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Original Article

Determination of the most efficient target tissue and helium pressure for biolistic transformation of oil palm (*Elaeis guineensis* Jacq.)

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Abstract

An efficient genetic transformation system for oil palm using particle bombardment was established. The transformation was performed using the pCAMBIA 1302 DNA which contains the green fluorescent protein (*mgfp5*) reporter gene and the selectable marker hygromycin phosphotransferase (*hph*) gene. Oil palm explants were bombarded under the following conditions: rupture disk to macrocarrier distance, 11 mm; macrocarrier to target tissue, 90 mm and using 1 μm gold particles as microcarrier. Four different pressures of helium were tested with three types of target tissues (mature embryo, embryogenic callus and young seedlings). From the transformation efficiency, calli were much more efficiently transformed in the biolistic process compared with mature embryos and seedlings. A 100% transformation efficiency for DNA delivery into callus oil palm explants was obtained at 850 psi helium pressures, for embryos a maximum 81.8% efficiency required 850 psi and for seedlings a maximum 75.9% efficiency required 1,550 psi. Using a confocal laser scanning microscope, and appropriate filters to block out the red fluorescence of chlorophyll, expression of the GFP gene was observed in all three bombarded explant types by a bright-green fluorescence. The *mgfp5* gene was still present more than 8 months after bombardment, hence it indicated the stability of transgene in those transformants.

Keywords: *Elaeis guineensis*, helium pressure, biolistic transformation, GFP

1. Introduction

The oil palm (*Elaeis guineensis* Jacq.), belongs to the plant Family Arecaceae, and is one of the most economically important crops in Thailand. The palm oil industry will continue to grow because of the many products derived from palm oil**.** In order to improve crop productivity and to add value to their products, genetic engineering, which has made possible a freer movement of genetic materials from one organism to another, could be used to produce better oil palms. The biolistic process, first reported by Sanford *et al*. (1987), was initially chosen as a method for oil palm transformation as it has been the most successful method for transforming monocotyledons to date (Parveez *et al.*, 2000; Majid and Parveez, 2007). The basic principle of the biolistic method is to use high velocity microprojectile particles coated with DNA to penetrate the outer tissue layers in order to introduce the genetic material into the cytoplasm of living cells, which then, hopefully, survive and may express the introduced gene. The gene for the green fluorescent protein (GFP) is an attractive reporter for non-destructive monitoring and is used to confirm the possibility of a successful transformation (Ahlandsberg *et al.*, 2001; Stewart, 2001). However, achieving high rates of DNA expression will initially require optimization of the parameters involved in transformation. Studies on optimizing the physical and biological parameters and promoters involved in assaying genetic transformation events have been reported for oil

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palms (Abdullah *et al.,* 2005; Chowdhury *et al.,* 1997; Parveez, 2000; Parveez *et al*., 1997; 1998; Ramli and Abdullah, 2003; Sanford *et al*., 1987). To date, a project of the Palm Oil Research Institute of Malaysia (PORIM) is to increase the level of unsaturation in palm oil for the synthesis of thermoplastics. In addition, the Malaysian Palm Oil Board (MPOB) and the Universiti Kebangsaan Malaysia (UKM) have been conducting research into the use of transgenic oil palm plants to improve oil quality, herbicide tolerance, insect resistance, and fungal resistance (Hashim *et al*., 2002). Consequently, several useful genes from such as *Bacillus thuringiensis* (*Bt*), cowpea trypsin inhibitor (*CpTI*), chitinase, ribosome-inactivating protein (*RIP*) have been successfully engineered into oil palm with transgenic plants produced (Abdullah *et al*., 2003; Lee *et al*., 2006). However the transformation efficiency was still rather low (Abdullah *et al.,* 2005).

Methods for the genetic engineering of oil palms will have to be developed in order to face future challenges in the field of agro-biotechnology. Accordingly, the ultimate goal of this research is to improve the biolistic transformation and selection efficiency in oil palm explants to achieve stable gene transformations.

2. Materials and Methods

2.1 Plant materials

Zygotic mature embryos and germinated seedlings from embryos of oil palm (*Elaeis guineensis* Jacq.), cultivar *Tenera*, were cultured on Murashige and Skoog (MS, 1962) medium in the absence of a growth regulator. Simultaneously, to establish callus cultures, oil palm embryos were cultured on MS medium supplemented with 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Patcharapisutsin and Kanchanapoom, 1996). All cultures were incubated at 25$\pm$2^{o}C air temperature in a culture room with a 16 h photoperiod and subcultured onto fresh medium every month. *In vitro* cultures, at least 3 months old are required to produce complete plants and calli for the bombardment experiments. After bombardment, calli were cultured on regeneration media.

