Journal of Oil Palm Research Vol. 29 (2) June 2017 p. 180 – 188

CONSTRUCTION OF A VECTOR CONTAINING HYGROMYCIN (HPT) GENE DRIVEN BY DOUBLE *35S* (2XCAMV*35S*) PROMOTER FOR OIL PALM TRANSFORMATION

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ABSTRACT

Transformation vector construction is one of the important disciplines for plant genetic transformation studies. A series of vector consisting of hygromycin phosphotransferase (hpt) gene as the selective marker and green fluorescent protein (GFP) as the visual reporter gene, under the control of double cauliflower mosaic virus 35S *(2XCaMV*35S*) promoter has been engineered for transformation into oil palm cells. These genes were cloned into different types of cloning and expression vectors. The cloning was carried out by using restriction enzyme digestion and ligation method. Five intermediate vectors have been created for insertion of 2XCaMV*35S*-HPT-35ST and 2XCaMV*35S*-GFP-35ST into modified pBINPLUS backbone vector for particle bombardment and* Agrobacterium*-based transformation protocols. All vectors were sequenced to confirm the integrity of DNA region. The vectors were later transformed into oil palm embryogenic calli using biolistic device. The viability of the vectors was initially evaluated by transient GFP fluorescence expression observed under fluorescence microscope. It was demonstrated that the 2XCaMV*35S *promoter was able to drive the expression of* gfp *as gene in oil palm calli.*

Keywords: vector construction, hygromycin, biolistic, embryogenic calli, GFP fluorescence.

Date received: 1 December 2016; **Sent for revision:** 6 December 2016; **Received in final form:** 9 February 2017; **Accepted:** 20 April 2017.

INTRODUCTION

Malaysia is one of the largest producers and exporters of palm oil and palm oil products in the world. This requires the palm oil industry to tackle the challenges in meeting the growing worldwide demand for palm oil as food, biodiesel and industrial non-food uses, such as oleochemicals. Improvement of oil palm crop by genetic engineering could have an important role to play in fulfilling the growing global need for oils and fats (Parveez *et al*., 2015a). Methods for the genetic engineering of oil palm will have to be developed in order to face future challenges. Transformation of oil palm has been achieved by using particle bombardment (Parveez, 2000), *Agrobacterium*-mediated gene delivery (Masli *et al*., 2009) and DNA microinjection (Masani *et al*., 2014). However, such transformation approaches are still considered difficult due to the tissue culture processes which exhibit slow cell growth and low response under *in vitro* condition during the selection and regeneration of transgenic plants (Hashim e*t al*., 2011; Masani *et al*., 2014). The transformation efficiencies previously recorded were rather low, between 0.7%-1.0% for *Agrobacterium* (Masli *et al*., 2009; Izawati e*t al*., 2015) and 1.0%-1.5% for biolistic (Parveez, 2000; Parveez *et al*., 2015b).

In last 30 years, improvements of gene constructs involving the choice of genes such as promoters, selectable markers and backbone vectors have been intensively studied. Most studies

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were started by comparing the strength of different promoters to transcribe the selectable marker gene in different tissues of specific plants. For oil palm, several promoters have been identified to drive the reporter genes at high expression levels, specifically the *35S* promoter of cauliflower mosaic virus (CaMV) and the maize ubiquitin promoter (*Ubi1*). Both promoters are constitutive promoters which have been characterised to deliver high transient gene expression in oil palm cells (Chowdhury *et al*., 1997; Masura *et al*., 2010). However, there has been no comparison between these promoters in the production of stably transformed oil palm cells.

Effective selection is an absolute requirement for successful transformation and regeneration of stable transgenic oil palm (Parveez *et al*., 2015a). Selection process requires suitable selection agents such as herbicide and antibiotic supplemented in selection media, allowing transformed cells to grow without any deleterious effects. A number of selectable marker genes have been tested for oil palm transformation, but the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* (*bar*) which confers resistance to the herbicide BASTA has been the most commonly used in oil palm transformation (Parveez, 2000; Masli *et al*., 2009; Nurfahisza *et al*., 2014; Parveez *et al*., 2015b). The *bar* gene has also been shown as a suitable selection agent for other monocot plants such as wheat (Melchiorre *et a*l., 2002), maize (Chen *et al*., 2008), rice (Zhao *et al*., 2011) and turfgrass (Song *et al*., 2013). While, the hygromycin phosphotransferase gene (*hpt*) of *E. coli* which confers resistance to hygromycin was also found to be the most efficient selection agent for oil palm transformation (Parveez *et al*., 1996). However, no report was published on production of stable transgenic oil palm using hygromycin as selection agent. Even though a few studies demonstrated regeneration of transgenic oil palm but no evidence of stable expression of transgenes has been achieved (Abdullah *et al*., 2005; Bhore and Shah, 2012). Nevertheless, the hygromycin antibiotic has been successfully used to obtain stable transgenic plants for legume, petunia, zoysia grass, finger millet and rice (Rosellini, 2012).

