BIOINFORMATION
Discovery at the interface of physical and biological sciences

open access www.bioinformation.net Hypothesis

Volume 6(6)

Construction of efficient and effective transformation vectors for palmitoyl-acyl carrier protein thioesterase gene silencing in oil palm

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Received May 12, 2011; Accepted May 13, 2011; Published June 06, 2011

Abstract:
Palm oil obtained from *E. guineensis* Jacq. Tenera is known to have about 44% of palmitic acid (C16:0). Palmitoyl-Acyl Carrier Protein Thioesterase (PATE) is one of the key enzymes involved in plastidial fatty acid biosynthesis; and it determines the level of the C16:0 assimilation in oilseeds. This enzyme's activity in oil palm is responsible for high (≥ 44 % in *E. guineensis* Jacq. Tenera and 25 % in *E. oleifera*) content of C16:0 in its oil. By post-transcriptional PATE gene silencing, C16:0 content can be minimized for nutritional value improvement of the palm oil. The objective of this study was the construction of novel transformation vectors for PATE gene silencing. Six different transformation vectors targeted against PATE gene were constructed using 619 bp long PATE gene (5' region) fragment (from GenBank AF507115). In one set of three transformation vectors, PATE gene fragment was fused with CaMV 35S promoter in antisense, intron-spliced inverted repeat (ISIR), and inverted repeat (IR) orientations to generate antisense mRNA and hair-pin RNAs (hpRNA). In another set of three transformation vectors with same design, CaMV 35S was replaced with Oil palm mesocarp tissue-specific promoter (MSP). The expression cassette of antisense, ISIR, and IR of PATE gene fragments were constructed in primary cloning vector, pHANNIBAL or its derivative/s. Finally, all 6 expression cassettes were sub-cloned into pCAMBIA 1301 which contains the Hygromycin^r and the GUS reporter genes for transformant selection and transformation detection respectively. The results of the RE analyses of the constructs and sequence analyses of PATE and MSP shows and confirms the orientation, size and locations of all the components from constructs. We hypothesize that 4 (pISIRPATE-PC, pIRPATE-PC, pMISIRPATE-PC and pMIRPATE-PC) out of 6 transformation vectors constructed in this study will be efficient and effective in palmitoyl-ACP thioesterase gene silencing in oil palm.

Keywords: African oil-palm, American oil-palm, Fatty acids, Genetic engineering, Oleic acid, Oil palm, Palmitic acid, Palm oil, Plastids, Vegetable oil.

Abbreviations: antiPATE - Antisense Palmitoyl-acyl carrier protein thioesterase, BCV - Binary cloning vector, cDNA - Complementary deoxyribonucleic acid, hpRNA - hair-pin RNA, ihpRNA - intron containing hair-pin RNA, IR - inverted repeat, ISIR - intron-spliced inverted repeat, MCS - Multiple cloning site, MSP - Oil palm mesocarp tissue-specific promoter, nt - Nucleotide/s, PATE - Palmitoyl-acyl carrier protein thioesterase, PCR - Polymerase chain reaction, PCV - Primary cloning vector, pDNA - Plasmid deoxyribonucleic acid, PTGS - Post-transcriptional gene silencing, RE - Restriction enzyme.

Background:
Vegetable oils and fats are integral part of human diet. The world population is growing with an alarming rate, and the demand for vegetable oils and fats is increasing steadily. Coconut, corn, cotton, oil palm, peanut, rapeseed, safflower, sesame, soybean, sunflower, and flax are the main vegetable oil producing crops **[1-2]**. The African oil palm (*E. guineensis* Jacq.), and the American oil palm (*E. oleifera*) are cultivated in the tropics for vegetable oil production. In the African oil palm there are three fruit forms namely, Dura, Tenera, and Pisifera. *Elaeis guineensis* Jacq. Tenera is a hybrid of Dura (♀) and Pisifera (♂), and is known for the high oil yield **[3]**. Hence, for the commercial cultivation farmers and plantation companies favor *E. guineensis* Jacq. Tenera. One hectare of oil palm in good growing conditions produces about 4.5 tonnes (t) oil per year, 0.50 t kernel oil and 0.45 t palm kernel cake **[4]**. This yield is almost three times the yield of coconut, and more than ten times that of soybean **[5]**. *Elaeis oleifera* a close relative of the *E. guineensis* is low oil yielding and not preferred for the commercial plantation, even though its oil contains more (69 %) oleic acid in comparison to commercially cultivated oil palm, *E. guineensis* Jacq. Tenera. In the world market of fats and oils, palm oil is considered as a market leader, constituting about 35 % of the world trade in fats and oils. Malaysia is the largest producer and exporter of the palm oil, and accounts for about 50 % of the world's palm oil output and 62 % of the net export trade in palm oil **[5-6]**. Palm oil has a great potential to fulfill the increasing demand for vegetable oil from both food and non-food industries. However, like other vegetable oils such as soybean oil, to get a good price in the market it is important to develop different varieties with desired fatty acid composition. To achieve this goal, conventional crop improvement

methods are not only tedious but also time-consuming **[5]**. Oil palm genetic engineering may save 80-90% of the time required for insertion of gene/s for new trait/s in it through conventional crop breeding **[7]**. Therefore, out of all existing methods available for crop improvement, perhaps genetic engineering is the best method to manipulate fatty acid profile in oil palm in a short period. Palm oil and palm kernel oil are the main commercial products of the oil palm fruits. Palm kernel oil is the main source of lauric acid (C12:0), which is mainly used to fulfill the needs of soap, detergent, and cosmetic industries **[8]**. Oil obtained from mesocarp of commercially cultivated oil palm (*E. guineensis* Jacq. Tenera) fruits contains 53.3% saturated fatty acids, while commercially less important *E. oleifera* contains 28% saturated fatty acids **[5]**. Among the saturated fatty acids, palmitic acid (C16:0) is predominantly accumulated. *Elaeis guineensis* Jacq. Tenera oil typically contains 44.0% C16:0, while in *E. oleifera* oil, it is 25.0% **[5]**. Lowering down the saturated fatty acid content in palm oil is one of the ways for palm oil nutritional value improvement.

The PATE enzyme is known to have C16:0-ACP substrate specificity **[9-11]**. Therefore, it is necessary to knockout the expression of PATE gene in order to minimize the percentage of C16:0 in palm oil. Palm oil will be healthier for the human consumption and will get more price in the market if the C16:0 percentage in palm oil could be reduced to make it low palmitate **[12-14]**. The silencing of PATE gene can be accomplished through any one of the several different methods of gene silencing such as, transcriptional gene silencing (TGS), cosuppression, post-transcriptional gene silencing (PTGS) using antisense, direct inverted repeats (IR), intron-spliced inverted repeat (ISIR) mediated gene silencing, and site-directed mutagenesis (SDM) **[15-19]**. Cosuppression and PTGS with antisense, IR and/or ISIR trans-genes markedly reduces the steady state mRNA levels of endogenous genes similar in transcribed sequence. These methods are proved to be very effective for gene silencing in oil producing, and other model plants like *Arabidopsis* **[18-24]**. A generic primary cloning vector, pHANNIBAL developed by CSIRO is very useful for the construction of novel transformation vectors with antisense, inverted repeats, and intron-spliced inverted repeats of the gene of interest **[18-19, 24-25]**. This paper reports the construction of novel transformation vectors with constitutive and oil palm fruit mesocarp-tissue-specific promoter for the oil palm PATE gene silencing using pHANNIBAL, pCAMBIA 1301 and 619 bp long 5' region of oil palm PATE gene fragments.

