# MOLECULAR ANALYSIS OF TRANSGENIC OIL PALM TO DETECT THE PRESENCE OF TRANSGENES

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**ABSTRACT**

*In order to meet the increasing demand of oleochemical industries, genetic engineering was initiated to modify the fatty acid composition of oil palm with the aim to increase the production of oleic acid to be used as a feedstock. Biolistic approach was used for inserting an antisense palmitoyl-ACP-thioesterase sequence into oil palm tissues to down regulate the activity of palmitoyl-ACP-thioesterase, and increase the accumulation of oleic acid at the expense of palmitic acid. Genomic DNA was isolated from transgenic oil palm and polymerase chain reaction (PCR) amplification of the, selectable marker gene (*bar*) and gene of interest, antisense palmitoyl-ACP-thioesterase, was successfully carried out. The presence of transgenes was confirmed by DNA sequencing.*

**Keywords:** transgenic oil palm, PCR amplification, *bar* gene, antisense PAT.

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## INTRODUCTION

Malaysia and Indonesia are the main producers and exporters of palm oil in the world. Currently, the demand for palm oil exceeds that of soyabean oil as the major edible oil. The high demand for palm oil is because of its nutritional value and competitive price as compared to other vegetable oils (Ramli, 2011). Besides food uses, palm oil is also widely used in non-food applications, such as in cosmetics, pharmaceuticals, lubricants, and various products of the oleochemical industries.

Since the demand for feedstock for the oleochemical industries is high, producing oil palm with high oleic acid has become one of the main priorities of MPOB's oil palm genetic engineering programme. Genetic engineering is targeted to produce high oleate transgenic oil palm by modifiying the fatty acids biosynthesis pathway. Palmitic acid is the predominant fatty acid in palm oil accounting for about 44% of the total fatty acid while about 39% is oleic acid making them the major fatty acids in palm oil (Sambanthamurthi *et al*., 2009). The high accumulation of palmitic acid in palm oil is due to a very active palmitoyl-ACP thioesterase (PAT) enzyme (Sambanthamurthi *et al*., 2002). Subsequently, efforts were made to down regulate the activity of the thioesterase by using antisense approach. A transformation vector carrying antisense PAT sequence was constructed to produce the high oleate transgenic oil palm via microprojectile bombardment. The transformation vector, designated as pAT1, contained the antisense PAT sequence driven by CaMV35S promoter (Abrizah *et al*., 2000; 2003). The antisense PAT which would integrate into the oil palm genome was expected to down regulate the activity of PAT and

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channel palmitic acid further along the pathway to produce more oleic acid (Parveez *et al*., 2003).

The transformation using microprojectile bombardment occurs randomly and selection should be carried out to isolate the transformant from the non-transformant tissues. Moreover, an ideal selection system should kill all non-transformed cells rapidly and it should also be simple and inexpensive to implement (Oneto *et al*., 2010). In this study, a DNA construct pAHC25 which contained herbicide Basta resistant selectable marker gene (*bar*) was co-bombarded during transformation for screening purposes to eliminate the non-transformants. Basta was used as the selective agent to kill non-transformed cells. Basta has also been used to select the transformed tissues in other crops such as maize (Oneto *et al*., 2010), wheat (Mariana *et al*., 2002) and rice (Christou *et al*., 1991).

Although transformants were generated on selection media, molecular analyses such as polymerase chain reaction (PCR) and DNA sequencing need to be performed to determine the status of regenerants. The objective of this study was to evaluate the transgenic status of putative transgenic oil palm plantlets produced using molecular analyses. Ten putative transgenic oil palms were selected and used to extract total DNA which was later subjected to molecular analyses. This communication reports the initial evidence of the integration of *bar* gene and antisense PAT sequence in the oil palm genome using PCR and DNA sequencing analyses.

## MATERIALS AND METHODS

### Plant Materials

Leaves from eight-year-old putative transgenic oil palms were harvested from biosafety screen house at Malaysian Palm Oil Board (MPOB), Bandar Baru Bangi, Selangor, Malaysia. The leaves were wiped with 70% ethanol and cut into small pieces. The leaves were ready for grinding or stored at -80°C.