The ultimate goal of this study is to improve the genetic transformation and selection efficiency in oil palm. Efforts have been initiated to investigate the potential use of the *hpt* gene as the selectable marker in the production of stable oil palm transformation. We also focused our effort to evaluate the potential use of double cauliflower mosaic virus *35S* (2XCaMV*35S*) promoter to control the *hpt* gene and reporter gene, *i.e.* green fluorescent protein (*gfp)* for improved expression in oil palm. The transgene expression regulated by 2XCaMV*35S* promoter has been demonstrated to be highly active in most tissues including roots, mature leaves and shoots. The promoter has been used for the production of stable transgenic plants such as grape (Li *et al*., 2001), citrus (Benyon *et al*., 2013), soyabean (Paz *et al*., 2004), rice (Zheng *et al*., 2009) and maize (Reyes *et al*., 2010).

In this article, a vector carrying the *hpt* gene as the selectable marker and *gfp* as the visual reporter gene, under the control of double cauliflower mosaic virus *35S* (2XCaMV*35S*) promoter was successfully constructed with the aim to increase the level of transgene expression and transformation efficiencies in oil palm. The suitability of the new vector to be used for oil palm transformation was verified.

MATERIALS AND METHODS

DNA Manipulation

DNA work was carried out according to standard protocols (Sambrook *et al*., 1989). NucleoSpin® Gel Extraction kit (Macherey-Nagel, Germany) was used to isolate specific restriction and polymerase chain reaction (PCR) fragments from agarose gels. DNA ligation was carried out according to the manufacturer's instructions (Promega). Transformation of DNA into competent *E. coli* strain DH5α was carried out according to standard protocols.

Polymerase Chain Reaction (PCR) Amplification

PCR was carried out using PTC-100™ Programmable Thermal Controller or PTC-200™ Programmable Thermal Controller (MJ Research, Inc.). Amplifications were carried out in 25 µl reaction mixture consisted of 1×PCR buffer, 200 µM dNTPs, appropriate primers and 10 ng of plasmid DNA as template. Each PCR reaction was electrophoresed on 1% agarose gel at 110V for 110 min. The image of the ethidium bromide fluorescence of each amplified band was captured by the CCD camera system under UV irradiation.

Vector Construction

Three plasmid vectors, pTF101.1 (Paz *et al*., 2004) containing *bar* gene driven by the 2XCaMV*35S* promoter, pCAMBIA 1303 (CSIRO, Australia) containing *hpt* gene and *gusA-mgfp5*-His6 reporter gene driven by CaMV*35S* promoter, pAMCFDV (Masani *et al*., 2014) containing *mgfp* gene driven by the CFDV promoter, were used as DNA template for PCR amplification. The backbone vector used for the insertion of DNA fragment of promoter-gene of interest-terminator is pBINPLUS/ARS/*Fse*1, a modified vector from pBINPLUS/ARS (Belknap *et al*., 2008). Meanwhile, PCRII-TOPO (Invitrogen) and pGreenII000 (Hellens *et al*., 2000) were used as cloning vectors for construction of intermediate

vectors. Overall cloning strategy for the vector construction is shown in *Figure 1*.

Plant Material

Oil palm embryogenic callus cultures derived from an *E. guineensis* var. *tenera* was used in this study. The 0.5 g fresh weights of embryogenic calli cultured on EC agar-solidified media containing MS macro and micronutrients (Murashige and Skoog, 1962) supplemented with 1 mg/napthalene acetic acid (NAA) and 30 g litre^{-1} sucrose (Parveez and Christou, 1998) were used and arranged in the centre of a Petri dish prior to bombardment. The

