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VECTOR CONSTRUCTION AND TRANSIENT EVALUATION IN OIL PALM CALLI VIA *Agrobacterium*-MEDIATED TRANSFORMATION

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ABSTRACT

Current oil palm transformation efficiency was relatively low compared to other monocots such as rice and sorghum. Thus, the construction of efficient transformation vectors is crucial for Agrobacterium*-mediated oil palm genetic engineering to elevate the transformation efficiency. In this study, six transformation vectors, which contain phosphinothricin acetyltransferase* (bar)*, modified green fluorescent protein* (mgfp) *and synthetic green fluorescent protein (*sgfp(S65T)*) genes driven by either maize ubiquitin (Ubi-1) or cauliflower mosaic virus (CaMV35S) promoters, were assembled in pBINPLUS/ARS or pCAMBIA0380 backbones. The efficiency of these vectors was evaluated in oil palm calli. Based on transient GFP signals, the pBINPLUS/ARS-based vectors produced a higher number of GFP signals than the pCAMBIA0380-based vectors. The CaMV35S promoter was more excellent than the Ubi-1 promoter in driving the expression of* gfp *genes. Furthermore, the* sgfp(S65T) *variant was more suitable for oil palm transformation than the* mgfp *variant because it produced more GFP signals in the oil palm calli than the* mgfp *variant. These results suggested that the pBAR65 vector, which carries the* bar *and* sgfp(S65T) *genes driven by the CaMV35S promoter in the pBINPLUS/ARS backbone, is the most suitable vector and could be used to develop an efficient* Agrobacterium*-mediated transformation system for oil palm.*

Keywords: *Agrobacterium*-mediated transformation, green fluorescent protein (GFP), transgenic oil palm, vector backbone.

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INTRODUCTION

Genetic engineering has been used worldwide to improve quality and introduce new traits in various plants, including food crops, medicinal plants, ornamentals and oil-producing plants such as the oil palm (*Elaeis guineensis* Jacq.). Like other economically important plants, efforts to improve oil palm planting materials through genetic engineering have been progressively carried out (Masani *et al*., 2018; Masura *et al*., 2017; Rasid *et al*., 2020). In any plant transformation works, studies were carried out to identify the most appropriate vector elements for that particular plant transformation system. For instance, Hiei and Komari (2006) reported that a combination of an ordinary *Agrobacterium tumefaciens* strain LBA4404 and a derivative of super binary pTOK233 vector was the most efficient in indica rice transformation as compared with the combination of a supervirulent *A. tumefaciens* strain EHA101 with a standard pIG121Hm binary vector. Likewise, an efficient transformation was also achieved in sorghum using the combination of a super binary vector and LBA4404 strain (Carvalho *et al*., 2004). However, in maize, a combination of a standard binary vector with EHA101 strain generated reproducible transformation events (Frame *et al*., 2002). Besides standard binary and super binary vectors, a ternary vector system was developed for maize transformation. This ternary vector system yielded a higher transformation efficiency than the super binary vector system (Anand *et al*., 2018).

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Besides vector, the choice of a promoter to drive the expression of a selectable marker, reporter gene and gene of interest (GOI) is also important. In monocotyledon plants such as wheat (Pérez-Piñeiro *et al.*, 2012) and geophytes (Koetle *et al.*, 2015), most of the transformation studies used maize ubiquitin (Ubi-1) and Cauliflower Mosaic Virus (CaMV35S) promoters. In oil palm, the CaMV35S and Ubi-1 promoters have been shown to drive high transient gene expression in oil palm cells (Fizree *et al.*, 2019; Masura *et al.*, 2010). In addition, Bahariah *et al*. (2017) have shown that a double CaMV35S promoter was also able to drive gene expression in oil palm cells.

Another critical element of a plant transformation vector is the selectable marker genes for the selection of the transgenic plant. Most of the selection systems for transgenic plants used the negative selectable marker genes, which confer resistance to antibiotics or herbicides (Masani *et al.*, 2018). Among the most commonly used selectable marker genes are the hygromycin phosphotransferase (*hpt*) (Hiei and Komari, 2008) and phosphinotrichin acetyl transferase gene (*pat* or *bar*) (Ishida *et al.*, 2015). The integration and expression of *hpt, pat* and *bar* genes produce enzymes that can metabolise the selection agent and allow the survival of transformed cells but suppress the growth of non-transformants (Yadava *et al.*, 2017). For oil palm, the most frequently used selectable marker gene is the *bar* gene (Hanin *et al.*, 2020; Masani *et al.*, 2018; Masura *et al.*, 2017; Parveez *et al.*, 2015). The *bar* gene was commonly used for the selection of transgenic oil palm because it allows easy identification of transformed oil palm calli as compared to dark non-transformed calli when selected on Basta herbicide, a commercial formulation that contains the ammonium salt of phosphinothricin. Meanwhile, selection using hygromycin antibiotic produced oil palm calli with a brown phenotype that caused difficulty in recognising the true transformants (Masani *et al.*, 2018).

Most of the transformation vectors also carry reporter genes. The most commonly used reporter genes are β-glucuronidase (*gusA*), luciferase (*Luc*), green fluorescent protein (*gfp*) and anthocyanin (Anami *et al.*, 2013). In addition, genes encoding the red fluorescent protein (RFP), the *DsRED* and its improved version, *DsRED2*, were also used in plant studies (Lin *et al.*, 2011). For oil palm transformation, the *gusA* (Masura *et al.*, 2010; 2011), *gfp* (Bahariah *et al.*, 2017; Majid and Parveez, 2016; Parveez and Majid, 2008; 2018) and *DsRED* (Fizree *et al.*, 2019), were used as the reporter genes.

This study discussed the construction of transformation vectors carrying different promoters and *gfp* genes in different backbones. These vectors carry the *mgfp* or *sgfp(S65T)* genes, each driven by the Ubi-1 or CaMV35S promoters in pBINPLUS/ARS-based or pCAMBIA-based backbone. These vectors were transformed into oil palm calli to evaluate the efficiency of each vector via transient GFP expression study. This study aimed to determine the most efficient vector for oil palm transformation to elevate the current transformation efficiency, which was considered relatively low compared with other monocots. The reported transformation efficiency was 0.7% to 1.0% for *Agrobacterium*-mediated transformation (Izawati *et al.*, 2012; 2015; Masli *et al.*, 2009) and 1.0% to 1.5% for biolistic (Parveez, 2000). Whereas for rice, 14.0% to 26.4% transformation efficiencies were achieved via *Agrobacterium*-mediated transformation for indica rice cultivar MR219 (Tan *et al.*, 2017). For sorghum, the reported transformation efficiency has reached 14.0% to 33.0% for *Agrobacterium*-mediated transformation and ranged from 1.0% to 20.7% for the biolistic method depending on vectors, *Agrobacterium* strains, selection agents and various parameters studied (Ahmed *et al.*, 2018).

MATERIALS AND METHODS

DNA Manipulation

Polymerase Chain Reaction (PCR) amplification and DNA manipulation were performed using plasmids listed in *Table 1*. The plasmid DNA was isolated using Nucleospin Plasmid EasyPure according to the manufacturer's protocol (Machery-Nagel, Germany). All restriction enzyme digestions of the DNA fragment were carried out according to the recommended protocol (New England Biolabs, USA). The DNA fragment was isolated using the NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Germany). In some cloning experiments, the vectors were blunted prior to a ligation reaction. The cohesive ends were filled in a reaction containing 1X *Pfx* Amplification Buffer (Invitrogen, USA), 0.2 mM dNTP mix, 0.5 mM $MgSO_4$, 1.25 units of Platinum *Pfx* DNA Polymerase (Invitrogen, USA) and sufficient amount of vector.

