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MUTAGENESIS OF THE PROMOTER OF THE OIL PALM *HOMOGENTISATE GERANYLGERANYL TRANSFERASE* GENE (*HGGT*) BY PCR-DRIVEN OVERLAP EXTENSION

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Abstract: PCR-driven overlap extension is a simple and versatile tool in performing site-directed mutagenesis. This technique contains two stages of PCR. The first stage is the initial PCR to generate mutated gene segments that overlapped, which are then used as a DNA template in the second stage PCR to produce full length product with the site-directed mutation. The internal primer serves to introduce nucleotide substitution for site-directed mutagenesis by complementary 3' ends on the intermediate segments. The full-length product amplied by the flanking primers in the second stage PCR includes the restriction enzyme sites for inserting the product into an expression vector for cloning purposes.

Keywords**:** mutagenesis, primer, PCR, oil palm

INTRODUCTION

Alteration and modification at the nucleotide level within genes can provide a better understanding of the structural elements critical for the gene and its function (Zoller et al., 1982; Urban et al., 2017). Site-directed mutagenesis generates targeted changes through the use of an oligonucleotide primer that introduce the desired modification including single or multiple nucleotide insertions, deletions or substitution (Hoa et al., 1989). In site-directed mutagenesis, segments of the target gene can be amplified from the template DNA using two flanking master primers (a and d) and two internal primers (b and c) that introduce the desired mutation. Primer design will be the key to the success of this method as the internal primers do not only contain the desired mutation but also create the nucleotide with overlapping segments(Lai et al., 2017). This will lead to the amplification of the product AD, when the strands of product AB and CD are denatured in the second stage PCR. In summary, master primer a and d will preferentially amplify product AD with the intended mutation. Once the second stage PCR is complete the final product with site-directed mutation can be inserted into an expression vector for the production of a large amount of the mutated DNA product.

METHODS

1. 1st Stage PCR (PCRs to generate product AB and CD)

Component	Amount per reaction (µl)
Nuclease free water	9
KAPA HI5 Hotstart ReadyMix®	12.5
Forward Primer A	0.75
Reverse Primer B	0.75
Forward Primer C	0.75
Reverse Primer D	0.75
DNA template	2

Thermocycler programme for 1st stage PCR

Cycle number	Denature	Anneal	Extend	Hold
1	95°C, 3 min	-	-	-
22-35	98°C, 20 sec	50-60°C, 15 sec	72°C, 1 min	-
36			72°C, 4 min	-
37				4°C, hold

Isolation of product AB and CD

The PCR products were separated on a 1% (w/v) agarose gel and undergone the process of gel extraction and purification using the Qiaquick Gel extraction Kit (Qiagen, Germany). The DNA fragments were excised from the agarose gel with a clean, sharp scalpel. Three volumes of buffer QC was added to the excised gel and incubated at 10°C for 10 minutes or until the gel completely dissolved. The tubes were vortexed every two to three seconds to help dissolve the gel. One volume of isopropanol was added to the sample and mixed. The mixture was then transferred to Qiaquick spin columns and centrifuged for one minuted at 179000 x g using the table-top microcentrifuge (Eppendorf centrifuge 5414 R). The flow-through was discarded and the column was washed using 750 µl of PE buffer. The column was centrifuged again for one minute at 17900 x g to remove the wash buffer. The column then placed into sterile 1.5 ml microcentrifuge tubes and 30 µl of EB buffer was added to column to elute the purified PCR product. Finally the column were let to stand for one minute and centrifuged again to collect the purified DNA.

2. 2nd Stage PCR (Full-length product AD)

Component	Amount per reaction (µl)
Nuclease free water	9
KAPA HI5 Hotstart ReadyMix®	12.5
Forward Primer A	0.75
Reverse Primer D	0.75
DNA template	2

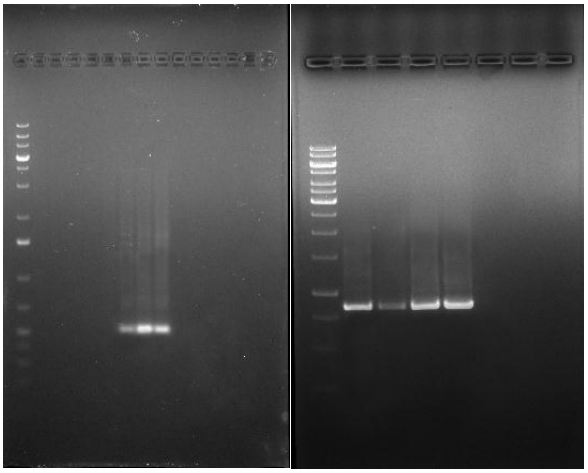
Thermocycler programme for 2nd stage PCR

Cycle number	Denature	Anneal	Extend	Hold
1	95°C, 3 min	-	-	-
22-35	98°C, 20 sec	50-60°C, 15 sec	72°C, 1 min	-
36			72°C, 4 min	-
37				4°C, hold

Isolation of product AD

The PCR products were separated on a 1% (w/v) agarose gel and undergone the process of gel extraction and purification using the Qiaquick Gel extraction Kit (Qiagen, Germany) as described above.

RESULTS AND DISCUSSION



1a **1b**

Figure 1a (PCR AB) and Figure 1b (PCR CD) shows the agarose gel electrophoresis analysis of the first stage PCR which are the PCR AB and PCR CD. Figure 1a shows the band of 500 bp which refers to the amplification using the forward primer A and reverse primer B. Figure 1b shows the band size of 750 bp which refers to the amplification by the forward primer C and reverse primer D. The band was then extracted from the gel and purified before sending for sequencing to confirm the substitution of the nucleotide in the *HGGT* gene by site-directed mutagenesis.

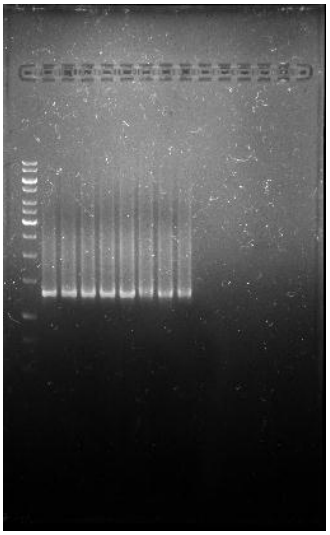


Figure 2 shows the agarose gel electrophoresis analysis of the second stage PCR which was PCR AD. The band 1200 bp refers to the amplification of the master forward primer a and reverse primer d. The band was then extracted from the gel and purified before sending for sequencing to confirm the substitution of the nucleotide in the *HGGT* gene by site-directed mutagenesis.

CONCLUSIONS

In conclusion, the result from the gel electrophoresis analysis and sequencing shows the successful desired substitution of the nucleotide in the HGGT promoter DNA and proved that the overlapped PCR extension can be a useful tool in introducing mutation in genes through site-directed mutagenesis.

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