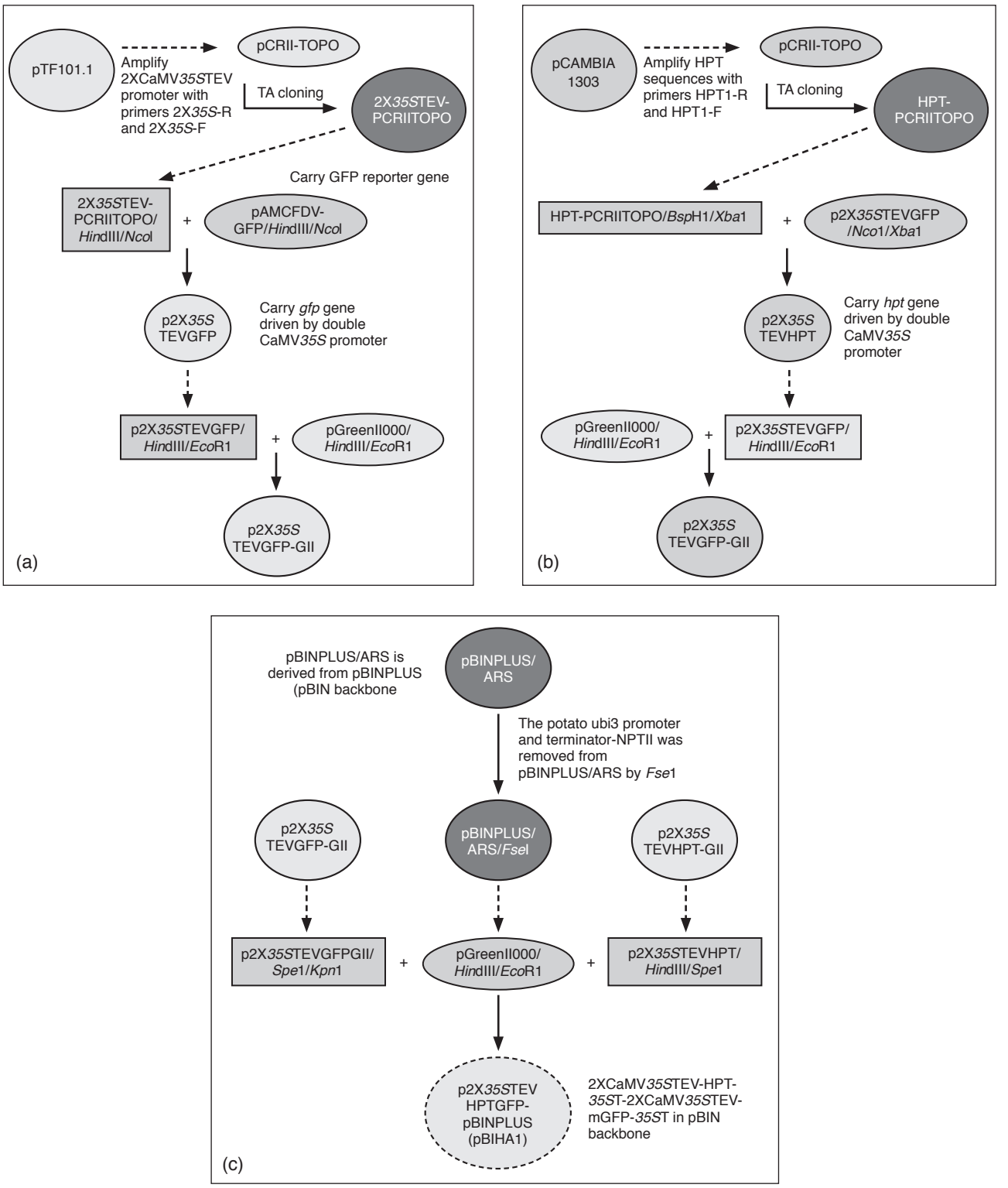

Figure 1. Cloning strategies of the 2X35S-TEVHPT-35ST (a) and 2X35S-mGFP-35ST (b) in pBINPLUS/ARS backbone to create pBIHA1 (c). Plus symbol (+) indicates the ligation of digested DNA fragments (represented by squares) to corresponding vectors restricted with appropriate restriction enzymes (represented by oval). Arrows shows the vectors resulted from the ligation (represented by circles).

bombarded embryogenic calli were incubated in the dark at 28°C and continuously sub-cultured onto fresh EC medium every month.

Biolistic Transformation of Oil Palm Calli

The plasmid DNA was precipitated onto gold according the instruction manual for the Biolistic PDS-1000/He (BioRad) device. To each aliquot of 100 µl gold particles, 20 µg DNA, 100 µl 2.5 M $CaCl_2$, and 40 µl 0.1 M spermidine were added in and vortexed vigorously for 3 min followed by centrifugation at 10 000 rpm for 10 s. The supernatant was removed as much as possible and the pellet used was washed twice with 500 µl 100% ethanol, mixed and centrifuged at 10 000 rpm for 60 s. Finally, the DNA coated gold particles were resuspended in 120 µl 100% ethanol. For each bombardment, about 6 µl of DNA coated gold particles was dispensed in the centre of a macrocarrier and dried under sterile condition and used immediately for particle bombardment. Embryogenic calli which were placed in the centre of solid medium were used as target tissues. Transformation was carried out under the following conditions; rupture disc pressure at 1100 psi, distance from rupture to macrocarrier and macrocarrier to stopping screen was at 6 mm and 11 mm, 75 mm distance from stopping plate to target tissue and 67.5 mm Hg vacuum pressure (Parveez, 1998).

Microscopic Detection of GFP

Expression of the *gfp* gene was examined in bombarded of oil palm embryogenic calli. Five replications were carried out and two controls were also incorporated; 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. Visualisation of GFP fluorescence on bombarded oil palm calli was carried out at several time intervals; 24 hr after the bombardment followed by five and seven days. Images of GFP-expressing cells were captured using a Fluorescence Microscope Multizoom AZ-100 (Nikon) with a GFP specific filter.