The mixture was incubated at 68°C for 15 min and purified using the NucleoSpin Gel and PCR Clean-up Kit. To avoid self-ligation of a vector, the 5′ phosphate group was detached with 5 units of Calf Intestinal Phosphatase (CIP) (Promega, USA) in a 40 µL reaction at 37°C for 60 min. Then, the dephosphorylated vector was purified using the NucleoSpin Gel and PCR Clean-up Kit. The insert was ligated into the vector using T4 DNA ligase (Promega). The ligation was carried out at 16°C overnight. The ligation of the insert into the pCRII-TOPO vector was carried out according

TABLE 1. LIST OF PLASMIDS USED IN THE CONSTRUCTION OF TRANSFORMATION VECTORS

Plasmid	Description	Reference/source
pBINPLUS/ARS/FseI	Binary vector for *Agrobacterium*-mediated plant transformation	Bahariah *et al.* (2017)
pAMDsRED	Carries the *DsRED* gene driven by CaMV35S promoter	Fizree *et al.* (2019)
pTF101.1	Binary vector for *Agrobacterium*-mediated plant transformation Contains TEV-*bar* fragment	Paz *et al.* (2004)
pCAMBIA0380	Binary vector for *Agrobacterium*-mediated plant transformation	Abcam (2021)
pGreenII0000	Binary vector for *Agrobacterium*-mediated plant transformation	Hellens *et al.* (2000a)
pME8	Contains maize ubiquitin promoter (Ubi-1) and transcriptional termination signal	Willis *et al.* (2008)
pAHC20	Contains maize ubiquitin promoter	Yunus *et al.* (2008)
pHBT-sGFP(S65T)	Contains *sgfp(S65T)* gene	Majid and Parveez (2007)
pAMCFDV-GFP	Carries the *mgfp* gene driven by CFDV promoter	Masani *et al.* (2014)
pCRII-TOPO	Commercial cloning vector	Invitrogen
p35SS65TGII	Carries the *sgfp(S65T)* gene driven by the CaMV35S promoter	MPOB

TABLE 2. LIST OF PRIMERS FOR AMPLIFICATION OF TEV-*bar* AND *mgfp* FRAGMENTS

Gene	Primer	Sequences	Restriction site
TEV-*bar*	TEVBAR-F	5′-GGCCGGATCCAATTAATTCTCAACACAACA-3′	*Bam*HI
	TEVBAR-R	5′-AATTGGATCCCTAGATTGTTGAGCAGATCTCG-3′	*Bam*HI
TEV-*bar*	TEVBAR2-F	5′-GGCCCTCGAGAATTAATTCTCAACACAACA-3′	*Xho*I
	TEVBAR2-R	5′-GGTCTAGACTAGATTGTTGAGCAGATCT-3′	*Xba*I
mgfp	mGFP2-F	5′-GCGGATCCCCATGGGTAAAGGAGAAGAACTTT-3′	*Bam*HI
	mGFP2-R	5′-GCGCGCGGATCCTTATTTGTATAGTTCATCCATGCC-3′	*Bam*HI

Note: The restriction sites are underlined.

to the manufacturer's protocol (Invitrogen, USA). The ligation mixture was transformed into competent *Escherichia coli* strain DH5α cells using the heat shock protocol and screened using colony PCR followed by restriction endonuclease analysis.

Polymerase Chain Reaction (PCR) Amplification

PCR amplification was used to amplify and add restriction enzyme sites in the TEV-*bar* insert, which was amplified from the pTF101.1 plasmid. Two sets of primers were used to add different restriction enzyme sites in the TEV-*bar* insert to construct two different intermediate vectors. Likewise, PCR amplification was also used to amplify and add restriction enzyme sites to the *mgfp* gene which was sourced from the pAMCFDV-GFP plasmid. All primers used in the PCR amplification are listed in *Table 2*. PCR was performed in a 25 µL reaction containing 1X Accuprime PCR Buffer I (Invitrogen, USA), 200 nm of each forward and reverse primers, 0.5 unit of Accuprime *Taq* DNA polymerase (Invitrogen, USA) and 10 ng of the plasmid. The PCR programme was set as follows: Pre-denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 1 min and final extension at 68°C for 10 min. After the PCR reaction was completed, the vials were placed on ice, added with 0.5 µL of *Taq* DNA polymerase and incubated at 72°C for 15 min to add the 3′ A-overhangs. The A-tailed PCR product was separated on a 1.0% agarose gel, purified using the NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Germany), and cloned into the pCRII-TOPO vector.

Vector Construction

All plasmids used for the construction of transformation vectors are listed in *Table 1*. The pTF101.1, pAMCFDV-GFP, pHBT-sGFP(S65T) or p35SS65TGII, pAHC20 and pAMDsRED were used as the source for tobacco etch virus translational enhancer (TEV)-infused *bar* gene, *mgfp* gene, *sgfp(S65T)* gene, Ubi-1 and CaMV35S promoters, respectively. The final backbone vectors for insertion of the promoter-GOI-terminator cassette were the pBINPLUS/ARS/*Fse*I and pCAMBIA0380. The pCRII-TOPO (Invitrogen) and pGreenII0000 were used as cloning vectors to construct intermediate vectors. Overall cloning strategies for all vectors are illustrated in *Figures 1, 2* and *3*.

Construction of pBINUBG and pZUBG transformation vectors. The construction procedure for transformation vectors carrying the *bar* and *mgfp* genes regulated by the Ubi-1 promoter in pBINPLUS/ARS and pCAMBIA0380 backbones is shown in *Figure 1*. The pTF101.1 plasmid was used as a template to amplify the TEV-*bar* fragment, and *Bam*HI sites were added at 5′ and 3′ sites of the fragment using the primers TEVBAR-F and TEVBAR-R (*Table 2*). The fragment (709 bp) was purified and cloned into the pCRII-TOPO vector to yield pTEVBAR3. The identity of the TEV-*bar* fragment in the pTEVBAR3 plasmid was verified by sequencing. Then, the TEV-*bar* fragment was digested from pTEVBAR3 using *Bam*HI and ligated into the *Bam*HI sites of pME8, which contained the Ubi-1 promoter to create pME8-TEVBAR. The resulting pME8-TEVBAR plasmid carried the *bar* gene controlled by the Ubi-1 promoter. Meanwhile, the pAMCFDV-GFP plasmid was used as a template to amplify the *mgfp* gene using primers mGFP2-F and mGFP2-R (*Table 2*), and *Bam*HI sites were also added at the 5′ and 3′ ends. The *mgfp* fragment was purified and cloned into the *Bam*HI sites of pME8 to create pME8-GFP. This plasmid carried the *mgfp* gene driven by the Ubi-1 promoter. After that, the *Spe*I-digested fragment of pME8-TEVBAR, which contained the Ubi-1 promoter and TEV-*bar* fragment, was cloned into the *Avr*II site of pME8-GFP to yield pME8BG, which carried both the *bar* and *mgfp* genes controlled by the Ubi-1 promoter. Then, the *Spe*I fragment of pME8BG was blunted and ligated into the *Sma*I site of pBINPLUS/ARS/*Fse*I to create pBINUBG. For the construction of transformation vectors in the pCAMBIA0380 backbone, the *Spe*I fragment of pME8BG was ligated into the *Spe*I site of pCAMBIA0380 to create pPZUBG. The resulting final vectors were digested with restriction enzymes to confirm their identity.

