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# Cloning and Sequencing Analyses of FATB Gene Family from Coconut (*Cocos nucifera*)

**Zaw Ko Latt[1,2]*, Yaodong Yang[2], and Jing Li[2]**

*[1]Biotechnology Research Department, Ministry of Education, Myanmar*
*[2]Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, China*

*Corresponding author
ZawKoLatt, Biotechnology Research Department, Ministry of Education, Kyaukse District, Mandalay Region, Myanmar, Tel: 95-9-973084176; Fax: 95-66-50622; Email: zawkolatt83@gmail.com

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**Abstract**

The coding region of CnFatB1, CnFatB2, and CnFatB3 of coconut was cloned in *E.coli* DH5α by heat shock method using pMD18-T. After identifying validated positive clones, FatB gene family was sequenced. The nucleotide sequence of CnFatB1 was related to *C.nucifera* acyl-ACP thioesterase FatB1 with a nodal value of 99, CnFatB2 showed that was related to C. *nucifera* acyl-ACP thioesterase FatB2 with a nodal value of 97 and CnFat3 was related to C. *nucifera* acyl-ACP thioesterase FatB3 with 98 for a nodal value respectively. Expressed vector was constructed by ligating CnFatB1, CnFatB2 and CnFatB3 to linearized pCAMBIA 1302 and transformed into *E.coli* by heat shock. Moreover, expression vector was also transformed into *Agrobacterium tumefaciens* GV3103. Potential colonies harboring FatB genes were screened by colony PCR, and confirmed by using expression vector as template for PCR and by enzyme digestion using BglII and BstEII. All results showed that CnFatB1, CnFatB2 and CnFatB3 were in expected size.

## INTRODUCTION

Coconut is one of the world's most versatile economically important tropical crops and is well known for its commercial and industrial applications in tropical and subtropical areas, such as in food and beverages and as a source of wood and handicrafts [1].

Oil extracted from coconut pulp is widely applied in cooking, soaps and cosmetics. Coconut is one of the few plants that store medium chain-length fatty acids (MCFAs) as the major portion of their energy reserves in the endosperm of seeds. In developed coconut fruit, more than 83.92% of the oil consists of MCFAs and long-chain fatty acids (C12:0, C14:0, C16:0 and C18:0), the majority of which is lauric acid (C12:0) ranging from 47.48% to 50.5% [2]. Coconut also has more MCFAs than soybean [3], oil palm and safflower, and animal fats such as butter, tallow, fish oil and lard [4-8]. It was also reported that MCFAs are abundant in the oil produced in fruits of coconut (i.e. predominantly C12 and C14 and a small amount (0.2-1%) of C6 fatty acids [9-11]. Thioesterases play a pivotal role in fatty acid synthesis owing to their role in catalysing the terminal reaction of fatty acid biosynthesis, which regulates the fatty acid composition of storage lipids, especially in plant seeds [12,13]. The expression of thioesterase genes displayed the highest levels in expanding tissues that are typically very active in lipid biosynthesis, such as developing seed endosperm and young expanding leaves [14,15]. The enzyme that determines fatty acid chain length is acyl-acyl carrier protein thioesterase (acyl-ACP TE). This enzyme catalyzes the terminal reaction of fatty acid biosynthesis, acyl-ACP thioester bond hydrolysis to release a free fatty acid and ACP. In discrete phyla and/or tissues of specific organisms (primarily higher plant seeds), thioester hydrolysis optimally produces medium-chain (C8-C14) fatty acids (MCFAs), which have wide industrial applications (e.g., producing detergents, lubricants, cosmetics, and pharmaceuticals) [16]. TEs that specifically hydrolyze medium- chain acyl-ACP substrates have been studied widely [16-18]. Several plant acyl-ACP TEs (e.g. CnFatB3) produced significant amounts of unsaturated fatty acids (UFAs) when expressed in *E. coli*. These include 10:1, 12:1, 14:1, and 16:1 fatty acids, which do not usually accumulate in *E. coli* or in the original host plant tissues from which the acyl-ACP TE was isolated [19].

