

# GENETIC TRANSFORMATION OF OIL PALM-BASED ON SELECTION WITH HYGROMYCIN

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## ABSTRACT

The *bar* gene conferring resistance to the herbicide Basta was used as a selectable marker in oil palm transformation system. However, the inefficiency in the selection system was believed to generate transgene escape and high rate of chimerism in oil palm. To overcome this limitation, an effective selectable marker for oil palm is required. The aim of this work is to evaluate the use of the hygromycin phosphotransferase (*hpt*) gene as the selectable marker for generation of stable oil palm transformation via biolistic method and subsequently improve the oil palm transformation efficiency. In this selection approach, the embryogenic calli were bombarded with the vectors carrying the *hpt* and a green fluorescent protein (*gfp*) reporter genes, which were driven by the 2X35S promoter assembled in pBINPLUS and pCAMBIA0380 (pPZP) backbones. Visualisation of GFP spots was observed using Fluorescence Microscope for confirmation of successful deoxyribonucleic acid (DNA) delivery. The calli were then cultured on regeneration medium added with hygromycin at 10 mg litre<sup>-1</sup> and consequently reduced to 5 mg litre<sup>-1</sup>. The presence of transgenes in the bombarded tissue was confirmed by polymerase chain reaction (PCR) amplification of *hpt* and *gfp* genes. These results demonstrate the potential of hygromycin as an alternative selection agent for oil palm transformation.

**Keywords:** hygromycin, selectable marker, biolistic.

**Received:** 30 July 2020; **Accepted:** 23 October 2020; **Published online:** 9 December 2020.

## INTRODUCTION

Genetic transformation of oil palm has progressed substantially in the last few decades (Parveez *et al.*, 2015). However, the transformation efficiency is low at 1.5% by using biolistic (Parveez, 2000), 0.7% by using *Agrobacterium* (Masli *et al.*, 2009) and relatively higher at 14% using oil palm protoplasts by microinjection (Masani *et al.*, 2014) methods, which needs to be further increased. Oil palm is a complicated plant with a long generation cycle, approximately 20-25 years of the economic lifespan (Zulkifli *et al.*, 2018). In producing transgenic material, it has become an important limitation which will require a long term strategy in executing sensitivity, consistency and repeatability tests of the oil palm tissue. Apart from this factor, the development of the effective transformation system, particularly efficient selectable markers for the selection of transformants is being carried out.

Initially, the use of herbicide selectable marker, the *bar* gene, has become an established practice in oil palm genetic transformation. It was first reported as the most efficient selectable marker in oil palm transformation (Parveez *et al.*, 2000; 2007). The *bar* gene isolated from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) was originally used to obtain tolerance to the antibiotic bialaphos and herbicide resistance in plants (De Block *et al.*, 1987). Resistance is conferred by encoding the enzyme phosphinothricin acetyltransferase, which is responsible for detoxifying phosphinothricin (PPT) by acetylation (Murakami *et al.*, 1986). Unfortunately, molecular analysis of the regenerated transgenic oil palm plants using *bar* selectable marker gene has suggested the presence of chimeric, causing them to survive under selection pressure (escape phenomenon) in majority of the analysed samples. This may be due to the non-optimal and appropriate selection system during the regeneration process of transgenic plants (Nurfahisza *et al.*, 2014; Masura *et al.*, 2017).

Effort has been made to establish an efficient transformation selection system for oil palm by using

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an alternative selectable marker gene, antibiotic hygromycin phosphotransferase (*hpt*). The *hpt* gene which was originally derived from *E. coli* confers resistance to hygromycin B (Gritz and Davies, 1983) that can interfere with translation process and disturb the protein synthesis (Gonzalez *et al.*, 1978). This can be more toxic and kills sensitive cells faster. As a result, it has been widely used as a resistance gene for plant transformation in different species, such as for cassava (Zhang and Puonti-Kaerlas, 2000), castor (Sujatha and Sailaja, 2005), dendrobium (Suwanaketchanatit *et al.*, 2007), grape (Fan *et al.*, 2008), rapeseed (Liu *et al.*, 2011) and spinach (Milojević *et al.*, 2012). The hygromycin antibiotic has also been successfully used as a selection agent in oil palm transformation, but no study has been reported describing the production of stable transformation plants. For instance, Parveez *et al.* (1996; 2007); Abdullah *et al.* (2005); Kanchanapoom *et al.* (2008); Bhore and Shah (2012); Kalawong and Te-chato (2012) and Fakhrana *et al.* (2019) showed that the antibiotic, hygromycin is one of the most sensitive selection agents for oil palm. Previous minimal inhibition studies demonstrated that the optimal concentrations of *hpt* gene varied with developmental stages of tissues. The antibiotic completely inhibited the growth of immature embryos, seedling, mature embryos and embryogenic calli at 8-6 mg litre<sup>-1</sup>, 10 mg litre<sup>-1</sup>, 20 mg litre<sup>-1</sup>, 30 mg litre<sup>-1</sup> and 50 mg litre<sup>-1</sup>, respectively (Table 1).

