

FUNCTIONAL ANALYSIS OF OIL PALM PALMITOYL-ACP THIOESTERASE (FatB) GENE VIA DOWN-REGULATION IN A MODEL PLANT: *Arabidopsis thaliana*

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ABSTRACT

Isolation of genes from the oil palm (*Elaeis guineensis*) has been routinely carried out to genetically alter the fatty acid content of palm oil to produce novel fatty acids and other metabolites. This study evaluated the function of an oil palm palmitoyl-ACP thioesterase (FatB) gene in the model plant *Arabidopsis thaliana*. *Arabidopsis thaliana* was transformed via the 'floral-dip' transformation method with either the gusA reporter gene (pCAMBIA 3301), an antisense palmitoyl-ACP thioesterase gene (pCB302-AT2), or the sense palmitoyl-ACP thioesterase gene (pCB302-AT1), together with the bar (Basta-resistant) selectable marker gene. Transformed seeds were selected by growing them on wet compost containing Basta. Basta-resistant plants were subjected to PCR and GUS analyses to prove the integration of the transgenes and their expression. Fatty acid composition of the transgenic seeds were analysed using gas chromatography. It was observed that the amount of palmitic acid (C16:0) in the sense transgenic seeds increased to 11.78 mol% compared to 8.91 mol% in the wild type control plants. Fatty acid analysis of the antisense transgenic seeds revealed that the amount of C16:0 in these lines decreased slightly from 8.91 mol% to 8.13 mol%. The results revealed the expected function of the oil palm palmitoyl-ACP thioesterase gene. Furthermore, this study also demonstrated the ability to test functionality of oil palm genes (from a monocotyledon) in a dicotyledonous plant, *Arabidopsis thaliana*.

Keywords: palmitoyl-ACP thioesterase (FatB), fatty acid modification, floral dip transformation, oil palm, *Arabidopsis thaliana*.

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INTRODUCTION

The oil palm (*Elaeis guineensis*) is the largest source of edible oils in the world, followed by the soyabean (Anon, 2007). Malaysia is the largest exporter of palm oil in the world. In 2009, Malaysia produced 17.56 million tonnes of palm oil. In that year, total

oil palm planted area in Malaysia was 4.69 million hectares. Among the options to sustain the palm oil industry are by increasing productivity (yield per unit area), increasing the quality of the oil, and producing higher value novel fatty acids, and these need to be carried out faster and more effectively than can be achieved by conventional breeding (Parveez, 1998). In order to genetically engineer the oil palm for increasing the quality of palm oil, isolation and characterization of useful genes are important. However, due to the long generation for the oil palm (25 years), initial evaluation of oil palm gene functions in a short generation model crop system, such as *Arabidopsis thaliana*, is essential.

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Plant genetic transformation developed more than 20 years ago was supported by the progress in whole plant regeneration via tissue culture techniques. Furthermore, the development in molecular biology techniques for isolating and expressing foreign marker or selectable marker genes in plant cells, and in improving various DNA delivery methods also aided the advancement of plant transformation. Various methods for transforming *Arabidopsis thaliana* have been developed, such as direct DNA uptake by protoplasts (Damm *et al.*, 1989) and co-cultivation of leaf or root explants with *Agrobacterium* (Valvekens *et al.*, 1988). Transforming the *Arabidopsis* plants directly (*in planta*) via seeds imbibition with *Agrobacterium* has also been made possible (Feldmann and Marks, 1987). These methods resulted in the recovery of transgenic progenies without the use of any tissue culture technique. Another *in planta* method was later developed by cutting young inflorescences and inoculating the wounded surfaces with *Agrobacterium* (Chang *et al.*, 1990), but the frequency of getting transgenic progenies is relatively low and inconsistent. Bechtold *et al.* (1993) later developed the vacuum infiltration method based on the assumption that T-DNA transfer takes place either at the end of gametogenesis or at the zygote stage. Adult plants were infiltrated with *Agrobacterium* at this reproductive stage. On average, 0.4% transformants were selected from the progenies of treated plants. Later, Clough and Bent (1998) developed a new method, the floral-dip, based on the same principle. They simplified the infiltration medium and substituted a surfactant (Silwet L-77) for vacuum infiltration, allowing penetration of the *Agrobacterium* into the plant tissues. The frequency of transformants obtained by this method is comparable to the infiltration method, *i.e.* about 0.5% of the progenies of the treated plants.