2.2 Biolistic transformation experiments

One day prior to bombardment, explants were subcultured to fresh medium to obtain the highest frequency of stable transformants (Parveez *et al*., 1997). In the bombardment process, the plasmid DNA was precipitated onto gold particles (1.0 mm diameter) and bombarded according to the protocols supplied for the Biolistic PDS-1000/He particle delivery system (BioRad, USA) using the conditions: rupture disk to macrocarrier distance, 11 mm; macrocarrier to target tissue, 90 mm. In the experiment a constant vacuum of 27 mm Hg was used, and the effect of 850, 1,100, 1,300 and 1,550 psi bombardment pressures were tested. Treatments were replicated three times.

Dishes containing unbombarded tissues and some bombarded with only gold particles were used as controls. The explants (embryos, seedlings and calli) were bombarded with a pCAMBIA 1302 plasmid (CAMBIA, Australia), consisting of the hygromycin phosphotransferase (*hpt*) gene as the selective marker and the green fluorescent protein (*mgfp5*) as the reporter gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

2.3 Selection of transformed explant

Transformed explants were selected by using the selection agents for oil palm which have been determined previously (Abdullah *et al.,* 2005). Explants were exposed to culture medium containing 50 mg/L hygromycin (Hyg) and subcultured onto fresh medium under selection every month. Bombarded tissues were subjected to Hyg selection for 2 cycles and the percentage of transformation efficiency was calculated. The resistant explants were subsequently transferred to fresh callus maintenance medium or to plant regeneration medium.

2.4 Microscopic detection of GFP

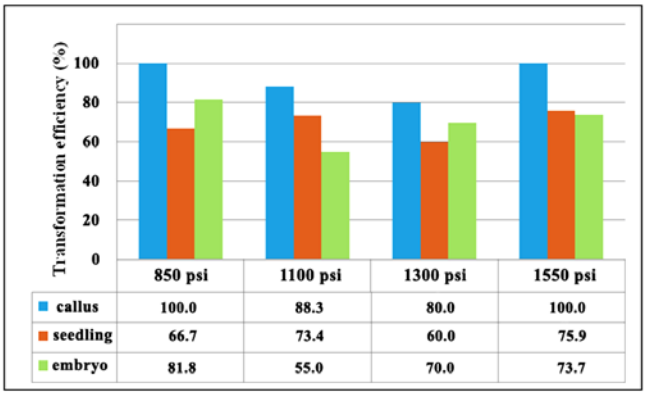
The expression of the *mgfp5* gene was monitored in embryos, seedlings and calli at 3 days, 1, 2, 3, 4 weeks 2, 3, 4, 5 and 6 months after the bombardment and photographed with a confocal laser scanning microscope (FV300, Olympus) fitted with GFP filter set for excitation between 455 and 490 nm and emission above 515 nm. Importantly, the difficulty of detecting GFP in chlorophyll fluorescent tissues was partly overcome by subjecting embryos and seedlings to dark conditions, overnight, prior to monitoring.

3. Results

3.1 Selection of appropriate target tissues and helium pressure levels

Embryogenic calli and seedlings, 1 day after subculture, or freshly mature embryo cultures, were bombarded with plasmid pCAMBIA1302 DNA. Bombardment without the pCAMBIA1302 vector were included in all experiments as a control. After being bombarded and kept on callus maintenance medium for 3 days, the cells were transferred to callus maintenance medium containing 50 mg/L Hyg. Four weeks after selection, Hyg-resistant explants continued to grow while no resistant explants were observed on control plates in any experiment. The control explants became black and died within 1 month after bombardment. Consequently, the percentages of survival explants were calculated 2 months after subculturing into the Hyg culture medium.

The transformation efficiencies obtained with mature embryos, embryogenic calli and seedlings as target tissues were compared (Figure 1). In only 2 sets of experiments, we obtained a 100% transformation efficiency but the average



	850 psi	1100 psi	1300 psi	1550 psi
callus	100.0	88.3	80.0	100.0
seedling	66.7	73.4	60.0	75.9
embryo	81.8	55.0	70.0	73.7

Figure 1. Comparison of mature embryo, embryogenic callus and seedling as target tissues used to produce transgenic oil palm plants. The patterns designate different experiments (3 replicates for each experiment).

total percentage of resistant explants was closer to 77%. The complete 100% survival of Hyg resistant explants was obtained in the bombarded callus using 850 and 1550 psi helium pressure. However, the transformation efficiency using helium pressures of 1100 and 1300 psi for callus bombardment were high but limited only 80.0-88.3%.