In this work, we aimed to evaluate the use of antibiotic *hpt* selectable marker gene to produce stably transformed transgenic oil palm. Thus, as part of the ongoing efforts to develop an oil palm transformation system using the antibiotic hygromycin resistance gene, two expression vectors carrying the *hpt* selectable marker gene and *gfp* reporter gene driven by 2XCaMV35S promoter in different vector backbones were evaluated in this study. The pBIHA1 vector in pBINPLUS backbone (Belknap *et al.*, 2008) which was previously reported (Bahariah *et al.*, 2017) and a newly constructed vector,

pBIHA-X in pCAMBIA backbone (Hajdukiewicz *et al.*, 1994) were examined for their ability to increase the transgene expression level and develop an efficient oil palm transformation system by using hygromycin selection.

## MATERIALS AND METHODS

### Vector Construction

The plasmids used for construction of pBIHA-X are listed in Table 2. The vector pBIHA-X is based on pCAMBIA0380, a pPZP family (Hajdukiewicz *et al.*, 1994), was constructed by using two intermediate vectors created in a previous study (Bahariah *et al.*, 2017). Plasmid pUC19 (Norrandar *et al.*, 1983) served as a vector for cloning DNA fragments. The p2X35STEVGFP-GII (5.2 kb) and p2X35TEVHPT-GII (5.5 kb) contain *gfp* gene from pAMCFDV-GFP and *hpt* gene from pCAMBIA1303, respectively. The genes were controlled by a 0.9 kb 2X35S promoter derived from the binary vector pTF101.1 (Paz *et al.*, 2004) and cloned in a pGreenII0000 cloning vector. In order to construct p2X35SHPTGFP-0380 (pBIHA-X) vector, two deoxyribonucleic acid (DNA) fragments of 2X35S-TEVHPT-35ST and 2X35S-TEVGFP-35ST were ligated to the *Hind*III and *Spe*I sites of pCAMBIA0380 to create 10 810 bp of p2X35TEVHPT-0380 (pBIHA-X).

### Plant Materials and Culture Conditions

The oil palm cultures used in this study were provided by Clonal Propagation Group, MPOB, Malaysia. Young leaflets of P164 ortet sample with a width of approximately 2-3 mm were cultured on a modified Murashige and Skoog medium, supplemented with 8-10 mg litre<sup>-1</sup> NAA for callus induction and proliferation (Murashige and Skoog, 1962). They were maintained in the dark with a

TABLE 1. THE HYGROMYCIN USED AS A SELECTION AGENT IN PREVIOUS OIL PALM TRANSFORMATION STUDIES

Oil palm tissues	General working concentration	References
Immature embryos	20-50 mg litre <sup>-1</sup>	Parveez <i>et al.</i> (1996)
Immature embryos	20-50 mg litre <sup>-1</sup>	Abdullah <i>et al.</i> (2005)
Immature embryos	20 mg litre <sup>-1</sup>	Parveez <i>et al.</i> (2007)
Seedling		
Mature embryos	50 mg litre <sup>-1</sup>	Kanchanapoom <i>et al.</i> (2008)
Embryogenic calli		
Immature embryos	8-6 mg litre <sup>-1</sup>	Bhore and Shah (2012)
Embryogenic calli	30 mg litre <sup>-1</sup>	Kalawong and Te-chato (2012)
Embryogenic calli	10 mg litre <sup>-1</sup>	Fakhrana <i>et al.</i> (2019)
Embryogenic calli	5 mg litre <sup>-1</sup>	In this study

temperature of  $28\pm 1^\circ\text{C}$ . Friable calli derived from the primary embryoid initiation were then isolated and inoculated in the liquid medium treated with  $1\text{ mg litre}^{-1}$  2,4-D and  $0.1\text{ mg litre}^{-1}$  NAA for culture maintenance (L-1). The embryogenic calli developed from eight months suspension calli were subsequently cultivated on L-1 solidified medium. Approximately 0.5 g of embryogenic calli with sizes, which ranged from 0.5 to 1.0 mm per plate, was incubated in the dark at  $28^\circ\text{C}$ , 24 hr prior to the bombardment. After bombardment, the embryogenic calli were cultured in L-1 medium with no selection for callus proliferation for four weeks in the dark at  $28^\circ\text{C}$ . The cultures were then transferred onto the selection medium, hormone-free maturation medium (MSB) supplemented with  $10\text{ mg litre}^{-1}$  of hygromycin selective agent at

$28^\circ\text{C}$  in dark condition, then transferred to medium regeneration (EC) under 16/8 hr light conditions until shoots developed. The culture medium was changed and subcultured repeatedly every four weeks. After a period of six months, the regenerated shoots were placed on shoot inducing (SI) medium and finally in the rooting medium (Table 3).

### Bombardment of Calli with *hpt* Gene Constructs

A total of  $20\text{ }\mu\text{g}$  of the plasmid DNA having *hpt* gene was precipitated onto  $0.6\text{ }\mu\text{g}$  gold particles. The DNA coated gold particles were used to bombard oil palm embryogenic callus cultures using the PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA USA). Bombardment was carried out at the following conditions; 1100 psi rupture