Methodology:

Plasmid vectors and bacterial strains:

Two cloning plasmid vectors, pHANNIBAL and pCAMBIA 1301 were used in the construction of 6 different transformation vectors for oil palm PATE gene silencing. The PCV, pHANNIBAL, a derivative of cloning vector pART7 was kindly provided by CSIRO, Australia **[25-26]**. The BCV, pCAMBIA 1301 was available in our laboratory (UKM, Bangi, Malaysia) **[27]**. The restriction enzymes map of PCV and BCV is shown in **Supplementary Figure 1**. The PCR cloning vector, pGEM®-T Easy was used for the cloning of PCR amplified antisense and sense PATE gene fragments. Bacterium, *E. coli* strain DH5-α was used for preparation of the competent cells to harbor the plasmids. Prepared competent cells were stored at –70 °C until the use.

Construction of transformation vectors for PATE gene silencing:

Plasmid, pPATE-RT is a recombinant PCR cloning vector (pGEM®-T Easy), which carries a 629 bp long (GenBank AF507115) fragment of *E. guineensis* Jacq. Tenera PATE gene previously isolated in our laboratory. We used this PATE gene fragment as template to synthesize antisense and sense PATE fragments in the vector construction. For the construction of 6 different transformation vectors for PATE gene silencing, standard gene cloning methods were used **[28]**. Construction of expression cassettes with antisense, ISIR and IR of PATE gene fragment was completed using pHANNIBAL. The expression cassettes from PCVs were then sub-cloned as a *Sac*I-*Pst*I fragment into pCAMBIA 1301.

Plasmid DNA extraction:

During preparation of constructs in PCV, selected and well-isolated colonies from the LB agar plates were inoculated aseptically and separately in universal bottles containing 10 ml LB medium, supplemented with 50 µg/ml Ampicillin (Ampicillin Sodium Salt, MF = C16H18N3NaO4S; Amersham Life Science). But, the BCV carries a Kanamycin resistant gene, and hence cultures were supplemented with 50 µg/ml Kanamycin. Cultures were incubated at 37 °C, 250 RPM for 16 hours. Alkaline lysis method was used for pDNA preparation. For confirmation of the intactness, and quality of the extracted pDNA, it was electrophoresed on 1% agarose gel.

PCR for RE sites addition and PATE amplification:

To incorporate PATE gene fragment in antisense orientation in pHANNIBAL, *Xba*I and *Bam*HI restriction sites were used. The forward primer [F-XbaI (5'-AGCTCTAGAATCTTTGGTCTTTCATTCCC-3')] and reverse primer [R-BamHI (5'- ATTGGATCCTTCCAATCAAGAAGGGTCC-3')] were designed with flanking sequence of the *Xba*I, and *Bam*HI restriction site (underlined nucleotides) respectively and used in amplification of the antisense PATE gene fragments. For amplification of 619 bp long sense PATE gene fragment, forward primer [F-XhoI (5'-ATTCTCGAGATCTTTGGTCTTTCATTCCC-3')] and reverse primer [R-EcoRI (5'-AACGAATTCTTCCAA TCAAGAAGGGTCC-3')] were designed with *Xho*I, and *Eco*RI restriction site (underlined) flanking sequence, respectively. The PCR for amplification of antisense and sense PATE gene fragments was completed under following conditions. Hot start 94 °C for 5 min, followed by 35 cycles of 33 sec at 94 °C, 1 min at 62 °C, 1.30 min at 72 °C and a final extension at 72 °C for 5 minutes. For the synthesis and amplification of the MSP, forward primer [PF-SacI/NotI (5'-GAGCTCGCGGCCGCGAATTTATCTGACAAAGGTGC-3')] with flanking nt sequence for *Sac*I, and *Not*I restriction sites (underlined) and reverse primer [PR-EcoRI (5'-CACGAATTCGAACTAGTAAGTGAAGA TCTTGG-3')] with flanking nt sequence for *Eco*RI RE were used. Amplification of MSP by PCR was carried out using the following conditions: 40 cycles of 33 sec at 94 °C, 1 min at 63 °C, 1 min and 30 sec at 72 °C, and a final extension of 1 cycle of 5 min at 72 °C.

PCR product purification:

The PCR products were purified from the PCR reaction mixture using NucleoSpin® Extraction Kit (Ready-to-use system for fast purification of nucleic acids), BD Biosciences Clontech, USA. The DNA bands of expression cassettes and plasmid DNA were excised from agarose gel with the help of surgical blade, and DNA was purified using NucleoSpin® Extraction Kit. Quantitative estimation of purified DNA was calculated using UV-160 A, UV-visible recording spectrophotometer (SHIMADZU).

Ligation reaction:

Ligation reactions for incorporation of 619 bp long antisense and sense PATE gene fragments and MSP into pHANNIBAL and ligation reactions to incorporate expression cassettes into pCAMBIA 1301 were assembled in 10.00 µl volume by adding: ~ 200 ng vector, ~ 600 ng insert, 10X Ligase buffer, 3 units of T-4 DNA Ligase; sterile distilled water was added finally to adjust the final volume of reaction to 10 µl. Recombination reactions for cohesive ends were incubated at 16 (±1) °C, whereas blunt end ligation reactions were incubated at 8 (±1) °C for 18 h.

Transformation of *E. coli* competent cells:

The preparation of frozen stocks of competent cells (using the protocol-I) and transformation of *E. coli* strain DH5-α competent cells with ligated product by using heat shock method was performed **[28]**.

Analysis of recombinant plasmids by restriction enzymes:

To confirm integration of antisense and sense PATE gene fragments, extracted recombinant plasmid DNA (20 µg pDNA) samples were double-digested with respective REs. For instance, for the confirmation of the antisense PATE gene fragment insert in pAPATE-H, it was double digested with *Xba*I-*Bam*HI by incubating digestion reactions at 37°C, for 4 h. For the characterization of the recombinant plasmids, pDNAs were double-digested with different combinations of RE to confirm the size, orientation and location of the inserts and the components of the cassettes.

Nucleotide sequencing:

To confirm the identity of the PATE gene fragment or MSP promoter in constructs, nucleotide sequencing of the inserts was carried out using automated DNA sequencer. The pair of primers used for synthesis of PATE (antisense or sense) and MSP by PCR technique was used in sequencing reactions for the sequencing of respective inserts. Nucleotide sequence was analyzed by using nucleotide-nucleotide blast (blastn) program **[29]**.