For seedlings, transformation efficiency was over a limited range of 60.0 to 75.9% at the different helium pressures with the highest number of surviving seedlings recovered after bombardment at 1,550 psi. For embryos, the transformation frequency was over the range of 55.0 to 81.8% with the highest viability of embryos after bombardment at 850 psi. The average total of resistant explants from three separate experiments was 92% for callus, 69% for seedlings and 70% for embryos. The resistant calli were subsequently transferred to fresh callus maintenance medium or to plant regeneration medium.

3.2 Visualization of GFP expression

GFP-derived fluorescence was observed in calli, embryos, leaves and roots (Figure 2) Transformed explants expressed bright-green fluorescence with the *mgfp5* construct. None of the control explants, fluoresced with the green spots. High levels of GFP were therefore expressed in all cultured oil palms.

4. Discussion

4.1 Determination of target tissues and helium pressure

When cultured on the Hyg selection medium, all the explants bombarded with only gold particles or unbombarded (used as controls) died. This result confirmed the reliability of the selection medium i.e., only transformed tissue survived. In contrast, tissue bombarded with the pCAMBIA vector at all helium pressure levels produced a 55-100% survival of Hyg resistant explants. These survival percentages of explants in the selective medium were used to monitor the transformation efficiency. The lowest transformation percentages of 55 and 60%, for embryo and seedling, respectively were too low. However, a satisfactory 75.9% transformation efficiency was obtained for seedling explants at 1,550 psi helium pressure. This result supports the previous experiments of Ramli and Abdullah (2003), who found the highest GFP expression in oil palm roots when bombarded at 1550 psi. In contrast, the highest transformation efficiency of 81.8% was obtained for embryos that were produced after bombardment at 850 psi. This was also in accordance with previous reports on transformation of oil palm utilizing immature embryos showed that the bombardment at 900 psi helium pressure was the best because it produced less damage and was amenable to both direct gene transfers (Lee *et al.*, 2006) and *Agrobacterium*-mediated gene transfer (Abdullah *et al.,* 2005). By far the best transformation efficiency of 100% was obtained from callus explants after bombardment at 850, however the high efficiency were also observed in 1,100, 1,300 and 1,550 psi bombardment pressures.

From these data, it is possible to conclude that the mature embryo and young seedling is not as suitable for gene transformation as the callus. Callus bombarded at 850 and 1550 psi is suitable for efficient biolistic transformation. This condition with an oil palm callus should become the standard protocol for biolistic transformation of oil palm, as the 100% efficiency obtained here is a significant improvement on all previously attempted oil palm gene transformations.

Furthermore, in the work of Lee *et al.* (2006), antibiotic selection was not used, even though their construct contained the *hptII* gene. The additional stress inflicted by dying cells that might reduce the nutrient supply to transgenic cells or by producing toxic compounds that would further impede the proliferation of transgenic cells and their differentiation into transgenic plants, is the reason why an antibiotic selective medium was not used in the experiment (Ebinuma *et al.,* 2001).

4.2 Detection of GFP expression analysis

Because GFP was part of the pCAMBIA 1302 plasmid, it was relatively easy to establish if transformation had occurred or not by looking for the green fluorescence. Bombarded calli, embryos and seedlings were separated into leaf and root parts and all showed many bright green spots from the GFP fluorescence inside the cells compared to the controls. Normally, the brightness of fluorescence was maintained at full intensity during the subculture. This indicated that explants and their plasmid were able to proliferate under Hyg selective conditions. However, the red autofluorescence of the chlorophyll interacted with the green fluorescence of the GFP in order to make the plants transformed with *Gfp*, appeared yellow under UV light. The use of an appropriate yellow or orange filter blocked the emitted red fluorescence