TABLE 2. PLASMIDS USED IN THIS STUDY

Plasmids	Description	Reference source
pBluescriptSK(-)	Cloning vector	Stratagene
PCR II TOPO	Cloning vector	Invitrogen
pUC19	Cloning vector	Norrander <i>et al.</i> (1983)
pGreenII0000	Intermediate cloning vector	John Innes Centre
pCambia0380	Binary Ti vector for <i>Agrobacterium</i> -mediated plants transformation	Hajdukiewicz <i>et al.</i> (1994)
pAMCFDV-GFP	Contain <i>mGFP</i> reporter gene and 35S <sub>T</sub> sequences	Masani <i>et al.</i> (2014)
pTF101.1	Contain 2XCaMV35S <sub>TEV</sub> promoter sequence	Paz <i>et al.</i> (2004)
pCambia1303	Contain hygromycin ( <i>hptII</i> ) gene	Hajdukiewicz <i>et al.</i> (1994)
p2X35S <sub>TEV</sub> GFP-GII	Intermediate cloning vector	Bahariah <i>et al.</i> (2017)
p2X35S <sub>TEV</sub> HPT-GII	Intermediate cloning vector	Bahariah <i>et al.</i> (2017)

TABLE 3. MEDIA COMPOSITION FOR SELECTION AND REGENERATION OF TRANSGENIC OIL PALM

Component	Medium					
	L-1	MSB	EC	SI	Rooting	
M+S Macro (ml litre <sup>-1</sup> )	50	50	50	50	50	
M+S Micro (ml litre <sup>-1</sup> )	10	10	10	10	10	
Y3 Vits (ml litre <sup>-1</sup> )	1	1	1	1	1	
NaFeEDTA (g litre <sup>-1</sup> )	0.0375	0.0375	0.0375	0.0375	0.0375	
Myo-inositol (g litre <sup>-1</sup> )	0.1	0.1	0.1	0.1	0.1	
L-Glutamine (g litre <sup>-1</sup> )	0.1	0.1	0.1	0.1	0.3	
L-Arginine (g litre <sup>-1</sup> )	-	0.1	0.1	0.1	-	
L-Asparagine (g litre <sup>-1</sup> )	-	0.1	0.1	0.1	-	
NAA ( $\mu\text{M}$ )	0.1	-	0.5	0.1	9.0	
2,4-D ( $\mu\text{M}$ )	1.0	-	-	-	-	
Activated charcoal (%)	-	-	-	-	0.25	
Plant agar (g litre <sup>-1</sup> )	8	8	8	8	-	
Sucrose (g litre <sup>-1</sup> )	30	30	30	30	60	
pH	5.7	5.7	5.7	5.7	5.7-6.0	
Antibiotic hygromycin (mg litre <sup>-1</sup> )	-	-	10	5	5	

Note: L-1 - proliferation medium; MSB - maturation medium; EC - regeneration medium: callus to shoot; SI - regeneration medium: shoots elongation; NAA -  $\alpha$ -naphthaleneacetic acid.

disc pressure, 6 mm rupture disc to macrocarrier distance, 11 mm macrocarrier to stopping plate distance, 75 mm stopping plate to target tissue distance and 67.5 mm Hg vacuum pressure (Parveez, 2000). Thirty to 40 replications were carried out. The experiment was repeated seven times with several modifications. After bombardment, the calli were observed for GFP expression at 24 hr and two weeks after bombardment, using Nikon AZ100 fluorescence microscope multizoom microscope equipped with specific GFP filter. The GFP signals provided an internal reference for the identification of successfully transformed tissues. The cultures were then incubated at 28°C under 16/8 hr light conditions. The culture medium was changed at every four weeks interval.

### Polymerase Chain Reaction (PCR) Analysis

Transformed embryoids that survived on hygromycin selection media were assessed and compared with wild type by PCR analysis. PCR amplification was carried out in a 25 µl reaction mixture, containing 100 ng of DNA template, 10 µM of each primer (reverse and forward primers flanking the *hpt* gene), 12.5 µl of 2XGoTaq® Green

Master Mix (Promega) and nuclease-free water to a final volume of 25 µl. The PCR amplifications carried out were as follows: initial denaturation (98°C for 1 min), denaturation (98°C for 5 sec), annealing (67°C for 5 sec), extension (72°C for 1 min) and elongation (72°C for 1 min). The putative transformed samples were further analysed by PCR for detection of *gfp* transgene using mGFP1 F/R primers. The sequences of reverse and forward primers of *hpt* and *gfp* genes are listed in Table 4. The PCR products were electrophoresed in a 1.0% agarose gel at 110V for 80 min.

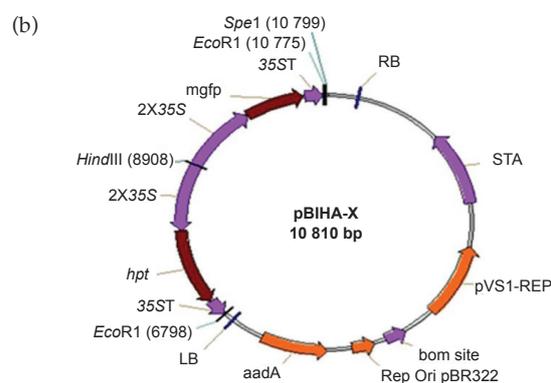
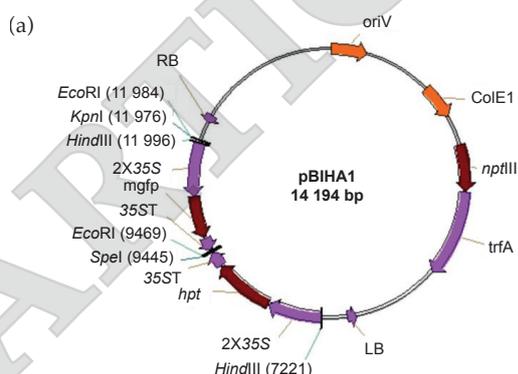
### RESULTS AND DISCUSSION

In order to investigate the potential use of *hpt* gene for selection of hygromycin resistant transgenic plants, two vectors namely pBIHA-X (10 810 bp) in pCAMBIA0380 backbone, which was successfully constructed in this study and pBIHA1 (14 194 bp) in pBINPLUS backbone which was previously reported (Bahariah *et al.*, 2017) were evaluated in this study (Figure 1).