RESULTS AND DISCUSSION

Construction of Transformation Vectors

A vector designated as pBIHA1, carrying the hygromycin phosphotransferase (*hpt*) gene as the selectable marker and a modified *gfp* (m*gfp*) as the visual reporter gene, each was under the control of double cauliflower mosaic virus *35S* (2XCaMV*35S*) promoter, was successfully constructed by assembling *hpt* and m*gfp* expression fragments in pBINPLUS/ARS/*Fse*1. In total, five intermediate vectors were constructed in order to produce the pBIHA1 vector (*Table 1*). All vectors were sequenced to confirm the integrity of DNA region.

The pBINPLUS/ARS/*Fse*1 backbone (9.6 kb) was modified from the binary plant transformation vector pBINPLUS/ARS (*Figure 2a*) that utilises the ubiquitin promoter and terminator sequences (*Ubi3*) to drive the *npt*II selectable marker gene. The modification was carried out by removing the potato ubi3 promoter-*npt*II-*ubi3* terminator DNA fragment from pBINPLUS/aRS (12.4 kb) by Fse1 digestion, followed by self-ligation to produce pBINPLUS/ARS/*Fse*I (*Figure 2b*). The pBINPLUS/

TABLE 1. LIST OF CONSTRUCTED VECTORS IN THIS STUDY

No.	Ligation	Vectors	Size (bp)	Description
1	PCR2X*35S*TEV+PCRII TOPO	2X*35S*TEV-PCRII TOPO	4 894	2XCaMV*35S*TEV-PCRII TOPO
2	pAMCFDV-GFP/*Hin*dIII/NcoI +2X*35S*TEV-PCRII TOPO/*Hin*dIII/NcoI	p2X*35S*TEVGFP	4 502	2XCaMV*35S*TEV-mGFP-*35S*T
3	PCRHPT1+PCRII TOPO	HPT- PCRII TOPO	5 013	HPT-PCRII TOPO
4	HPT-PCRII TOPO/*Bsp*HI/*Xba*I +p2X*35S*TEVGFP/*Nco*I/*Xba*I	p2X*35S*TEVHPT	4 805	2XCaMV*35S*TEV-HPT-*35S*T
5	pGII000/*Hin*dIII/*Eco*RI + p2X*35S*TEVHPT/*Hin*dIII/*Eco*RI	p2X*35S*TEVHPT-GII	5 462	2XCaMV*35S*TEV-HPT-*35S*T -pGII000
6	p2X*35S*TEVGFP/*Hin*dIII/*Eco*RI + pGII000/*Hin*dIII/*Eco*RI	p2X*35S*TEVGFP-GII	5 159	2XCaMV*35S*TEV-mGFP-*35S*T-pGII000
7	p2X*35S*TEVGFP/*Hin*dIII/*Spe*I + p2x*35S*TEVGFP-GII/*Spe*I/*Kpn*I +pBINPLUS/ARS/*Fse*I/*Hin*dIII/*Kpn*I	p2X*35S*TEVHPTGFP-pBINPLUS (pBIHA1)	14 194	2XCaMV*35S*TEV-HPT-35ST-2XCaMV*35S*TEV-mGFP-*35S*T-pBINPLUS
8	pBINPLUS/ARS/*Fse*I	pBINPLUS/ARS/*Fse*I	10 082	pBINPLUS/ARS/*Fse*I

Note: 1-6: Intermediate vectors, 7: Final transformation vector and 8: Backbone vector.

ARS/*Fse*l contains a unique *Fse*1 restriction site for the insertion of foreign gene sequences. Binary plant vector pBINPLUS/ARS was based on the pBINPLUS vector (Van Engelen *et al*., 1995), an improved vector derived from pBIN19 (Bevan, 1984). The pBINPLUS/ARS vector comprises a number of user friendly features such as two rare restriction sites (*Fse*I) around the multiple cloning sites for easier subcloning of target genes. The vector replicates at high copy numbers in *E. coli*, leading to a high-yield DNA concentration for routine cloning procedure as well as for bombardment experiments. The pBINPLUS/ARS has been successfully used to introduce genes of interest into potato, tobacco, apple and tomato (Belknap *et al*., 2008).

