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

AYUB NOR HANIN1*; MAT YUNUS ABDUL MASANI1*; OMAR ABDUL RASID1 and GHULAM KADIR AHMAD PARVEEZ1

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 pG121Hm 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).
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 plants. 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 MgSO4, 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
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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/FseI 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 Figure 1, 2 and 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequences</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEV-bar</td>
<td>TEVBAR-F 5’-GGCCGGATCCAATTAATTCTCAACACAACA-3’</td>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TEVBAR-R 5’-AATTGGATCCCTAGATTGTTGAGCAGATCTCG-3’</td>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td>mgfp</td>
<td>mGFP2-F 5’-GGCGGATCCCATGGGTAAAGGAGAAGAACTTT-3’</td>
<td>BamHI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mGFP2-R 5’-GCGCGCGGATCCTTATTTGTATAGTTCATCCATGCC-3’</td>
<td>BamHI</td>
<td></td>
</tr>
</tbody>
</table>

Note: The restriction sites are underlined.
Construction of pBINUBG and pZUBG 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 HindIII and BamHI. The mgfp gene with Nos terminator was released from the pHBT-sGFP(S65T) by double digestion with BamHI and EcoRI. Then, both DNA fragments were ligated into the HindIII and EcoRI sites of the pGreenII0000 plasmid to yield pUbi-sGFP(S65T)-GII. Then, the Ubi1-TEV-bar fragment was excised from pME8-TEVBAR by SpeI digestion and ligated into the SpeI 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 Ubi1 promoter. Next, the pUbiS65TEVBAR-GII plasmid was digested with HindIII and XbaI sites of pBINPLUS/ARS/FseI to create pNH1 vector. In addition, the HindIII and XbaI digested fragment was also ligated into the HindIII and XbaI sites of pCAMBIA0380 plasmid to create pNH2 vector. The
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 XhoI site (TEVBAR2-F) at 5' and the XbaI 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 purified and cloned into pCRII-TOPO vector at 3’ ends of the fragment. The fragment (709 bp) at 5' and the XbaI site (TEVBAR2-R) XhoI site (Table 2) were designed to add the primers (I site). The PCR template to amplify the TEV-bar fragment was released from p35SS65TGII plasmid (FseI and HindIII sites of pCAMBIA0380 to create pBAR65. Then, the CaMV35S-TEV-bar fragment was released from the pTEVBAR4 plasmid to create p35S-TEVBAR plasmid. Finally, the identity of the pBAR65 vector was analysed via digestion with HindIII and EcoRI.

Next, the 35S-sgfp(S65T)-35ST fragment was released from p35SS65TGII plasmid (Table 1) via KpnI and EcoRI double digestion. Finally, both fragments were ligated into the HindIII and KpnI sites of the pBINPLUS/ARS/Fsel plasmid to create pBAR65 vector. Then, the CaMV35S-TEV-bar-35ST fragment was released from pBAR65 with HindIII digestion and ligated into HindIII site of pCAMBIA0380 to create pBARSGFP. Finally, the identity of the pBAR65 vector was analysed via digestion with HindIII while the identity of the pBARSGFP vector was analysed via digestion with BamHI.

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).
incubated at 28°C in the dark for 3 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 MgSO₄ solution (10 mM MgSO₄, 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 OD₆₀₀ 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≤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 pBARS GFP (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 pBARS GFP 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 BamH I 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 BamH I 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 bp (TEV-bar fragment) (Figure 4b and 5b). For pNH1 vector (16 186 bp), digestion with BamH I 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 BamH I 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 HindIII, 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 pBARS GFP vector (9681 bp) was analysed via digestion with BamH I. 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 each 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, pBARS GFP, 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 pBARS GFP 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 pPZF 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 BamHI; b) pPZUBG digested with BamHI; c) pNH1 digested with BamHI; d) pNH2 digested with BamHI; e) pBAR65 digested with HindIII and f) pBARSGFP digested with BamHI. Numbers represent individual clones. Lane M is the 1 kb Plus DNA Ladder (Invitrogen).
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, Sripiya 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%). Sripiya 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 to 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), Sripiya 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
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). 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 pBINUG (Figure 7). Likewise, vectors in the pCAMBIA0380 backbone also showed a similar trend as observed for pNH2 and pPZU65. 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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