Palm oil has a unique fatty acid composition which is predominantly around 44% palmitic acid and 39% oleic acid. Studies on transgenic plants have demonstrated the possibility of shifting the profile of saturated fatty acids by the expression of an acyl-ACP thioesterase gene (Jones *et al.*, 1995). It is expected that down-regulating the activity of the above gene, either by the antisense or RNAi approach, could also result in fatty acid changes in oil-bearing crops. For example, down-regulating the oleoyl-CoA desaturase activity in soyabean resulted in an increase in oleic acid content from 21.5% to 78.9% as a result of a reduction in linoleic acid content from 55% to 3% (Broglie *et al.*, 1997). Similarly, oleic acid content in cottonseed oil was increased from 15% to 78% by down-regulating the oleoyl-CoA desaturase activity (Liu *et al.*, 2002). It was reported that the high activity of palmitoyl-ACP thioesterase in oil palm mesocarp may result in the accumulation of a high amount of palmitic acid (Sambanthamurthi and Oo, 1990). Chromatographic analysis of oil

palm thioesterases revealed that palmitoyl-ACP thioesterase and oleoyl-ACP thioesterase are two separate proteins (Abrizah, 1995). This observation is very important as the activity of palmitoyl-ACP thioesterase could be manipulated without any effect on oleoyl-ACP thioesterase activity. Therefore, reducing palmitic acid content by down-regulating the activity of palmitoyl-ACP thioesterase should not concurrently reduce the activity of oleoyl-ACP thioesterase which can reduce the oleic acid content. The current article will describe the evaluation of the function of an oil palm *palmitoyl-ACP thioesterase* gene in *Arabidopsis thaliana* using the floral-dip transformation approach.

MATERIALS AND METHODS

Transformation Vectors

In order to study the function of the oil palm *palmitoyl-ACP thioesterase* gene, the activity of the gene was separately down-regulated and up-regulated in *Arabidopsis thaliana* using the floral-dip transformation approach. The gene was previously cloned into pCB302.1 and pCB302.3 vectors (Xiang *et al.*, 1999). Two expression constructs were constructed, in which the thioesterase cDNA was placed in the sense or antisense orientation behind a CaMV 35S promoter to produce pCB302-AT1 and pCB302-AT2, respectively (Abrizah *et al.*, 2003). Both binary vectors used in this experiment contained the Basta (active ingredient: glufosinate ammonium) resistance gene, *bar*, which encodes phosphinothricin acetyltransferase (de Block *et al.*, 1987). Concurrently, to evaluate transformation success, transformation with a binary plasmid pCAMBIA 3301 (Roberts *et al.*, 1997), carrying *bar* and *gusA* genes under the control of the CaMV35S promoter, was carried out.

Agrobacterium tumefaciens Transformation

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.*, 1983) was transformed by the following: plasmid pCAMBIA 3301, pCB302-AT1 and pCB302-AT2 via electroporation (Bio-Rad GENE PULSER). The *Agrobacterium* were grown overnight at 27°C on 2XYT medium (1% NaCl, 1% tryptone and 0.5% yeast extract; pH 7). Two tubes containing 1.5 ml of bacteria were aliquoted and centrifuged. The resulting pellets were then re-suspended in 0.5 ml ice-cold 10% glycerol and re-centrifuged. These pellets were again resuspended in 20 μ l 10% glycerol and the contents of both tubes were combined. pCAMBIA 3301 DNA (1 μ l) was added into 40 μ l cells of *Agrobacterium* and left on ice for 2 min. The DNA-bacteria were pulsed on an ice-cold 0.2 cm Bio-Rad cuvette. The Gene Pulser was set at 25 μ F capacitance and 2.5 KV charge, with the pulse controller at 400 Ω resistance. Immediately after the

pulse, 1 ml of SOC broth (containing glucose, 20 mM; magnesium chloride, 10 mM; magnesium sulphate, 10 mM potassium chloride, 2.5 mM; sodium chloride, 10 mM; tryptone, 20 g litre⁻¹ and yeast extract, 5 g litre⁻¹; pH 7) was added into each tube. The mixture was incubated at 27°C for 4-6 hr before plating on 2XYT agar containing 10 g/ml chloramphenicol and 50 g/ml 2XYT. Plates were cultured overnight and 10 resistant colonies were selected randomly and inoculated into 10 ml 2XYT medium containing the above-mentioned antibiotics. The transformed cells were confirmed after isolation and digestion of the plasmids.

Small-scale Plasmid Isolation

Ten microlitres of each overnight culture were inoculated into 10 ml of 2XYT (10C50K) medium. The overnight culture was transferred to microfuge tubes, and the bacteria cells were pelleted by centrifuging (4000 g, 5 min and 4°C). Isolation was carried out using the alkaline lysis method according to Sambrook *et al.* (1989).

The pellet was resuspended in 200 µl of ice-cold solution I (25 mM Tris-HCl, 10 mM Na₂-EDTA, 50 mM glucose at pH 8.0 {5 mg ml⁻¹ lysozyme}) and the mixture was kept at room temperature (RT) for 5 min. Three hundred microlitres of solution II (0.2 M NaOH, 1% sodium dodecyl sulphate) were added, mixed by gently inverting the tube and incubated on ice for 5 min followed by adding 250 µl of solution III (5 M KAc), and mixing vigorously before further incubation on ice for 5 min. The lysate was spun down (15 000 rpm, 15 min and 4°C) and the supernatant was transferred into a new microfuge tube. Six hundred microlitres of phenol: chloroform (1:1) were added to each tube, mixed for 2 min and spun (15 000 g, 5 min and 4°C). The aqueous phase was transferred to 600 µl of chloroform, mixed for 2 min and centrifuged (15 000 rpm, 5 min and 4°C). Again, the aqueous phase was transferred to a new tube, and one volume of isopropanol and 100 µl of 10 M NH₄Ac were added, mixed gently and left at RT for 15 min. DNA was pelleted (15 000 rpm, 15 min and RT), rinsed with 70% ethanol and dried at RT for 30 min.

