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DESIGNING gRNAs TARGETING OIL PALM PHYTOENE DESATURASE (*EgPDS*) GENE AND CONSTRUCTION OF VECTORS FOR OIL PALM CRISPR/CAS9 STUDY

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ABSTRACT

*The CRISPR/Cas9 system is a precise and versatile genetic tool used to induce site-specific manipulation in the targeted gene. This study elaborates on the strategy to select the gRNAs targeting the oil palm phytoene desaturase (*EgPDS*) gene, which complements CRISPR-GE and sequence analysis. Sixty-one gRNAs were selected from 167 gRNA candidates generated by the CRISPR-GE tool. These gRNA candidates were further analysed to meet the specific criteria, including the guanine (G) and cytosine (C) content of 35%-60% and the presence of preferable gRNA features. From 61 gRNAs, seven gRNAs were selected, which represent three different parts of the* EgPDS *gene in the genome. The efficiency of each gRNA candidate to cause cleavage on the target gene was confirmed by* in vitro *cleavage analysis. All seven gRNA candidates were then cloned into the pYLsgRNA-OsU6 cassette before being incorporated seamlessly into the final transformation vector, pYLCRISPR/Cas9P35S-H, via Gibson assembly. Three CRISPR/Cas9 transformation vectors targeting the* EgPDS *gene, namely pYLEgPDS1-35S-H, pYLEgPDS2-35S-H and pYLEgPDS3-35S-H, were successfully constructed. These constructs will be transformed into oil palm protoplasts via polyethylene glycol (PEG)-mediated transformation and subsequently into oil palm embryogenic calli via biolistic and* Agrobacterium*-mediated transformation to determine the efficacy of the multiplex CRISPR/Cas9 system in oil palm genome.*

Keywords: CRISPR/Cas9, gRNA, *in vitro* cleavage assay, oil palm, phytoene desaturase.

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INTRODUCTION

Oil palm (*Elaeis guineensis*) is a monocot perennial oil-producing fruit tree with a significant socio-economic prospect in Africa and Asia. Currently, the demand for crude palm oil in various industries is increasing (Parveez *et al.*, 2021). Thus, it is urgent to improve the yield and quality of palm oil better than what conventional techniques have achieved. Previously, significant achievements in oil palm biotechnology have been reported in biochemical research, including identification, isolation of promoters and functional genes from oil palm (Masani *et al.*, 2018). Numerous transformation constructs using the previously isolated promoters and genes for various target products have been extensively developed (Masani *et al.*, 2018). The development of oil palm transformation methods and molecular analyses of oil palm putative transformants was actively carried out (Rahman *et al.*, 2020). In 2013, Malaysian Palm Oil Board

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(MPOB) released the whole genome sequence of African oil palm species (Singh *et al.*, 2013), which has opened an opportunity to shift into oil palm genome editing.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) was first introduced in 2013 (Charpentier and Doudna, 2013). It has been successfully applied in various plant species, including recalcitrant crop species (Ma *et al.*, 2015). Therefore, this system offers a promising solution for oil palm genome editing. Evaluation studies of CRISPR/Cas9 in oil palm are essential to mitigate certain restrictions such as inefficient guide RNA (gRNA) design, off-target activity, and selection of vector system that can affect the CRISPR/Cas9 efficiency. CRISPR/Cas9 utilises the Cas9 protein and gRNA, which interact to form a complex that can identify target sequences with high selectivity (Charpentier and Doudna, 2013). The gRNA is responsible for locating, and the Cas9 will cleave the target DNA, producing double-strand breaks (DSBs). The DSBs can induce various types of mutations (*e.g.*, base insertion, deletion and substitution) through the error-prone non-homologous end-joining pathway and specific modification through the homology-directed repair pathway. Predictably, when the induced mutations are located in the genes coding region, the corresponding genes may entirely or partially lose their functions (Ma and Liu, 2016).

The binding of Cas9 nuclease to the target sequence occurs only with the presence of the protospacer adjacent motif (PAM) sequence. In CRISPR/Cas9 system, the *Streptococcus pyogenes* Cas9 (*Sp*Cas9) nuclease cleaves at 3-4 nucleotides upstream of the PAM sequence 5'-NGG-3' ("N" confers any nucleotide base). In addition, the 20-nucleotide sequence of gRNA is also crucial in determining the efficiency of designed gRNA (Doench *et al.*, 2014). For example, guanine is favoured directly after the PAM sequence (nucleotide position 20), whereas cytosine is unpreferable. This is because guanine has a better affinity for Cas9 binding over cytosine (Wang *et al.*, 2014a). The efficacy of each gRNAs, accessibility at a specific position for Cas9 binding and presence of stem-loops were also predicted based on their secondary structure (Bruegmann *et al.*, 2019). Furthermore, off-target activity also should be avoided. Few mismatches between gRNA and the target sequence are intolerable as Cas9 still cuts the off-target region. Thus, various gRNA design tools using a computerised prediction method have been developed (Wang *et al.*, 2020).

Analyses of successfully targeted mutations have revealed that the expression levels, expression timing, and variants of gRNA and Cas9 need to be highly regulated (Ma *et al.*, 2015). Therefore, the promoters of these genes and the target site are the critical factors for genome-editing efficiency in oil palm. Various modified CRISPR/Cas9 editing vector systems that can carry multiple gRNAs in a single construct are available. This technology is known as a multiplex CRISPR/Cas9 system. A multiplex genome editing can be achieved by simultaneous insertion and expression of multiple gRNAs. The generation of multiple DSBs followed by the deletion of large fragments between these multiple gRNA-targeted sites on the same chromosome has been achieved in many species (Wang *et al.*, 2014b). Hence, using a multiplex CRISPR/Cas9 genome editing system may foretell the successful genome editing in oil palm.

This study highlights the preliminary work of the CRISPR/Cas9 study in oil palm, including the gRNAs selection strategy, targeting the oil palm phytoene desaturase (*EgPDS*) gene and *in vitro* cleavage assay to determine the functionality of designed gRNAs. We also elaborated on the construction of CRISPR/Cas9 transformation vectors carrying multiple gRNAs to kickstart the CRISPR/Cas9 study in oil palm.

MATERIALS AND METHODS

Selection of gRNA Candidates Targeting *EgPDS* Gene

The identification of possible CRISPR/Cas9 target sites in the 1.7 kb coding sequence of *EgPDS* was performed using the CRISPR-GE online tool (Ma *et al.*, 2015). The generated targets were gRNA sequences that are 20 bases in length, followed by the PAM sequence (20n-NGG). gRNA candidates were identified based on the CRISPR-GE targetDesign tool. The off-target analysis was carried out using NCBI BLAST. The gRNA candidates' sequence was BLAST with a 'somewhat similar sequence' option to omit candidates with high similarity with other possible off-target sequences in the genome.

Secondary Structure Analysis of *EgPDS* gRNA Candidates

The secondary structure prediction was carried out using RNA Folding Form v2.3 (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). The gRNA sequence without its PAM linked to the nucleotides of the scaffold sequence (GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT) was submitted for analysis. gRNA candidates having at least stem-loop 2 and stem-loop 3 were selected for further experiments.

In vitro Transcription of gRNA Targeting *EgPDS* Gene

The gRNA candidates were PCR amplified using Guide-it™ sgRNA *In vitro* Transcription and Screening Systems (Takara Bio, USA) following the manufacturer's protocol. The PCR reaction contained the designed forward primers (0.5 μM), the Guide it Scaffold template (1.0 μM), 1× PrimeSTAR Max Premix (12.5 μL) and RNase free water to bring the total of 20 μL. The PCR amplification of the gRNA template was carried out using the following program: 33 cycles of 98°C for 10 s, 68°C for 10 s. Subsequently, the PCR product was incubated at 37°C for 4 hr for *in vitro* transcription of gRNAs. The transcribed gRNAs were purified using Guide-it IVT RNA Clean-Up Kit (Takara Bio, USA) according to the manufacturer's protocol. RNase-free water, 20 μL was used in the final elution.