Construction of pNH1 and pNH2 transformation vectors. The overall cloning strategy is shown in *Figure 2*. The Ubi-1 promoter was released from the pAHC20 plasmid by double digestion with *Hind*III and *Bam*HI. The *sgfp(S65T)* gene with Nos terminator was released from the pHBT-sGFP(S65T) by double digestion with *Bam*HI and *Eco*RI. Then, both DNA fragments were ligated into the *Hind*III and *Eco*RI sites of the pGreenII0000 plasmid to yield pUbi-sGFP(S65T)-GII. Then, the Ubi1-TEV-*bar* fragment was excised from pME8-TEVBAR by *Spe*I digestion and ligated into the *Spe*I site of pUbi-sGFP(S65T)-GII to create pUbiS65TEVBAR-GII plasmid. This intermediate vector carries the *sgfp(S65T)* and TEV-*bar* genes controlled by the Ubi-1 promoter. Next, the pUbiS65TEVBAR-GII plasmid was digested with *Hind*III and *Avr*II. The digested fragment was ligated into the *Hind*III and *Xba*I sites of pBINPLUS/ARS/*Fse*I to create pNH1 vector. In addition, the *Hind*III and *Avr*II digested fragment was also ligated into the *Hind*III and *Avr*II sites of pCAMBIA0380 plasmid to create pNH2 vector. The

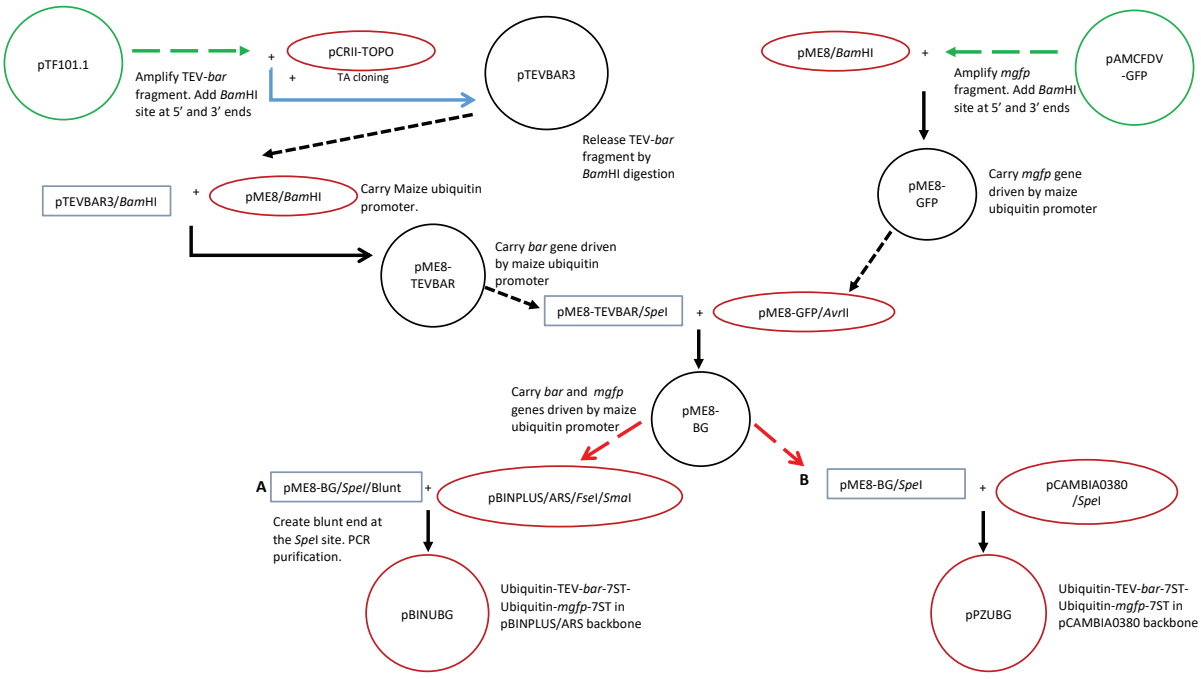

*Figure 1. Overall cloning strategy for the construction of ubiquitin-TEV-*bar*-7ST-ubiquitin-*mgfp*-7ST in pBINPLUS/ARS backbone (pBINUBG) and pCAMBIA0380 backbone (pPZUBG). Symbol plus (+) shows the ligation of digested DNA fragments (represented by squares) to corresponding vectors digested with appropriate restriction enzymes (represented by an oval). Arrows indicate the vectors resulting from the ligation (represented by circles).*

integrity of the vectors was verified by digestion with *Bam*HI.

Construction of pBAR65 and pBARSGFP transformation vectors. The overall cloning strategy for pBAR65 and pBARSGFP vectors is shown in *Figure 3*. The pTF101.1 plasmid was used as a template to amplify the TEV-*bar* fragment. The PCR primers (*Table 2*) were designed to add the *Xho*I site (TEVBAR2-F) at 5′ and the *Xba*I site (TEVBAR2-R) at 3′ ends of the fragment. The fragment (709 bp) was purified and cloned into pCRII-TOPO vector to yield pTEVBAR4. The pAMDsRED plasmid was used as the source for the CaMV35S promoter and 35S terminator (35ST). The *DsRED* gene was removed by digestion with *Xho*I and *Xba*I and replaced with TEV-*bar* gene from the pTEVBAR4 plasmid to create p35S-TEVBAR plasmid. Then, the 35S-TEV-*bar*-35ST fragment was released from the p35S-TEVBAR plasmid by digestion with *Hind*III and *Eco*RI.

Next, the 35S-*sgfp(S65T)*-35ST fragment was released from p35SS65TGII plasmid (*Table 1*) via *Kpn*I and *Eco*RI double digestion. Finally, both fragments were ligated into the *Hind*III and *Kpn*I sites of the pBINPLUS/ARS/*Fse*I plasmid to create pBAR65 vector. Then, the CaMV35S-TEV-*bar*-35ST-CaMV35S-*sgfp(S65T)*-35ST fragment was released from pBAR65 with *Hind*III digestion and ligated into *Hind*III site of pCAMBIA0380 to create pBARSGFP. Finally, the identity of the pBAR65 vector was analysed via digestion with *Hind*III while the identity of the pBARSGFP vector was analysed via digestion with *Bam*HI.

Mobilisation of Transformation Vectors into *A. tumefaciens* Strains

The transformation vectors were mobilised into *A. tumefaciens* strains EHA105 by electroporation using the Gene Pulser Electroporation system (Bio-Rad, USA) as indicated in the manufacturer's protocol. The transformed *A. tumefaciens* cells were spread onto yeast extract and beef (YEB) agar added with 100 mg/L rifampicin, 100 mg/L streptomycin and 50 mg/L kanamycin (YEB-RSK) and incubated at 28°C for 2-3 days. After that, single colonies were screened by colony PCR using primers specific to the *bar* gene and restriction endonuclease analysis.

Plant Materials

Oil palm embryogenic calli (EC) that were derived from the cabbage of *E. guineensis* Jacq. var. *Tenera* was supplied by the Breeding and Tissue Culture Unit, MPOB. The calli were cultured on Y3A-4 agar (Masani *et al.*, 2022) for two weeks prior to infection with *Agrobacterium* suspension cells.