The final pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0), gently mixed, and treated with 75 µl RNase (5 mg ml⁻¹, 15 min and 37°C). DNA concentration was determined by measuring optical density (O.D.) A_{260}/A_{280} and analysed on an agarose gel.

Arabidopsis thaliana Plant Growth before Infiltration

Arabidopsis seeds (ecotype Columbia) were harvested and sown in wet compost in plastic pots. The pots were kept at 4°C for stratification.

The pots were later placed in a Conviron growth chamber (Model TC30) set at 16 hr day photoperiod, a temperature ranging from 20°C to 25°C, with additional artificial light (105:E/m 2/s), and sub-irrigated by maintaining a layer of tap water under the pots, until germination. Plants to be used in transformation must be as vigorous as possible. The optimal stage for floral-dipping is when the first siliques are formed and the secondary floral stems start to appear in the plants.

Arabidopsis thaliana Floral-dip Transformation with *Agrobacterium tumefaciens* LBA4404

Arabidopsis were transformed according to the method developed by Clough and Bent (1998). *Agrobacterium tumefaciens* strain LBA4404 carrying the binary plasmids pCAMBIA 3301, pCB302-AT1 and pCB302-AT2 was used in all the experiments for which data are shown. Unless otherwise noted, the bacteria were grown to a stationary phase in liquid culture at 25°C-28°C, 250-280 rpm on sterile 2XYT medium. The cultures were typically started from a 1:100 dilution of smaller overnight cultures, and grown for roughly 18-24 hr. The cells were harvested by centrifugation for 20 min at RT at 5500 g, and then resuspended in the floral-dip medium to a final OD₅₀₀ of approximately 0.80 prior to use. The floral dip inoculation medium contained 5.0% sucrose and 0.05% Silwet L-77. The inoculum was poured into a beaker, and the plants were inverted into this suspension so that all above-ground tissues were submerged. After 3-5 s of gentle agitation, the dipped plants were removed from the beaker, placed in a plastic tray and covered with a tall clear-plastic dome to maintain humidity. The plants were left in a low light or in dark overnight, and returned to the growth chamber the next day. The domes were removed 12-24 hr after treatment, and the plants were allowed to grow for a further three to five weeks until the siliques had turned brown and dry. The seeds were harvested by gently pulling the grouped inflorescences through the fingers (to dislodge them) over a piece of clean paper, and stored in a microcentrifuge tube prior to screening.

Screening of Transformants

The harvested seeds were sown on wet compost in plastic pots. The compost was moistened with water containing *Basta* (final concentration: 10 ppm). Selection with herbicide is more efficient (to avoid escape plants) as compared to selection with antibiotics. The pots were kept at 4°C for stratification, and later placed in the Conviron growth chamber (Model TC30) set at 16 hr day photoperiod, a temperature of 20°C to 25°C, with additional artificial light (105:E/m 2/s). Sub-irrigation by maintaining a layer of tap water

containing *Basta* under the pots was carried out until germination. Once the plants produced normal green cotyledons and the first two leaves, they were sprayed with *Basta* solution (final concentration: 50 ppm). The plants were sprayed again with the same *Basta* solution after two weeks. The resistant plantlets were transferred into individual pots when they were sufficiently developed (four- to five-leaf stage) to prevent cross-pollination and/or seed contamination. The surviving plants were left to grow until the siliques turned brown and dry. The T2 seeds were harvested as described previously, and stored in a microcentrifuge tube.

GUS Assay on Transgenic *Arabidopsis thaliana* Leaves

The T2 seeds were sown on *Basta*-containing compost and grown as described earlier until plantlets developed. The leaves were harvested for GUS assay and PCR analysis. The GUS assay buffer consisting of 0.1 M NaPO₄ buffer (pH 7.0), 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.01 M EDTA, 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid (dissolved in dimethyl formamide at 50 mg ml⁻¹) and 1 μl ml⁻¹ Triton X-100 (Klein *et al.*, 1988) together with 20% v/v methanol was filter-sterilized and stored at -20°C in the dark. Leaves from the T2 and untransformed plants (control) were stained overnight (20 hr) with the GUS buffer at 37°C, and the blue pigmentation was scored optically using a Nikon SMZ-U stereoscopic zoom microscope and photographed with a Nikon UFX-DX system.

Preparation of Total DNA from Regenerated Plants

Genomic DNA was isolated from the leaves of the transgenic plants, and one untransformed plant was used as the negative control. Total DNA was isolated using the method of Ellis (1993). Leaf pieces (0.5-1.0 g) were placed in a mortar and liquid nitrogen was added. The frozen leaves were ground with a pestle and 8 ml EB2 buffer (500 mM NaCl, 100 mM Tris-Cl, pH 8.0 and 50 mM EDTA, pH 8.0) were added. After further grinding, 400 μl 20% w/v SDS were added and the mixture was ground again. Eight millilitres of phenol mix (1:1 phenol:chloroform) were then added, mixed by gentle inversions and centrifuged (15 000 g, 5 min, RT). The aqueous phase was transferred into a new centrifuge tube and mixed with 16 ml of absolute ethanol. DNA was removed by centrifugation (15 000 g, 15 min, RT). The pellet was washed with 70% ethanol and dissolved in 300 μl TE buffer.