Construction of transformation vectors using MSP:

The MSP was isolated previously in our laboratory from *Elaeis oleifera* **[30]**. Three transformation vectors (with antisense, ISIR and IR of PATE gene fragment) were constructed for PATE gene silencing using MSP. From the already prepared expression cassette, CaMV 35S promoter was replaced with MSP to control expression of the constructs designed against oil palm PATE gene. MSP was used in the constructs to express constructs in oil palm mesocarp tissue-specific manner.

Transfer of expression cassettes from PCV into BCV:
The *Sac*I and *Pst*I restriction sites were used to separate expression cassettes with antisense, ISIR and IR of PATE gene fragment from the respective PCV. The separated and purified expression cassettes were incorporated as *Sac*I-*Pst*I fragment into BCV (pCAMBIA 1301). The BCV carries a Kanamycin resistant gene, and hence transformed bacteria were selected on LB-agar plates supplemented with 50 µg/ml Kanamycin.

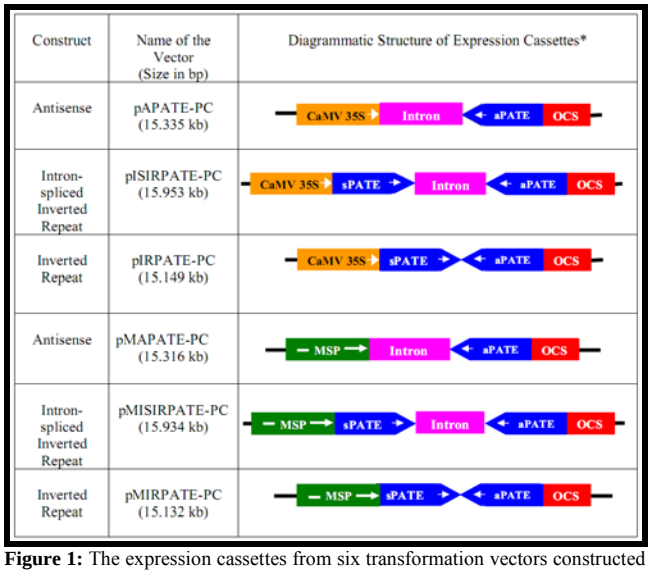

Figure 1: The expression cassettes from six transformation vectors constructed for PATE gene silencing. aPATE, antisense Palmitoyl-ACP Thioesterase gene fragment (619 bp); CaMV 35S, Cauliflower mosaic virus 35S promoter; MSP, Oil palm mesocarp tissue-specific promoter; sPATE, sense Palmitoyl-ACP Thioesterase gene fragment (619 bp); OCS, Octopine synthase terminator. *Figure drawing is not to the scale.

Results:
Six different types of transformation vectors for PATE gene silencing were constructed. A set of three constructs (with antisense, ISIR and IR of PATE) was constructed using constitutive (CaMV 35S) promoter. Another set of three constructs was with the same design of expression cassettes except that the CaMV35S promoter was replaced with MSP. **Figure 1** shows the six different constructed expression cassettes and their design for PATE gene silencing. The expression cassette with antisense PATE gene fragment was prepared using 619 bp long PATE gene fragment from plasmid, pPATE-RT and pHANNIBAL. Based on the restriction enzymes map of PCV and PATE gene fragment, antisense PATE gene fragment was incorporated in pHANNIBAL using *Xba*I and *Bam*HI REs without discarding intron sequence. The intron sequence was kept as it is because it will be spliced out during the mRNA maturation; and hence its removal was not necessary. The strategy used for the construction of transformation vector (pAPATE-PC) with CaMV 35S promoter driven antisense PATE gene fragment is shown in **Supplementary Figure 2**. Double-digestion of pAPATE-H with *Sac*I-*Cla*I released ~ 2173 bp long DNA fragment (lane 5, **Figure 2A**). This fragment contains CaMV 35S promoter and an intron. Double-digestion of pAPATE-H with *Xho*I-*Cla*I released DNA fragments of ~ 812 bp in length (lane 6, **Figure 2A**). This fragment is of intron. The DNA fragment of ~ 1447 bp in size was released from pAPATE-H as a result of its double-digestion with *Xho*I-*Xba*I (lane 7, **Figure 2A**). This fragment comprises an intron, and antisense PATE gene fragment. The double-digestion of pAPATE-H with *Sac*I-*Pst*I released 3531 bp long entire expression cassette with CaMV 35S driven antisense PATE gene fragment (lane 8, **Figure 2A**). After completion of the REs analysis of prepared antisense PATE gene fragments expression cassette, it was sub-cloned into pCAMBIA 1301 to have a selection marker, reporter gene, and other elements of the BCV.

The REs analysis of the plasmid, pAPATE-PC was carried out to confirm the location, orientation, and length of the expression cassette and its elements. Digestion of plasmid, pAPATE-PC with *Eco*RI released 1373 bp long DNA fragment of CaMV 35S promoter (lane 3, **Figure 2B**). As expected double digestion of pAPATE-PC with *Eco*RI-*Pst*I released two DNA fragments, smaller one was of 1373 bp long CaMV 35S promoter; and another bigger one was of 2164 bp in size (lane 4, **Figure 2B**). This fragment contains intron, antisense PATE gene fragment and OCS terminator of the expression cassette. The double-digestion of pAPATE-PC with *Sac*I-*Kpn*I released one DNA fragment of 1377 bp in size (lane 5, **Figure 2B**). This DNA fragment is of CaMV 35S promoter. Double-digestion of pAPATE-PC with *Sac*I-*Pst*I released the entire 3531 bp long 'antisense PATE gene fragment expression cassette' (lane 6, **Figure 2B**). Once the antisense PATE gene fragment is inserted in pHANNIBAL using *Xba*I and *Bam*HI sites, the resulting plasmid can be used to make expression cassettes with ISIR and IR of PATE for the PATE gene silencing. For the construction of hpRNA generating transformation vector for PATE gene silencing, construct should have PATE gene fragments sequence either in ISIR or in an IR in its expression cassette. The strategy used for the construction of transformation vector (pISIRPATE-PC) with CaMV 35S promoter driven ISIR of PATE gene fragment is shown in **Supplementary Figure 3**. The REs analysis of pISIRPATE-H and pISIRPATE-PC is depicted in **Figure 2C & 2D**, respectively. The pISIRPATE-H was used in construction of expression cassette with IR of PATE gene fragment. The intron fragment was taken out from pISIRPATE-H to make an expression cassette with direct IR of PATE gene fragments **(Supplementary Figure 4)**. The REs analysis was carried out for both pIRPATE-H and pIRPATE-PC. The REs analysis results for pIRPATE-H and pIRPATE-PC are shown in **Figure 2E & 2F**, respectively.