In vitro Cleavage Assay

The components required for the *in vitro* cleavage reaction are the oil palm control PCR fragment containing the target sites (wild type), the previously transcribed gRNAs and the Cas9 enzyme (Guide-it Recombinant Cas9 Nuclease, Takara Bio, USA). The amplification of the control target fragments was carried out using specific primers for each gRNA (*Table 1*). The PCR products were then purified by NucleoSpin® Gel and PCR Clean-up (Macherey Nagel, Germany). *In vitro* cleavage assay was carried out for a single gRNA and multiple gRNA (two to three gRNAs targeting one PCR fragment) according to the manufacturer's protocol (Guide-it™ sgRNA *In vitro* Transcription and Screening Systems, Takara Bio, USA). The gRNAs were first incubated with the recombinant Cas9 nuclease for 5 min at 37°C. Then, the Cas9-gRNAs were mixed with Cas9 reaction buffer, BSA, RNase free water and the control PCR fragment (250 ng) containing the target sequence, incubated at 37°C for 1 hr, then 80°C for 5 min. The cleavage reaction was observed on 2% agarose gel.

Preparation of Target Adapters and Ligation into pYLsgRNA-OsU6 Cassettes

The design of target adapter primers (*Table 1*) for cloning gRNA sequences into pYLsgRNA-OsU6a and pYLsgRNA-OsU6b cassettes was carried out using primerDesign in CRISPR-GE (Ma *et al.*, 2015). The target adapter primers were designed such that the 5'end of the adapters contained a compatible *Bsa*I digested end site of the corresponding U6 promoters. The corresponding U6 promoters were either OsU6a for gRNA1, gRNA3, gRNA5 and gRNA7 or OsU6b for gRNA2, gRNA4 and gRNA6. The OsU6a and OsU6b promoters were derived from pYLsgRNA-OsU6a and pYLsgRNA-OsU6b cassettes, respectively, which were obtained from Prof. Liu YG Lab (South China Agricultural University). The target adapters were prepared by dissolving 5 μL of 100 μM forward primer (FP) and 5 μL of 100 μM of reverse primer (RP) for each gRNA cassette in one tube. The tubes were heated in 100°C boiled water for 1 min, and cooled to room temperature for 2 hr. Then, the double-stranded target adapters were ligated into either pYLsgRNA-OsU6a or pYLsgRNA-OsU6b, which were previously linearised with the *Bsa*I enzyme according to the standard protocol (New England Biolabs). The ligation reaction was carried out at 37°C for 10 min using T4 DNA ligase (Promega, USA).

Construction of pYLEgPDS1-35S-H, pYLEgPDS2-35S-H and pYLEgPDS3-35S-H

The integrated gRNA expression cassettes carrying the target adapters were then amplified by overlapping PCR in two sets of reactions with different primer combinations (*Table 2*). In the first PCR (PCR GXL), the previous ligation products were used as the template in a 15 μL PCR reaction containing 1x PRIMESTAR GXL buffer, 2.5 mM dNTP mix, 0.2 μM of each FP and RP primers (*Tables 1* and *2*) and 0.75 U PRIMESTAR GXL DNA Polymerase (PrimeSTAR® GXL DNA Polymerase, Takara Bio USA). The PCR cycling condition was 98°C for 1 min, 25 cycles of 98°C for 15 s; 63°C for 30 s; 68°C for 45 s, final extension at 68°C for 2 min. The PCR GXL products were then electrophoresed on 2% agarose gel and purified using NucleoSpin® Gel and PCR Clean-up (Macherey Nagel, Germany). The purified DNA fragments were used as the template for the subsequent PCR amplification.

In the second PCR, PCR GXL1, the combination of PCR products from PCR GXL (reaction 1 and reaction 2; *Table 2*) was used as a template for PCR amplification using U-F and gR-R primers (*Table 1*), using the same cycling program. The PCR product was purified and used as the template for PCR GXL2. In PCR GXL2, the position-specific primers (*Table 2*) were used to create the overlapping ends that allow multiple gRNA expression cassettes to be ligated into a final vector. The sequence of the primers used is shown in *Table 1*. The PCR cycle was, 95°C for 30 s; 36 cycles of 95°C for 10 s, 58°C for 15 s and 68°C for 30 s; the final extension at 68°C for 2 min. Three μL of PCR product were gel electrophoresed and visualised under the UV gel imager to confirm the amplification of a correct fragment. The remaining PCR product was then purified using ethanol precipitation

TABLE 1. LIST OF PRIMERS

Primer name	Sequence (5'->3')	Purpose
EgPDSgRNA1FP	CCTCTAATACGACTCACTATAGG**CCATAAAGTTAGCCCTCTTC** GTTTAAGAGCTATGC	Forward primer contains a T7 promoter (underlined), additional guanine (red) and gRNA sequence (bold) used to PCR transcribed the gRNA template for *in vitro* assay
EgPDSgRNA2FP	CCTCTAATACGACTCACTATAGG**CATTCTTTCGTAATTGTGCA** GTTTAAGAGCTATGC	
EgPDSgRNA3FP	CCTCTAATACGACTCACTATAGG**CAATGAGATGCTGACATGGC** GTTTAAGAGCTATGC	
EgPDSgRNA4FP	CCTCTAATACGACTCACTATAGG**CAAGCTTATGTGGAGGCCC** GTTTAAGAGCTATGC	
EgPDSgRNA5FP	CCTCTAATACGACTCACTATAGG**CTTGAGATCCATTCCTCTGC** GTTTAAGAGCTATGC	
EgPDSgRNA6FP	CCTCTAATACGACTCACTATAGG**TTGGGGACCTCTGTAGAGGG** GTTTAAGAGCTATGC	
EgPDSgRNA7FP	CCTCTAATACGACTCACTATAGG**AGAAATACTTGGCTTCAATG** GTTTAAGAGCTATGC	
EgPDSF1	AAGTGGAACGGTGCAGAGA	Amplification of *EgPDS* gene for gRNA1/gRNA2
EgPDSR1	GAGGGAGCAAAACTAAGACAAAATC	
EgPDSF2	CGGTTCGTGATATTAAAGGTGCAGTGCC	Amplification of *EgPDS* gene for gRNA3/gRNA4
EgPDSR2	GGTAAGCTGAGAGCATCCATTAAC	
EgPDSF3	GGGATGGCGAAGTATCCTCTTAC	Amplification of *EgPDS* gene for gRNA5/gRNA6/gRNA7
EgPDSR3	GCATTTCGACAATCAGCCCATCCATC	
AdFP1	gccgCCATAAAGTTAGCCCTCTTC	Target adapter forward and RP for cloning of the guide sequence into the pYLsgRNAOsU6 cassette. The lowercase sequences are the overhangs complementary to the overhangs of the cloning vector digested by *Bsa*I Also used in colony PCR to screen for positive clones in vector construction of pYLEgPDS1-35S-H, pYLEgPDS2-35S-H and pYLEgPDS3-35S-H
AdRP1	GGTATTTCAATCGGGAGAAGcaaa	
AdFP2	gttgCATTCTTTCGTAATTGTGCA	
AdRP2	GTAAGAAAGCATTAACACGTcaaa	
AdFP3	gccgCAATGAGATGCTGACATGGC	
AdRP3	GTTACTCTAGACTGTACCGcaaa	
AdFP4	gttgCAAGCTTATGTGGAGGCCC	
AdRP4	GTTCGAATACACCTCCGGGcaaa	
AdFP5	gccgCTTGAGATCCATTCCTCTGC	
AdRP5	GAACTCTAGGTAAGGAGACGcaaa	
AdFP6	gttgTTGGGGACCTCTGTAGAGG	
AdRP6	AACCCCTGGAGACATCTGGGcaaa	
AdFP7	gccgAGAAATACTTGGCTTCAATG	
AdRP7	TCTTTATGAACCGAAGTTACcaaa	
U-F	CTCCGTTTTACCTGTGGAATCG	Universal position-specific primers used for the vector construction of CRISPR/Cas9 vectors
gR-R	CGGAGGAAAATTCCATCCAC	
U-GAL	ACCGGTAAGGCGCGCCGTAGTGCTCGACTAGTATGGAATCGGCAGCAAAGG	
Pgs-GA2	CAGGGAGCGGATAACAATTTCACACAGGCACATCCACTCCAAGCTCTTG	
U-GA2	GTGCCTGTGTGAAATTGTTATCCGCTCCCTGGAATCGGCAGCAAAGG	
Pgs-GA3	CCACGCATACGATTTAGGTGACACTATAGCGCATCCACTCCAAGCTCTTG	
U-GA3	CGCTATAGTGTCACCTAAATCGTATGCGTGGTGGAATCGGCAGCAAAGG	
Pgs-GA4	GTCGCTAGTTATTGCTCAGCGGCCAAGCTCATCCACTCCAAGCTCTTG	
U-GA4	GAGCTTGGCCGCTGAGCAATAACTAGCGACTGGAATCGGCAGCAAAGG	
Pgs-GAR	TAGCTCGAGAGGCGCGCCAATGATACCGACGCGTATCCATCCACT CCAAGCTCTTG	