The pBIHA-X (p2X35SHPTGFP-0380) plasmid was constructed to contain the *hpt* selectable marker and *gfp* reporter genes driven by 2XCaMV35S in

TABLE 4. PRIMER SEQUENCES FOR POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Primer name	Primer sequence
mGFP1	Forward: 5'-CCGGCCATGGGTTAAAGGAGAAGAACTTTTCAC-3' Reverse: 5'-CCGGTCTAGATTATTTGTATAGTTCATCCATGCC-3'
Hpt 1	Forward: 5'-GGTCATGAAAAAGCCTGACACCGCG-3' Reverse: 5'-GGCCTCTAGACTATTTCTTTGCCCTCGGACG-3'



Note: The arrow indicates the orientation of each DNA fragments assembled. LB: left border of T-DNA; 2X35S: double cauliflower mosaic virus 35S promoter; *hpt*: gene for hygromycin phosphotransferase; *mgfp*: modified green fluorescent protein; 35ST: 35S terminator gene; RB: right border of T-DNA.

Source: Bahariah *et al.* (2017).

Figure 1. Schematic diagram of pBIHA1 and pBIHA-X carrying the *hpt* and *gfp* genes driven by 2XCaMV35S assembled in (a) pBINPLUS, and (b) pCAMBIA backbones used for biolistic transformation.

pCAMBIA0380 backbone (10.8 kb). The construction was carried out by employing two intermediate plasmids, p2X35STEVEHPT-GII (5.5 kb) and p2X35STEVEGFP-GII (5.2 kb), created in the earlier study (Bahariah *et al.*, 2017). The p2X35STEVEHPT-GII fragment was ligated to the *Hind*III and *Eco*RI sites of pCAMBIA0380 to form 8.9 kb of p2X35STEVEHPT-0380. Then, the 2X35STEVEHPT

fragment of p2X35STEVEHPT-0380 was inserted into p2X35STEVEGFP-0380 by digestion with *Hind*III and *Spe*I to produce a vector p2X35STEVEHPTGFP-0380, designated as pBIHA-X (Figure 2a). In order to confirm gene insertions into cloning vectors, the vector was analysed by double restriction endonuclease assay using *Hind*III and *Eco*RI (Figure 2b).