Plant acyl-ACP thioesterases fall into two distinct but related classes known as FatA and FatB [20,21]. Thioesterases, known as oleoyl-ACP thioesterases, that prefer oleoyl-ACP as a substrate with limited activity on 16:0- and 18:0-ACP define the FatA class. These thioesterases are assumed to be ubiquitousin plant tissues and responsible for the fatty acid composition found in membranes and most temperate plant oils. The FatB class contains all of the specialized thioesterases described to date from plants which accumulate specific saturated fatty acids, as well as an apparently ubiquitous thioesterase with a broader specificity for longer chain saturated and unsaturated substrates with a preference for 16:0-ACP and an unknown

*in vivo* function. It is notable that a distinct acyl-ACP *in vivo* functions. It is notable that a distinct acyl-ACP thioesterase has been cloned with a preference for each substrate from 8:0-ACP through 18:1-ACP with the exception of 18:0-ACP, although an 18:0-ACP thioesterase has been biochemically characterized from leek epidermal tissue [22]. Enzymes from both the FatA and FatB classes have limited activity on 18:0-ACP, but none of them prefer that substrate. Three acyl-ACP thioesterases; CnFatB1 (JF338903), CnFatB2 (JF338904), CnFatB3 (JF338905)) from coconut have been isolated and characterised [13].

The objectives of this research work were to clone FatB family gene of Coconut; CnFatB1, CnFatB2 and CnFatB3 and analyze the sequencing of these three genes.

## MATERIALS AND METHODS

### Isolation of FatB gene family

To isolate FatB gene family of coconut, the coding region of CnFatB1, CnFatB2 and CnFatB3 were isolated by PCR using pMD18-T which contains these gene families as a template with respective primers as shown in (Table 1). The primers used for isolation and cloning of CnFatB1, CnFatB2 and CnFatB3 were designed by using Vector NTI. The conditions of PCR were as follows; cycling conditions of 30 rounds of amplification; initial denaturation at 94ºC for 5min,denaturation at 94ºC for 30 sec, annealing at 56ºC for 30 sec and extension at 72ºC for 1.20 min followed by final extension at 94ºC for 7min. The PCR products were checked on a 1% agarose gel.

### Transformation of pMD18-T-FatB into competent E.coli DH5α

The PCR products of FatB genes were checked by agarose gel electrophoresis and recovered from the gel by DNA purification kit. The amplified products of CnFatB1, CnFatB2 and CnFatB3 were ligated to pMD18-T. 5μl of solution I, 4 μl of DNA and 1 μl of pMD18-T were put into tube, mixed and incubated the mixture at 4ºC overnight. It was designated as pMD18-T-FatB. It was transformed into competent *E.coli* DH5α by heat shock method.

50μl of competent *E.coli* was mixed with 5 μl of ligation mixture and put on ice for 30 min. After 30 min, the mixture was incubated at 42ºC for 90 seconds, immediately transferred into ice and let for 1-2 min. 500 μl of Luria-Bertani (LB) broth was added to the mixture and incubated at 37ºC for 45min by shaking at 180 rpm. After that, the culture broth was centrifuged at 8000 rmp for 1 min and the supernatant was discarded after taking 100 μl of supernatant. The pellet was resuspended in 100 μl of LB and spread on LB media containing ampicillin. Finally the plates were incubated at 37ºC overnight. After overnight incubation, it was observed for the appearance of colonies.

### Valiadation of FatB gene family by bacterial clone analysis and enzyme digestion

Potential bacterial clones harboring FatB genes were screened using colony PCR. Single *E. coli* colonies were picked up using a sterile toothpick and added to a 20μl PCR reaction mixture with respective primers for CnFatB1, CnFatB2, and CnFatB3. The bacteria were identified by PCR.

pMD18-T-FatB was extracted from *E.coli* DH5α after inoculation of single colony of *E.coli* DH5α into LB broth containing ampicillin at 37ºC for overnight. The DNA extracted from identified positive colonies was validated with double enzyme digestion using BstEII and BglII.