TABLE 2. PRIMERS USED IN THE CONSTRUCTION OF *EgPDS* CRISPR/CAS9 VECTORS

Target	Target adapter	Template	PCR GXL		PCR GXL1	PCR GXL2		Construct
			Reaction 1	Reaction 2		Sites	Primer	
gRNA1	AdFP1+AdRP1	Osu6a-gRNA1	gR-R/AdFP1	U-F/AdRP1	U-F/gR-R	Site B-L Site 2	U-GAL+ Pgs-GA2	pYLEgPDS1-35S-H
gRNA2	AdFP2+AdRP2	Osu6b-gRNA2	gR-R/AdFP2	U-F/AdRP2	U-F/gR-R	Site 2 Site B-R	U-GA2+ Pgs-GAR	
gRNA3	AdFP3+AdRP3	Osu6a-gRNA3	gR-R/AdFP3	U-F/AdRP3	U-F/gR-R	Site B-L Site 2	U-GAL+ Pgs-GA2	pYLEgPDS2-35S-H
gRNA4	AdFP4+AdRP4	Osu6b-gRNA4	gR-R/AdFP4	U-F/AdRP4	U-F/gR-R	Site 2 Site B-R	U-GA2+ Pgs-GAR	
gRNA5	AdFP5+AdRP5	Osu6a-gRNA5	gR-R/AdFP5	U-F/AdRP5	U-F/gR-R	Site B-L Site 2	U-GAL+ Pgs-GA2	pYLEgPDS3-35S-H
gRNA6	AdFP6+AdRP6	Osu6b-gRNA6	gR-R/AdFP6	U-F/AdRP6	U-F/gR-R	Site 2 Site 3	U-GA2+ Pgs-GA3	
gRNA7	AdFP7+AdRP7	Osu6a-gRNA7	gR-R/AdFP7	U-F/AdRP7	U-F/gR-R	Site 3 Site B-R	U-GA3+ Pgs-GAR	

and the concentration was determined using a spectrophotometer. These purified PCR products containing gRNA cassettes were ligated into the linearised pYLCRISPR/Cas9P35S-H (Ma *et al.*, 2015) that was previously digested with *Bsa*I. The recombination reaction was carried out using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd, China) following the manufacturer's protocol.

RESULTS AND DISCUSSION

Identification of gRNA Candidates for *EgPDS* Gene

Since the *PDS* gene was broadly used to verify the feasibility of the CRISPR/Cas9 system in many plant species (Ma *et al.*, 2015; Zhang *et al.*, 2016), including oil palm (Yeap *et al.*, 2021), we selected *EgPDS* gene to study the efficiency of CRISPR/Cas9 system in oil palm. The *EgPDS* gene sequence was obtained from the oil palm EG5 database. The *EgPDS* genomic sequence is located in chromosome 13 and consists of 14 exons and 13 introns (*Figure 1a*). Out of 167 listed gRNA candidates, 61 gRNAs with GC content more than 35% or less than 60% range, no poly-T and less than seven gRNA-pairing targets were selected for further analysis. Then, the gRNA candidates with the presence of guanine (G) at nucleotide 20, cytosine (C) at position 16 and purine residues (A/G) at the last four nucleotide sequences, and also the absence of C at nucleotide 3, were selected for off-target analysis (data not shown). The GC percentage of gRNAs was positively correlated with CRISPR/Cas9 on-target efficiency. The guanine-rich sequences can fold into stable noncanonical structures called G-quadruplexes *in vivo*, contributing to the DNA and RNA stability (Zhang *et al.*, 2015).

Sequence Verification of gRNA Candidates for *EgPDS* Gene

Analysis to detect the presence of preferable features in the 61 gRNA candidates was carried out based on previous studies (Wang *et al.*, 2014a; Wong *et al.*, 2015), and seven sgRNAs were chosen for further experiments. The selected gRNA1-gRNA7 are targeting exon 1, exon 2, exon 6, exon 12 and exon 13 of *EgPDS* (*Figure 1a*). The selected motifs of seven gRNAs were summarised in *Table 3*. Doench *et al.* (2014) reported that, within the gRNA sequence, cytosine was disfavoured at nucleotide 3, whilst guanine, cytosine and adenine were most favoured at nucleotide 20, 16 and in the middle of the gRNA, respectively. Therefore, all gRNA1-gRNA7 do not have cytosine at position 3 in the gRNA sequence. In addition, gRNA6 and gRNA7 are the gRNAs that contain guanine at position 20. gRNA7 also has cytosine at position 16, which was reported to be a favourable motif for gRNA sequence (Wu *et al.*, 2014). Furthermore, gRNA1, gRNA2, gRNA4 and gRNA5 have adenine in the middle of the gRNA sequence. In addition, purine residues in the last four nucleotides of the gRNA sequence were also preferable because it can promote the strong binding between gRNA and Cas9 (Bruegmann *et al.*, 2019; Wang *et al.*, 2014b). Thus, all gRNA selected contains purine residue except gRNA1. Repetitive TTTT, UUU and GGGG were also avoided in selecting gRNA candidates except for gRNA6 (having GGGG) because it may significantly lead to insufficient CRISPR/Cas9

activity (Wong *et al.*, 2015). In addition, the GGGG sequence is also prone to form a guanine tetrad that causes the gRNA sequence to be less accessible for target sequence recognition and binding. However, since 4.9% of functional gRNAs contain repetitive Gs (Wong *et al.*, 2015), gRNA6 was selected for further analysis. We also selected gRNAs targeting both strands, with gRNA5 and gRNA6 targeting the antisense strand. Both strands have been reported to have no significant influence on the CRISPR/Cas9 activity (Doench *et al.*, 2014).