Preparation of *Agrobacterium* Suspension Cells

Agrobacterium tumefaciens strain EHA105 glycerol stocks, which carry the transformation vectors, were streaked on YEB-RSK plates and

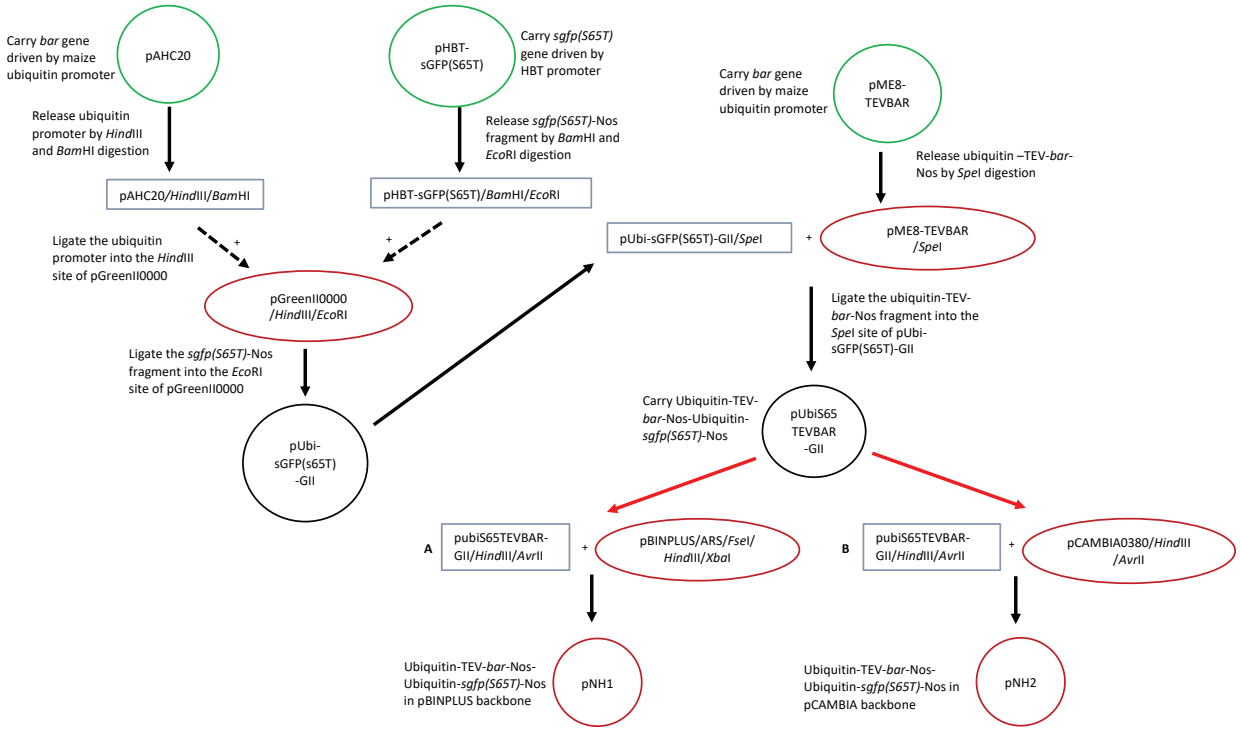

*Figure 2. Overall cloning strategy for the construction of ubiquitin-TEV-*bar*-Nos-ubiquitin-*sgfp(S65T)*-Nos in pBINPLUS/ARS backbone (pNH1) and pCAMBIA0380 backbone (pNH2). Symbol plus (+) shows the ligation of digested DNA fragments (represented by squares) to corresponding vectors digested with appropriate restriction enzymes (represented by an oval). Arrows indicate the vectors resulting from the ligation (represented by circles).*

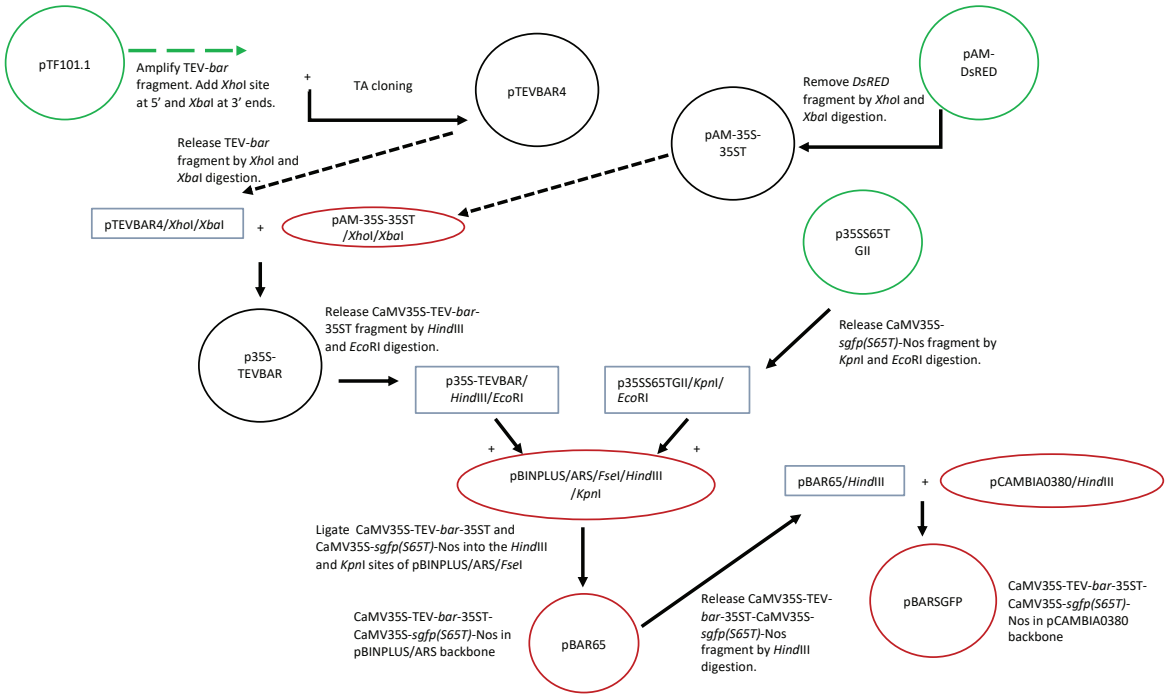

*Figure 3. Overall cloning strategy for the construction of CaMV35S-TEV-*bar*-35ST-CaMV35S-*sgfp(S65T)*-Nos in pBINPLUS/ARS backbone (pBAR65) and pCAMBIA0380 backbone (pBARSGFP). Symbol plus (+) shows the ligation of digested DNA fragments (represented by squares) to corresponding vectors digested with appropriate restriction enzymes (represented by an oval). Arrows indicate the vectors resulting from the ligation (represented by circles).*

incubated at 28°C in the dark for three days. A single colony of *A. tumefaciens* was inoculated into 10 mL of liquid YEB-RSK medium and incubated for two overnights at 28°C (200 rpm). Next, about 5 mL of the bacteria culture was transferred into 45 mL YEB-RSK media and incubated at 28°C (200 rpm) until the Optical Density at 600 nm (OD_{600}) reached approximately 1.0. Then, the cells were pelleted by centrifugation at 6000 rpm for 7 min (16°C), washed with $MgSO_4$ solution (10 mM $MgSO_4$, pH 5.2) and re-suspended with 2.5 mL Y3A-4G liquid medium [Y3A-4 media (Masani *et al.*, 2022) supplemented with 6% glucose (Y3A-4G)]. This cell suspension was gradually added to 30 mL Y3A-4G liquid medium supplemented with 200 µM acetosyringone until OD_{600} was 0.2 and incubated at 22°C (120 rpm) overnight.