### Sequencing and phylogenetic analysis

Sequencing of CnFatB1, CnFatB2 and CnFatB3 was performed by selecting validated positive colonies of *E.coli*. The phylogenetic tree was constructed by computing the lowest BIC value. The parameter of K2 +G was 59 in the use of a neighbor-joining method in MEGA6. Bootstrap with 500 replicates was used to establish the confidence limit of the tree branches.

### Construction of expression vector

Plant expression vector pCAMBIA1302 was digested with BglII and BstEII and two bands, GFP gene (~538bp) and the remaining part of vector were checked by 1% agarose gel electrophoresis. GFP gene was deleted from the vector after enzyme digestion.

pMD18-T-FatB was also digested with the same enzymes. After enzyme digestion, FatB genes (1200 bp) were purified by DNA purification kit.

For enzyme digestion, 1μl of 10xNE buffer, 0.2μg of DNA and 0.2 μl of BglII were mixed and filled $H_2O$ to final 10μl. The reaction mixture was incubated at 37ºC for 15 min. After 15 min incubation, 0.2μl of BstEII was added and incubated again at 60ºC for 15 min. After enzyme digestion, DNA in expected size of pCAMBIA1302 and CnFatB1, CnFatB2, and CnFatB3 were visualized by agarose gel electrophoresis and purified by DNA purification kit.

The purifiedCnFat1, CnFatB2 and CnFatB3 were ligated to linearized pCAMBIA1302 under the reaction of T4 DNA ligase. The reaction mixture used was shown in (Table 2).

The reaction mixture was incubated at 16ºC for overnight and transformed into competent *E.coli* DH5α as described in above procedure. The recombinant expression vector was validated by colony PCR using the same conditions as described above.

**Table 1:** Primers used for isolation and validation after cloning of CnFatB1, CnFatB2, CnFatB3 of coconut (*Coco snucifera*).

| Primers | Used |
|---|---|
| ATGGTTGCTTCAGTTGCCGCTTCGTCAAGCACTTCCAGCTGAAGTGGGAC | Forward primer – CnFatB1<br>Reverse primer- CnFatB1 |
| ATGGTTGCTTCAATTGCCGCCTC<br>TCATGCACTACCACCTGGAGTTGG | Forward primer- CnFatB2<br>Reverse primer- CnFatB2 |
| ATGGTCGCCTCCGTTGCTGCCTCTCATTTACTCTCAGTTGGGTGCAGACC | Forward primer- CnFatB3<br>Reverse primer- CnFatB3 |

**Table 2:** Reaction conditions for ligation of CnFatB1, CnFatB2, CnFatB3 into linearized 1302 under T4 DNA ligase.

| Component | 20µl reaction |
|---|---|
| 10x T4 DAN ligase buffer | 2µl |
| Vector DNA | 1:3 vector to insert |
| Insert DNA | 1:3 vector to inset |
| Nuclease free water | To 20µl |
| T4 DNA ligase | 1µl |

### Transformation of *A. tumefaciens* with expression vector

Expression vector was transformed into *A.tumefaciens* by freeze thaw method. A single colony of the *Agrobacterium* strain was inoculated into 3 ml of LB in a 15 ml snap-cap tube. Growth was allowed at 30°C for overnight by shaking at 200 rpm. For *A.tumefaciens* GV3101, gentamycin or rifampicin was added to LB media. Overnight culture was inoculated into 50 ml of LB in a 250 ml flask with 0.5 ml (1/100 volume) and incubated at 30°C until mid-log (OD600 was between 0.5 and 1.0). This took ~4-5 hours to get the cells to this stage. The culture was chilled 5-10 minutes on ice, centrifuged at 3000 rpm for 5 minutes at 4°C in chilled sterile centrifuge tubes. Supernatant was discarded; the tubes were inverted for 30-60 seconds. Pellet was resuspended in 1 ml of ice cold 20 mM $CaCl_2$. 0.1 ml of bacterial suspension was dispended into each of two pre-chilled 1.5 ml. microfuge tubes on ice. One is a control.