In this study, 2XCaMV*35S* promoter was used as a driving promoter of *hpt* and *mgfp* genes with the aim to exhibit strong constitutive activity in oil palm tissues and subsequently result in efficient selection for stable transgenic oil palm. Higher expression also has been demonstrated in transgenic plants regulated by 2XCaMV*35S* promoter such as grape (Li *et al*., 2001) and citrus (Benyon *et al*., 2013). Constitutively transgene expression driven by this promoter also resulted in the production of stable transgenic plants for soyabean (Paz *et al*., 2004), strawberries (Qin *et al*., 2008), rice (Zheng *et al*., 2009), maize (Reyes *et al*., 2010) and citrus (Mondal *et al*., 2012). The DNA fragment of 2X*35S* including TEV leader sequence, was amplified from pTF101.1 using the primers (restriction site underlined): 2X*35S*-F (5′-GGAAGCTTCCTGCAGGTCAACATGGT-3′) and 2X*35S*-R (5′-GGCCATGGTAGATCCCCCGTT CG TAAAT-3′). A 0.9 kb 2X*35S*TEV PCR product was cloned into PCRIITOPO (3.9 kb) vector to generate a 4.9 kb 2X*35S*TEV-PCRIITOPO. The positive clones were selected and confirmed by restriction enzyme digestions and DNA sequencing. TEV leader sequence is non-encoding 5′-end of Tobacco Etch Virus (TEV) sequence which has been shown to enhance the translation of genes in transgenic plants (Saunders and Lomonossoff, 2013). The TEV leader sequence has been shown to increase the transgene expression activity seven to 10-fold compared to the expression of transgene without TEV leader sequence (Saunders and Lomonossoff, 2013).

The 0.9 kb 2X*35S*TEV promoter fragment was extracted from plasmid 2X*35S*TEV-PCRIITOPO and ligated to the *Hin*dIII and NcoI sites of pAMCFDV-GFP (3.9 kb) to create p2X*35S*TEVGFP (4.5 kb). The positive clones for p2X*35S*TEVGFP were identified by *Hin*dIII and *Eco*R1 digestion which produced two DNA fragments of 1.9 kb 2X*35S*TEV-GFP-*35S*T and 2.6 kb pUC19 as shown in *Figure 3a*. The 2X*35S*TEVGFP fragment was later inserted into the *Hin*dIII and E*co*R1 sites of pGreenII0000 to generate a 5.2 kb p2X*35S*TEVGFP-GII (*Figure 1a*) which was identified by digestion with *Hin*dIII and *Eco*R1 (*Figure 3b*). The p2X*35S*TEVGFP-GII was constructed to introduce a unique restriction enzyme site for further cloning procedure. The cloning vector, pGreenII0000, is a mini binary vector that has the advantage of being relatively small (3.3 kb) for easy sub cloning procedure due to the presence of unique multiple cloning sites. The plasmid also replicates at high copy numbers in *E. coli* (Hellens *et al*.,