Agrobacterium tumefaciens-Mediated Transformation of Oil Palm Calli

Transformation of oil palm calli was carried out as described by Masli *et al.* (2009) and Izawati *et al.* (2012; 2015) with modifications. First, about 0.5 g of oil palm calli were transferred into 30 mL Y3A-4G liquid medium added with 200 µM acetosyringone and incubated at 22°C with shaking at 120 rpm overnight. Then, the calli were sonicated for 1 min. After that, the Y3A-4G medium was replaced with 30 mL of *Agrobacterium* suspension and vacuum infiltrated at 400 psi for 1 hr. Then, the calli were transferred to a sterile filter paper placed onto a co-cultivation medium (CCM) (Y3A-4 medium containing 200 mM acetosyringone) and co-cultivated for three days in the dark at 22°C. Then, the infected calli were washed 3-4 times with a washing solution (Y3A-4 liquid medium containing 500 mg/L cefotaxime) to eliminate unwanted bacteria. After washing, transient GFP signals were observed using Nikon AZ-100 fluorescent microscope.

Statistical Analysis

Data obtained in this study were analysed using the SPSS version 20 (SPSS Inc., USA). The significant differences among treatments were determined using a one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. Data presented in this study were from the means of six replicates ± the standard error of the means.

RESULTS AND DISCUSSION

Construction of Transformation Vectors in pBINPLUS/ARS and pCAMBIA0380 Backbones

Overall, six transformation vectors carrying *bar* and different variants of *gfp* genes (*mgfp* and *sgfp(S65T)*) controlled by either Ubi-1 or CaMV35S

promoters in pBINPLUS/ARS and pCAMBIA0380 backbones were successfully constructed. The vectors were designated as pBINUBG (16 244 bp) (*Figure 4a*), pPZUBG (12 970 bp) (*Figure 4b*), pNH1 (16 186 bp) (*Figure 4c*), pNH2 (12 886 bp) (*Figure 4d*), pBAR65 (12 952 bp) (*Figure 4e*) and pBARSGFP (9681 bp) (*Figure 4f*). The vectors, pBINUBG and pPZUBG, carry *mgfp* and *bar* genes under the control of Ubi-1 promoter in pBINPLUS/ARS and pCAMBIA0380 backbones, respectively. Likewise, the pNH1 and pNH2 vectors also carry the *bar* gene controlled by the Ubi-1 promoter, but the *mgfp* gene was replaced with *sgfp(S65T)* gene and was constructed in pBINPLUS/ARS and pCAMBIA0380, respectively. Meanwhile, the pBAR65 vector carries the *bar* and *sgfp(S65T)* genes controlled by CaMV35S promoter in the pBINPLUS/ARS backbone. The pBARSGFP vector carries the same insert as the pBAR65 vector (the *bar* and *sgfp(S65T)* genes controlled by the CaMV35S promoter) but was constructed in pCAMBIA0380 backbone. Different promoters, *gfp* genes and backbones were used to evaluate which of these components were the best for oil palm transformation.

The resulted final vectors were digested with suitable restriction enzymes to confirm their identity. Digestion of pBINUBG plasmid (16 244 bp) with *Bam*HI yielded five fragments with the size of 10 443 bp (backbone and other elements), 2353 bp (Ubi-1 promoter), 2010 bp (Ubi-1 promoter), 723 bp (*mgfp* gene) and 715 bp (TEV-*bar* fragment) (*Figure 4a* and *5a*). Meanwhile, digestion of pPZUBG (12 970 bp) with *Bam*HI produced five fragments with the size of 7123 bp (backbone and other elements), 2353 bp (Ubi-1 promoter), 2056 bp (Ubi-1 promoter), 723 bp (*mgfp* gene) and 715 (TEV-*bar* fragment) (*Figure 4b* and *5b*). For pNH1 vector (16 186 bp), digestion with *Bam*HI produced five fragments with the size of 12 068 bp (backbone and other elements), 2009 bp (Ubi-1 promoter), 1038 bp (*sgfp(S65T)* gene-Nos terminator fragment), 715 bp (TEV-*bar* fragment) and 356 bp (Nos terminator) (*Figure 4c* and *5c*). Whereas pNH2 (12 886 bp) digestion with *Bam*HI produced four fragments with the size of 9124 bp (backbone and other elements), 2009 bp (Ubi-1 promoter), 1038 bp (*sgfp(S65T)* gene-Nos terminator fragment) and 715 bp (TEV-*bar* fragment) (*Figure 4d* and *5d*). Finally, the identity of the pBAR65 vector (12 952 bp) was analysed via digestion with *Hind*III, which produced 10 083 bp (backbone and other elements) and 2869 bp fragments (CaMV35S promoter-TEV-*bar*-35S terminator-Nos terminator-*sgfp(S65T)* gene-CaMV35S promoter) (*Figure 4e* and *5e*). Next, the identity of the pBARSGFP vector (9681 bp) was analysed via digestion with *Bam*HI. This digestion produced fragments with the size of 7222 bp (backbone and other elements), 1429 bp (CaMV35S promoter-TEV-bar-35S terminator) and 1030 bp (CaMV35S promoter- *sgfp(S65T)* gene-Nos terminator (*Figure 4f* and *5f*).

Evaluation of Transformation Vectors in Oil Palm Calli

In this study, all the constructed vectors carried *gfp* gene as the reporter gene. In plant genetic engineering, a reporter gene is used as a tool to monitor the transfer and expression of foreign genes in the recipient host. The *gfp* gene was used as the reporter gene because it allows for non-destructive detection without any substrates and chemicals in any species (Parveez and Majid, 2018). The GFP signals were detected from calli transformed with every construct, indicating that the constructs were viable and functioning in oil palm cells (*Figure 6*).

The GFP signals were scored as the number of GFP-expressing calli. The number of GFP signals was the highest in calli transformed with the pBAR65 vector, followed by pNH1, pBARSGFP, pBINUBG, pNH2 and pPZUBG (*Figure 7*). Previously, the use of *gfp* as a reporter gene in oil palm has been reported (Bahariah *et al.*, 2017; Majid and Parveez, 2007; 2016; Parveez and Majid, 2008; 2018). The reports suggested that the use of *gfp* gene as a reporter gene could facilitate the detection of successful transformation because the selection and regeneration of transformed oil palm tissues are very time-consuming (Masani *et al.*, 2018).