Secondary Structure Analysis of gRNA Candidates

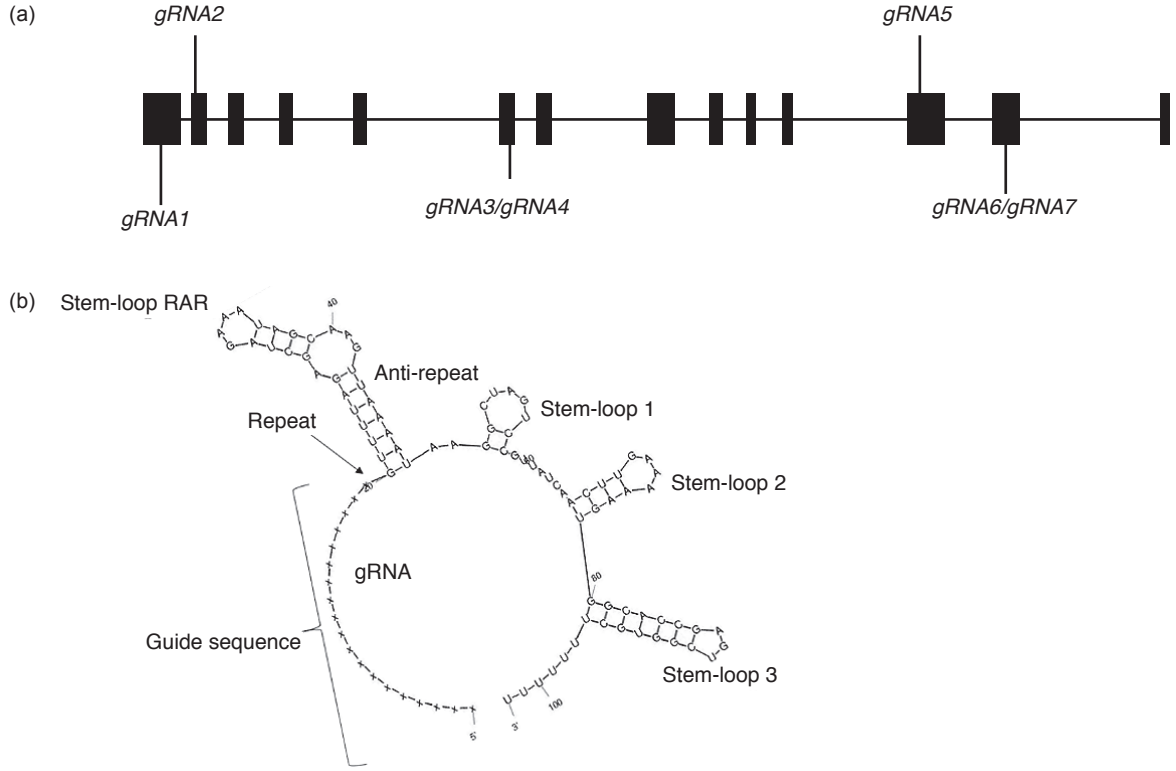
The secondary structure of gRNA has also been reported to interfere with the gene-editing mechanism (Wong *et al.*, 2015). Generally, a gRNA secondary structure consists of a CRISPR RNA (crRNA) sequence that comprises 20 nucleotides of gRNA and 12 nucleotides repeat region, and *trans*-activating RNA (tracrRNA) sequence derived from a combination of anti-repeat region and three stem-loop structures. These structures, so-called artificial tetraloop, are linked together to form a single RNA transcript structure (*Figure 1b*). The tetraloop is the repeat and anti-repeat region or also known as RAR stem-loop (GAAA), stem-loop 1 (CUAG), stem-loop 2 (GAAA) and stem-loop 3 (AGU). As shown in *Figures 1c-1i*, all seven gRNAs contain at least stem-loop 2 and stem-loop 3 structures, with gRNA2 and gRNA5 having all the required structures. The RAR stem-loop stimulates the precursor CRISPR RNA (pre-crRNA) processing by the RNase III enzyme and activates the crRNA-guided DNA cleavage by Cas9, whilst stem-loops 2 and 3 aid in the formation of a stable Cas9-gRNA-DNA complex (Liang *et al.*, 2016). Although stem-loop 1 has been shown as a beneficial structure for the functionality of the Cas9-gRNA-DNA complex, 82% of functional gRNA absent stem-loop 1, indicating that it may not be linked to editing efficiency (Liang *et al.*, 2016).

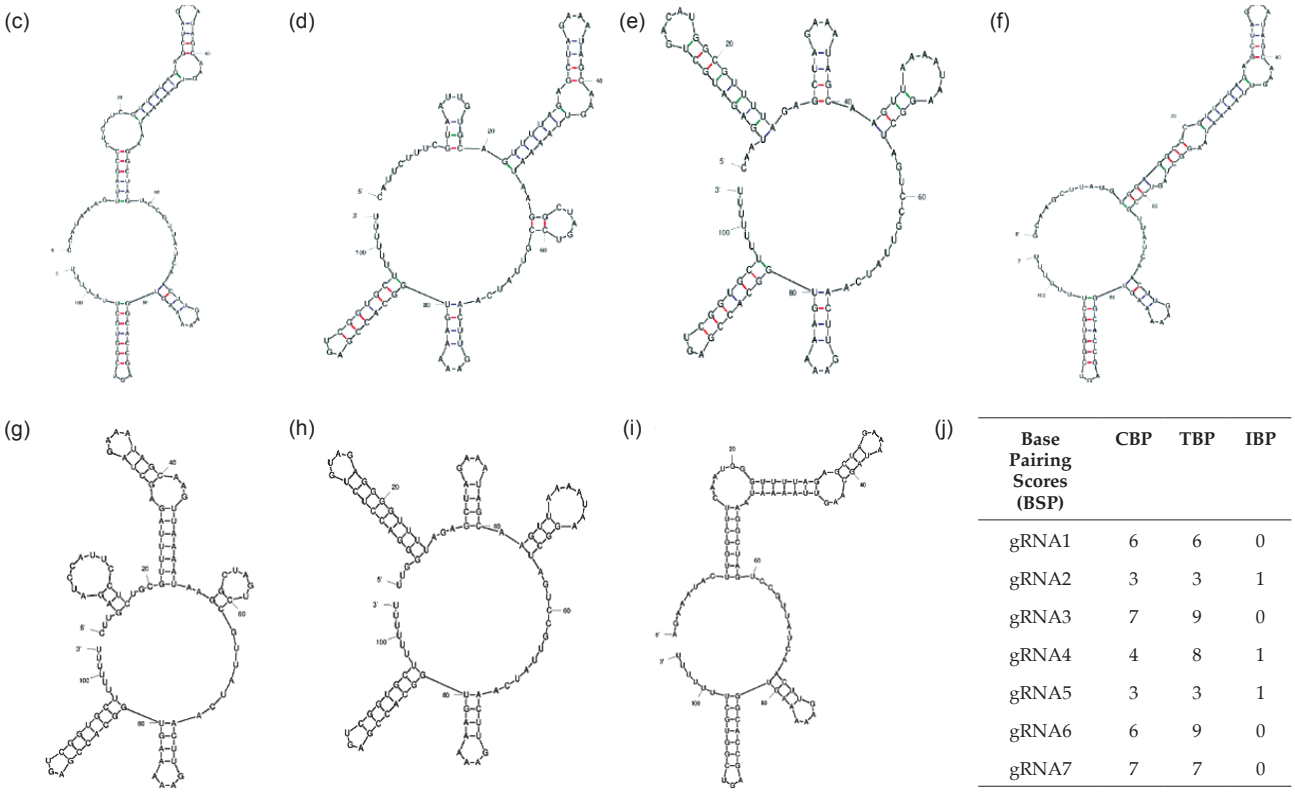
We also assessed the gRNA accessibility at the seed region (10-12 nucleotide proximal to PAM sequence) at positions 18-20 and 51-53 of the gRNA secondary structure (*Figure 1*; *Table 3*). The accessibility of nucleotides 18-20 and 51-53 is crucial in the target recognition process to allow the binding of Cas9-gRNA to the gRNA complementary site (Doench *et al.*, 2014). Therefore, the 18-20 and 51-53 nucleotides should be unpaired for the binding to occur. However, Bruegmann *et al.* (2019) reported successful mutation using gRNA with partially accessible nucleotides 18-20 and 51-53. As shown in *Figures 1c-1i*, only gRNA5 has accessible nucleotide 18-20, whilst the other gRNA candidates were only partially accessible. For positions 51-53, we found that gRNA1, gRNA4 and gRNA7 (*Figures 1c, 1f* and *1i*, respectively) are partly paired to the seed region and formed an extended stem-loop secondary structure. Furthermore, gRNA4 was shown to have 'C' as the last nucleotide of the seed region which has a solid propensity to pair with AAG at positions 51-53 of the gRNA, forming an extended stem-loop secondary structure (Wong *et al.*, 2015). This extended stem-loop structure will restrict the base accessibility at 51-53 and reduce the chance of proper binding of Cas9-gRNA to the target site. However, CRISPR/Cas9 editing in poplar showed that successful editing was still achieved with 'C' paired with AAG at positions 51-53 of the gRNA (Bruegmann *et al.*, 2019).

