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# Green fluorescent protein as a visual selection marker for oil palm transformation

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ABSTRACT

The aim of the study was to regenerate transgenic oil palm expressing the *gfp* gene throughout the whole plant by transforming oil palm cultures with organelle-targeted and non-organelle targeted *gfp* genes. In this study, oil palm embryogenic calli and immature embryos were separately bombarded with different constructs carrying either organelle-targeted (mitochondria, plastid or endoplasmic reticulum) or non-organelle targeted *gfp* gene in combination with the herbicide Basta resistance (*bar*) gene for regenerating transgenic oil palm expressing *gfp*. Transformed embryogenic calli were successfully regenerated on media containing Basta but failed to express the *gfp* gene in the regenerated plants. On the other hand, the bombarded immature embryos were either directly germinated or regenerated into whole plantlets via callogenesis on medium containing Basta. The regenerated plantlets demonstrated *gfp* expression only as patches on some parts of the plants such as roots and leaves and not in the whole plant, contrary to what was expected. Even though the plantlets failed to show *gfp* gene expression in the whole plant, molecular analyses revealed the presence of *bar* and *gfp* transgenes in the transgenic plants. Based on results obtained from this study, it could be concluded that the failure was due to the inability of *gfp* gene to express in the regenerated transgenic plants. In order to overcome this failure, further interventions are proposed which include the use of stronger promoters, use of different *gfp* versions and finally to use alternative transformation methods.

## 1. Introduction

Oil palm (*Elaeis guineensis*) originally from West Africa is now a major economic crop in Malaysia (Kushairi et al., 2017). As a major oil producing crop, oil palm could benefit from transgenic technology to produce high value fatty acids and metabolites for increasing its sustainability. Another advantage of transgenic technology for oil palm is the reduced time to genetically improve the crop as compared to classical breeding due to its long regeneration period (7-10 years) (Sambanthamurthi et al., 2009). The targets of oil palm genetic modification are as follows: high oleic acid, high stearic acid, high palmitoleic acid, high ricinoleic acid, high lycopene (carotenoid) and synthesis of biodegradable plastics (Masura et al., 2017; Parveez et al., 2015a).

Production of transgenic oil palm carrying selectable marker genes mediated via biolistics, *Agrobacterium* and microinjection of protoplasts has been reported; however the transformation efficiency was less than 1.5% (Masani et al., 2014; Masli et al., 2009; Parveez et al., 2000). Increasing the transformation efficiency is essential and is being carried out using biolistics, *Agrobacterium* and microinjection of protoplasts methods and selection agents such as herbicide Basta, 2-dioxyglucose and mannose (Bahariah et al., 2013; Izawati et al., 2015; Nurfahisza et al., 2016). Based on minimal inhibitory concentration studies on oil palm embryogenic calli, the herbicide Basta and antibiotic hygromycin are the most effective selection agents (Parveez et al., 2007). Besides using common herbicides and antibiotics, green fluorescent protein (GFP), a visual selection system was also evaluated for production of transgenic oil palm. It was anticipated that the transformation efficiency would be increased due to the non-destructive selection nature of the GFP system (Majid and Parveez, 2007). Increases in transformation efficiency using *gfp* have been demonstrated in plants such as tobacco, Spadone pear and *Chamaecyparis obtuse* (Chen et al., 2005; Taniguchi et al., 2005; Yancheva et al., 2006). The *gfp* gene originated from jellyfish (Chalfie et al., 1994) and is the most popular visual selection system for plant transformation as it does not require any chemical or substrate for detection and is species independent.

Transformation of 11 vectors carrying *gfp* genes in oil palm embryogenic calli and immature embryos was previously carried out; however, despite the observation of prolonged and high intensity *gfp* gene expression in the tissues, the transformed cells failed to regenerate

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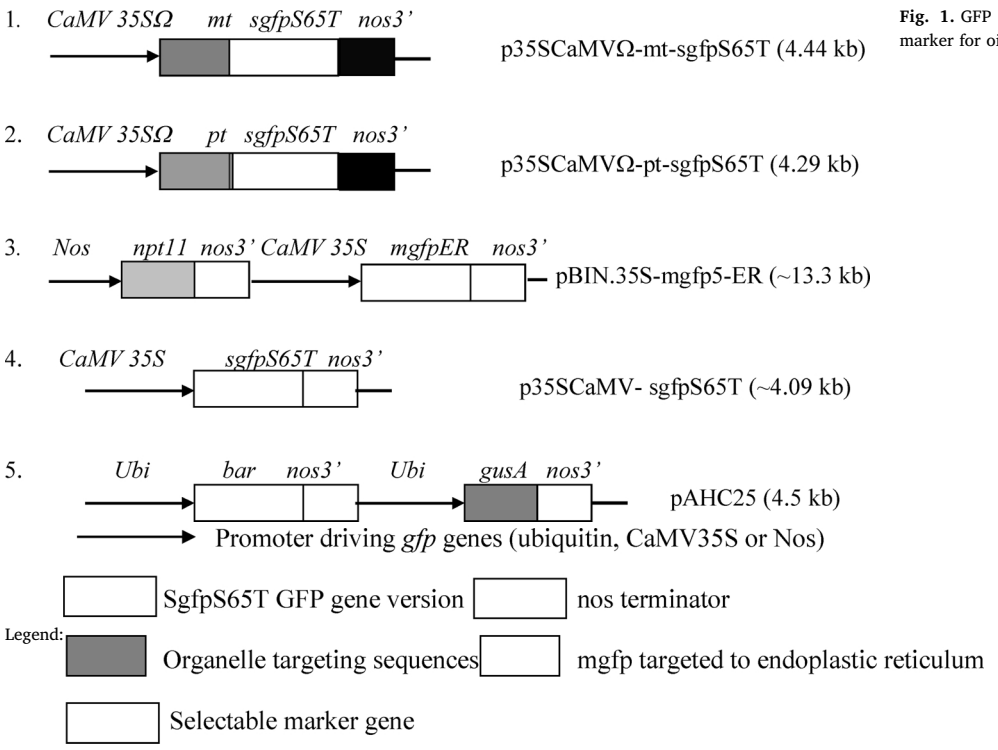