In this study, two types of backbone were used for vector construction; pBINPLUS/ARS and pCAMBIA0380 vectors. The pBINUBG, pNH1 and pBAR65 vectors were constructed in the pBINPLUS/ARS backbone. Meanwhile, pPZUBG, pNH2 and pBARSGFP were constructed in the pCAMBIA0380 backbone. The pBINPLUS/ARS vector (12 462 bp) was modified from the pBINPLUS vector and was successfully used to genetically engineer both monocots and dicots plants such as potatoes, tobacco and apple (Belknap *et al.*, 2008). For oil palm transformation, the pBINPLUS/ARS backbone was used to construct the pBIHA1 vector, which carries the *mgfp* gene driven by a double CaMV35S promoter (Bahariah *et al.*, 2017). The pCAMBIA0380 vector (6812 bp) was derived from the pPZP vector (Abcam, 2021). The pCAMBIA0380 vector has been used to transform plants such as rice, potato and fungus (Lu *et al.*, 2015; Oliva *et al.*, 2014; Sayari *et al.*, 2019; Zhou *et al.*, 2013). Both pBINPLUS/ARS and pCAMBIA0380 differ in size, backbone and origin of replication elements for replication in *Agrobacterium* (Abcam, 2021; Belknap *et al.*, 2008). Although both vectors have been successfully used to transform both monocots and dicots plants, there is no side-by-side comparison on the efficiency of these vectors in *Agrobacterium*-mediated oil palm transformation. For the transformation of oil palm using biolistic,

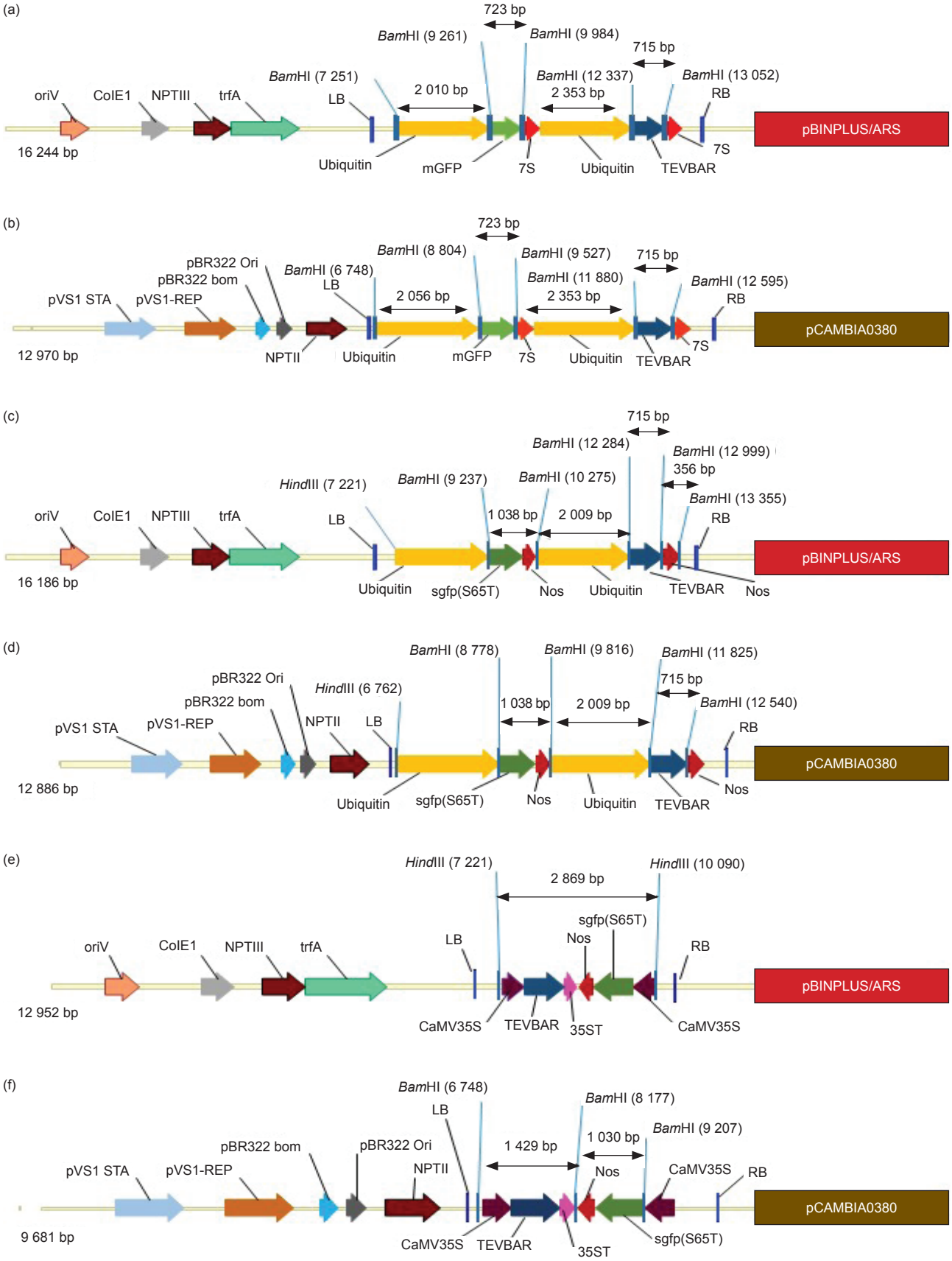

Figure 4. Schematic diagram of pBINUBG (a), pPZUBG (b), pNH1 (c), pNH2 (d), pBAR65 (e) and pBARSGFP (f) transformation vectors. The arrows indicate the orientation of each DNA fragment. The restriction site and the numbers of each site represent the approximate location in the vectors. Ubiquitin: maize Ubi-1 promoter; mgfp: *modified* gfp (mgfp) *gene; 7S: soybean beta conglycinin 7S 3′ transcriptional termination sequence;* TEVBAR: *tobacco etch virus translational enhancer (TEV)-infused bar gene;* sgfp(S65T): *synthetic* gfp *gene with replacement of serine at position 65 with a threonine; Nos: nopaline synthase termination sequence; CaMV35S: cauliflower mosaic virus 35S promoter; 35ST: 35S termination sequence;* NPTII: *kanamycin resistance gene; LB: Left border; RB: right border.*