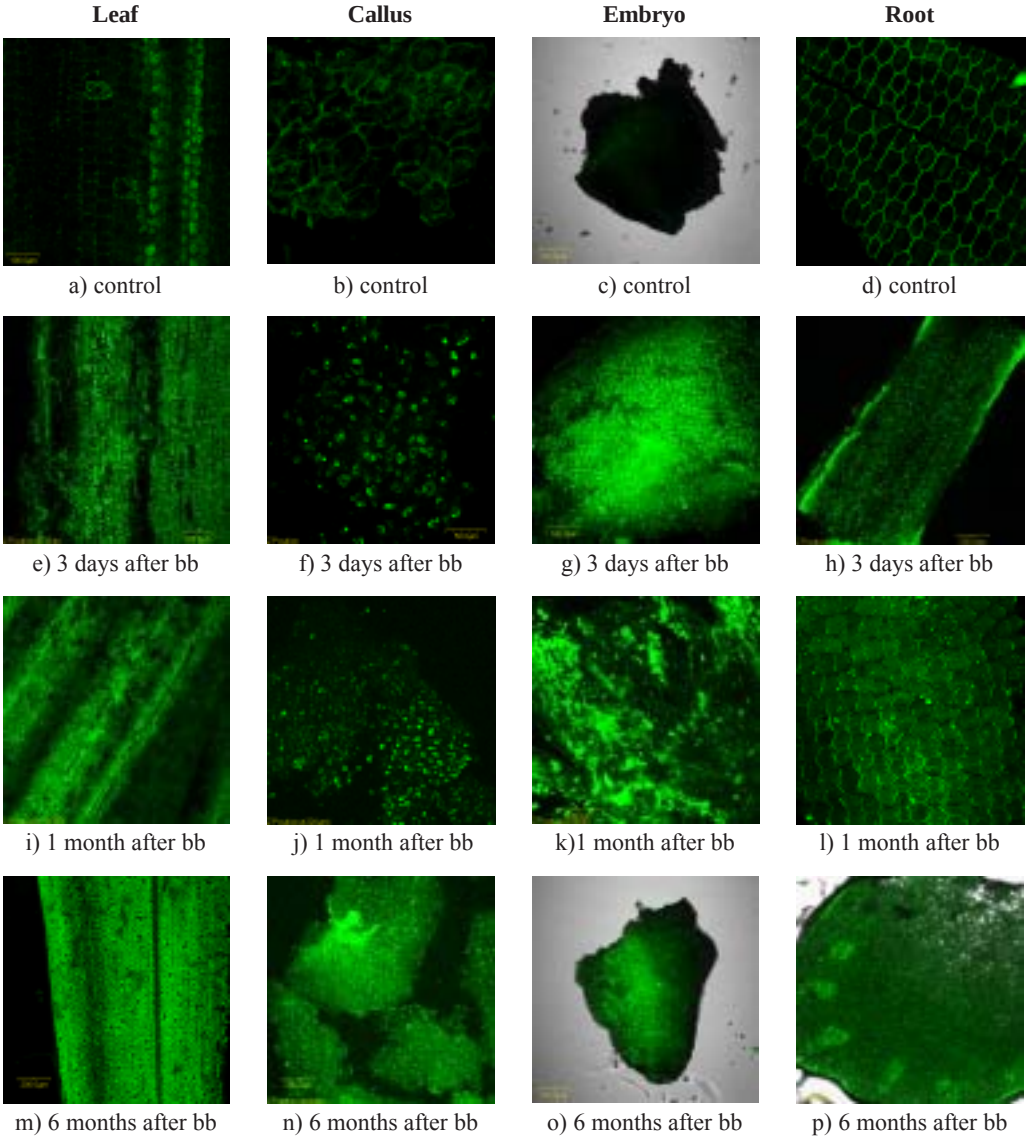


Figure 2. GFP Expression in transformed oil palm explants compared to the control (a-d), transformed explants 3 days after bombardment (e-h), transformed explants 1 month after bombardment (i-l), and transformed explants 6 months after bombardment (m-p) show many bright green spots under a confocal laser scanning microscope. bb: bombardment.

to allow the identification of the transformed plants as those expressing green fluorescence (Elliott *et al.*, 1999). By preventing chlorophyll fluorescence in leaves, GFP fluorescence could be easily detected, however, roots did not require the use of a filter to detect the green fluorescence. In addition, the autofluorescence (red) could be screened out under macroscopic conditions, hence the oil palm leaves could be screened without interference from non-transformed tissue. Thus, the GFP-based visual selection has made it possible and simple to detect and select transgene-carrying tissues.

The ability of the above tissues to express GFP activities more than 8 months after bombardment further indicated that the transgene had been successfully and stably integrated into the genome of the putative transformants (Majid and Parveez, 2007). Moreover, further monitoring of the GFP reporter gene in the regenerating plants is now being investigated in order to evaluate the stable expression of the GFP reporter gene in bombarded oil palm. In addition, the stable integration of the gene after integrating into the cells of oil palm plants has been proved via PCR analysis. Cells with fluorescence from long term surviving tissue will later be subjected to Southern blot analysis to further confirm the integration and copy number of the transferred gene. Further research will continue to investigate the regeneration rate in transformed callus of oil palm and improve the transformation and selection process so that transformation technologies will become more robust.