Next, we evaluated the gRNAs based on the base-pairing scores of the gRNA secondary structure. A stable complex between the gRNA sequence and the other nucleotide bases can affect the base pairing of the guide sequence with its target DNA (Liang *et al.*, 2016). The base pairing scores are the total base-pairing scores (TBP), consecutive base-pairing scores (CBP) and internal base-pairing scores (IBP). The TBP score is the total base pair in the guide sequence with other sequences of the gRNA, while the CBP score is the number of bases in the guide sequence with other sequences. Meanwhile, the IBP score is the internal base pair in the gRNA. Based on Liang *et al.* (2016), gRNA sequences with no more than 12 TBP scores, a maximum of seven CBP and six IBP, were preferred as efficient parameters for selecting gRNA. Therefore, in this study, gRNA candidates were selected with a maximum of nine TBP for gRNA3 and gRNA6, a maximum of seven CBP for gRNA7, and a maximum of one IBP for gRNA2, gRNA4 and gRNA5. The overall base-pairing scores are shown in *Figure 1j*.

Off-target Analysis of gRNA Candidates

Within the genome, the presence of off-target sites that share sequence similarities with the actual target sites may lead to the possibility of off-target editing. However, if the off-target sequences contain mismatches in the seed region of the gRNA, it will destroy the chance of target DNA binding and the Cas9 cleavage activity (Charpentier and Doudna, 2013). In this work, each gRNA candidate sequence was aligned and compared with all known sequences in the oil palm genome using NCBI BLAST (data not shown). As a result, the seed sequences of gRNA3, gRNA4, gRNA6 and gRNA7 were unique in the oil palm genome, thus, minimising off-target activity. Furthermore, the overall similarity percentage for each gRNA candidate with possible off-target sequences in the genome was not more than 43%, and even though single and double mismatches were found in gRNA1, gRNA2 and gRNA5 (data not shown), the capability to trigger the off-target activity could





(j)

Base Pairing Scores (BSP)	CBP	TBP	IBP
gRNA1	6	6	0
gRNA2	3	3	1
gRNA3	7	9	0
gRNA4	4	8	1
gRNA5	3	3	1
gRNA6	6	9	0
gRNA7	7	7	0

Figure 1. (a) Structural organisation of the EgPDS *gene. Exons and introns are shown as black boxes and lines, respectively. The location of each gRNA candidate in the exon is shown in the diagram. (b) Schematic representation of the general secondary structure of gRNA. (c)-(i) The predicted secondary structures of gRNA candidates (gRNA1-gRNA7) targeting* EgPDS *gene. (j) Base pairing scores (BSP) for gRNA1-gRNA7.*

be low since the off-target sequence is tolerated to certain degrees depending on their position along with the gRNA-DNA interface (Jiang *et al.*, 2013). A recent study has also reported that CRISPR/Cas9 off-target activity is not as boundless as speculated before, and random targeting of non-coding regions in the genome usually has no functional consequences (Wu *et al.*, 2014).

TABLE 3. SELECTED FEATURES OF SEVEN *EgPDS* gRNA CANDIDATES

EgPDS gRNA	GC content	Guanine at position 20	Cytosine at position 16	Adenine at position 9 to 11	No cytosine at position 3	Purine residues in the last 4 nucleotides	Target strand	RAR stem-loop (GAAA)	Stem-loop 1 (CUAG)	Stem-loop 2 (GAAA)	Stem-loop 3 (AGU)	Unpaired seed region	Unpaired 18-20 nt	Unpaired 51-53 nt
gRNA1 CCATAAAGTTAGCCCTCTTCAGG	45%	No (C)	No	Yes	Yes (A)	No	Sense	Present	Absent	Present	Present	Partly paired	Unpaired	Partly paired to seed region
gRNA2 CATTCTTTCGTAATTGTGCACGG	35%	No (A)	No	Yes	Yes (T)	2 (tgca)	Sense	Present	Present	Present	Present	Partly paired	No (Partly paired)	Partly paired (51-53)
gRNA3 CAATGAGATGCTGACATGGCCGG	50%	No (C)	No	No	Yes (A)	2 (tggc)	Sense	Absent	Absent	Present	Present	Partly paired	No (Fully paired)	Partly paired (51-53)
gRNA4 GCAAGCTTATGTGGAGGCCCAGG	60%	No (C)	No	Yes	Yes (A)	1 (gccc)	Sense	Present	Absent	Present	Present	Partly paired	No (Partly paired)	Partly paired to seed region
gRNA5 CTTGAGATCCATTCCTCTGCAGG	50%	No (C)	No	Yes	Yes (T)	1 (ctgc)	Antisense	Present	Present	Present	Present	Partly paired	Unpaired	Partly paired (51-53)
gRNA6 TTGGGGACCTCTGTAGAGGGCGG	60%	Yes (G)	No	No	Yes (G)	4 (aggg)	Antisense	Absent	Absent	Present	Present	Partly paired	No (Fully paired)	Partly paired (51-53)
gRNA7 AGAAATACTTGGCTTCAATGAGG	40%	Yes (G)	Yes	No	Yes (A)	3 (aatg)	Sense	Present	Absent	Present	Present	Partly paired	Unpaired	Partly paired to seed region

In vitro Cleavage Analysis of gRNA Candidates

In vitro cleavage assay is important to select the most efficient gRNA *in vitro* before deploying the gRNA into the plant (Bente *et al.*, 2020). In this study, approximately 694-1600 bp of PCR products were amplified to have the target sites (gRNAs) located asymmetrically within the target region to produce cleavage products with unequal sizes. This allows easy visualisation of subsequent cleavage of genomic *EgPDS* gene fragments. As shown in *Figures 2a, 2b* and *2c*, we tested seven gRNAs that were complementary to three target regions of 736 bp, 694 bp and 1600 bp fragments of *EgPDS* loci. The forward primer that composted the T7 promoter, target gRNA sequence and the scaffold template was used to amplify 130 bp of gRNA (*Figures 2d* and *2e*). These DNA fragments were transcribed to RNAs and combined with Cas9 to cleave the three target *EgPDS* regions. The determination of cleavage efficiency was performed by comparing the intensity of the uncut fragment with the control fragment. Based on *Figure 2f*, the cleavage efficiency of individual gRNAs was varied, with gRNA5 showing 100% cleavage efficiency. For multiplex gRNAs, a combination of three gRNAs (gRNA5, gRNA6, and gRNA7) has resulted in 100% cleavage efficiency. The results also showed that all gRNAs were able to cleavage target DNA sequences of the *EgPDS* gene into two to three fragments, with the expected sizes visible on the gel. A single (gRNA1 or gRNA2) and multiple (gRNA1 and gRNA2) *in vitro* cleavage assays were carried out for gRNA1 and gRNA2, producing DNA fragments with sizes of 551 bp, 395 bp, 341 bp and 185 bp (*Figure 2f*). For gRNA3 and gRNA4, *in vitro* cleavage analysis of 694 bp fragment produced four DNA fragments as 407 bp, 356 bp, 338 bp and 287 bp (*Figure 2f*). Meanwhile, multiple *in vitro* cleavage assays for gRNA5, gRNA6 and gRNA7 produced six bands with expected sizes of 1196 bp, 1419 bp, 850 bp, 750 bp, 431 bp and 181 bp (*Figure 2f*). Taken together, all the seven gRNAs were able to cleavage the corresponding target regions into the expected DNA fragments.