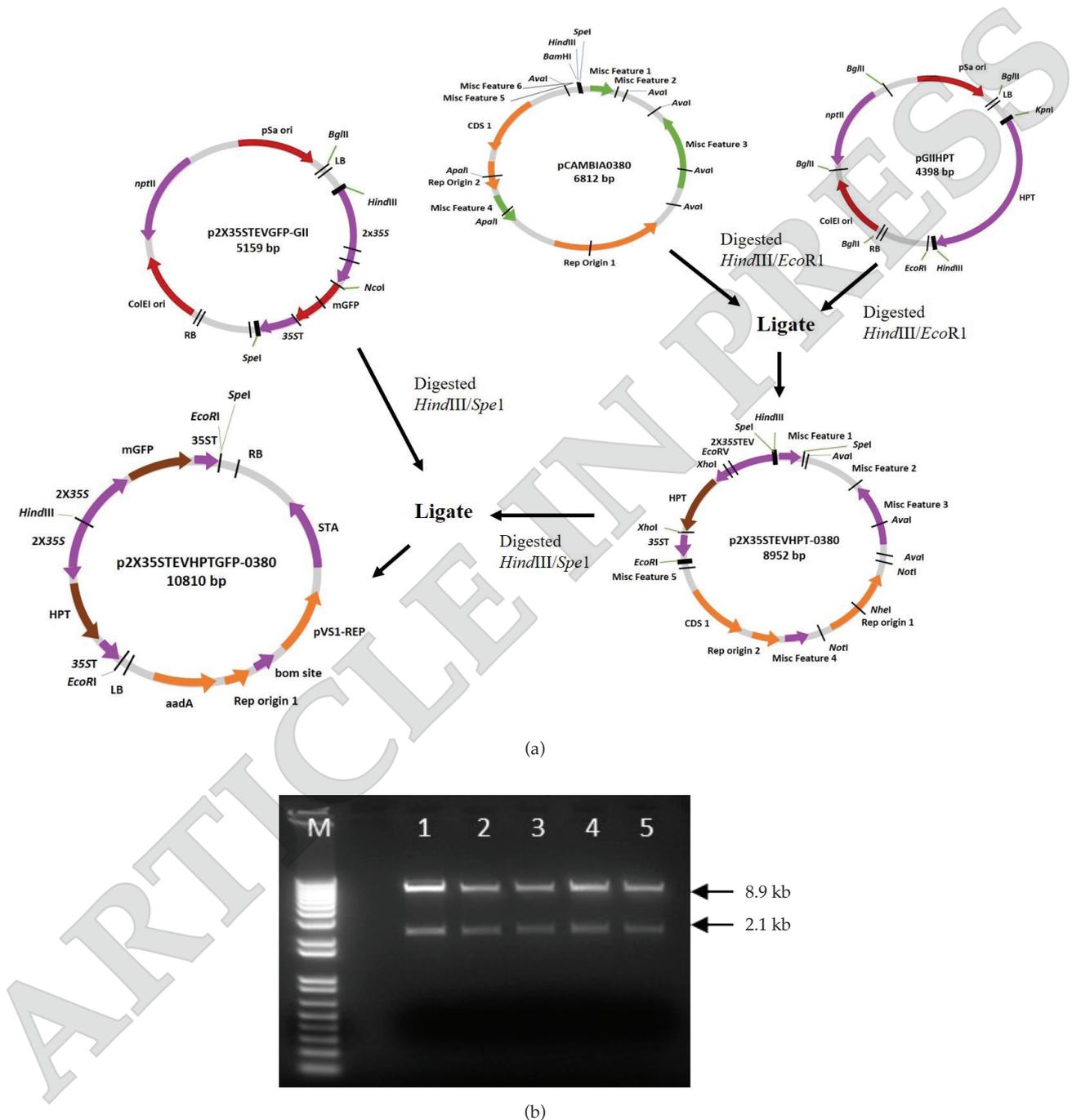


Figure 2. Construction of pBIHA-X (p2X35SHPTGFP-0380). (a) The *gfp* reporter gene was released from pAMCFDV-GFP using the *Hind*III and *Nco*I digestion, and ligated into the *Hind*III and *Nco*I site of 2X35STEVE-PCRITTOPO to create p2X35STEVEGFP. This was then recombined with *hpt* gene fragment amplified from pCAMBIA1303 to construct p2X35STEVEHPT. Then, the fragments were digested with *Hind*III, *Eco*RI, and cloned into the *Hind*III and *Eco*RI site of pGreenII0000 to generate the p2X35STEVEHPT-GII and p2X35STEVEGFP-GII. These plasmids were finally ligated to the *Hind*III and *Eco*RI sites of pCAMBIA0380 to create p2X35STEVEHPT-0380 and with *Hind*III and *Spe*I to form a vector of p2X35STEVEHPTGFP-0380 (pBIHA-X). (b) Restriction endonuclease analysis of pBIHA-X vector. An equal amount of DNA of each plasmid was digested with *Hind*III and *Eco*RI. Lane M: 1 kb plus DNA ladder, lanes 1-5: Positive clones for p2X35STEVEHPT-0380. Arrows indicate the size of 2.1 kb inserted DNA fragment of 2X35S-mGFP-Nos and 8.9 kb of p2X35STEVEHPT-0380.

The viability of pBIHA-X and pBIHA1 vectors was evaluated by transforming oil palm embryogenic calli using biolistic mediated transformation as described previously (Parveez *et al.*, 2000). After 24 hr post bombardment, the *gfp* reporter gene expression was visually observed in oil palm calli using AZ100 microscope. The GFP signals were clearly visible in embryogenic calli and reached a peak of more than 30 spots per tissue clump, indicating the *gfp* gene in both constructed vectors was integrated and expressed in the transformed cells (Figure 3). However, two weeks after bombardment, the number of visible spots expressing *gfp* declined considerably to less than two spots per clump of tissues with *gfp* spots grew bigger for some samples. Similar observations were reported in previous studies by Majid and Parveez (2007; 2016). The reduction in the number of GFP spots in oil palm embryogenic calli suggested that GFP degradation and only a few events were stably integrated and expressed. It could also be due to the incidence of transgene silencing mechanisms

(Schubert *et al.*, 2004; Chee *et al.*, 2018). There has been no direct comparison between different vector backbones (pCAMBIA and pBINPLUS) that carry the selectable marker genes. No difference in the GFP expressing cells was observed, suggesting the more prevalent role of the promoter driving the gene as compared to the plasmid backbone (Parveez and Majid, 2008).

Transformed calli were selected in selection medium containing 10 mg litre<sup>-1</sup> hygromycin after rested for four weeks. The concentration was chosen based on a previous study on minimal inhibitory concentration (MIC) of hygromycin selection agent for oil palm embryogenic calli (Fakhrana *et al.*, 2019). The study showed that a higher concentration of hygromycin was needed to inhibit the proliferation of embryogenic calli. Therefore, in this study, the embryogenic calli were subcultured every four weeks onto fresh media containing 10 mg litre<sup>-1</sup> concentration of hygromycin. Early observation found that the development of embryoid and shoots was normal during regeneration of half-