### Total DNA Extraction

Total DNA was extracted from the leaves according to Doyle and Doyle (1990) with modifications. The 4 g of fresh leaves were ground to a powder with mortar and pestle in the presence of liquid nitrogen. The 20 ml of CTAB buffer (2% CTAB; 1.4M NaCl; 0.2% 2-mercaptoethanol, 20 mM EDTA; 100 mM Tris-HCl, pH8.0) was added to the powder and incubated at 60°C for 30 min. The 20 ml of chloroform-isoamyl alcohol (24:1) were later added and thoroughly mixed, followed by centrifugation at 12 000 rpm for 15 min at room temperature. The aqueous phase was transferred into a new centrifuge tube and 0.6 volumes of cold isopropanol was added and mixed gently to precipitate the DNA. The mixture was incubated at -20°C for 1 hr before being centrifuged at 12 000 rpm, 4°C for 15 min. The pellet was then washed with 10 ml wash buffer (76% ethanol; 10 mM ammonium acetate) for 1 hr and centrifuged at 12 000 rpm for 15 min. The pellet was dried at room temperature and resuspended in 4 ml TE buffer (10 mM Tris-HCl: 1 mM EDTA; pH 7.4). Then, about 10 $\mu$g ml$^{-1}$ RNase was added into the DNA solution and incubated for 20 min at room temperature. The DNA solution was diluted with 2 ml 7.5 M ammonium acetate (pH 7.7) and incubated on ice for 30 min. The DNA mixture was centrifuged at 12 000 rpm, 4°C for 15 min and the supernatant was transferred into a new tube. About 2.5 volumes of 100% cold ethanol was added and gently mixed to precipitate the DNA before storing at -20°C overnight. The DNA mixture was later centrifuged at 12 000 rpm for 15 min at 4°C and the pellet formed was air dried before being suspended in 1 ml TE buffer. The concentration and purity of the isolated DNA were determined by using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

### PCR Amplifications

PCR reactions were carried out in mixtures containing 25-100 ng of plant genomic DNA, 1X Promega Go Taq® Green Master Mix (Promega), 0.4 $\mu$M each of forward and reverse primers. Three sets of primers were used in this experiment. The first set of primers was named as POR-12 and POR-38 with sequence 5′-CCCTCATCCATAGCACA-3′ and 5′- CAGGGAGCAAAGAAGCA-3′, respectively. For the second set, the sequence of forward primer (BarF1) was 5′-GGTCTGCACCATCGTCAACC-3′ and reverse primer (BarR2) was 5′-GTCATGCCAGTTCCCGTGCT-3′. The third set, the sequence of forward primer (TE-F) was 5′-GGCCGGCGCGCCACCATGCTTGCTGA-3′ and the reverse primer (TE-R) was 5′-GCGCGGCGCGCCTGCCACATCATGCAC-3′. The PCR reactions were performed by using PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). The POR-12 and POR-38 primers were used to amplify an endogenous DNA sequence in oil palm. The PCR was started with a denaturation step at 95°C for 2 min. The reactions were performed in 40 cycles of 95°C for 30 s followed by 50 s at 45°C and elongation for 1 min 30 s at 72°C. The final extension at 72°C was done for 5 min. Meanwhile, the primers BarF1 and BarR2, were used to detect the presence of selectable marker gene (*bar* gene) by using a touchdown programme set at 95°C for 5 min for denaturation followed by 10 cycles of 94°C for

45 s, annealling at 70 °C for 45 s and reduced by 0.5°C per cycle and 72°C at 1 min. Another 20 cycles were carried out at 94°C for 45 s, 65 °C for 45 s and 72 °C for 1 min. The final extension was at 72°C for 2 min. Finally, the presence of PAT gene in the transformant was detected using the primers TE-F and TE-R. The amplification was carried out with an initial denaturation at 95°C for 5 min followed by 29 cycles of denaturation at 92°C for 50 s followed by 57°C for 50 s and elongation at 72°C for 1 min. The final extension was at 72°C for 2 min. The PCR products were examined on 1.2% of agarose gel.

**Cloning of PCR Products and DNA Sequence Analysis**

The PCR products separated on agarose gel were cut and extracted from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen, Germany). The purified PCR products were ligated into the PCRII-TOPO vector and transformed into competent cells, *Escherichia coli* strain DH5$\alpha$ according to the manufacturer's instructions (Invitrogen, USA). The digestion with *Eco*RI was performed to confirm the ligation of the insert. The DNA was sequenced by First Base Laboratories Sdn Bhd and analysed using the vector NTI software (Invitrogen, USA).