Polymerase Chain Reaction (PCR) Analysis

Primers, BARF1 and BARR1, were used to amplify the *bar* gene resulting in a band size of about

550 bp. Amplification of the *bar* gene was carried out using a modified touch-down PCR procedure (Parveez, 1998). PCR reactions were carried out in the following mixture: DNA (50 ng total DNA or 5 ng plasmid DNA) + reaction buffer (containing 50 mM KCl and 10 mM Tris-Cl, pH 8.3) + dNTPs (200 μM each) + 1.5 mM MgCl₂ + primers and taq DNA polymerase. The touch-down procedure was as follows: hot start at 95°C (300 s), denaturation at 94°C (45 s), annealation at 70°C (45 s; -0.5°C per cycle for 10 cycles) and elongation at 72°C (60 s), followed by 24 cycles of 94°C (45 s), 65°C (45 s) and 72°C (60 s) and finally at 72°C for 120 s. Amplified DNA fragments were checked by electrophoresis on a 1.4% agarose gel in 1 X TBE buffer.

Fatty Acid Analysis of Transgenic *Arabidopsis thaliana* Seeds Using Gas Chromatography (GC)

Seeds (0.1 g) from the transformed and untransformed control plants were ground using a mortar and pestle. Methanol (4 ml) was added twice, and each sample was transferred into 100 ml conical flasks with stoppers. Chloroform (16 ml) was added and incubated with shaking (150 g for 2 hr). The mixture was poured into a funnel lined with Whatman No. 1 filter paper, and the filtrate was collected into a separating funnel. The NaCl (0.9%) was later added to the solution and was briefly stirred, and the mixture was left standing overnight. It was then drained into a glass vial and evaporated off using a rotary evaporator set at 60°C and rotating at 30-35 rpm.

Preparation of Fatty Acid Methyl Esters (FAMES)

Hexane (1 ml) was added to the lipid extract in the flask and vortexed for 1 min. The extract was transferred into a test tube and 100 μl sodium methylate were added before the mixture was vortexed for another 1 min. The mixture was then centrifuged at 2000 rpm for 2 min and left to stand for 5 min. It was dried under nitrogen gas, followed by reconstitution with 1 ml hexane.

Instrumentation

GC analyses were performed on an Agilent GC6890N (Agilent, USA) equipped with a split/splitless injector at 150°C. FAMES 1:5 v/v in hexane solution (1 μl) were placed into the GC. An Agilent autosampler, AOL series (Agilent 7683 Series), and a flame ionization detector (FID) at 150°C were used (H₂ flow 40 ml min⁻¹, air flow 350 ml min⁻¹ and makeup He 45 ml min⁻¹). Data acquisition was performed by MSD and MSD Data Analysis software (Agilent, USA). A Supelco column, SP 2380 (0.25 mm × 30 m × 0.20 μm film), was used for the analyses. The temperature programme was as follows: 50°C initial

temperature for 2 min, final temperature 250°C, ramp rate 4°C/min for 10 min. Helium was used as a carrier gas (1 ml min⁻¹). Peaks on the chromatogram were identified based on standard FAMES. Peaks are integrated and the percentage of peak area/total area represents directly the percentage of fatty acid (Christie, 1989).

RESULTS AND DISCUSSION

Agrobacterium tumefaciens Transformation

The cells of transformed *Agrobacterium tumefaciens* strain LBA4404 with three different plasmids, pCAMBIA 3301, pCB302-AT1 and pCB302-AT2, were selected on 2XYT medium. Ten resistant colonies were selected randomly and inoculated into 10 ml 2XYT medium containing chloramphenicol. Plasmid DNA was isolated from the overnight grown cultures using the alkaline lysis miniprep method. The plasmid DNA was digested with appropriate restriction enzymes and confirmed by agarose gel electrophoresis. All the digested plasmids showed the expected band numbers and sizes. The confirmed plasmids were later used to transform *Arabidopsis thaliana*.

Arabidopsis thaliana Transformation via Floral-dip Method

Agrobacterium tumefaciens strain LBA4404 carrying the pCAMBIA 3301, pCB302-ATI or pCB302-AT2 plasmids were grown overnight in 2XYT (10C50K) medium, and harvested by centrifugation. The *Agrobacterium* cells were resuspended in the floral-dip medium prior to floral dipping. *Arabidopsis thaliana* plants, at the right stages, were taken out from the growth chamber and used for the floral-dip treatment. After dipping, the plants were grown in the growth chamber until maturity. At the early stage of post-transformation, maintaining humidity was crucial for successful transformation. The treated plants showed normal growth and successfully produced seeds. No plant showed any negative effect or died after the floral dipping treatment. The seeds (T1) were collected from the treated plants when the siliques were brown and dry. At this stage, it was important to ensure that there was no accidental mixing of seeds from the different treated plants to be used for determining transformation efficiency. The seeds were collected and stored in microcentrifuge tubes until used for selection.