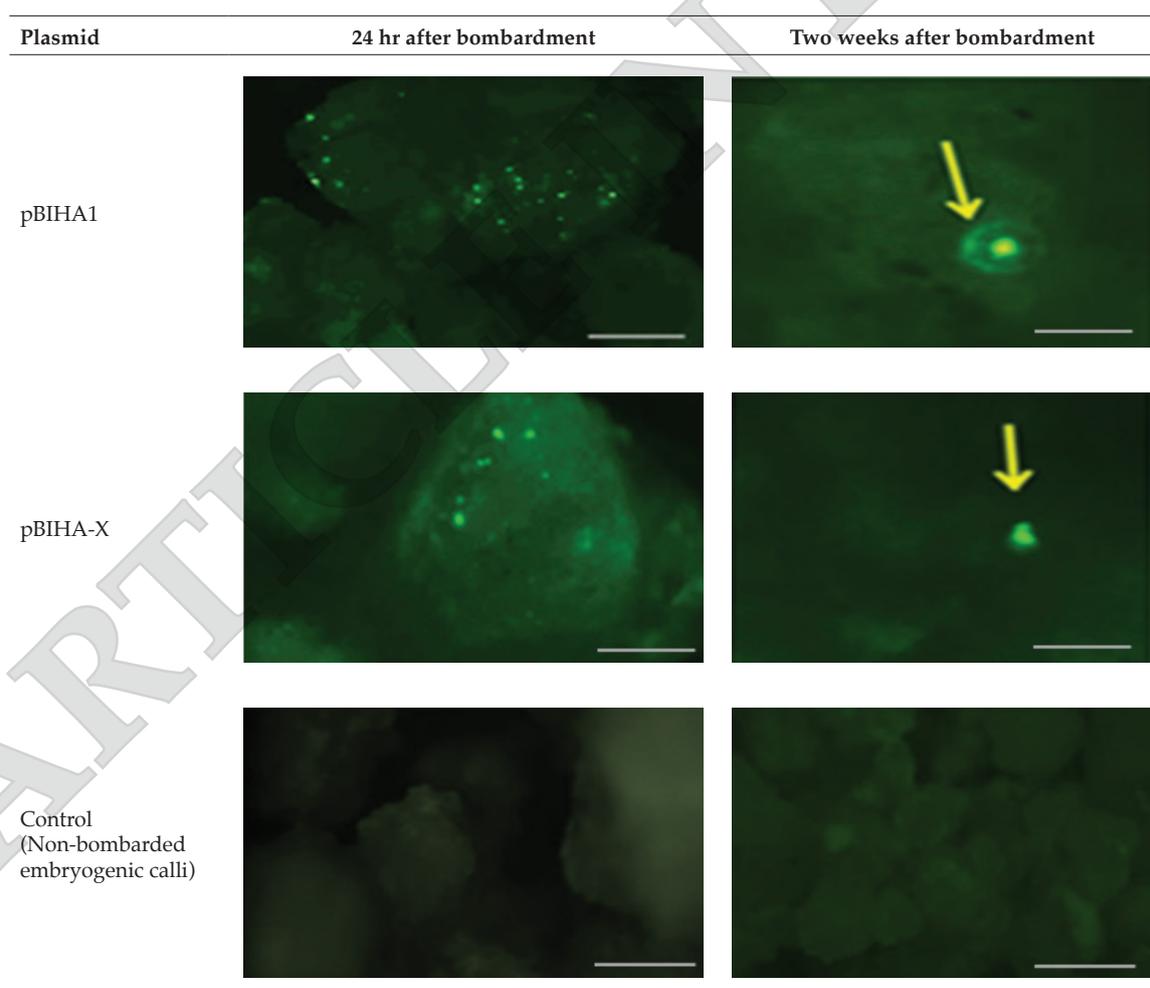


Figure 3. The green fluorescent protein (GFP) expression in transformed oil palm calli at 24 hr and two weeks after bombardment. Transformed tissues expressing GFP and non-bombarded tissues as control are on the right side. Successful transformation of calli bombarded with pBIHA1 plasmid carrying *hpt* and *gfp* genes in pBINPLUS backbone; calli bombarded with pBIHA-X plasmid in pCAMBIA backbone. Yellow arrows indicate GFP spots and scale bar equals 10 mm (24 hr) and 20 mm (two weeks).

year period. However, after six months of culture, the proliferation was completely stunted and the cultures became necrotic. The embryoid and shoots turned brown and some failed to survive after eight months (Figure 4). The results showed that hygromycin dramatically delayed the inhibition and the growth of oil palm cells at 10 mg litre<sup>-1</sup> selection concentration. This observation suggested that oil palm embryogenic calli are highly sensitive to the hygromycin. This finding is consistent with previous studies that showed the presence of antibiotic selection pressures at exceeding maximum levels could inhibit the growth of the transformed plants and kill the nontransformed cells, thus, leading to delay in regeneration (Wilmink and Dons 1993). Subsequently, we then utilised stepwise decreasing concentration of hygromycin from 10 to 5 mg litre<sup>-1</sup> at nine months. The hygromycin concentration at 5 mg litre<sup>-1</sup> was used as in agreement of an earlier study by Fakhra et al. (2019). The study showed the concentration of hygromycin needs to gradually decrease throughout the callus maturation process to allow the transformed cells to regenerate to normal plants (Fakhra et al., 2019). Similar

observations were reported in *Setaria viridis* (Van Eck et al., 2017) and wheat (Gils, 2017).