Construction of Multiplex gRNA-CRISPR/Cas9 Vectors Targeting *EgPDS* Gene

A schematic representation for the simplified construction procedure of CRISPR/Cas9 vectors carrying multiple gRNAs targeting the *EgPDS* gene is shown in *Figure 3*. In order to integrate the gRNA sequence into pYLsgRNA-OsU6a and pYLsgRNA-OsU6b, the target-gRNAs must contain guanine nucleotide as a definite transcription initiation site (Ma *et al.*, 2015). The selection of target-gRNAs in the genome is usually 5′-GN(19)NGG, so-called regular target gRNAs (Ma and Liu, 2016). However, the number of possible regular target gRNAs was found to be limited in the coding region of the *EgPDS* gene. As shown in *Figure 3*, six out of seven gRNAs were irregular targets with starting adenine (gRNA7), thymine (gRNA6) and cytosine (gRNA1, gRNA2, gRNA3, gRNA5). It was previously reported that the target-gRNAs with additional 'G' nucleotide at the 5' end (derived from the vector ligation site) could also lead to CRISPR/Cas9 editing in plants and produce similar editing efficiency (Ma *et al.*, 2015).

CRISPR/Cas9 multiplex genome editing system was proven to be highly effective in producing high frequencies of targeted mutation in many plant species, including those with very low transformation efficiency (Ma *et al.*, 2015). Previous CRISPR/Cas9 study in oil palm reported successful mutations of the oil palm PDS gene by using a single vector cassette for Cas9 or individual gRNA in oil palm immature embryos (Yeap *et al.*, 2021). However, in this study, we employed the multiplex CRISPR/Cas9 vector system consisting of gRNA expression cassettes, namely pYLsgRNA-OsU6a and pYLsgRNA-OsU6b and a binary vector pYLCRISPR/Cas9P35S-H. As shown in *Figures 4a* and *4b*, the assembly of gRNA expression cassettes in the pYLCRISPR/Cas9P35S-H vector was based on the position of gRNAs in the PDS sequence. The OsU6a and OsU6b promoters were originated from an indica rice cultivar 93-11 and a japonica rice cultivar *Nipponbare* (Ma *et al.*, 2015), monocotyledon plant species. Thus, the promoters were expected to work in oil palm. Furthermore, the activity of OsU6 promoters has been tested in rice and proven to be highly expressive and could also be possibly efficient for genome editing in oil palm (Bahariah *et al.*, 2021).

Gibson assembly method was used to assemble the gRNA expression cassettes into the pYLCRISPR/Cas9P35S-H vector. Sequentially, a series of PCR amplification (*Table 2*) denoted as PCR GXL, PCR GXL1 and PCR GXL2 were carried out to create overlapping ends that allow the cloning of multiple gRNA expression cassettes into the final vector, pYLCRISPR/Cas9P35S-H. The construction of the CRISPR/Cas9 vector was initiated with the ligation of double-stranded gRNA into the gRNA expression cassettes. The pYLsgRNA-OsU6a and pYLsgRNA-OsU6b were previously linearised using *Bsa*I and purified before being ligated with the double-stranded target-gRNAs. During the linearisation of the gRNA expression cassette, over-digestion with excess *Bsa*I and prolonged reaction time were avoided because it could produce abnormal constructs and reduce the cloning efficiency (Ma *et al.*, 2015). The ligated target-gRNA-cassettes were