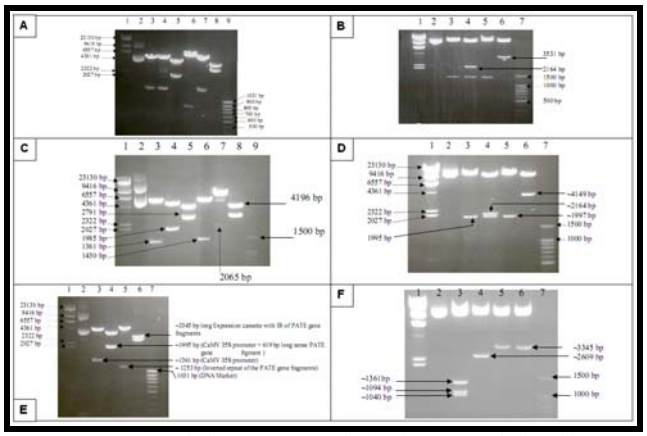

Figure 2: Agarose gel showing REs analysis of constructed plasmid vectors. **(A)** REs analysis of pAPATE-H. Lane 2, undigested pAPATE-H; lane 3, digested with *Sac*I- *Xho*I; lane 4, digested with *Sac*I-*Eco*RI; lane 5, digested with *Sac*I-*Cla*I; lane 6, digested with *Xho*I-*Cla*I; lane 7, digested with *Xho*I-*Xba*I; lane 8, digested with *Not*I; and lane 9, 100 bp DNA markers. **(B)** REs analysis of pAPATE-PC. Lane 2, undigested pAPATE-PC; lane 3, digested with *Eco*RI; lane 4, digested with *Eco*RI-*Pst*I; lane 5, digested with *Sac*I-*Kpn*I; lane 6, digested with *Sac*I-*Pst*I; and lane 7, 100 bp DNA markers. **(C)** REs analysis of pISIRPATE-H. Lane 2, undigested pISIRPATE-H; lane 3, digested with *Sac*I-*Xho*I; lane 4, digested with *Sac*I-*Eco*RI; lane 5, digested with *Sac*I-*Cla*I; lane 6, digested with *Xho*I-*Cla*I; lane 7, digested with *Xho*I-*Xba*I; lane 8, digested with *Not*I; and lane 9, 100 bp DNA markers. **(D)** REs analysis of pISIRPATE-PC. Lane 2, undigested pISIRPATE-PC; lane 3, digested with *Eco*RI; lane 4, digested with *Eco*RI-*Pst*I; lane 5, digested with *Sac*I-*Kpn*I; lane 6, digested with *Sac*I-*Pst*I; and lane 7, 100bp DNA markers. **(E)** REs analysis of pIRPATE-H. Lane 2, undigested pIRPATE-H; lane 3, digested with *Sac*I-*Xho*I; lane 4, digested with *Sac*I-*Bam*HI; lane 5, digested with *Xho*I-*Xba*I; lane 6, digested with *Not*I; and lane 7, shows the 100 bp DNA markers. **(F)** REs analysis of pIRPATE-PC. Lane 2, undigested pIRPATE-PC; lane 3, digested with *Sac*I-*Xho*I; lane 4, digested with *Sac*I-*Xba*I; lanes 5, digested with *Sac*I-*Pst*I; and lane 7, 100 bp DNA markers; in A-F, Lane 1 shows λ DNA *Hind*III markers.

The MSP was isolated previously in our laboratory and cloned in a PCR cloning vector, pGEM®-T Easy **[30]**. This plasmid DNA was designated as pSESQ-PRO. Before PCR amplification of MSP its presence was confirmed in pSESQ-PRO by digesting it with *Eco*RI RE. Since *Eco*RI sites are located adjacent to the MSP DNA insert in pSESQ-PRO. The 1399 bp long MSP DNA fragment was released from the plasmid (pSESQ-PRO) as a result of its digestion with *Eco*RI (figure not shown). The released DNA fragment contains 1293 bp of MSP, 96 bp of the gene, and 10 bp from the MCS of PCR cloning vector. In construction of the pMAPATE-PC, antisense expression cassette of PATE gene fragment from pAPATE-H was used by replacing CaMV 35S promoter with MSP. The strategy used in construction of pMAPATE-PC is shown in **Supplementary Figure 5**. The REs analysis of plasmids, pMAPATE-H and pMAPATE-PC is shown in **Figure 3A & 3B**. The ISIR of

PATE gene fragment under MSP was constructed using pMAPATE-H and PCR amplified sense PATE fragment from pPATE-RT **(Supplementary Figure 6)**. To confirm the presence of MSP and components of ISIR of PATE in expression cassette, REs analysis was carried out for pMISIRPATE-H and pMISIRPATE-PC. The results are shown in **Figure 3C & 3D**. The pMISIRPATE-H carries the ISIR of PATE gene fragments. Hence to make IR of PATE gene fragments in the expression cassette, intron was taken out by using *Kpn*I and *Cla*I REs **(Supplementary Figure 7)**. The sticky ends were made blunt by using T-4 DNA polymerase; and blunt ends were ligated to make IR of existing PATE gene fragments from the pMISIRPATE-H. The ligated plasmid was designated as pMIRPATE-H. The REs analysis of this plasmid and transformation vector (pMIRPATE-PC) was carried out; and the results are depicted in **Figure 3E & 3F**.