1 ug of plasmid DNA was added to one tube and nothing to the other the control and mixed well. The tubes were breezed in liquid $N_2$, thenthe tubes were allowed to thaw for 5 minutes at 37°C.

1 ml of LB was added to each tube, and incubated for ~2 hours by shaking at 200 rpm of 30°C. Culture broth was centrifuge at 8000 rpm for 1 min, supernatant was removed. Pellets were resuspended in 100 µl of LB and spread onto LB containing Kanamycin and Gentamycin. All of the suspension were plated on appropriate antibiotic-LB plates and incubate for two days at 30°C. Transformed colonies should be visible on the second day of incubation.

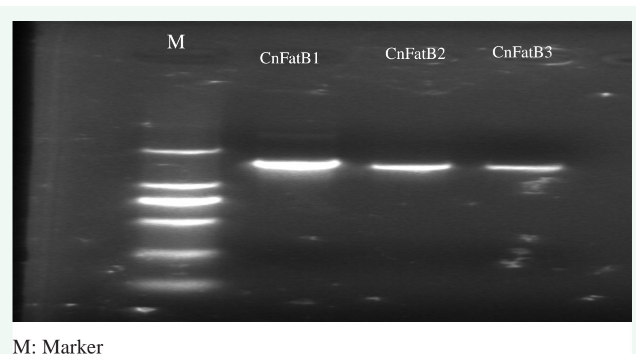
## RESULTS AND DISCUSSION

### Isolation of FatB gene

The coding region of CnFatB1, CnFatB2 and CnFatB3 of coconut were amplified using pMD18-T as template with respective primers and 1200 bp in size of each gene were obtained as shown in (Figure 1).

### Cloning of FatB genes

Figure 2 showed the general procedure for cloning of CnFatB genes of coconut using pMD18-T and pCAMBIA after constructing as expression vector by cloning in *E.coli* DH5α. Purified CnFatB1, CnFatB2 and CnFatB3 were ligated to pMD18-T and transformed into *E.coli* DH5α. Potential colonies harboring FatB genes were screened by colony PCR. Two positive colonies for CnFatB1, four positive colonies for CnFatB2 and two colonies for CnFatB3 were obtained. It was shown in (Figure 3a).




M: Marker

**Figure 1** CnFatB1, CnFatB2 and CnFatB3 of Coconut (*Cocos nucifera*) were isolated using pMD18-T as template by PCR (pMD18-T that was already inserted with FatB gene (CnFatB1, CnFatB2 and CnFatB3) family and cloned into *E.coli* DH5α. Recombinant pMD18-T was extracted from validated *E.coli* DH5α). Expected size (1200 bp) of these three genes was seen by 1% agarose gel electrophoresis after PCR.

pMD18-T-Fat B was extracted from *E.coli* and used as template to validate the FatB genes in plasmid by using the same primers. CnFatB1, CnFatB2 and CnFatB3 were in uniform size from colony PCR and from using pMD18-T-FatB as template. It was shown in (Figure 3b).

After enzyme digestion of pD18-T-FatB with BglII and BstEII, the products were visualized by 1% agarose gel electrophoresis. CnFatB1, CnFatB2 and CnFatB3 were seen in expected size as shown in (Figure 4). The results showed that the size of CnFatB1, CnFatB2 and CnFatB3 were in consistent with the expected size and also consistent from all examination.

### Sequencing and phylogenetic tree analysis

By sequencing, CnFatB1 was related to *Cocos nucifer* FatB1 with a nodal value of 99, CnFatB2 was related to *Cocos nucifer* FatB2 with 97 for a nodal value and CnFatB3 was related to *Cocos nucifer* FatB3 with a nodal value of 98. Phylogenetic analysis was conducted according to the similarity of the FatA and FatB conserved domain sequences from different species. The analysis indicated that CnFatB1 was highly related to CnFatB1 (JX275886.1 and JF338903.1) of *Cocos nucifera*, CnFatB2 was highly related to CnFatB2 (JF338904.1) of *Cocos nucifera*, and CnFatB3 was highly related to CnFatB3 (JF338905.1) of *Cocos nucifera*. The phylogenetic tree was shown in (Figure 5).