Selection of Transformed *Arabidopsis thaliana*

Seeds from the floral-dip treated plants were grown on wet compost until two-leaf stage. The seeds were dispersed in a 0.1% agarose solution to ensure they were evenly distributed. Even

distribution of the seeds was important during selection. If there were clusters of plants, there was a possibility that some of the untransformed plants might become escapes and survive selection. At this two-leaf stage, Basta solution was sprayed on the plants in the growth chamber. After 48-72 hr, the non-transformed plants started browning while the putative transformants remained green. After one week, the transformed plants grew normally but produced few leaves compared to untransformed plants without Basta, whereas the untransformed plants became brown and remained at the two-leaf stage (Figure 1). As shown in Figure 1, the putative transformed plants could be easily distinguished from the untransformed plants due to their distinct leaf size and colour. Selection at the right time and at an appropriate concentration of Basta was important to ensure that the selection pressure was not too high to kill the transformed plants. After two weeks, the transformed plants were sprayed again with Basta to confirm their resistance. Almost all the transformed plants that survived the first Basta selection survived the second selection and grew to maturity. The second stage of selection was important to ensure the elimination of escapes. The T2 seeds were collected separately from each of the Basta-resistant plants when the siliques were brown and dry. Selection using Basta spraying is very efficient for high-throughput selection [such as the sand-bed/Basta approach by Bouchez *et al.* (1993)],

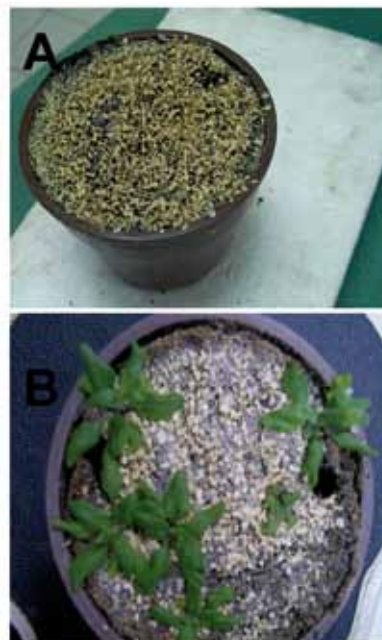


Figure 1. Screening of *Arabidopsis thaliana* by Basta spraying. A) Control - untransformed plants became brown and died after seven days. B) Transgenic plants (green and few leaves stage) surrounded by untransformed plants (which became brown and died after seven days) from treated plant seeds.

allowing for the efficient generation of thousands of independent *Arabidopsis* transformants (Clough and Bent, 1998).

The transformation efficiency of transgenic plants was calculated based on the number of transformed plants (Basta-resistant and proven by molecular and GUS-staining analyses) obtained over the total number of T1 seeds harvested and germinated. The number of all dead plants was calculated to determine the total number of seeds harvested. In this study, the transformation rate was about 0.4%. The result was the mean of data from four different transformation experiments. The transformation efficiency is about the same as that reported by the inventors of the technique (Clough and Bent, 1998), *i.e.* 0.5%. This efficiency was equivalent to, or even at some stage better than, the vacuum infiltration method. There are other factors which could help increase the transformation efficiency as described by Clough and Bent (1998) and Desfeux *et al.* (2000).

The floral-dip method, in combination with Basta selection, has proven to be an efficient method, and eliminates the need for vacuum infiltration using Silwet L-77 as a surfactant. Furthermore, the floral-dip method is more convenient than the vacuum infiltration method because it allows for the large-scale treatment of *Arabidopsis thaliana* plants in growth chambers without the need for a vacuum apparatus, or for uprooting and re-planting of the plants (Clough and Bent, 1998). Silwet was chosen by the inventors (Clough and Bent, 1998) because it was shown to be more effective in reducing surface tension compared to most other surfactants. Furthermore, it can be used at low concentrations which reduce phytotoxicity and enhance the entry of bacteria into relatively inaccessible plant tissues (Whalen *et al.*, 1991). Clough and Bent (1998) have determined three main requirements for successful transformation of *Arabidopsis thaliana* via the floral-dip method: 1) correct plant development stage (maximum number of unopened floral bud clusters), 2) sugar concentration, and 3) surfactant type.

The T2 seeds were harvested on Basta-wet compost and grown until plantlets developed. The plantlets produced were later subjected to DNA isolation for PCR analysis and GUS assay.

PCR Analysis on the Transgenic Plants

Genomic DNA was isolated from the leaves of the transgenic plants and from one untransformed plant acting as the negative control. All the transformants were derived from plants transformed with plasmids pCAMBIA 3301, pCB302-AT1 or pCB302-AT2, binary vectors carrying the *bar* gene and driven by CaMV 35S promoters, and selected on Basta.