## RESULTS AND DISCUSSION

**The Purity and Quality of Genomic DNA**

Total DNA of 10 putative transgenic oil palms was successfully isolated and the DNA obtained was shown to have good purity. This was indicated by the ratio of absorbance at 260 nm to 280 nm ranging from 1.8 to 2.0. The DNA was subjected to PCR analysis to amplify the endogenous DNA sequence (POR) as to further confirm that the DNA was sufficiently pure to be used as DNA template to amplify the transgene. The result showed a few non-specific bands. The presence of these non-specific bands could be due to the low annealing temperature used for this PCR programme. Nevertheless, the expected endogenous DNA sequence (1.1 kb) was successfully amplified from all of the DNA samples (*Figure 1*). This result showed that the isolated DNA samples were of good quality and suitable for PCR analyses of the transgenes.

**Detection of *Bar* Gene and Antisense PAT Sequence**

PCR is the simplest molecular technique for early detection of the transgenes in putative transgenic plants. Thus, PCR amplification of the selectable marker gene and subsequently the gene of interest is often used as an indication of transgenic status of regenerants (Bhat *et al*., 2002). Since these putative transgenic oil palms were successfully selected on herbicide Basta, the PCR analysis of *bar* gene was carried out. The *bar* gene was also commonly used in transgenic plant studies such as in maize (Yan *et al*., 2010), sweet potato (Zang *et al*., 2009) and snapdragon (Hoshino *et al*., 1998).

The presence of *bar* gene in the putative transgenic oil palms was confirmed by the production of PCR product with a size of 460 bp (*Figure 2*). This 460 bp was a result of the amplification of a part of the *bar* gene. The product was not detected in water (negative control) and untransformed oil palm. Meanwhile, the construct pAHC25 which carried the *bar* gene driven by constitutive promoter maize ubiquitin was used as the positive control. Among the transformants, only one sample, TE3-8(1) showed a strong band while a few more samples showed

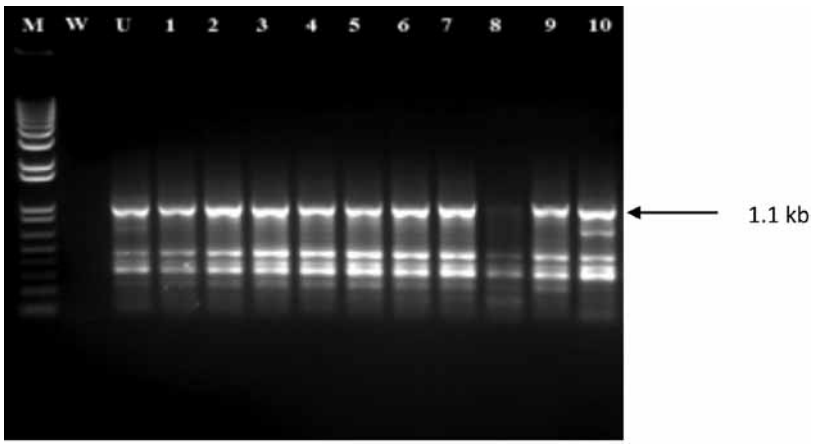


*Figure 1. Polymerase chain reaction (PCR) amplification of endogenous DNA sequence was examined by electrophoresis in a 1.2% agarose gel. The PCR product is shown with an arrow and the size was 1.1 kb. Lane M= 1 kb plus DNA ladder (Invitrogen, USA), W= water (negative control), U= untranformed oil palm and lane 1-10 = 10 different samples of transgenic oil palm [lane 1:TE 3-8(1), lane 2: TE 3-8(2), lane 3: TE 3-8(3), lane 4: TE 20-2(1), lane 5: TE 20-2(2), lane 6: TE 20-2(3), lane 7: TE 20-3(1), lane 8: TE 20-3(2), lane 9: TE 20-3(3) and lane 10: TE 17-2(2)].*