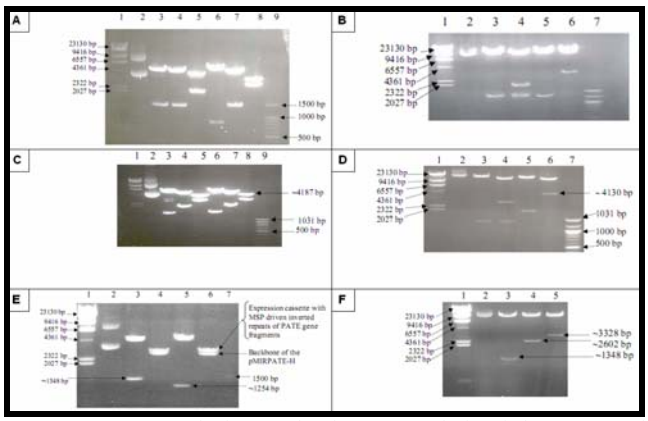

Figure 3: Agarose gel pictures showing REs analysis of the constructed plasmid vectors in which PATE gene fragments were kept under the control of MSP. **(A)** REs analysis of pMAPATE-H. Lane 2, the undigested DNA of pMAPATE-H; lane 3, digested with *Sac*I-*Eco*RI; lane 4, digested with *Sac*I-*Kpn*I; lane 5, digested with *Sac*I-*Cla*I; lane 6, digested with *Eco*RI-*Cla*I; lane 7, digested with *Eco*RI-*Xba*I; lane 8, digested with *Sac*I-*Pst*I; and lane 9, 100 bp DNA markers. **(B)** REs analysis of pMAPATE-PC. Lane 2, undigested pMAPATE-PC; lane 3, digested with *Eco*RI; lane 4, digested with *Eco*RI-*Pst*I; lane 5, digested with *Sac*I-*Kpn*I; lane 6, digested with *Sac*I-*Pst*I; and lane 7, 100 bp DNA markers. **(C)** REs analysis of pMISIRPATE-H. Lane 2, undigested pMISIRPATE-H; lane 3, digested with *Sac*I-*Eco*RI; lane 4, digested with *Sac*I-*Kpn*I; lane 5, digested with *Sac*I-*Cla*I; lane 6, digested with *Eco*RI-*Cla*I; lane 7, digested with *Eco*RI-*Xba*I; lane 8, digested with *Not*I; and lane 9, 100 bp markers. **(D)** REs analysis of pMISIRPATE-PC. Lane 2, undigested plasmid DNA of pMISIRPATE-PC; lane 3, digested with *Eco*RI; lane 4, digested with *Eco*RI-*Pst*I; lane 5, digested with *Sac*I-*Kpn*I; lane 6, digested with *Sac*I-*Pst*I, and lane 7, 100 bp markers. **(E)** REs analysis of pMIRPATE-H. Lane 2, Undigested plasmid DNA of pMIRPATE-H; lane 3, digested with *Sac*I-*Eco*RI; lane 4, digested with *Sac*I-*Pst*I; lane 5, digested with *Eco*RI-*Xba*I; lane 6, digested with *Not*I; and lane 7, 100 bp markers. **(F)** REs analysis of pMIRPATE-PC. Lane 2, undigested pMIRPATE-PC; lane 3, digested with *Eco*RI; lane 4, digested with *Sac*I-*Xba*I; lane 5, digested with *Sac*I-*Pst*I.; and lane 6, 1 kb DNA ladder. In A-F, Lane 1 shows λ DNA *Hind*III markers.

Discussion:

The presence of *Xho*I, *Eco*RI, *Kpn*I, *Cla*I, *Hind*III, *Bam*HI, *Xba*I, *Sac*I, and *Pst*I REs in pHANNIBAL DNA was confirmed prior to synthesis and insertion of antisense PATE into pHANNIBAL. It was necessary to confirm the identity of the plasmid, and to confirm uniqueness of REs so that the strategy can be implemented and the expected sizes of the fragments can be recognized. Our results confirmed the uniqueness of 9 REs sites (*Xho*I, *Eco*RI, *Kpn*I, *Cla*I, *Hind*III, *Bam*HI, *Xba*I, *Sac*I, and *Pst*I) and the identity of the pHANNIBAL **[25]**. In construction of novel transformation vectors two (CaMV 35S and MSP) promoters were used. The use of the tissue-specific gene promoters enables tissue-specific expression of the gene/s. Therefore kernel and mesocarp tissue-specific gene promoters were isolated previously in our laboratory after isolation of tissue-specific genes by differential display method **[30-32]**. Fatty acid biosynthesis pathway in oil palms (*E. guineensis* Jacq. Tenera and *E. oleifera*) can be manipulated genetically at different levels using key genes and tissue-specific promoters. The reduction in level of the saturated fatty acid content in palm oil is one of the ways to improve nutritional value of palm oil. To achieve this goal, down-regulation of the PATE gene holds the key, since reducing palmitate content lowers down level of the saturated fatty acids significantly in palm oil.

The research findings reported by Smith *et al*. (2000) showed that PTGS using antisense and/or cosuppression construct usually leads to modest proportion of silencing and silenced individuals in comparison to gene silencing induced by constructs with ISIR and IR **[19, 25]**. Therefore, a new generation of transformation vectors with ISIR, and IR of PATE gene fragments for PATE gene silencing was constructed. The construction of 2 transformation vectors with antisense construct (pAPATE-PC and pMAPATE-PC) was carried out along with the constructs with ISIR (pISIRPATE-PC and pMISIRPATE-PC) and IR (pIRPATE-PC and pMIRPATE-PC) of PATE for PATE gene silencing to compare the efficiency of the constructs in PATE gene silencing in oil palm. The CaMV 35S promoter was used for three reasons. Firstly, to determine whether there is an effect of PATE gene silencing on fatty acid profile in the leaves instead of waiting for flowering and fruiting of the transgenic oil palm to be obtained. Secondly, to determine the effect of PATE gene silencing on fatty acid profile of the palm oil obtained from mesocarp. And, the third reason was to compare the efficiency of CaMV 35S promoter with MSP **[30]**.

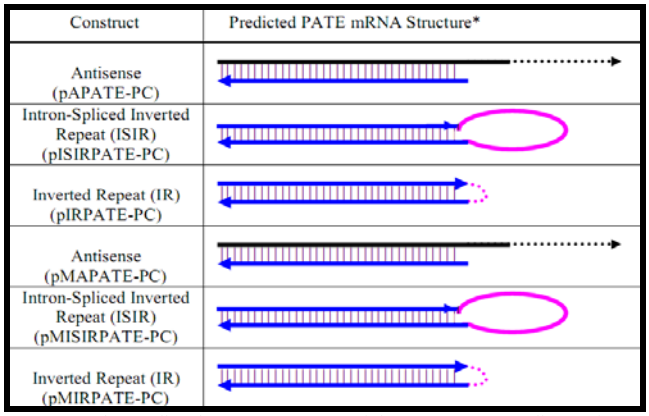

Figure 4: The predicted mRNA structure of the constructs encoding antisense PATE and self-complementary hpRNA (from ISIR and or IR of PATE gene fragments) to efficiently silence PATE gene (in oil palm). In the 6 constructs targeted against PATE gene, in 3 constructs expression of PATE is driven by CaMV 35S promoter; and in another 3 constructs expression is driven by MSP. Thick blue arrows indicates the 619 nt PATE sequence; long, black, thick lines in predicted RNA structure for antisense indicate endogenous PATE gene mRNA. Pink color loops indicate the loop formed by intron; pink dots indicate the nucleotides from intron junction sequences left after intron deletion; and short lines within the stem of hairpin structures indicates base pairing. *Note this figure is not drawn to the scale.

