 568/ 637 nm) (Australia) using a standard filter set (excitation at 488 nm, emission at HQ 515/30 nm for GFP: excitation at 543 nm and emission at Em570 LP nm for red). The green and red channels were imaged at the same time, but saved as separate images. Optical sections were taken along the optical axis and projected into a single image. Final merging of images was performed using LaserSharp 2 000 software (Bio-Rad, Australia). The slide with samples bombarded with mitochondrial targeted GFP plasmid was further stained with MitoTracker Red CMXRos (excitation at 578 nm, emission at 599 nm).

RESULTS AND DISCUSSION

Visualization of GFP in oil palm tissue. Each GFP plasmids were bombardments with a minimum of five replicates for oil palm embryogenic calli and 10 replications for immature embryos. No green fluorescence protein (GFP) spots were detected on the bombarded oil palm calli within the first 7 hours after bombardment. However, GFP spots were easily detected 16 hours post bombardment using the Leica MZ12.5 fluorescent microscope, even with a magnification of as low as 10X (Figure 2). Figure 2a distinguishes green fluorescence spots from non-GFP spots on bombarded calli. GFP spots were also detected on bombarded immature embryos (Figure 2b). The presence of GFP spots indicates successful transfer of the GFP gene into oil palm tissues. However, detection of GFP 16 only hours post bombardment is considered as slow when compared to bombarded orchid calli where GFP expression could be detected as early as 2 hours after bombardment (Tee et al., 2003). As both observations were based on bombardment using the same plasmid, the difference in GFP gene expression time may be due to the response of recipient cells to the transgene.

Confocal laser scanning microscopy (CLSM) was used to determine the exact location of the GFP gene expression within the transformed cells. Subcellular distribution of GFP spots derived from bombardment using targeted and non-targeted GFP gene were observed within 5 days post-bombardment. Prior to CLSM, the Leica MZ12.5 fluorescent microscope was used to locate GFP fluorescing tissues. CLSM results showed that localization of the targeted and non-targeted GFP were all within the expected locations (Figures 3 and 4). Figure 3a shows TYG.GFP from non-targeted plasmid located on membrane of cell surfaces and within the nuclei, indicating that GFP was distributed throughout the cytoplasm with a tendency to accumulate in nucleoplasm. Moreover, it was not detected in vacuoles (Figure 3c). Similar observations were reported in other plants (Chiu et al., 1996; Elliott et al., 1998; Haseloff et al., 1997; Ponappa et al., 2000 and Tee et al., 2003). GFP could diffuse passively into the nucleoplasm (Jang et al., 1999) because it is a small (27 kDa) monomer (Yang et al., 1996). It is smaller than the diffusional exclusion limit of nuclear pores (Gorlich and Mattaj, 1996). Figures 3 (3b & 3d) show the endoplasmic reticulum targeted GFP (mGFP5-ER) distributed outside the nucleus, validating endoplasmic reticulum localization of GFP in oil palm cells. Similar localization of mGFP-ER was reported by Haseloff et al., (1997).

Plastid- and mitochondria-localization of TYG.GFP from p35S-pt-sGFP(S65T) and p35S-mt-sGFP(S65T) bombarded oil palm calli, respectively, are illustrated in Figure 4. Chloroplast were detected in the channel (a) due to chlorophyll autofluorescence. Figure 4a(i) shows green fluorescent spots indicating the localization of GFP. No fluorescence was detected on untransformed cells under the green channel (not shown). Figure 4a(ii) shows the overlay of red autofluorescence (chlorophyll) and GFP that appeared yellowish green, which represented co-localization of green-plastid in the presence of GFP. Therefore, without employing specific staining, green plastid containing GFP could be visualized in p35S-pt-sGFP(S65T) bombarded oil palm calli. In addition, images from merged channel and light microscope mode [Figure 4a(ii) and a(iii)] clearly demonstrate the distribution of green fluorescence in a distinct granular appearance throughout the cytoplasm and aside from nucleus, vacuoleous or other cytoplasmic compartments. These observations confirm plastid localization of the GFP. Similar localization was observed in Arabidopsis cells transformed with p35S-pt-sGFP(S65T) (Niwa et al., 1999).

Finally, localization within mitochondria was identified using MitoTraker Red that stains mitochondria to red. Thus, co-localization of GFP [green channel b(i)] and red mitochondrial staining (b) confirmed the actual location of the mitochondrial-targeted GFP within transformed cells. Figure 4b(ii) shows the merged image of oil palm calli expressing mitochondria targeted GFP and demonstrates the subset of green and red fluorescence which appeared as green yellowish fluorescence. This confirmed the exact location of mt-sGFP(S65T). However, the fluorescence was in excess in the nucleoplasm and this was suspected to be due to over staining or as a result of broken cells.

CLSM images of bombarded embryogenic calli illustrated in Figures 3 and 4 evidently revealed correct localization of the targeted and non-targeted GFP. This strongly indicates that the detection of GFP was consistent. Therefore, gene delivery as well as detection of GFP in oil palm transformed calli using fluorescent stereomicroscopy was reliable.
Expression of non-targeted and targeted-GFP in oil palm tissue. Expression patterns of GFP from non-targeted and targeted plasmids was evaluated. Six GFP gene-containing constructs were transformed into oil palm embryogenic calli. The number of GFP-expressing cells were counted and plotted against time (Figure 5). On the first day after bombardment, the average number of GFP-expressing cells was less than 200 for p35S-GFP, pCAMBIA 1302, pGEM-Ubi-sgfpS65T and pHBT.sgfp. However, embryogenic calli bombarded with plasmids HBT-sgfpS65T and 35S-sgfpS65T had an average number of GFP-expressing cells between 600 to ±800. These sGFP(S65T)-containing plasmids consistently showed higher GFP expression compared to the other plasmids, from the first day after bombardment up to more than 5 months after bombardment. Moreover, the 3 plasmids showing the highest number of GFP expressing cells in both experiments were plasmids carrying the sGFP(S65T) version of GFP. This suggests that the sGFP(S65T) variant of GFP gene may be the most efficient in oil palm cultures, compared to sGFP or mGFP5.