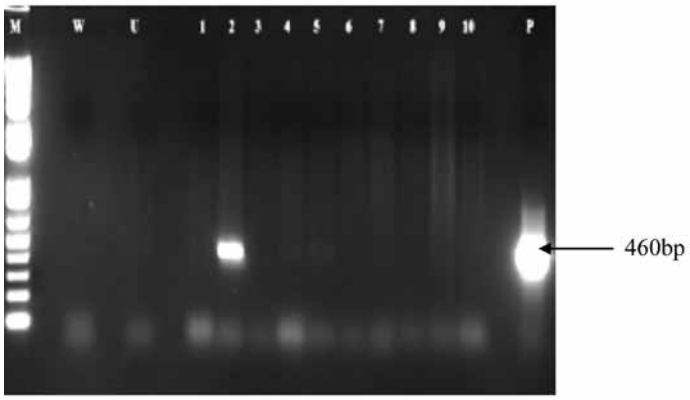


*Figure 2. Polymerase chain reaction (PCR) amplification of bar gene was examined by electrophoresis in a 1.2% agarose gel. The PCR product (460 bp) specific for bar gene is indicated by arrow. Lane M= 1 kb plus DNA ladder (Invitrogen, USA), W= water (negative control), U= untranformed oil palm, lane 1-10 = 10 different samples of transgenic oil palm [lane 1: TE 17-2(2); lane 2:TE 3-8(1), lane 3: TE 3-8(2), lane 4: TE 3-8(3), lane 5: TE 20-2(1), lane 6: TE 20-2(2), lane 7: TE 20-2(3), lane 8: TE 20-3(1), lane 8: TE 20-3(2) and lane 10: TE 20-3(3)) and P = plasmid (pAHC25)].*

faint bands demonstrating the presence of *bar* gene. The size of the amplified product corresponded to the size of the amplified product from the positive control (pAHC25).

In this study, the antisense approach was used to suppress the expression of PAT gene to reduce the production of palmitic acid and increase the oleic acid content. This antisense approach has also been successfully used to down regulate genes of interest in other plants such as Brassica (Knutzon *et al*., 1992), cottonseed (Liu *et al*., 2000), tomato (Liu *et al*., 2010) and tobacco (Duan *et al*., 2013). In order to determine the putative transgenic oil palms that carried the antisense PAT sequence, PCR analysis was carried out. Since the PAT gene is an endogenous gene, the positive transgenic samples were expected to amplify two bands with different but known sizes. The PCR products for antisense PAT and endogenous PAT gene were about ~900 bp and ~480 bp respectively. As expected, the non-template control did not show any band and the untransformed oil palm showed the amplification of only one product at ~480 bp corresponding to the endogenous PAT gene (*Figure 3*). From the results of PCR analysis, again one sample [TE 3-8(1)] showed a strong amplification of the ~900 bp band, while another few showed faint bands which revealed the present of antisense PAT sequence.

Some of the samples did not show the amplified bar gene and antisense PAT sequence. This could be due to the escape phenomenon. As shown by Mariana *et al*. (2002), around 30% of plants selected using Basta were escape events. This escape phenomenon was suggested to be due to low concentration of herbicide Basta during selection. The escapes could be reduced by increasing the concentration of herbicide Basta or prolonging the time needed during selection. Alternatively, the escape phenomenon could also be due to the temporal expression of bar gene which enabled the cells to survive under selection pressure (Subhash and Farida, 2012). It could also be due to the fact that


*Figure 3. Polymerase chain reaction (PCR) amplification of antisense PAT with a size of about ~900 bp and the endogenous thioesterase gene at ~480 bp. Lane M= 1 kb plus DNA ladder (Invitrogen, USA), W= water (negative control), U= untranformed oil palm, lane 1-10 = 10 different samples of transgenic oil palm and P = plasmid (pAT1). [Lane 1: TE 20-2(1);lane 2: TE 20-2(2), lane 3: TE 20-2(3), lane 4: TE 20-3(1), lane 5: TE 20-3(2), lane 6: TE 3-8(1), lane 7: TE 3-8(2), lane 8: TE 3-8(3), lane 8: TE 20-3(3) and lane 10: TE 17-2(1)].*

the transgene was not stably integrated into plants and subsequently lost during the regeneration process (Subhash and Farida, 2012). Besides that, the generated plant could be chimeric carrying transgenic and non-transgenic tissues causing it to survive even under rigorous selection (Berthomieu *et al*., 1994). In *Citrus*, very high frequencies of escapes and chimeras were reported about 90% of the regenerated lines (Domínguez *et al*., 2004).

**Identification of *bar* Gene and PAT Sequence**

For further confirmation, sequence analysis of PCR products was carried out to confirm that the inserted transgene was actually amplified during PCR analysis (Subhash and Farida, 2012; Fong, 2006). The sequence of PCR product was aligned with the original sequence of *bar* gene in the transformation vector to determine the similarity of