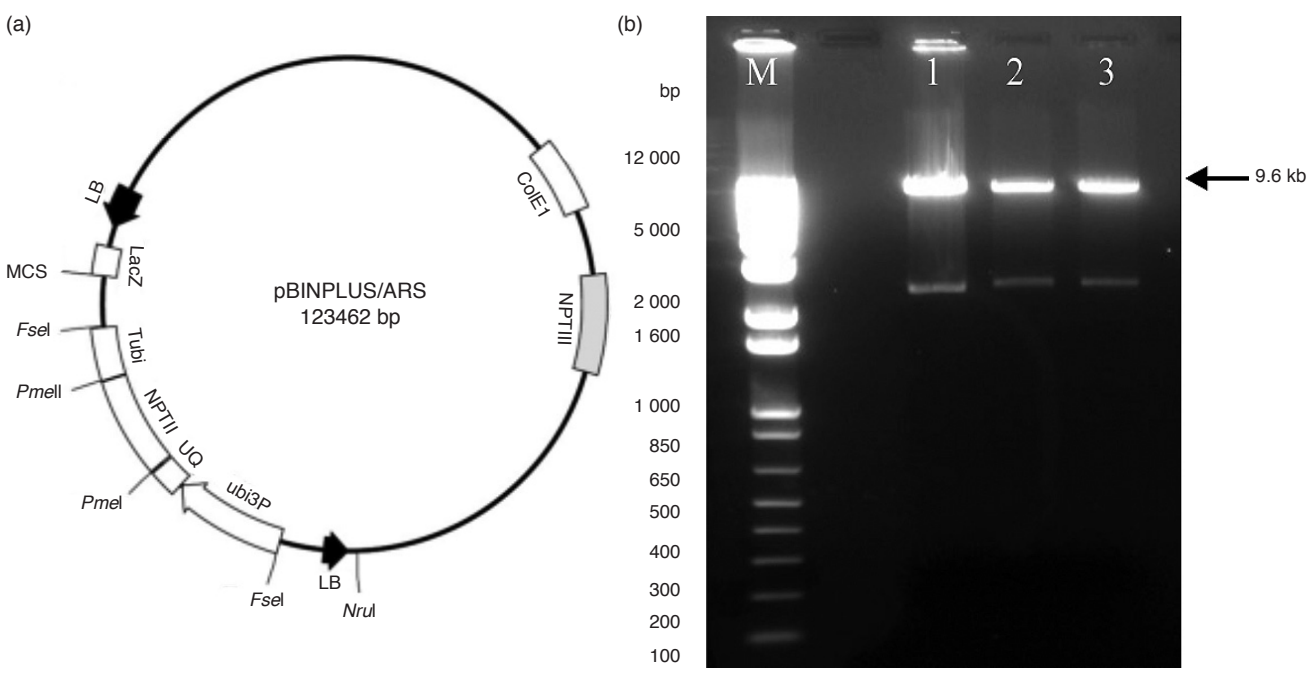

*Figure 2. Construction of pBINPLUS/ARS/*FseI *by removing the potato ubi3 promoter-NPTII- ubi3 terminator DNA fragment from pBINPLUS/ARS (12.4 kb). (a) pBINPLUS/ARS; (b) lanes 1-3: purified plasmid DNA pBINPLUS/ARS was digested with* Fse1. *Lane M: 1 kb plus DNA ladder. Arrow indicates the size of fragment at 9.6 kb.*

2000) making the plasmid DNA isolation is more convenient for identification of positive clones by restriction enzyme analysis.

In order to allow for selection of transformed oil palm tissues with the antibiotic hygromycin, the *hpt* gene fragment was amplified from pCambia1303 using primers (restriction site underlined): HPT1-F (5′-GGTCATGAAAAAGCCTGACACCGCG-3′) and HPT1-R (5′-GGCCTCTAGA CTATTTCTTTG CCCTCG GACG-3′). The 1.0 kb PCR product was gel purified and cloned into PCRIITOPO vector to produce HPT-PCRIITOPO (5.0 kb). Then, the HPT fragment was excised with BspHI and *Xba*1, gel purified and ligated into the *NcoI* and *Xba*1 sites replacing the *gfp* gene of p2X*35S*TEVGFP to generate p2X*35S*TEVHPT (4.8 kb). The positive clones for p2X*35S*TEVHPT were confirmed by *Hin*dIII and *Eco*R1 digestion (*Figure 3c*). The 2X*35S*TEVHPT was later digested with *Hind*III and *Eco*R1, gel purified and cloned into the similar sites of pGreenII0000 to generate a 5.5 kb of p2X*35S*TEVHPT-GII (*Figure 1b*). Selection of positive clones for p2X*35S*TEVHPT-GII was carried out by *Hin*dIII and *Eco*R1 digestion which produced 2.2 kb 2X*35S*TEV-HPT-35ST and 3.3 kb pGreenII000 DNA fragments (*Figure 3d*).

For construction of the final transformation vector (*Figure 1c*), a ligation procedure was performed using 3 DNA fragments in order to assemble the 1.9 kb 2X*35S*TEVGFP and 2.2 kb 2X*35S*TEVHPT in a single vector. The *Hin*dIII and *Spe*1 restricted 2X*35S*TEVHPT DNA fragment of p2X*35S*TEVHPT-GII and *Spe*I and *Kpn*I restricted 2X*35S*TEVGFP DNA fragment of p2X*35S*TEVGFP-GII were ligated to the *Hin*dIII and *Kpn*1 sites of pBINPLUS/ARS/*FseI* to create p2X*35S*TEVHPTGFP-pBINPLUS (~14.2 kb) designated as pBIHA1 (*Figure 4*). The p2X*35S*TEVHPTGFP-pBINPLUS vector containing dual gene cassettes adjacent; the expression cassette with *gfp* gene and selection cassette with *hpt* gene under the control of constitutive 2XCaMV*35S* promoter. This vector was designed in such a way for providing the potential for genomic integration of dual genes to be introduced into the plant tissues at a single locus. Subsequently, resulting in stable integration of the transgene at a transcriptionally active gene locus, which will be particularly useful for genetic engineering applications (Masani *et al*., 2009).

Visualisation of GFP Expression

The viability of vector pBIHA1 was evaluated in oil palm embryogenic calli via biolistic transformation. This was carried out to ensure that the construct carries an effective and functional *gfp* gene. Even though *gfp* gene expression was unstable in oil palm cells possibly due to toxicity (Parveez and Majid, 2008), the gene is still an attractive reporter gene for oil palm transformation as it is a non-destructive visual reporter marker that could be used to confirm the possibility of a successful transformation. Early evidence of successful transformation is very important since the selection process in oil palm transformation is laborious and time-consuming procedure. It was shown that putative transgenic oil palm embryoids could only be obtained after 6-10 months of culture. Longer time is needed for obtaining the transgenic shoots or plantlets when transformed calli were cultured on selection media (Parveez *et al*., 2015b; Izawati *et al*., 2015). In this study, the *gfp* expression cassette was placed next to *hpt*