The REs analysis of the PCVs and six (pAPATE-PC, pISIRPATE-PC, pIRPATE-PC, pMAPATE-PC, pMISIRPATE-PC, and pMIRPATE-PC) transformation vectors confirmed the length, orientation, presence and location of the MSP/CaMV 35S promoter and PATE gene fragment in the respective expression cassettes. The identity of the 619 bp long PATE gene fragments was confirmed by its sequencing from pAPATE-H and pAPATE-PC. Nucleotide sequence of antisense PATE gene fragment from pAPATE-PC showed 100 % homology with the template PATE sequence (Figure not shown). This result of sequence analysis confirmed the identity of the PATE gene fragments. All six transformation vectors targeted against PATE gene are ready for the oil palm genetic transformation experiments. The genetic engineering of the oil palm to minimize C16:0 can be done using constructs constructed in this study. Immature zygotic embryos (IZEs), non-embryogenic and embryogenic-callus can be used as target tissues in the oil palm genetic transformation **[33-34]**. The pHANNIBAL is a derivative of pART7 **[19, 25-26]**. Both, the pART7 and pHANNIBAL are originally designed in such a way that the entire expression cassette cartridge can be removed from them as a *Not*I fragment to bring in directly into the specially designed BCV, pART27 **[25-26]**. However, the strategies used in this study highlights that various designs of expression cassettes constructed using pHANNIBAL (PCV) can be sub-cloned into pCAMBIA 1301. Hence, strategies used to construct transformation vectors in this study also highlight the compatibility of pHANNIBAL and pCAMBIA 1301.

The research findings of the Wesley *et al*., (2001) suggest that ihpRNA and hpRNA constructs containing sense and anti-sense arms ranging from 98 to 853 nt leads to efficient silencing in a wide range of plant species **[25]**. In our all 6 constructs, the size of the PATE gene fragment used is 619 nt. Hence these constructs should be equally effective and efficient **[19, 25]**. Based on the

nt's complementary nature, the predicted RNA structure of the constructs encoding antisense PATE and self-complementary hpRNA (from ISIR and or IR of PATE gene fragments) to efficiently silence PATE gene (in oil palm) is depicted in **Figure 4**. The PCV (pHANNIBAL) is a generic vector that allows simply, single PCR product from a gene of interest to be easily converted into a highly effective and efficient ihpRNA silencing construct **[35]**. Because of this PCV's proven effectiveness and efficiency of resulting construct, this vector is a choice in constructing effective and efficient constructs for PTGS of targeted gene/s. This PCV is used in peanut to facilitate its genetic engineering for alleviating peanut allergy **[36]** and in other plants for PTGS of different genes **[24, 35, 37]**. By realizing the efficiency and effectiveness of the construct designs which pHANNIBAL enables, pHANNIBAL-like silencing vectors are also developed for gene silencing in fungi **[38]**. Therefore, we strongly believe that the integration of expression cassettes (containing ISIR and IR of PATE gene fragments) into oil palm genome will lead to the effective PATE gene silencing. As a result of it, C16:0 content will be minimized significantly in the palm oil. It is reported that the silencing effect of such kind of constructs are stably inherited over many generations **[35]**, and oil palm should not be exception for it.

The optimized physical and biological parameters reported by Parveez *et al*. (2000) for the particle bombardment mediated genetic engineering of oil palm can be used to accelerate the genetic transformation of oil palm with constructs reported in this study to knockout the PATE gene expression **[7]**. Recently, constitutive and oil palm leaf-specific promoters (and their potential applications) has been reported by Masura *et al*. **[39]** and Masani *et al*. **[40]**, respectively. These types of promoters can be used to drive expression cassettes of PATE gene for its silencing. However, we have used MSP which is known to regulate gene expression in oil palm fruit-tissue-specific manner **[30]**. Therefore, ISIR, IR and antisense construct of PATE gene fragments from pMISIRPATE-PC, pMIRPATE-PC, and pMAPATE-PC will express only in the fruit mesocarp tissues of the oil palm after its transformation. Whereas, ISIR, IR and antisense construct of PATE gene fragments from pISIRPATE-PC, pIRPATE-PC, and pAPATE-PC will express constitutively. This will help to determine and compare the efficiency and effectiveness of the 2 promoter used. However, we strongly believe that genetic engineering of oil palm with pISIRPATE-PC, pIRPATE-PC, pMISIRPATE-PC and pMIRPATE-PC vectors will facilitate development of low saturated fatty acids producing oil palms **[7]**. Based on the literature and our understanding, we hypothesize that 4 (pISIRPATE-PC, pIRPATE-PC, pMISIRPATE-PC and pMIRPATE-PC) out of 6 transformation vectors constructed in this study will be efficient and effective in PATE gene silencing in oil palm. Nevertheless, these constructs with efficient and effective construct design for PATE gene silencing are bound to facilitate the efforts of genetic engineering in oil palm to produce palm oil with low saturated fatty acids.

Conclusion:

Six different transformation vectors namely, pAPATE-PC, pISIRPATE-PC, pIRPATE-PC, pMAPATE-PC, pMISIRPATE-PC, and pMIRPATE-PC are constructed for the PTGS of the PATE gene. The results of the RE sites analyses and nucleotide sequence analyses of the PATE gene fragments and MSP confirms that 619 bp long PATE gene fragments insert are at the right locations and orientations in the constructs. The PATE gene fragment insert is in antisense orientation in pAPATE-PC and pMAPATE-PC, in ISIR orientation in pISIRPATE-PC and pMISIRPATE-PC, and in direct IR orientation in pIRPATE-PC and pMIRPATE-PC. In all six transformation vectors, all the elements of the respective expression cassettes targeted against PATE gene are at the right place and orientation. But, to take this research forward, transformation of *E. guineensis* Jacq. Tenera and *E. oleifera* with constructed constructs needs to be done in order to obtain transgenic oil palms for the low C16:0 palm oil in a reasonable time.

Disclosure:

Authors attest that there are no conflicts of interest to declare.

Acknowledgments:

Authors are grateful to the Ministry of Science, Technology and Innovation (MOSTI) of Malaysian Government for research funding [Grant Code: IRPA: 01-02-02-0014 / PR00 15-07-07], and to the Commonwealth Scientific and Industrial Research Organization [CSIRO], Australia for providing the primary cloning vector, pHANNIBAL.

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Edited by P Kangueane

Citation: Bhore & Shah. Bioinformation 6(6): 212-220 (2011)

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Supplementary material:

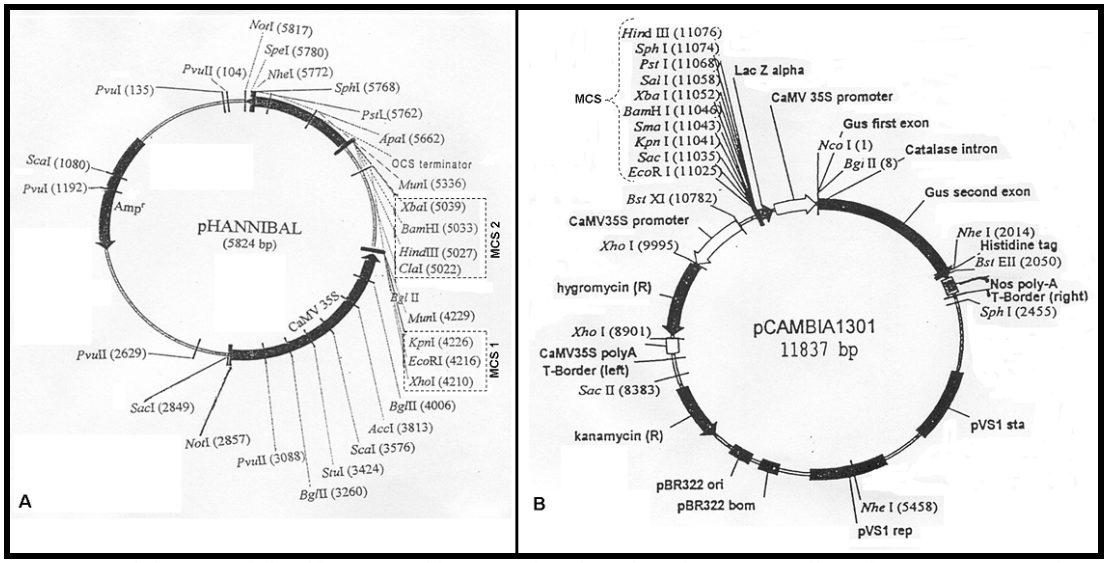

Figure 1: Restriction maps of plasmid vectors used in construction of transformation vectors to silence the PATE gene expression; **(A)** Primary cloning vector (PCV), pHANNIBAL provided by CSIRO, Australia **[19, 25]**; **(B)** Binary cloning vector (BCV), pCAMBIA 1301 (Source: provided by UKM Laboratory) [27]. MCS stands for multiple cloning sites.

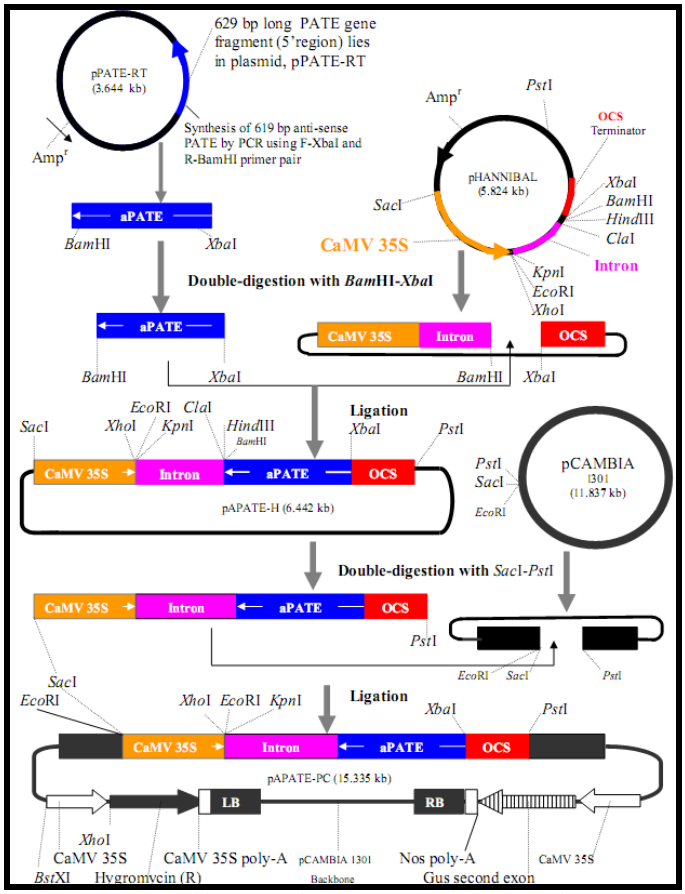

Figure 2: Strategy used in construction of transformation vector with CaMV 35S driven antisense PATE gene fragment. Plasmid, pPATE-RT is a plasmid which carries 629 bp long 5' region of the PATE gene. Plasmid, pHANNIBAL was used as PCV; and plasmid, pCAMBIA 1301 was used as BCV. Refer to **Supplementary Figure 1A and B** for restriction map of pHANNIBAL and pCAMBIA 1301. LB, left (T-DNA) border; RB, right (T-DNA) border; aPATE, antisense PATE; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Figure is not drawn to the scale.

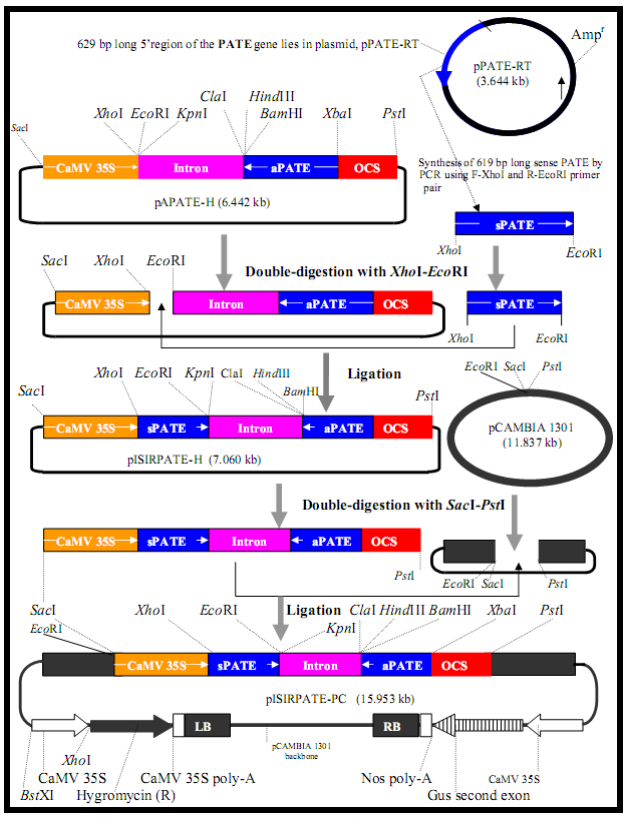

Figure 3: Illustration of the strategy used in construction of transformation vector with CaMV 35S driven ISIR of PATE gene fragments. Plasmids, pAPATE-H and pCAMBIA 1301 were used as PCV, and BCV, respectively. LB, left (T-DNA) border; RB, right (T-DNA) border; s/aPATE, sense/antisense PATE; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Note that this figure is not drawn to the scale.