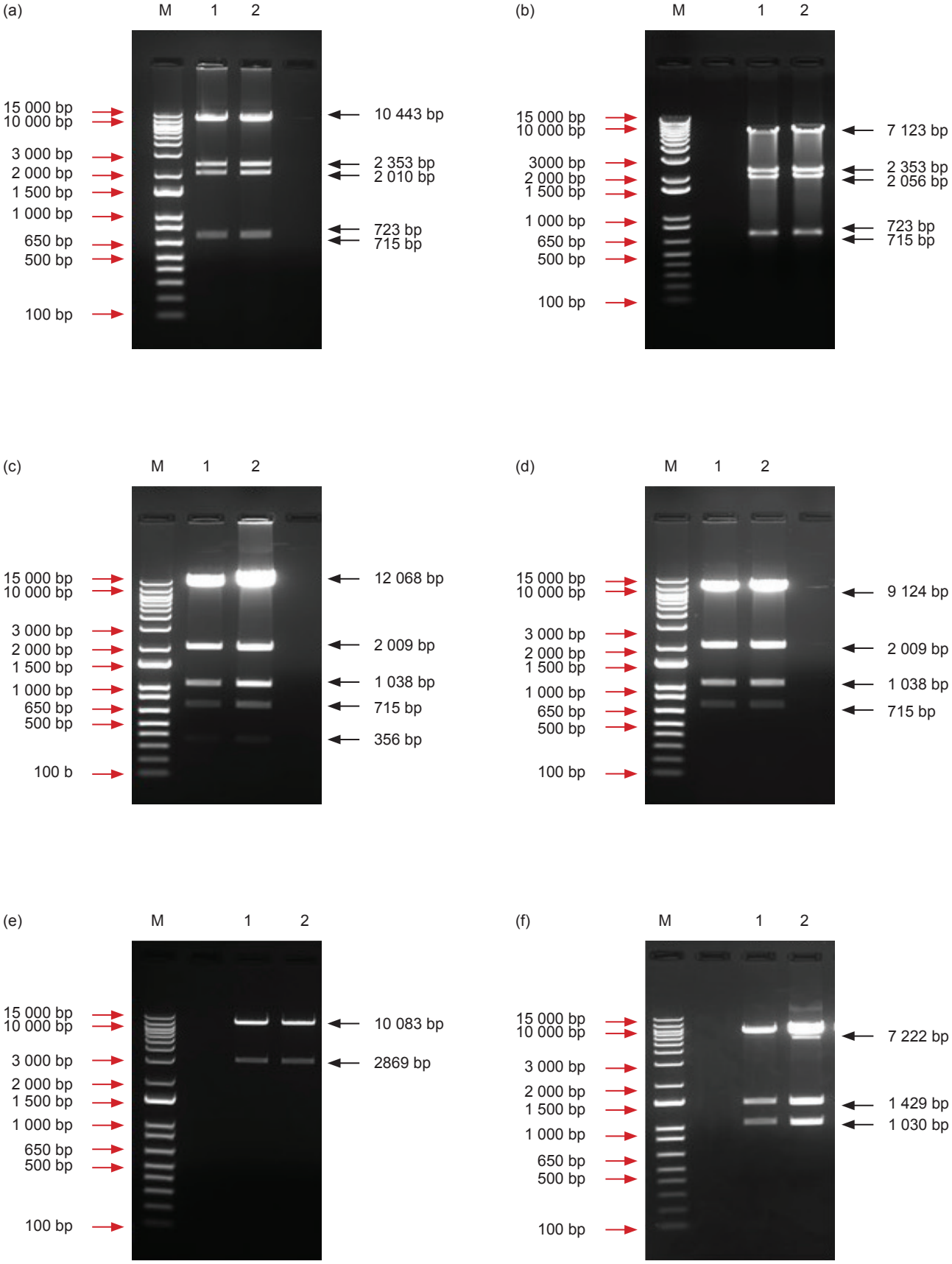

Figure 5. Restriction endonuclease analyses of pBINUBG, pPZUBG, pNH1, pNH2, pBAR65 and pBARSGFP. (a) pBINUBG digested with Bam*HI; (b) pPZUBG digested with* Bam*HI; (c) pNH1 digested with* Bam*HI; (d) pNH2 digested with* Bam*HI; (e) pBAR65 digested with* Hind*III and (f) pBARSGFP digested with* Bam*HI. Numbers represent individual clones. Lane M is the 1 kb Plus DNA Ladder (Invitrogen).*

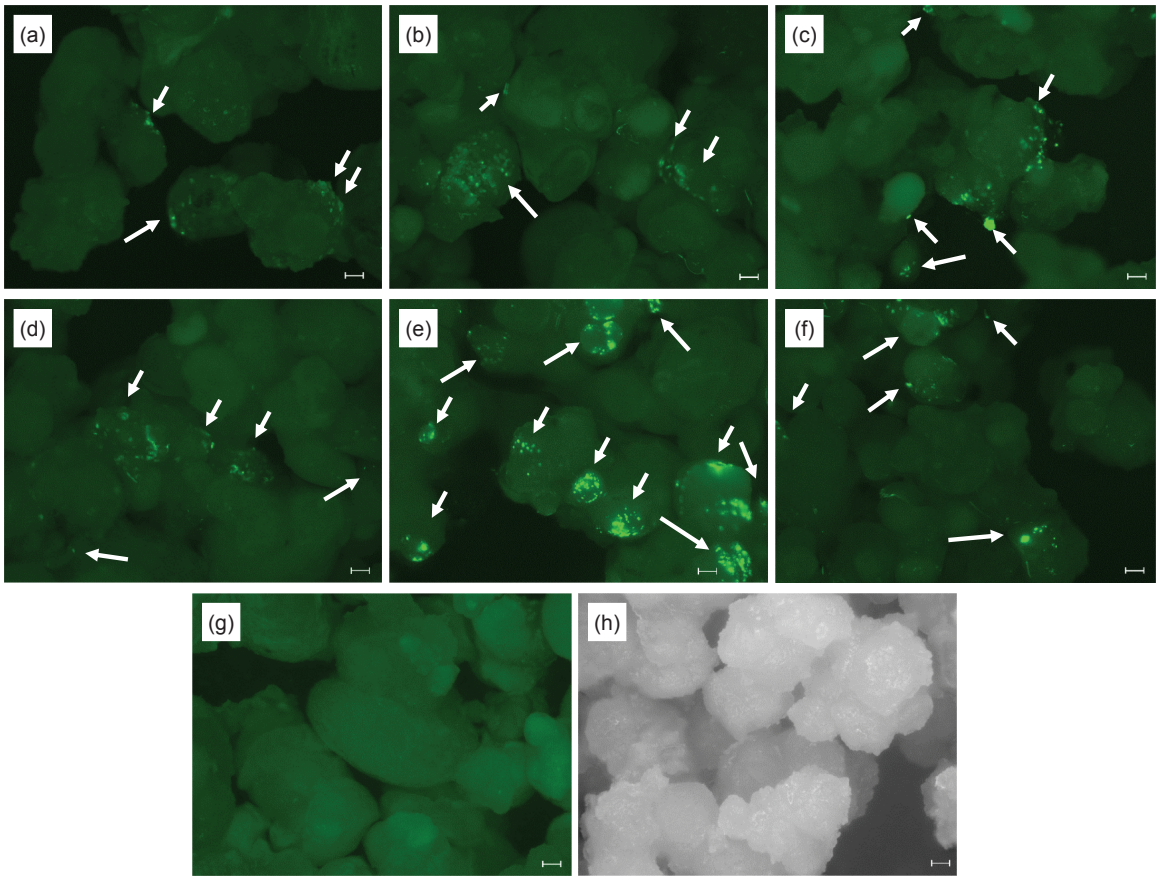

Figure 6. Visualisation of GFP signals in oil palm calli after the co-cultivation period. The oil palm calli were transformed with A. tumefaciens *strain EHA105 carrying the pBINUBG (a), pPZUBG (b), pNH1 (c), pNH2 (d), pBAR65 (e) and pBARSGFP (f) transformation vectors compared with the untransformed (g). The bright-field image of oil palm calli is shown in (h) the white arrow indicates GFP expressing calli. Scale bar = 1000 µm.*

Parveez and Majid (2008) have reported that the vector backbone and size affected the expression of *gfp* genes. Thus, direct comparison of pBINPLUS/ARS and pCAMBIA0380 backbones in this study will provide beneficial information that can be used to select the best vector backbone for *Agrobacterium*-mediated oil palm transformation.