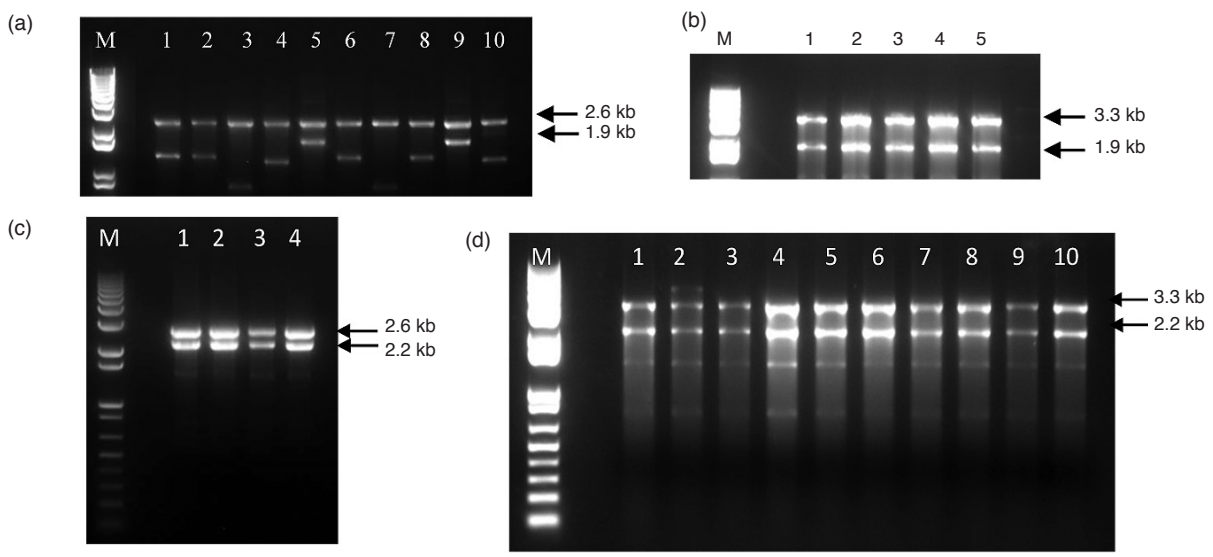

Figure 3. Screening of positive clones for intermediate vectors. Equal amount of DNA of each intermediate vector was digested with Hin*dIII and* Eco*R1. Arrow indicates the size of the fragments produced for positive clones in each gel as predicted. Lane M: 1 kb plus DNA ladder. Restriction endonuclease analyses of (a) lanes 5 and 9: positive clones for p2X35S*TEVGFP*; (b) lanes 1- 5: positive clones for p2X35S*TEVGFP-GII*; (c) lanes 1- 4: positive clones for p2X35S*TEVHPT *and (d) lanes 1- 10: positive clones for p2X35S*TEVHPT-GII.*

expression cassette for confirmation of transgene delivery and determination of transformation efficiency. Result showed that the bombarded oil palm calli produced visible GFP spots as early as 24 hr post-bombardment, and reached a peak of more than 30 spots per tissue clump in five to seven days post-bombardment (*Figures 5a* and *5b*). No GFP activity was observed in control tissues (*Figure 5c*). The results clearly suggested that the construct was successfully transferred into the oil palm cells and the *gfp* reporter gene within the construct was functional.

CONCLUSION

A new vector designed as pBIHA1 carrying the *hpt* and *gfp* genes driven by the double cauliflower mosaic virus *35S* is currently available to be used in both biolistic and *Agrobacterium*-