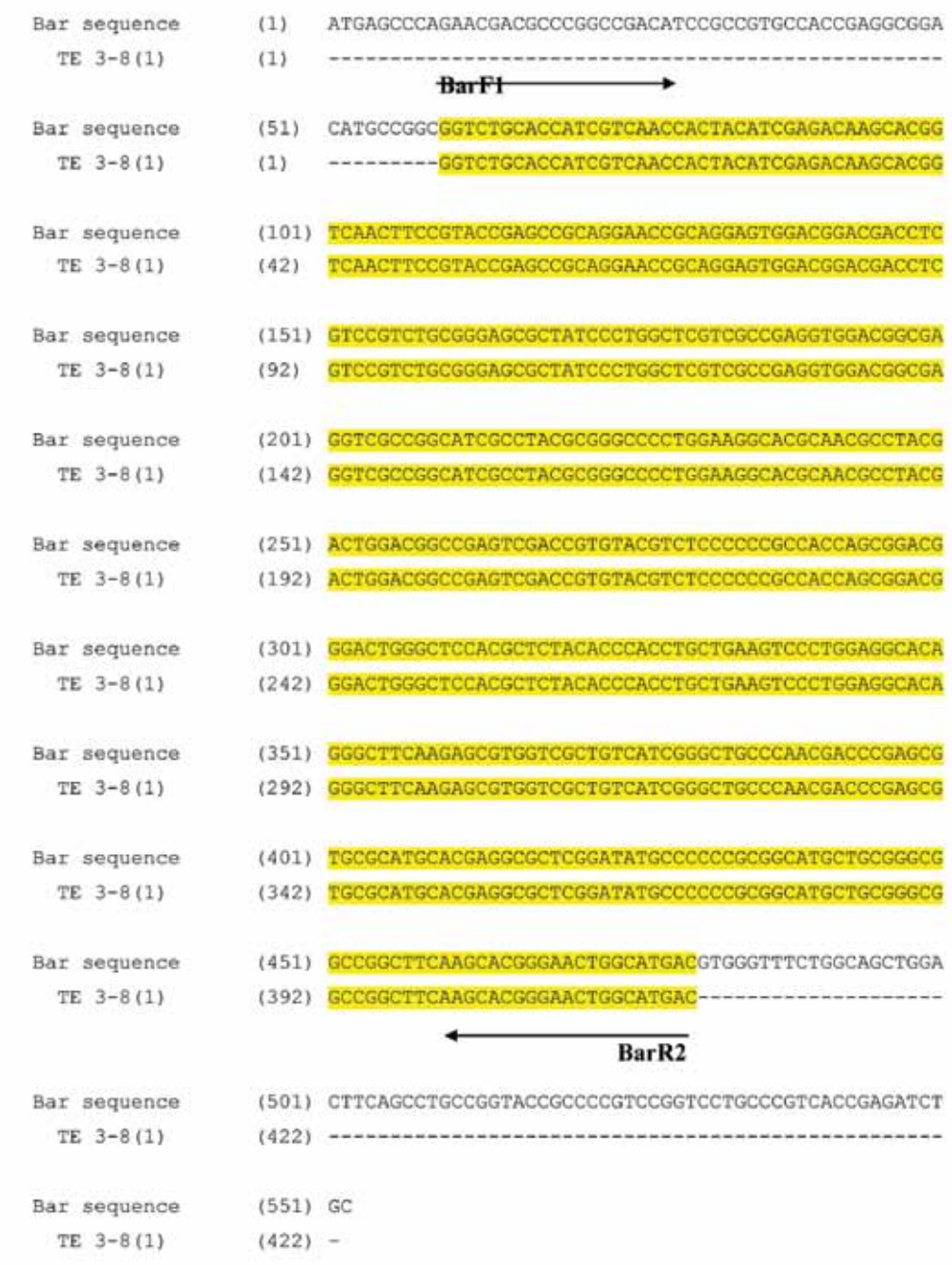


*Figure 4. The alignment of bar sequence with polymerase chain reaction (PCR) product of sample TE-3-8(1) using BarF1 and BarR2 showed 100% similarities. The arrows are the primers used during PCR amplification.*

both sequences. The amplified *bar* fragment from TE 3-8(1) was shown to have 100% similarity (*Figure 4*) to the original *bar* sequence in the vector. This result confirmed that the amplified product, which was presumably inserted into TE 3-8(1), was indeed the *bar* gene.