To investigate the presence of the *hpt* gene in transformed oil palm tissue, genomic DNA from regenerated embryoid was used for PCR analysis using a specific *hpt* primers (Table 4). The result of the PCR analysis is shown in Figure 5. Seven plants out of 33 samples tested, were positive for *hpt* gene (21%). From the results obtained, 27% of the samples bombarded with pBIHA1 vector and 18% of the samples bombarded with pBIHA-X were *hpt* positive. The results showed that a 1000 bp fragment of *hpt* gene was successfully amplified. These positive DNA samples were later subjected to PCR amplification using primers specific for *gfp* gene. A total of five out of seven *hpt* positive samples tested were able to amplify the 450 bp *gfp* gene fragment (Figure 6). The results indicated that only five lines were confirmed to carry both transgenes, *gfp* and *hpt*, while the other 26 lines did not show any PCR product. These results could further support the presence of gene escapes due to the chimeric nature of these lines or incomplete insertion of the target genes into the plant genome.

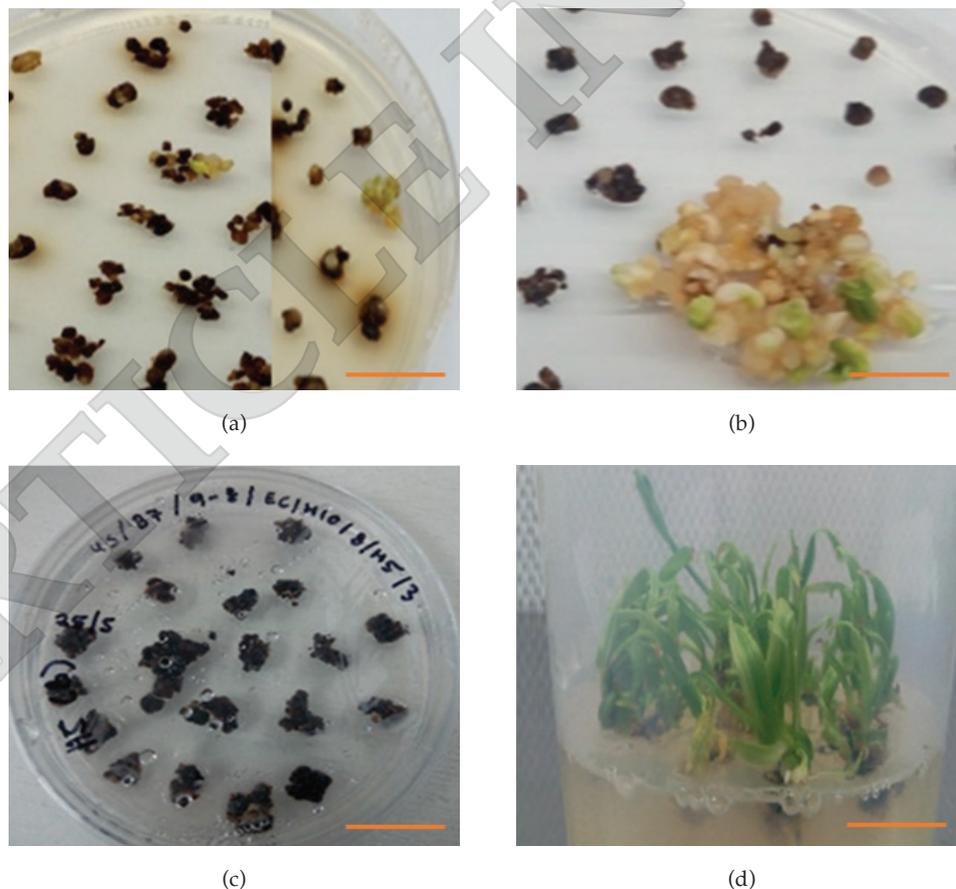


Figure 4. The bombarded calli with pBIHA-X plasmid grown on media containing hygromycin selection agent. (a) Oil palm calli were selected on 10 mg litre<sup>-1</sup> hygromycin media for eight months (b) at three months after decreasing to 5 mg litre<sup>-1</sup> hygromycin, (c) non-bombarded calli in selection medium and (d) non-bombarded calli on medium without selection. Yellowish and greenish were observed for the transformant (a, b), while for the untransformed cells turn black (c). Scale bar: a (bar 1 cm), b (bar 4 mm), c (bar 1 cm) and d (bar 2 cm).