From the observation of transient GFP signals, vectors in the pBINPLUS/ARS backbone (pBAR65, pNH1 and pBINUBG) produced more GFP signals as compared with vectors in the pCAMBIA0380 backbone (pBARSGFP, pNH2 and pPZUBG) regardless of promoter type and *gfp* variant (*Figure 7*). This result suggested that the pBINPLUS/ARS backbone is more suitable for oil palm transformation than the pCAMBIA0380 backbone. The pBINPLUS/ARS and pCAMBIA0380 backbones differ in the origin of replication (ORI). The pBINPLUS/ARS plasmid is modified from the pBINPLUS replicates in *Agrobacterium* using the RK2 ORI (Belknap *et al.*, 2008). Meanwhile, the pCAMBIA0380 vector modified from the pPZP vector (Hellens *et al.*, 2000b) replicates using the pVS1 ORI. Previously, Sripriya *et al.* (2011) reported that a binary plasmid with the RK2 ORI produced a higher transformation frequency in rice (86%) as compared with a binary plasmid with the pVS1 ORI (20%). Sripriya *et al.* (2011) also reported that the relative copy number of plasmid with the RK2 ORI was 10-15 copies per cell compared to plasmid with the pVS1 ORI (3 copies per cell). However, Zhi *et al.* (2015) reported that the copy number of plasmid with the pVS1 ORI was higher (20 copies/cell) than the RK2 ORI (10-12 copies/cell) in maize, while Oltmanns *et al.* (2010) reported that both RK2 and pVS1 ORI produced 7-10 copies of plasmids per cell in *Arabidopsis* and maize. The differences in copy number of plasmid per cell might be attributed to the different strains of *Agrobacterium* used in their study and also the type of binary vector (binary or super-binary). Despite the differences in copy number between both ORIs, Oltmanns *et al.* (2010), Sripriya *et al.* (2011) and Zhi *et al.* (2015) agreed that a higher copy number of plasmid per cell resulted in a higher transformation frequency and vice versa. The higher transformation frequency from a high copy number plasmid was due to a rise in the processing and transfer of T-DNA from *Agrobacterium* to a plant cell (Zhi *et al.*, 2015). Thus, it could be postulated that a similar phenomenon might occur in the oil palm cells based on our results.

Besides the effect of ORI on the copy number of plasmid per cell, the size of ORI was reported to affect plasmid performance. The original RK2

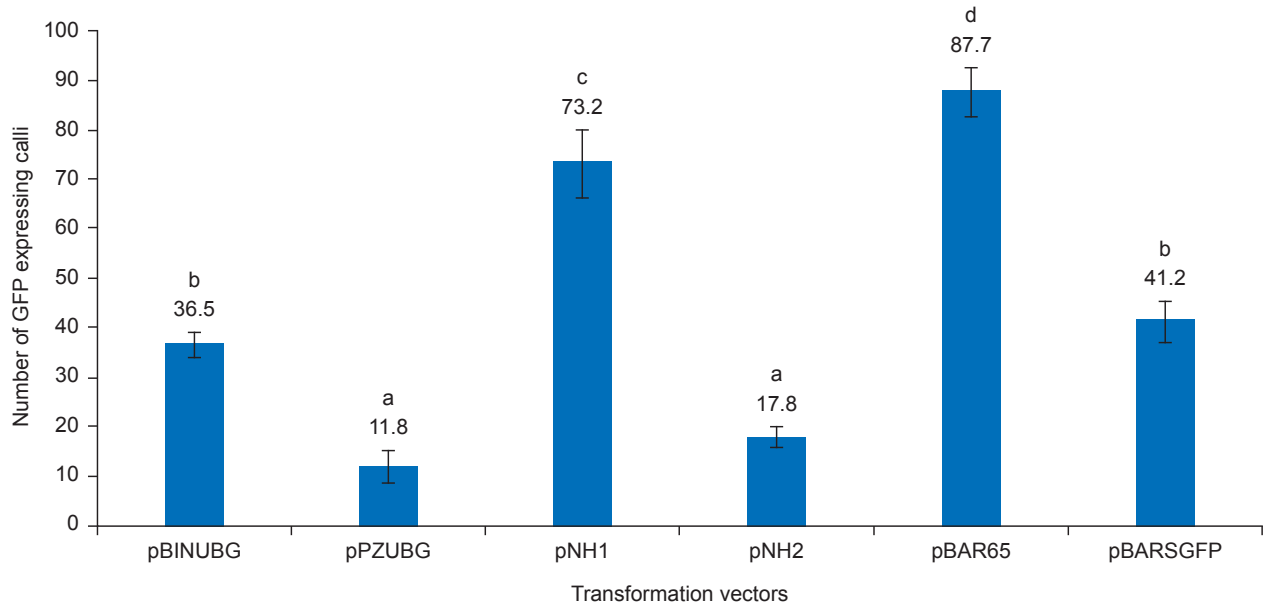

Figure 7. Numbers of GFP expressing calli transformed with A. tumefaciens *strain EHA105 carrying pBINUBG, pPZUBG, pNH1, pNH2, pBAR65 and pBARSGFP after the co-cultivation period. The data presented is the mean of six replicates, and the error bar represents the standard error of the mean. Means with the same letter are not significantly different at* $p < 0.05$ *as determined by Duncan's Multiple Range Test (DMRT).*

ORI (19.7 kb) was reported to adversely affect the plasmid performance due to its large size and the presence of non-essential DNA sequences (Anand *et al.*, 2018). The pBINPLUS/ARS backbone (Accession no: DQ320121) contains a smaller size of the RK2 ORI (OriV) which is only 618 bp as compared to the pVS1 ORI (1001 bp) in the pCAMBIA0380 backbone (Accession No.: AF234290). Hence, the small ORI in pBINPLUS/ARS contributed to the superior performance of the pBINPLUS/ARS-based vectors over the pCAMBIA0380-based vector.

Besides the backbone, two types of promoters were used to drive the expression of *gfp* and *bar* genes, namely CaMV35S and Ubi-1 promoters. The transient GFP signals were the highest from calli transformed with vectors that carry *sgfp(S65T)* and *mgfp* genes driven by the CaMV35S promoter in the pBINPLUS/ARS backbone (pBAR65) or pCAMBIA0380 backbone (pBARSGFP). This result indicated that the CaMV35S promoter was superior in driving the expression of *gfp* genes in oil palm calli. This result was in agreement with previous studies that reported that oil palm calli bombarded with a construct carrying *gfp* gene driven by the CaMV35S promoter had the highest number of GFP signals as compared with the Ubi-1 promoter (Majid and Parveez, 2007; 2016; Parveez and Majid, 2008; 2018). A recent study by Fizree *et al.* (2019) using *DsRED* gene also showed that the CaMV35S promoter was the strongest promoter compared with other promoters.

Another interesting result from this study was that the *gfp* gene variant also influenced the number of GFP expressing calli. The number of GFP expressing calli was higher in calli transformed with the *sgfp(S65T)* gene as compared with the *mgfp* gene, although both genes were driven by the Ubi-1 promoter in the same backbone (pBINPLUS/ARS) as shown by the pNH1 and pBINUBG (*Figure 7*). Likewise, vectors in the pCAMBIA0380 backbone also showed a similar trend as observed for pNH2 and pPZUBG. This finding was similar to the results reported by Majid and Parveez (2007) and Parveez and Majid (2008), where they also discovered that the vector carrying the *sgfp(S65T)* variant produced more GFP signals as compared with *mgfp*. Furthermore, the GFP signals for *sgfp(S65T)* were brighter than those of *mgfp*. The *sgfp(S65T)* variant was modified from the original GFP from jellyfish by replacing the serine at position 65 with threonine (Niwa, 2003). This variant has been shown to produce more and brighter signals in plant cells compared with the native GFP (Niwa, 2003). Thus, it can be concluded that the *sgfp(S65T)* variant is more efficiently expressed in oil palm cells.

CONCLUSION

In summary, the pBINPLUS/ARS backbone was identified as a more suitable backbone than the pCAMBIA0380 backbone for *Agrobacterium*-mediated transformation of oil palm. The CaMV35S promoter was superior in driving the *gfp* gene expression in oil palm cells. The *sgfp(S65T)* GFP variant was more suitable for oil palm transformation than *mgfp*. The effectiveness of each vector in producing the most stable transgenic oil palm is still being evaluated.

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