For PAT transgene sequence analysis, the 900 bp PCR product was shown to have 99.8% similarity to the original antisense PAT sequence (*Figure 5*). Two different nucleotides in the sequence from sample TE 3-8(1) were very likely due to error during PCR process. Furthermore, the multicycle nature of PCR

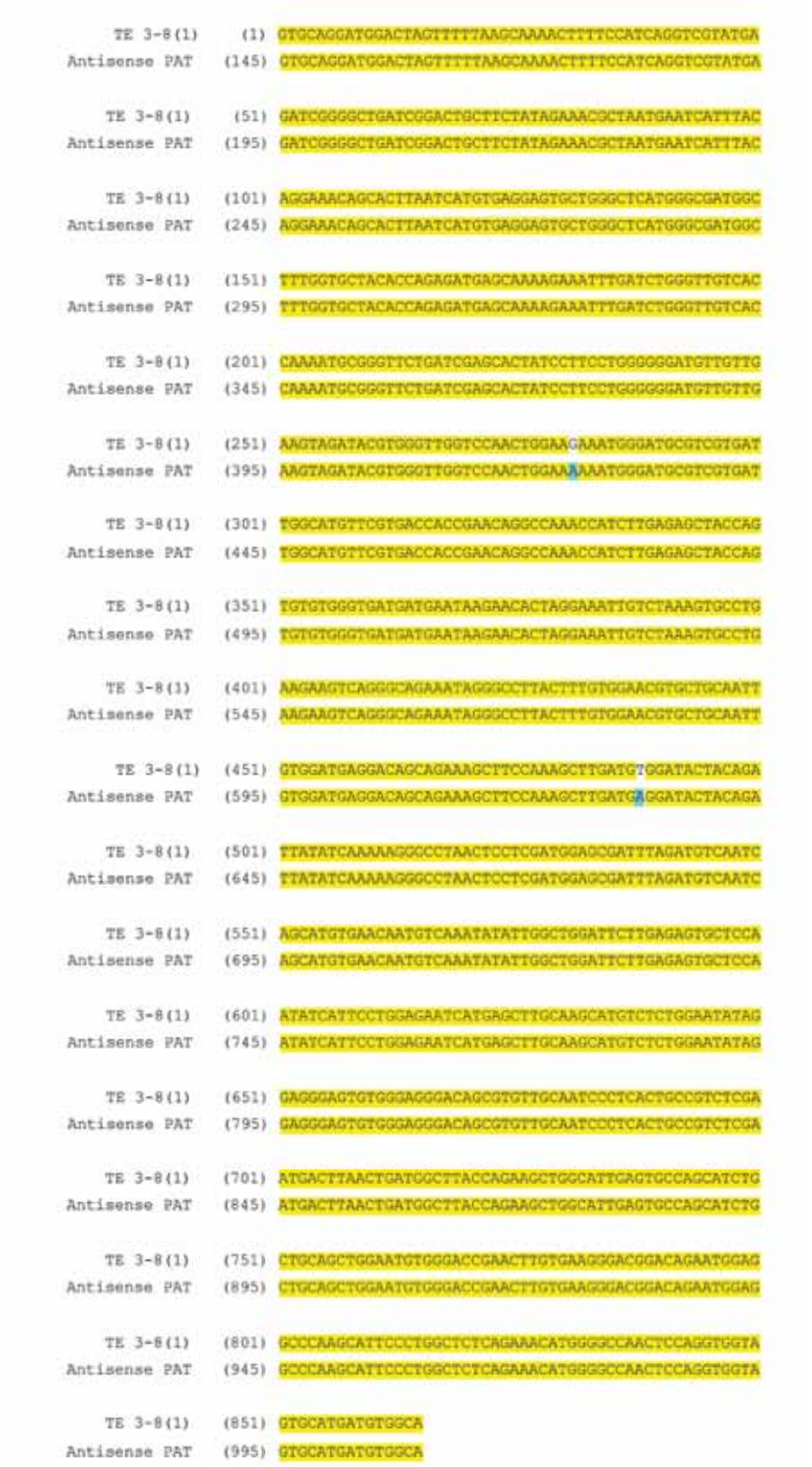


*Figure 5. The alignment of original antisense palmitoyl-ACP thioesterase (PAT) sequence with amplified product for sample TE 3-8(1). The two sequences were shown to share 99.8% similarities.*

allows errors to multiply and accumulate rapidly (Wagner and Alan, 1999). Since the sequence results obtained were just to verify the similarities with the original sequence, the error rate of less than 1% had no effect on the results. Thus, the amplified PCR product was confirmed to be antisense PAT.

## CONCLUSION

The presence of *bar* gene and antisense PAT sequence in some of the transgenic oil palm samples was proven by PCR analysis. The identity of the amplified sequences exactly matched the inserted gene as confirmed by DNA sequence analysis. Southern blot and RT-PCR analysis will be carried out to further confirm the integration and expression of transgenes, respectively. Consequently, gas chromatography analysis will also be carried out to determine the expected changes in the fatty acid composition.

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