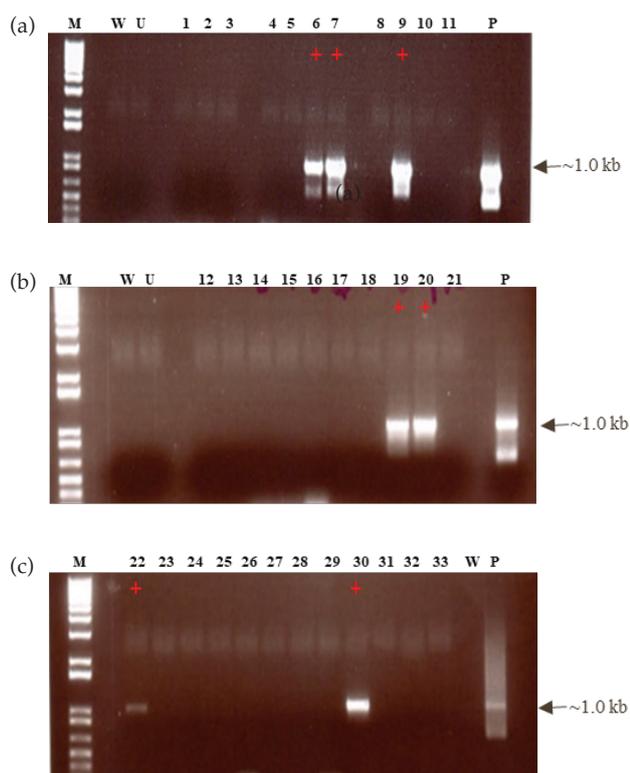


Figure 5. Polymerase chain reaction (PCR) analysis of the putative transgenic embryoid bombarded with plasmids, pBIHA1 (a) and pBIHA-X (b, c) to detect the presence of *hpt* gene. Lane M: 1 kb plus marker ladder; lane W: water as negative control; lane U: untransformed plants; lane P: positive control (pBIHA-X); lanes 1-33: transformed plants and lanes 6, 7, 9, 19, 20, 22, 30 were positive amplified a 1 kb DNA fragment using primers corresponding to a *hpt* gene.

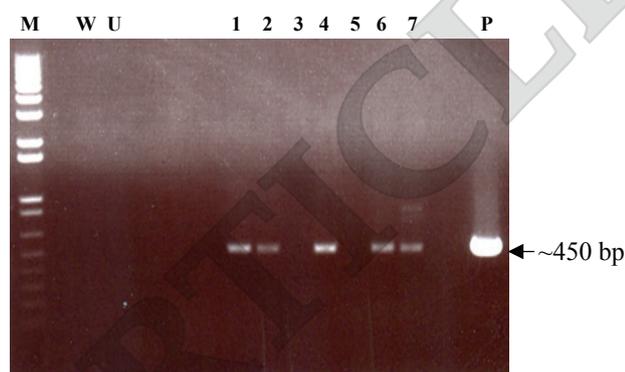


Figure 6. Polymerase chain reaction (PCR) analysis of genomic DNA from putative green fluorescent protein (GFP) transformed oil palm calli to detect the presence of *gfp* gene. PCR amplified a 450 bp fragment using mGFPIF/R primers. Lane M: 1 kb plus marker ladder; P: positive control (pBIHA-X); U: untransformed plants, W: water as negative control; 1-7: transformed plants.

Overall, the study demonstrated the suitability of the *hpt* gene in both vectors, pBIHA-X and pBIHA1 for biolistic transformation of oil palm. Both vectors were driven by the same promoter, the double enhanced CaMV35S promoter, which was amplified from pCAMBIA1303. Previously, the CaMV35S

promoter was found to be the most effective promoter for driving *gfp* gene in oil palm tissues (Parveez and Majid, 2008). The vectors transformed to express the *gfp* gene and stable integration of both *gfp* and *hpt* transgenes into oil palm embryogenic calli was detected by PCR and transient expression assay. A low concentration of selection agent may be required for callus regeneration. The concentration of 10 mg litre<sup>-1</sup> is probably too high for selection. This will not only kill the nontransformed cells but also result in toxicity that might inhibit the growth of transformed cells. Some species are very sensitive to hygromycin and only a low concentration was used for their transformant selection, such as 3.0 mg litre<sup>-1</sup> for rapeseed leaf petiole cells (Liu *et al.*, 2011) and 7.5 mg litre<sup>-1</sup> for *Centaurea montana* leaf explants (Abou-Alaiwi *et al.*, 2012). The sensitivity of plant tissues to the selective agent depends on many factors, including the tissue types, size of explants, developmental stage, chemical properties and concentration of the selective agents (Bowen, 1993). This could lead to variation in the optimal concentration for selection between plant species. For example, the hygromycin concentration at 25 mg litre<sup>-1</sup> for *S. viridis* (Van Eck, 2018), 50 mg litre<sup>-1</sup> for banana (Barlett *et al.*, 2008), 20 mg litre<sup>-1</sup> for cotton (Meng *et al.*, 2007) and 30 mg litre<sup>-1</sup> for rice (Kumar *et al.*, 2005) were determined to be the optimal concentration of this selective agent. In addition, the selection system to differentiate transformed and nontransformed cells are requisite to regenerate the truly genetically transformed cell.

## CONCLUSION

The production of transgenic oil palm is difficult and time-consuming. An alternative approach to tighten the selection system for stable transformation of oil palm was evaluated using an antibiotic hygromycin selectable marker gene, which might be useful and efficient to address these challenges. Oil palm embryogenic calli were bombarded with plasmids carrying *hpt* selectable marker and *gfp* reporter genes and selected on medium containing hygromycin. The GFP expression was detected on the transformed tissues that survived the selection, indicating successful transformation. Successful regeneration of the bombarded tissues was achieved through the application of stepwise decreasing concentration of hygromycin selection from 10 to 5 mg litre<sup>-1</sup>. The non-bombarded embryogenic calli were completely stunted and became necrotic. The regenerated transgenic plants were confirmed through PCR. The rate of *hpt* positive plants was at 21%. The majority of these *hpt* positive plants were also shown to carry *gfp* transgene. Expression studies of the *hpt* and *gfp* genes in transgenic oil palm will be carried out to assess the expression level of the transgenes. In

addition, the integration of the transgenes will also be verified to further confirm their integration and copy number in the regenerated plants.

### ACKNOWLEDGEMENT

The authors would like to thank the MPOB for permission to publish this article. We also would like to acknowledge Mrs Nurul Huda Ideris and all members of the Transgenic Technology Laboratory of MPOB for their assistance.

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