In conclusion, embryogenic callus is the best target tissue for biolistic transformation of oil palm when compared to mature embryos and young seedlings. The transformation strategy described in this manuscript using bombardment at 850 psi was efficient at producing transformed calli. The

presence of the *mgfp5* gene was detected in all explants by CLSM. For stable assays, the presence of transgenes in the hygromycin resistant explants was confirmed by PCR. It indicated that the transgene was integrated into the genome of the transformants. In the future, we expect that this method will make it possible to transfer a gene that will improve the value of oil palm varieties.

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References

Abdullah, R., Zainal, A., Heng, W.Y., Li, L.C., Beng, Y.C., Phing, L.M., Sirajuddin, S.A., Ping, W.Y.S. and Joseph, J.L. 2005. Immature embryo: A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. Electronic Journal of Biotechnology. 18, 24-34.

Abdullah, R., Chari, C., Yap, W.S.P. and Yeun, L.H. 2003. Transgenic Oil Palm With Stably Integrated *Cpti* Gene Confers Resistance To Bagworm Larvae. *In*: I.K. Vasil, editor. Plant Biotechnology 2002 and Beyond: A Celebration and A Showcase. Kluwer Academic Publishers. pp. 163-165.

Ahlandsberg, S., Sathish, P., Sun, C. and Jansson, C. 2001. A set of useful monocotyledon transformation vectors. Biotechnology Letters. 23, 1871-1875.

Chowdhury, M.K.U., Parveez, G.K.A. and Saleh, N.M. 1997. Evaluation of five promoters for use in transformation of oil palm (*Elaeis guineensis* Jacq.). Plant Cell Reports. 16, 277-281.

Ebinuma, H., Sugita, K., Matsunaga, E., Endo, S., Yamada, K. and Komamine, A. 2001. Systems for the removal of selection marker and their combination with a positive marker. Plant Cell Reports. 20, 383-392.

Elliott, A.R., Campbell, J.A., Dugdale, B., Brettell, R.I.S. and Grof, C.P.L. 1999. Green-fluorescent protein facilitates rapid in vivo detection of genetically transformed plant cells. Plant Cell Reports. 18, 707-714.

Hashim, M., Osman, M., Abdullah, R., Pillai, V., Abu, Bakar, U.K., Hashim, H. and Daud, H.M. 2002. Research and development of transgenic plants in Malaysia: an example from an Asian developing country. Food and Nutrition Bulletin. 23, 367-375.

Lee, M.-P.,Yeun, L.-H. and Abdullah, R. 2006. Expression of *Bacillus thuringiensis* insecticidal protein gene in transgenic oil palm. Electronic Journal of Biotechnology. 9, 117-126.

Majid, N. A and Parveez, G.K.A. 2007. Evaluation of green fluorescence protein (GFP) as a selectable marker for oil Palm transformation via transient expression. Asia Pacific Journal of Molecular Biology and Biotechnology. 15, 1-8.

Murashige, T. and Skoog, F. 1962. A revised method for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15, 473-497.

Parveez, G.K.A. 2000. Production of transgenic oil palm (*Elaeis guineensis* Jacq.) using biolistic techniques. *In*: S.M., Jain and S.C., Minocha, editor. Molecular Biology of Woody Plants. Kluwer Academic Publishers. pp. 327-335.

Parveez, G.K.A., Masri, M.M., Majid, A.Z.N.A., Yunus, A.M.M., Fadilah, H.H. and Cheah, S.-C. 2000. Transgenic oil palm: production and projection. Biochemical Society Transactions. 28, 969-972.

Parveez, G.K.A., Chowdhury, M.K.U. and Saleh, N.M. 1998. Biological parameters affecting transient GUS gene expression in oil palm (*Elaeis guineensis* Jacq.) embryogenic calli via microprojectile bombardment. Industrial Crops and Products. 8, 17-27.

Parveez G.K.A., Chowdhury, M.K.U. and Saleh, N.M. 1997. Physical parameters affecting transient GUS gene expression in oil palm (*Elaeis guineensis* Jacq.) using the biolistic device. Industrial Crops and Products. 6, 41-50.

Patcharapisutsin, W. and Kanchanapoom, K. 1996. Somatic embryogenesis and plantlet regeneration from oil palm (*Elaeis guineensis* Jacq.) callus. Journal of the Science Society of Thailand, 22: 13-20.

Ramli, Z. and Abdullah, S.N.A. 2003. Development of a transient promoter assay system for oil palm. Journal of Oil Palm Research. 15, 62-69.

Sanford, J.C., Klein, T.M., Wolf, E.D. and Allen, N. 1987. Delivery of substances into cells and tissues using a particle bombardment process. Journal of Particulate Science and Technology. 5, 27-37.