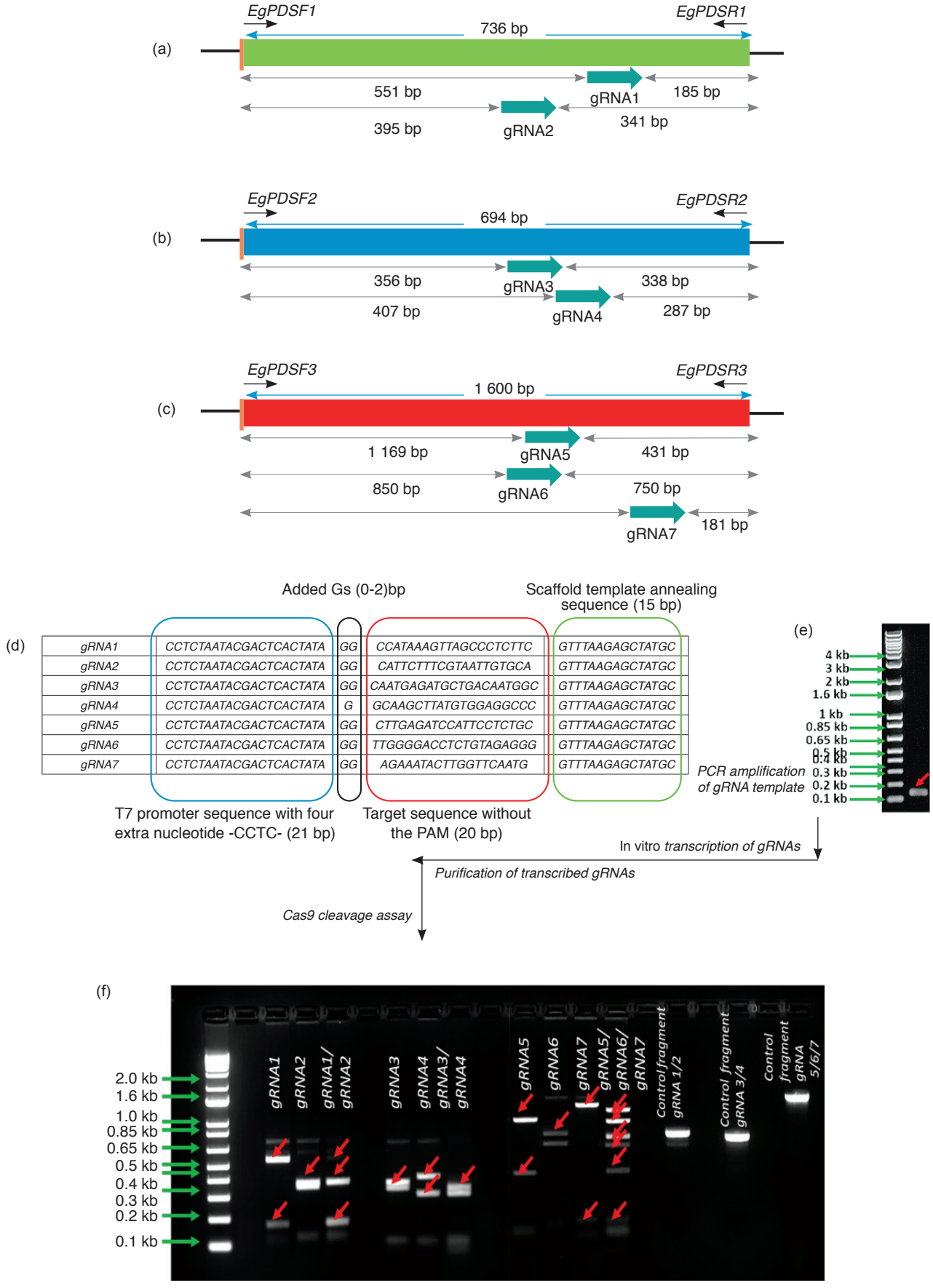


Figure 2. In vitro *cleavage assay reaction of seven gRNA candidates. (a)-(c) Schematic representation of three regions of* EgPDS *gene loci targeted by gRNA1-gRNA7. (d) The designed forward primers (56-58) nucleotide were used to transcribe the gRNA template carrying the target sequence and its scaffold. (e) The amplified gRNA template was observed on the gel as 130 bp amplification for each gRNA. (f)* In vitro *cleavage assay was carried out using the transcribed gRNAs coupled with Cas9 enzyme against the control template in single and multiple gRNA reactions.*

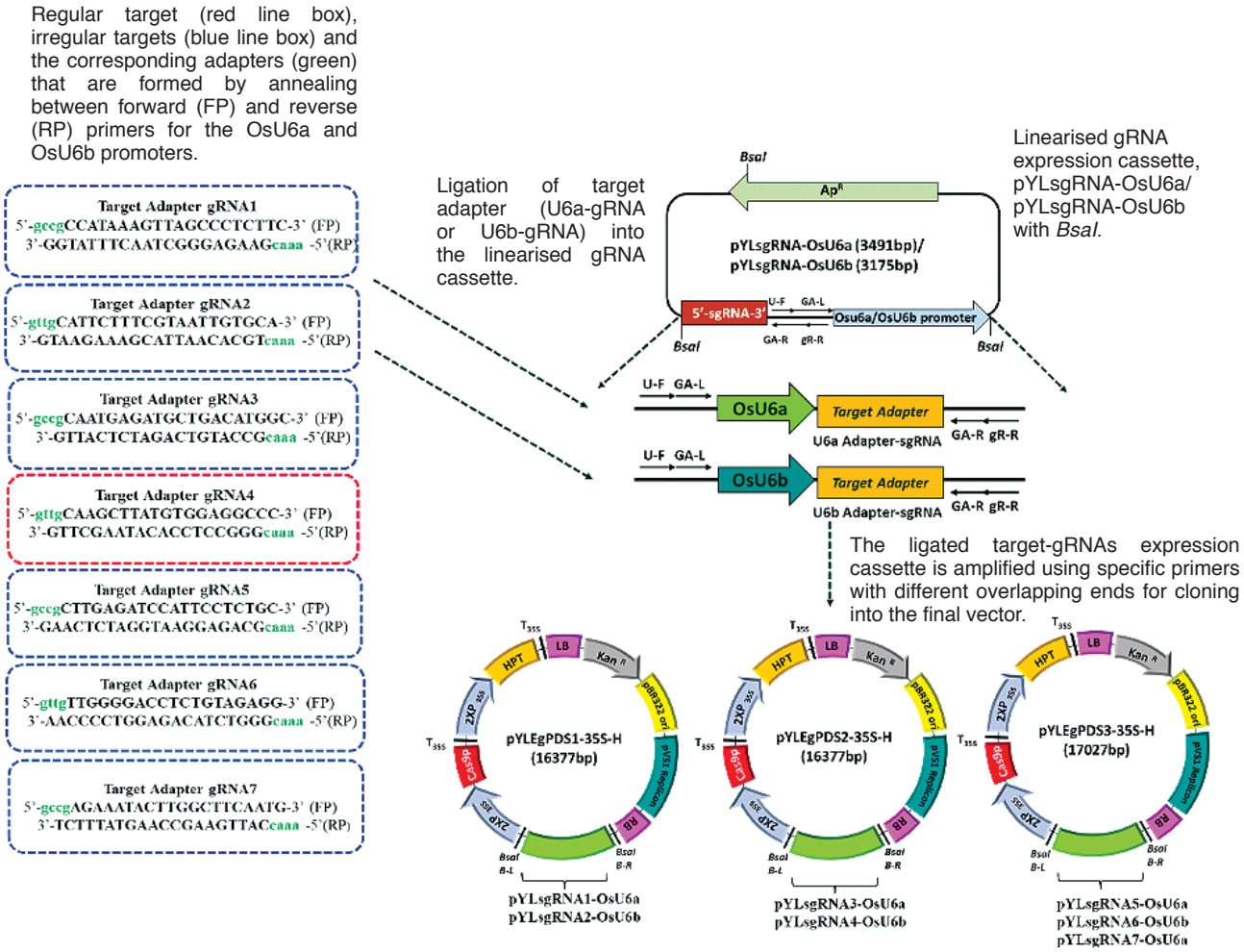


Figure 3. Schematic representation of a step-by-step procedure for constructing CRISPR/Cas9 vectors carrying multiple gRNAs (gRNA1-gRNA7) targeting the EgPDS *gene. The blue and red line boxes show the regular and irregular target adapters sequence for each gRNA candidate. These target adapters were ligated into the* BsaI *site of their corresponding gRNA expression cassettes; pYLsgRNA-OsU6a or pYLsgRNA-OsU6b. Then, the ligated target-gRNA expression cassette was amplified using nested PCR primers U-F/gR-R and position-specific primers (GA-L/GA-R) to create different overlapping ends for cloning into the final transformation vector pYLCRISPR/Cas9P35S-H via Gibson assembly reaction. The gRNA expression cassettes were arranged into the final vector as follows; pYLEgPDS1-35S-H carry two gRNA expression cassettes (gRNA1/gRNA2), pYLEgPDS2-35S-H have two gRNA expression cassettes (gRNA3/gRNA4), and pYLEgPDS3-35S-H carry three gRNA expression cassettes (gRNA5/gRNA6/gRNA7).*

PCR amplified using nested PCR primers U-F/gR-R and target adapter primers in PCR GXL. PCR GXL produced approximately 500 bp OsU6a-gRNA (OsU6a-gRNA1, OsU6a-gRNA3, OsU6a-gRNA5 and OsU6a-gRNA7) and 400 bp OsU6b-gRNA (OsU6b-gRNA2, OsU6b-gRNA4 and OsU6a-gRNA6) for reaction 1 and 150 bp gRNA-scaffold for reaction 2 (*Figure 4c*). The amplified PCR products from PCR GXL were used in the second amplification (PCR GXL1) to yield the 650 bp fragment for OsU6a-gRNA-scaffold and 550 bp fragment for OsU6b-gRNA-scaffold (*Figure 4d*). The PCR products were then used as templates in PCR GXL2. In PCR GXL2, position-specific primers (*Tables 1* and *2*) were used to amplify the overlapping ends of PCR products (*Figure 4e*) for assembling multiple gRNA expression cassettes in the respective positions of the vector backbone pYLCRISPR/Cas9P35S-H. PCR fragment of each target-gRNA cassette was purified and ligated into the linearised pYLCRISPR/Cas9P35S-H/*BsaI* (*Figure 4f*), and successfully cloned DNA plasmids were confirmed by restriction analysis with *AscI* (*Figure 4g*). A fragment of 1150 bp was observed for plasmids carrying two gRNA cassettes designated as pYLEgPDS1-35S-H and pYLEgPDS2-35S-H. Meanwhile, *Asc*I digested plasmid produced approximately 1800 bp fragment was designated as pYLEgPDS3-35S-H, which carries three gRNA expression cassettes.