The high level of GFP expression obtained from bombardment using the sGFP(S65T) gene is possibly due to the particular codon usage in the construct, which was suggested to enhance higher level of protein synthesis in Arabidopsis plants (Haseloff et al., 1997; Chiu et al., 1996). The sGFP(S65T) gene also has its S65T chromophore mutated (SYG at position 65 was mutated to TYG) which induces rapid chromophore formation and enhances fluorescent signal (Heim et al., 1994). The sGFP(S65T) gene, with the above advantage, has been reported to have broad applications in plants, such as in detection of weak promoter activity, visualization of protein targeting into nucleus or plastids, and in analysis of signal transduction pathways in transgenic plants or other living cells (Chiu et al., 1996).

Surprisingly the sGFP(S65T) gene driven by the 35S CaMV promoter showed higher numbers of GFP expressing cells...
Figure 4. Visualization of subcellular location of organelle-targeted sGFP(S65T) in oil palm calli. Confocal microscopic images of transformed calli cells expressing plastid (a) and mitochondria (b) targeted GFP gene. From left to right, red channel for chlorophyll (autofluorescence), green channel for GFP (green), merged image of red and green channels, and transmission image. The yellow or yellowish green color in the merged image indicates co-localization of GFP in the chloroplasts (a) or mitochondria (b). b, transformed calli treated with mitoTraker red dye.

as compared to the Ubiquitin promoter. This is in contrast to earlier observations where the Ubiquitin promoter gave higher gus A gene expression in oil palm as compared to the 35S CaMV promoter (Chowdhury et al., 1997). Nevertheless, HBT, a chimeric promoter also showed a higher GFP expression than Ubiquitin promoter. This might be due to the fact that HBT promoter, which is based on maize C4PPDK gene, has an addition of 35S enhancer that makes the expression much stronger (Sheen et al., 1995).

Within 2 weeks after bombardment, the number of GFP-expressing cells started to reduce for most of the plasmids. However, for plasmids HBT-sgfpS65T and 35S-sgfpS65T, the average number of GFP-expressing cells was highest on the second day (600 to ±800), but became lower (400-600) on the third day after bombardment. The highest number of GFP-expressing cells for sgfp(S65T) gene version was on the second day after bombardment. This might suggest the highest transient expression could occur on the second day, which was observed earlier in oil palm transient expression using the GUS gene (Parvez, 1998), and also in other plants such as orchid (Tee et al., 2003), barley (Carlson et al., 2001) and soybean (Ponappa et al., 2000). Furthermore, reduction in the numbers of GFP-expressing cells continued over the first 2 weeks and in most cases, reached zero by the end of the second week after bombardment. Therefore, it was assumed that most of the earlier GFP-expressing cells were actually a result of transient expression of the GFP gene.

When immature embryos were bombarded with GFP gene constructs (both targeted and non-targeted), the number the cells expressing GFP reduces over time (Figure 6). The reduction in the number of cells expressing GFP was observed to be almost similar to the reduction rate for embryogenic calli bombarded with non-targeted GFP gene constructs. Even though the rate of GFP expressing cells reduction varies from one construct to another, it was observed that almost all the cells expressing GFP disappeared after 3 weeks post-bombardment. Irrespective of the type of GFP gene constructs used (organelle targeted or not targeted), the number of cells expressing GFP was reduce to almost zero within 3 weeks.

Figure 6 reveals that p35S-mt-sGFPS65T had the highest average of cells with GFP spot count, followed by pHBT-pt-sGFPS65T and pTO134. pBIN-Ubi-mGFP5-ER and pBIN-35S-mGFP5-ER had the lowest number of cells with GFP spot, while pGEM-Ubi-sGFPS65T showed more drastic decrease in GFP spot within the first 2 weeks post-bombardment compared to the other GFP gene constructs. Regardless of promoter and vector used, sGFPS65T-containing GFP gene constructs consistently showed the highest GFP gene expression. Therefore, GFPS65T expression was higher than mGFP5, while 35S and HBT promoters were stronger than Ubiquitin promoter. This was consistent with observations made in transformed embryogenic calli.
Figure 5. GFP expression pattern of non-targeted plasmids in bombarded calli. The expression pattern was measured based on numbers of cells with GFP spots that counted at different time intervals after bombardment. D: day, W: week and M: month. The highest numbers of green fluorescent spots occurred 2 day after bombardment for 35S.sGFP(S65T). Y-axis showed the highest number cells expressing GFP.

Figure 6. GFP expression from mitochondrial & plastid-targeted sgfp.S65T, non-targeted sgfp.S65T and ER-targeted mgfp5 plasmids in bombarded immature embryos at different time intervals of post-bombardment. Y-axis showed the highest number cells expressing GFP.

CONCLUSION

Detection of GFP in bombarded oil palm tissue was simple and reliable, especially when using GFP gene constructs driven by the 35S CaMV promoter. However, the expression observed was mainly transient in nature. Stable expression of the GFP gene could be monitored up to 8 months after bombardment. This initial observation shows that GFP gene constructs could direct the transgene expression at least up to the transient expression level. In order to evaluate the stable expression of the GFP genes constructs in transgenic oil palm, regeneration of the transformed calli and immature embryos needs to be carried out. Further molecular analysis to confirm the integration of the transgene in oil palm cultures also needs to performed.

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