The amplification of the *bar* gene was used for verification of transformation. The transformants, and the positive controls, showed amplification of the *bar* gene (Figure 2). No bands were amplified from the untransformed control plant. PCR analysis demonstrated that all the transformed Basta-resistant plants contained the *bar* gene used for selection (about 550 bp). However, amplification of the transgenes using PCR is not definitive evidence of stable integration of transgenes into a host genome. As such, Southern blot hybridization analysis, using high molecular weight DNA of hybrids between the transgene and host genomic DNA, was carried out (data not shown).

GUS Assay of the Transformed Plants

The leaves from both transformed (T2) and untransformed plants were collected, soaked in GUS assay buffer and incubated at 37°C for 20 hr. The leaves from transgenic plants started to show GUS gene expression as early as 5 min after soaking in GUS assay buffer. After 20 hr, the leaves were

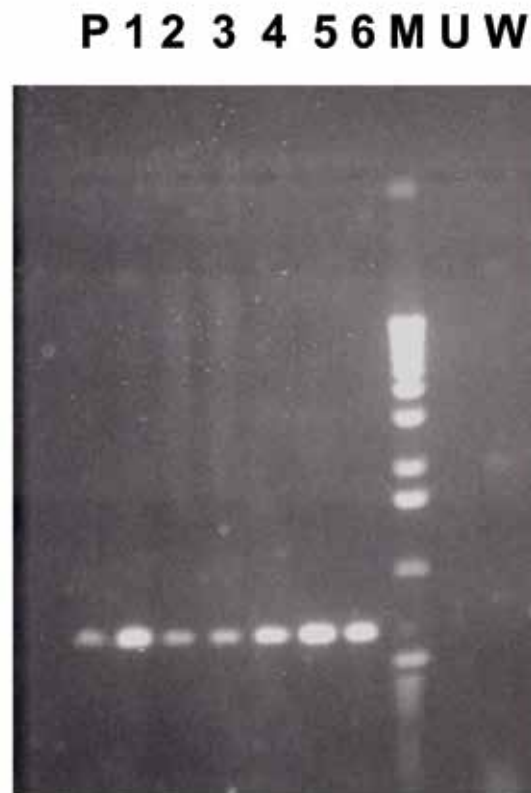


Figure 2. PCR analysis of transgenic *Arabidopsis thaliana*. A PCR band for the *bar* gene was detected from each of the transformed plants but not from the untransformed plant and water. Lane M = 1Kb DNA marker (BRL), P = transforming plasmid: pCAMBIA 3301, U = untransformed plant, W = water control and 1-6 = transformed plants.

scored optically using a Nikon SMZ-U stereoscopic zoom microscope and photographed with a Nikon UFX-DX system. The leaves from transgenic plants showed very strong *gusA* gene expression, *i.e.* dark blue pigmentation, as compared to the leaves from the control plant which showed no blue pigmentation (Figure 3). This showed that the transgenic plants which survived Basta selection also carried and expressed the *gusA* gene, which was transformed via the *Agrobacterium* floral-dip method. This also showed that the transgenic plants did not only carry the *bar* gene used for selection but also carried and expressed the *gusA* gene which was also present on the same pCAMBIA 3301 plasmid.

Fatty Acid Analysis of Transgenic *Arabidopsis thaliana* Seeds

Transgenic *Arabidopsis* plants resulting from transformation using pCB302-AT1 and pCB302-AT2 plasmids were further grown until the T3 generation was obtained. The T3 generation was produced to obtain homozygous lines from the transgenic plants, carrying the antisense or sense genes, based on Basta herbicide selection. As these transgenic plants had been incorporated with the *palmitoyl-ACP*

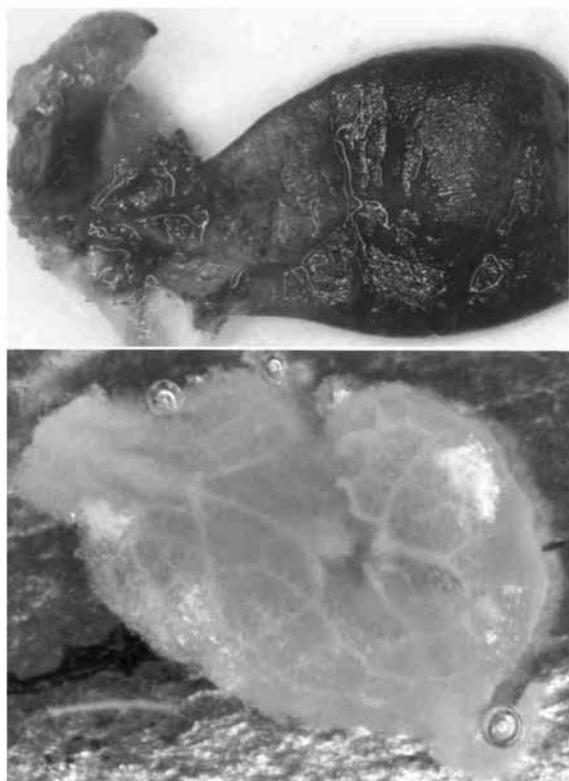


Figure 3. *GUS* gene expression in *Arabidopsis thaliana* leaves after transformation with plasmid pCAMBIA 3301. Leaf from Basta-resistant transformed plant (top) and leaf from untransformed control plant (bottom).

thioesterase gene, fatty acid composition of the resulting seeds was evaluated to determine the functionality of the gene. Total fatty acids were extracted from mature seeds of T3 transgenic *Arabidopsis* and from the wild type (WT). Total fatty acids were analysed as FAMES using a modified extraction method. In all cases, a minimum number of three runs were carried out, with three replications, and quantified against known standards (Table 1). All results were expressed as mole percentages (mol%) of total fatty acids, with a maximum standard error (SE) of 3% of the mean.