Three CRISPR/Cas9 transformation vectors, pYLEgPDS1-35S-H, pYLEgPDS2-35S-H and pYLEgPDS3-35S-H, were successfully constructed for transformation into oil palm. For pYLEgPDS1-

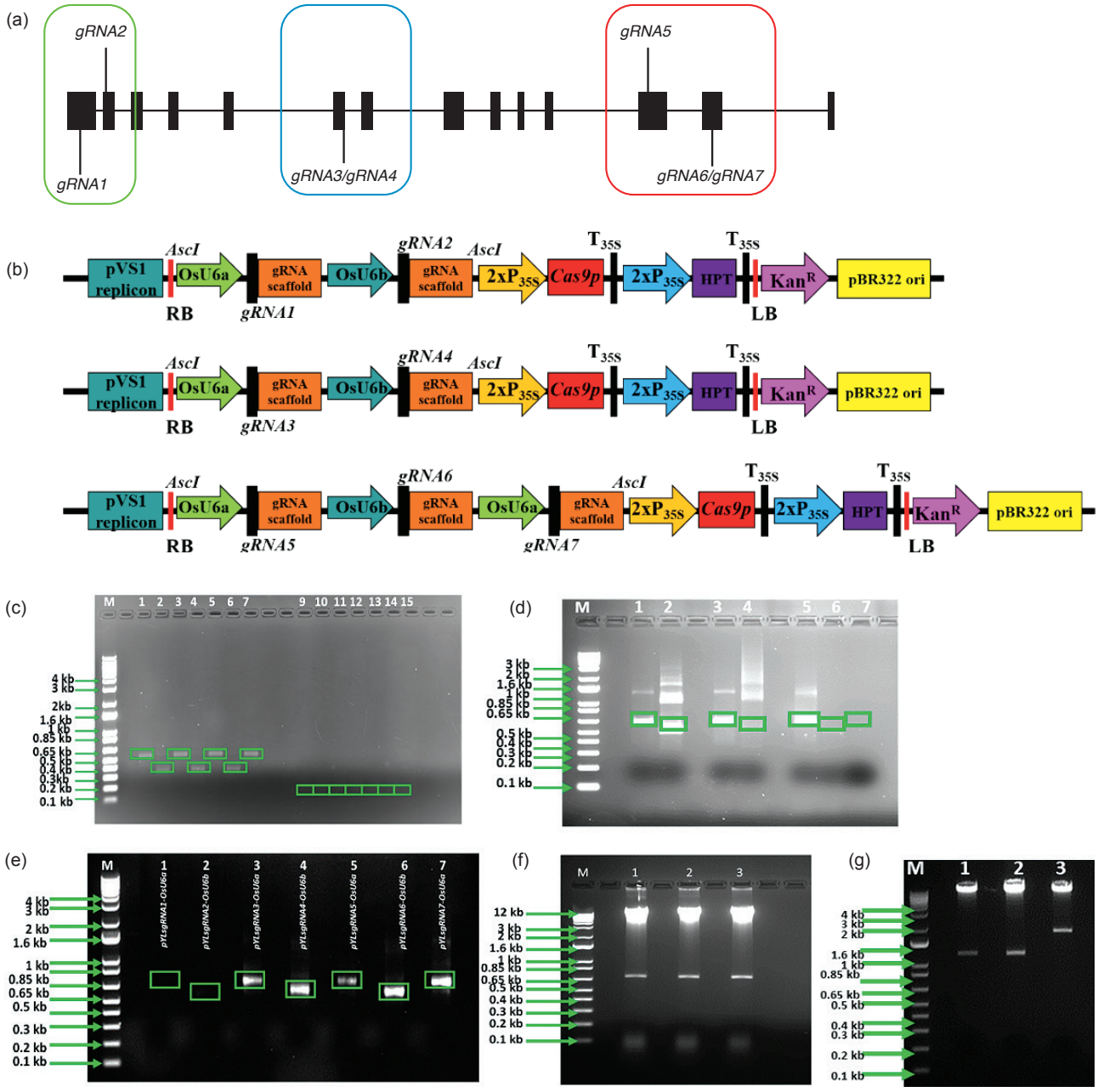


Figure 4. Construction of CRISPR/Cas9 vectors using multiplex genome editing system. (a) Structural organisation of the EgPDS *gene, exons and introns are shown as black boxes and lines, respectively. The green line box indicates the target gRNAs in pYLEgPDS1-35S-H vector, the blue line box indicates the target gRNAs in pYLEgPDS2-35S-H and the red line box indicates the target gRNAs in pYLEgPDS3-35S-H. (b) Schematic arrangement of OsU6 promoters driving-sgRNA cassettes, pYLsgRNAOsU6a and pYLsgRNAOsU6b for each gRNA candidate (gRNA1-gRNA7) in the final vector (pVS1 replicon: pVS1 for replication, RB: Right border of T-DNA, 2XP35S: Double cauliflower mosaic virus 35S promoter, Cas9p: CRISPR associated protein 9, HPT: hygromycin phosphotransferase, Kan^R: Kanamycin resistance gene, pBR322: pB322 vector, T_{35S}: Cauliflower mosaic virus 35S terminator, LB: Left border). (c) PCR GXL of gRNA expression cassettes; pYLsgRNA-OsU6a and pYLsgRNA-OsU6b amplified using primers gR-R/gRNA target adapter forward primer for reaction 1, U-F/gRNA target adapter RP in reaction 2. (d) PCR GXL1 of gRNA expression cassettes; pYLsgRNA-OsU6a and pYLsgRNA-OsU6b on amplified using primers U-F/gR-R. (e) PCR GXL2 of gRNA expression cassettes on amplified using position-specific primers (f) Restriction analysis of pYLCRISPR/Cas9P35S-H with* BsaI. *(g) Verification of the final CRISPR/Cas9 vectors using restriction analysis with* AscI.

35S-H, two gRNAs cassettes carrying gRNA1 (pYLsgRNA-OsU6a) and gRNA2 (pYLsgRNA-OsU6b) were arranged alternately in the pYLCRISPR/Cas9P35S-H. These two gRNAs were located in the first part of the *EgPDS* gene targeting exon 1 and exon 2. The second vector, pYLEgPDS2-35S-H, carries gRNA3 and gRNA4, both targeting exon 6 and the third vector, pYLEgPDS3-35S-H, carries gRNA5, gRNA6 and gRNA7, targeting the terminal region of the *EgPDS* gene on exon 12 and 13. These CRISPR/Cas9 vectors were designed to possibly increase the editing efficiency of the oil palm genome targeting the *EgPDS* gene. The use of multiple gRNAs targeting a single gene has been proven to increase the mutation frequency in tomato (Brooks *et al.*, 2014).

CONCLUSION

Seven gRNAs located at exon 1, exon 2, exon 6, exon 12 and exon 13 of chromosome 13 targeting the *EgPDS* gene in the oil palm genome were successfully designed and validated. These gRNAs have several preferable features, including the GC content of 35%-60%, preferable gRNA sequence motifs, secondary structure with at least stem-loop 2 and stem-loop 3, and minimum off-target effects. Furthermore, *in vitro* cleavage assay confirmed the cleavage ability of these gRNAs on the corresponding target regions of the *EgPDS* genomic sequence. Inserting gRNAs into pYLsgRNA-OsU6 cassettes and then assembling the gRNA cassettes into pYLCRISPR/Cas9P35S-H have successfully produced three CRISPR/Cas9 expression vectors. Thus, the CRISPR/Cas9 vectors carrying seven gRNAs targeting the oil palm *EgPDS* gene are now available for CRISPR/Cas9 studies in oil palm.

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