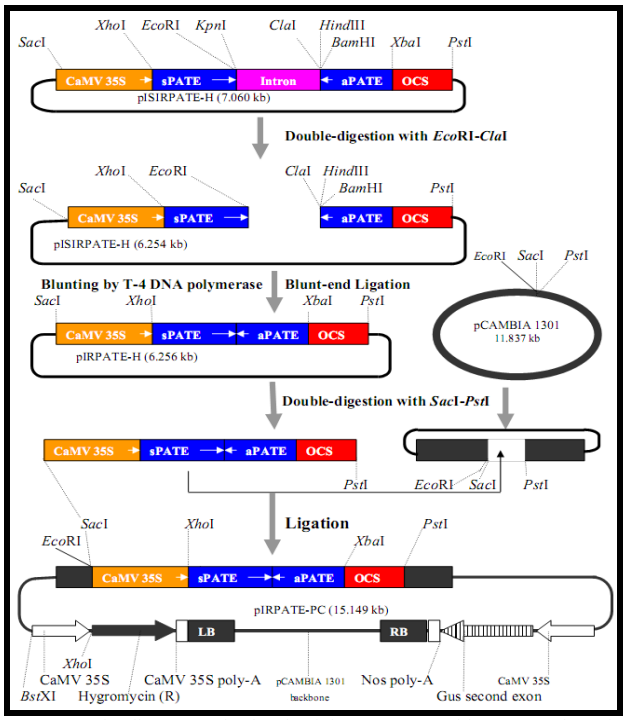

Figure 4: The diagrammatic sketch of the strategy used in construction of transformation vector with CaMV 35S driven IR of PATE gene fragments. Plasmids, pISIRPATE-H and pCAMBIA 1301 were used as PCV and BCV, respectively. LB, left (T-DNA) border; RB, right (T-DNA) border; s/aPATE, sense/antisense PATE gene fragments; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Note this figure is not drawn to the scale.

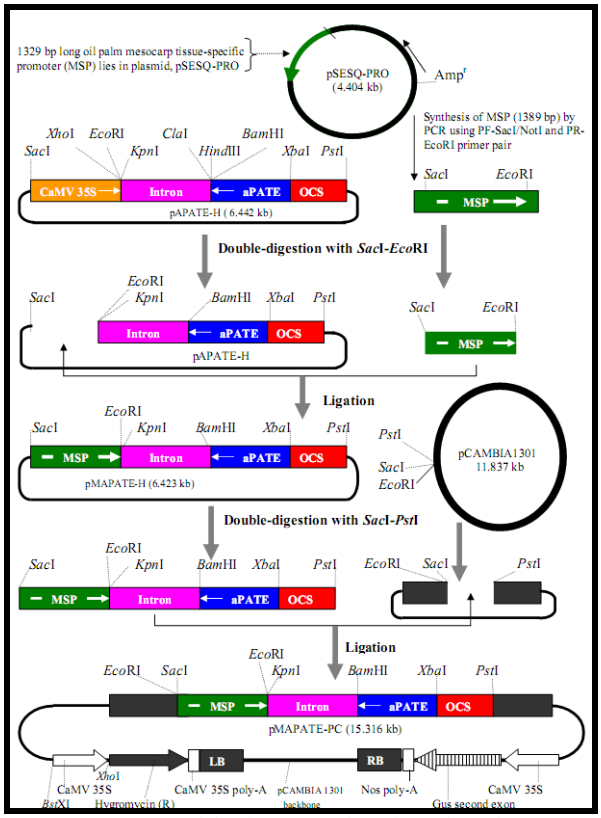

Figure 5: Strategy used for the construction of transformation vector with MSP driven antisense PATE gene fragment. Plasmids, pAPATE-H and pCAMBIA 1301 were used as PCV and BCV, respectively. LB, left (T-DNA) border; RB, right (T-DNA) border; MSP, mesocarp tissue-specific promoter; aPATE, antisense PATE gene fragment; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Note that this figure is not drawn to the scale.

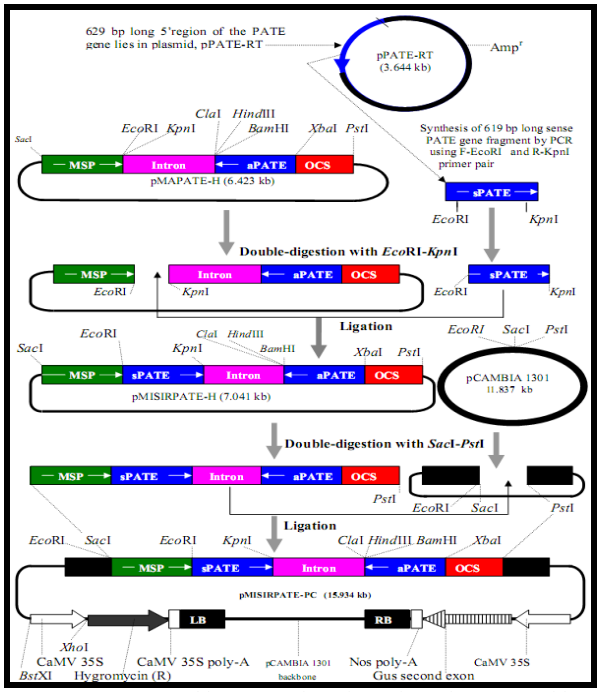

Figure 6: Strategy used in construction of transformation vector with MSP driven ISIR of PATE gene fragments. Plasmid, pMAPATE-H was used as a primary cloning vector, whereas plasmid, pPATE-RT was used as a template for the synthesis of 619 bp long sense PATE gene fragment. LB, left (T-DNA) border; RB, right (T-DNA) border; MSP, mesocarp tissue-specific promoter; s/aPATE, sense/antisense PATE; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Note that figure is not drawn to scale.

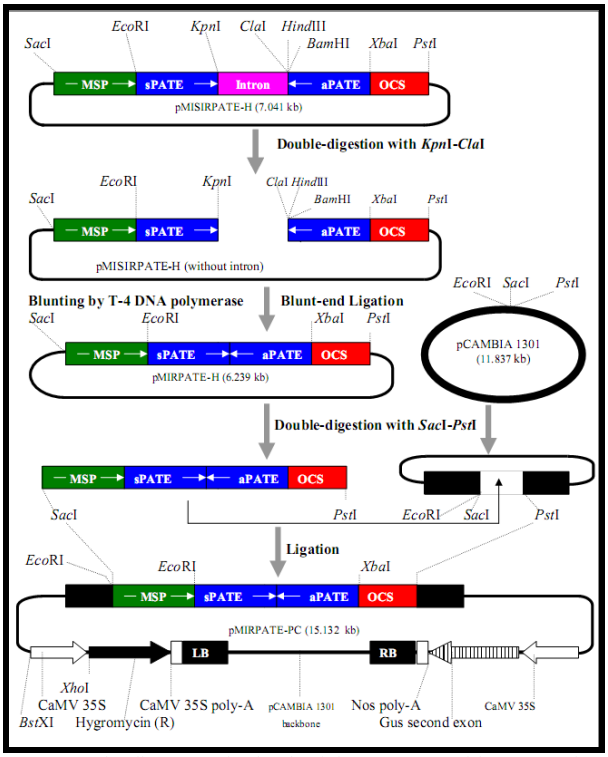

Figure 7: The diagrammatic sketch of the strategy used in construction of transformation vector with IR of PATE gene fragments under the control of MSP. Plasmid, pMISIRPATE-H was used as PCV. LB, left (T-DNA) border; RB, right (T-DNA) border; s/aPATE, sense/antisense PATE gene fragments; OCS, octopine synthase terminator; and CaMV 35S, cauliflower mosaic virus 35S promoter. Note that diagrammatic sketch is not drawn to the scale.