**Fig. 1.** GFP plasmids used in evaluating GFP as a selectable marker for oil palm transformation.

into transgenic oil palm expressing *gfp* in the whole plant (Majid and Parveez, 2007, Parveez and Majid, 2008). Owing to the possibility that the failure to regenerate oil palm expressing *gfp* was because the selection system could not effectively separate transformed cells from majority of untransformed cells, in subsequent experiments, transformation of oil palm calli using the *gfp* gene was carried out in the presence of the Basta resistance (*bar*) gene. Transformed oil palm calli were later selected and regenerated on medium containing the herbicide Basta. However, the regenerated transgenic oil palm failed to express the *gfp* gene in the whole plantlets (Majid and Parveez, 2016). Green fluorescence was only observed in some parts of the plantlets instead of in the whole plant. Based on similar observation in other plants, maize protoplast, *Arabidopsis* cells and barley (Haseloff et al., 1997; Murray et al., 2004; Sheen et al., 1995) where high-level *gfp* gene expression in transgenic plant cells interfered with plant regeneration and failed to regenerate *gfp* gene expressing plants, it was proposed that the failure to express the *gfp* gene in the whole plantlets may be due to toxicity of the green fluorescent protein in the oil palm cells. The toxicity was suggested to be due to the production of fluorescent-related free radicals in the nucleoplasm. Therefore, when the *gfp* gene was targeted to the endoplasmic reticulum, the toxicity problem was resolved (Haseloff et al., 1997). In addition, localization of *gfp* within the endomembrane system and proplastids, was believed to aid the post-translational maturation of *gfp*. Since then, various modifications of the *gfp* gene have been reported, such as increasing the fluorescent signal and solubility, varying emission spectrum and targeting to subcellular compartments as reviewed by Zimmer (2002).

Based on the failure to regenerate transgenic oil palm expressing *gfp* (Majid and Parveez, 2016) and reports on the successful overcoming of similar problems in other transgenic plants, such as in *Arabidopsis* and soybean (Haseloff et al., 1997; Ponappa et al., 2000), in this current study transformation of oil palm embryogenic calli and immature embryos with *gfp* gene targeted to three different organelles (endoplasmic reticulum, mitochondria and plastid) was proposed. Therefore, the objective of this study was to regenerate transgenic oil palm expressing the *gfp* gene throughout the whole plant by transforming oil palm cultures (embryogenic calli and immature zygotic embryos) using one non-organelle targeted (control) and three organelle-targeted *gfp* genes. Besides the *gfp* gene, the oil palm cultures were co-transformed with a Basta resistance gene (*bar*) for selection of transformants on medium containing the herbicide Basta to assist in the regeneration of transgenic oil palm expressing the *gfp* gene. For the immature embryos, direct germination of immature embryos into whole plants was also carried out for early determination of *gfp* gene integration and expression.

## 2. Materials and Methods

### 2.1. Plant materials

Oil palm embryogenic calli derived from calli originating either from leaf or cabbage of oil palm, variety *tenera,* were cultured on callus induction media (CIM) [MS micro-and macro- salts (Sigma-Aldrich, USA) (Murashige and Skoog, 1962) + Y3 vitamins (Eeuwens, 1976) + 0.1 g/l L-glutamine and myo-Inositol (Sigma-Aldrich, USA) + 5 µM 2, 4-D (Sigma-Aldrich, USA) + 0.25% (w/v) activated charcoal + 3% (w/v) sucrose (Sigma-Aldrich, USA) + 0.7% (w/v) agar (Sigma-Aldrich, USA)] and incubated at 28 °C in the dark and subcultured to fresh CIM every four weeks (Parveez et al., 2000). Any callus formed was subcultured onto the same CIM until embryogenic calli were formed.

Immature embryos (IE) were isolated from oil palm, variety *tenera* fruits at 10-12 weeks after anthesis. Individual fruits were soaked for 15 min each in tap water containing a few drops of undiluted Tween 20 (Sigma-Aldrich, USA) followed by sterilization in 50 mL 100% ethanol (BDH Prolabo, France). Excess ethanol was removed from the fruits by drying in a laminar flow cabinet for about 30 min. The dried fruits were cut and IE were isolated and cultured onto Callus Induction Media 1 (CIM-1) (Texeira et al., 1993).

### 2.2. GFP Gene Constructs

The following *gfp* gene constructs were used in this study: p35SCaMV-sgfpS65T (Sheen et al., 1995) a non-organelle-targeted construct and three constructs where the GFP gene was targeted to a specific organelle: p35SCaMVΩ-mt-sgfpS65T (mitochondria-mt) (Niwa