From the fatty acid analysis, it was found that the amount of C16:0 (palmitic acid) in the seeds of transgenic *Arabidopsis* transformed with pCB302-AT1 sense plasmid increased by 2.87 mol% from 8.91 mol% in the wild type control plants up to 11.78 mol% (Figure 4). It was also revealed that besides C16:0, the contents of C18:2, C18:3 and C20:0 also increased while the amounts of C18:0 and C18:1 were reduced. For the transgenic *Arabidopsis* transformed with pCB302-AT2 antisense plasmid, the amount of C16:0 was reduced by 0.78 mol% from the wild type. It was also observed that besides the decrease in C16:0, the contents of C18:0, C18:1 and C18:3 also decreased while the amounts of C18:2 and C20:0 increased.

The results presented here demonstrate that the expression of the oil palm *palmitoyl-ACP thioesterase* gene in transgenic *Arabidopsis thaliana* plants transformed by the pCB302-AT1 plasmid resulted in an accumulation of C16:0 in the mature seeds (Figure 4). It could also be seen that there was a clear correlation between the expression levels (gene activity) of the oil palm thioesterase gene and the levels of palmitic acid, the product of *FatB* thioesterase activity. The reduction and increase in palmitic acid in the antisense and sense constructs, respectively, demonstrate that, for this particular fatty acid, the oil palm gene functions as expected in *Arabidopsis*. It was also observed that besides C16:0, the oil palm *palmitoyl-ACP thioesterase* gene also affected other fatty acids. The differences observed could be due to the homology of the oil palm gene with other fatty acid genes in *Arabidopsis* and/or oil palm thioesterase may behave differently in *Arabidopsis thaliana*. Most importantly, the results showed that the oil palm *palmitoyl-ACP thioesterase* gene was functional in *Arabidopsis thaliana*.

The transgenic plants were normal in phenotype, similar to the other non-transformed *Arabidopsis* plants. This demonstrated that the changes in fatty acid content did not affect the growth and development of the transgenic plants. The fertility of the transgenic plants was not affected as T3 generation could easily be obtained.

Fatty acid thioesterases are divided into two types, namely, FatA and FatB. FatA is predominantly

TABLE 1. TOTAL FATTY ACID COMPOSITIONS OF MATURE SEEDS OF EITHER WILD TYPE (WT) OR HOMOZYGOUS LINES (pCB302-AT1 and pCB302-AT2) EXPRESSING THE OIL PALM FatB THIOESTERASE

| Fatty acid | C16:0 | C18:0 | C18:1 | C18:2 | C18:3 | C20:0 | Others |
|-------------|-------|-------|-------|-------|-------|-------|--------|
| WT | 8.91 | 19.15 | 28.84 | 13.28 | 19.59 | 2.19 | 9.49 |
| pCB-302-AT1 | 11.78 | 18.41 | 27.34 | 17.21 | 21.64 | 5.09 | 3.89 |
| pCB-302-AT2 | 8.85 | 15.31 | 7.69 | 17.07 | 15.21 | 10.51 | 34.59 |

Note: Values are means of triplicate measurement.

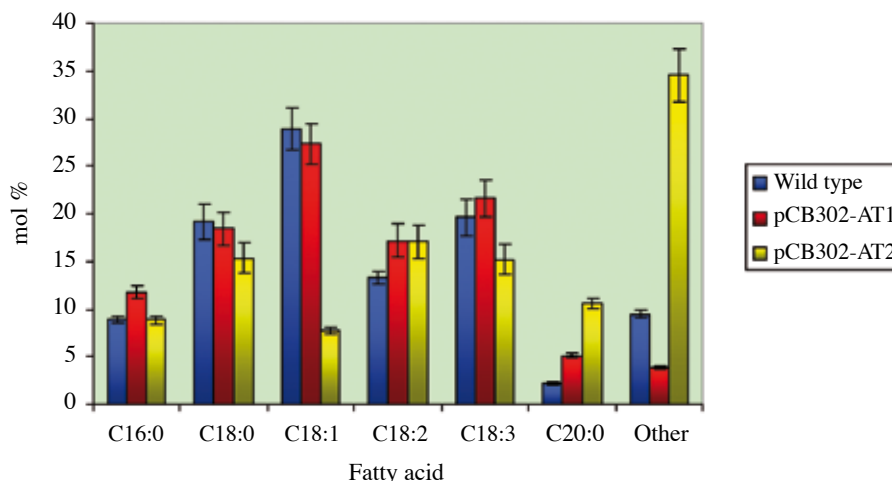


Figure 4. Fatty acid composition of *Arabidopsis thaliana* var. *Colombia* of the wild type and of the transgenics of pCB302-AT1 and pCB302-AT2.