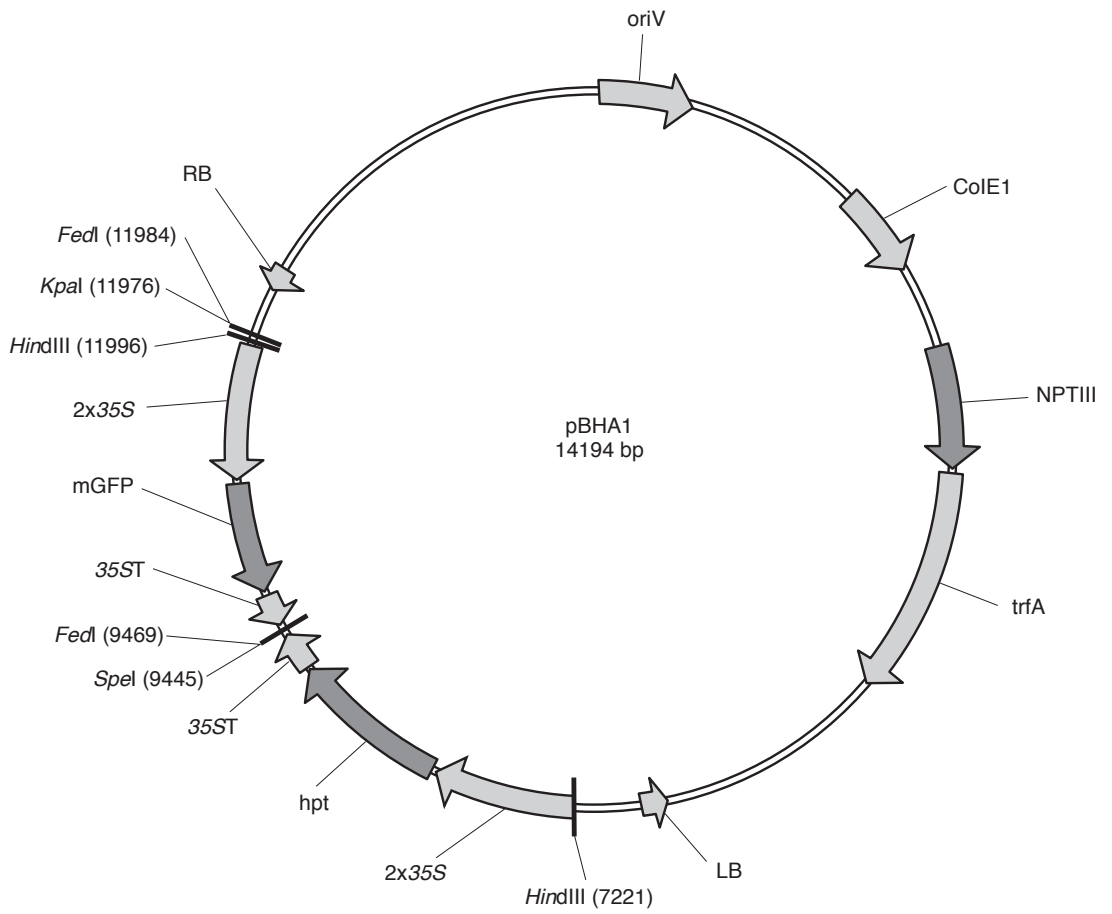

Figure 4. Restriction map of pBIHA1 vector. The restriction sites and the number of each site show the position in the vector. The arrow shows the orientation of each of DNA fragments assembled. LB: left border of T-DNA; 2X35S: double cauliflower mosaic virus 35S promoter; hpt: gene for hygromycin phosphotranferase; mGFP: modified green fluorescent protein; 35ST: 35S terminator gene and RB: right border of T-DNA.

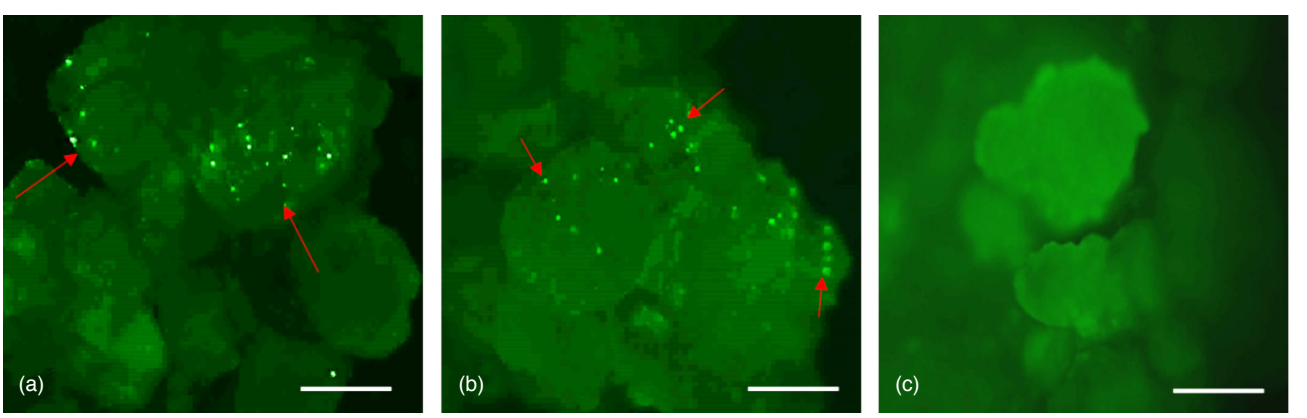

Figure 5. Visualisation of mGFP fluorescence in bombarded oil palm embryogenic calli with pBIHA1 vector. a, b: 24 hr after bombardment and c: non-bombarded embryogenic calli as control. Red arrows indicate GFP spots and scale bar equals 300 um.

based transformation protocols for oil palm. Further research will continue to investigate the regeneration rate of pBIHA1 transformed calli cultured on hygromycin selection media towards developing an efficient oil palm transformation system. In addition, the stable integration of the gene into the cells of oil palm plants will later be verified by PCR and Southern blot analyses to further confirm the integration and copy number of the transferred gene, respectively. In the future, we expect that this vector will make it possible to carry and transfer a gene that will improve the value of oil palm varieties.

ACKNOWLEDGEMENT

The authors would like to thank the Director-General of MPOB for permission to publish this article and Dr R W Bellnap (USDA Agricultural Research Services) for the supply of pBINPLUS/ARS vector use in this study. We also would like to acknowledge all members of the Transgenic Technology Group of MPOB for their assistance.

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