involved in hydrolyzing C18:1-ACP (oleoyl-ACP), and C18:1 is the intermediate precursor of most fatty acids found in phospholipids and triglycerides produced in eukaryotic systems (Gibson *et al.*, 1994). It is also highly conserved in almost all higher plants. FatB, on the other hand, is highly variable as it shows preference to a wider range of fatty acids, from C14:0 to C18:0. For example FatB1 in California bay shows the strongest preference for C12:0-ACP and a modest preference for C14:0-ACP (Voelker and Davies, 1994). However, FatB1 in *Arabidopsis* and *Cuphea hookeriana* has preference for C16:0-ACP (Jones *et al.*, 1995). Using this knowledge, strategies to change the fatty acid composition of a given plant to accumulate a specific type of fatty acid could be drawn up. Studies on fatty acid biosynthesis in developing California bay seeds established that accumulation of medium-chain fatty acids was possible via genetic engineering, by the manipulation of a lauroyl-ACP thioesterase (Davies *et al.*, 1991). One of the most successful examples of modifying plant oil composition has been the production of high laurate canola oil using tandem gene constructs and creating plants with multiple copies of the lauroyl-ACP thioesterase gene (Voelker *et al.*, 1996).

The expression pattern of acyl-ACP thioesterase from *Arabidopsis* (*AtFATB1*) and transgenic plants with variable expression of this gene have also been

reported (Dörmann *et al.*, 2000). The expression of the *AtFATB1* cDNA under the control of the rapeseed seed-specific promoter (napin) resulted in an accumulation of high amounts of palmitate in the *Arabidopsis* seeds (an increase from 10.0 mol% in the wild-type up to 38.6 mol% in the transgenic seeds). A similar observation was reported when a C16:0-ACP specific thioesterase cDNA (*ChFATB1*) isolated from the medium-chain fatty acid accumulating species *Cuphea hookeriana* was expressed in canola seeds (Jones *et al.*, 1995). The palmitic acid level was increased from about 7 mol% to 13 or 27 mol%. Recently, the same observation was made when a *FATB1* gene from the woody oil plant *Jatropha curcas* L. was over-expressed in *Arabidopsis*, with the resulting transgenic plants demonstrating increased levels of saturated fatty acids, especially palmitate (C16:0), and simultaneously reduced levels of unsaturated fatty acids were observed (Wu *et al.*, 2009).

In this study, it was demonstrated that the antisense expression of the oil palm FatB resulted in a reduction in palmitic acid content. A similar observation was made when the *Arabidopsis* FatB1 was down-regulated. However, the antisense expression of the *AtFATB1* cDNA under the control of the CaMV 35S promoter resulted in a reduction of the C16:0 content in its seeds and flowers, but with

no changes to the leaf fatty acids (Dörmann *et al.*, 2000). It is expected that if the antisense FatB1 was controlled by the rapeseed seed-specific promoter (napin), reduction of palmitic acid could only be detected in the seeds as demonstrated by the sense over-expression.

Another interesting observation was that when the oil palm FatB was over-expressed or down-regulated in *Arabidopsis*, the respective changes in fatty acid content were not only limited to palmitic acid, but also occurred in the other fatty acids. This may be due to the expression of a gene from a different plant source. A similar observation was also made when the *Arabidopsis* FatB1 was expressed in canola seeds (Jones *et al.*, 1995). Besides palmitic acid, myristic (C14:0), stearic (C18:0) and arachidic (C20:0) acids were also increased. When a FatB1 gene from *Cuphea wrightii* was over-expressed in *Arabidopsis*, the content of lauric (C12:0), myristic and palmitic acids were increased, with a major increase observed for myristic (Leonard *et al.*, 1997). Similarly when the *Arabidopsis* FatB1 was over-expressed in its seeds (Dörmann *et al.*, 2000), besides palmitic acid, myristic (C14:0) and stearic (C18:0) acids were also increased.

CONCLUSION

Successful transformation of *Arabidopsis thaliana* was demonstrated using the floral-dip transformation method. The transformants were selected by spraying Basta, and confirmed by PCR analysis and GUS staining. Some transgenic plants transformed with the oil palm palmitoyl-ACP thioesterase gene were also produced and analysed for fatty acid composition at the T3 generation. It was demonstrated that transgenic *Arabidopsis thaliana* plants expressing the oil palm sense transgene showed increased levels of palmitic acid, while the plants transformed with the antisense gene demonstrated a reduction in the palmitic acid. This showed that there was a positive correlation between the expression levels (gene activity) of the oil palm palmitoyl-ACP thioesterase gene and the levels of palmitic acid. These results also showed the functionality of the oil palm thioesterase gene in transgenic *Arabidopsis* plants. Finally, the experiments described above demonstrated that it is feasible to manipulate seed oil composition via expression of the thioesterase transgenes. It is anticipated that similar results will be obtained in oil palm, both in terms of sense over-expression and antisense reduction, using the constructs generated in this work.

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