### Construction of expression vector and transformation in *E.coli*

After digestion of pMD18-T-FatB and pCAMBIA1302 with BglII and BstEII, CnFatB1, CnFatB2, CnFatB3 were ligated to linearized pCMABIA1302 in which GFP was deleted as shown in (Figure 6) and transformed into *E.coli*. In this study, it was not needed to use GFP as reporter gene. But GFP excited by ultraviolet light will emit green fluorescence light [23]. GFP gene employed as reportor gene is widely applied in plant genetic transformation [24-26] due to its advantages of stable fluorescent light emission, convenient detection and non-species specificity, no poisoning to plant as well as being capable of in vivo detection [27].

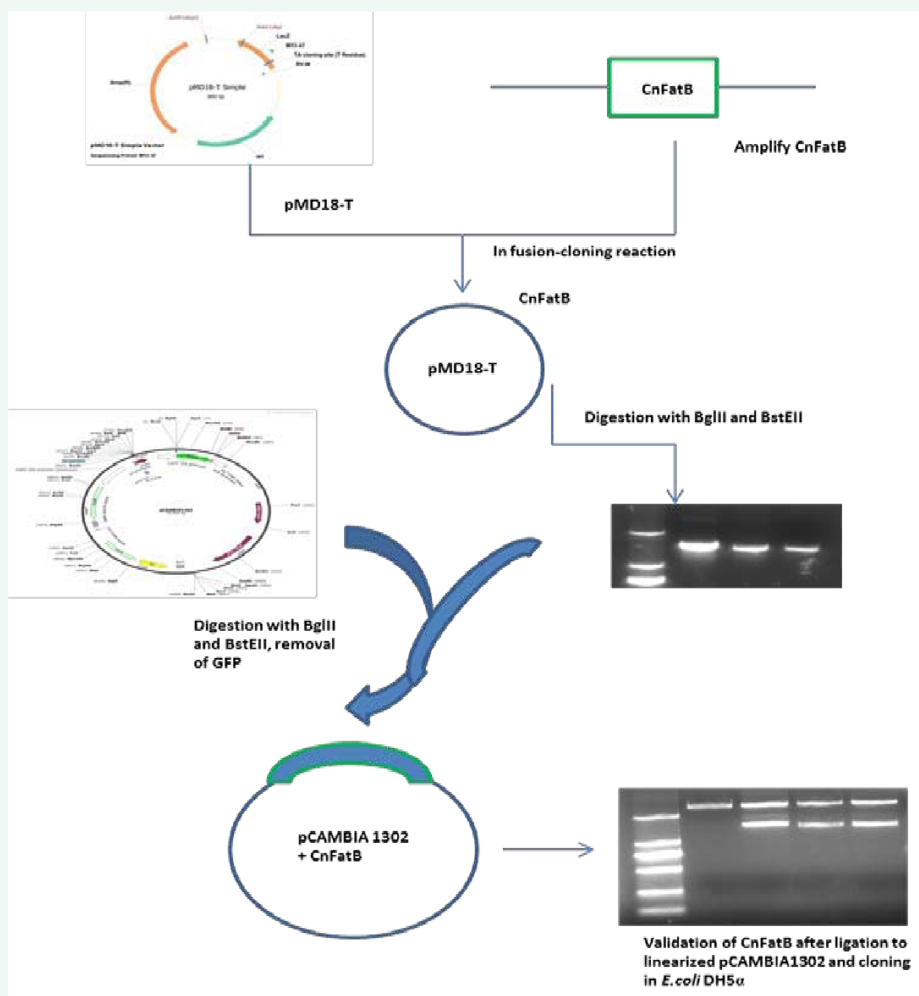



**Figure 2** General procedure of cloning CnFatB gene family of coconut (*Coco snucifera*) in *E.coli* DH5α using pMD18-T and pCAMBIA1302. Isolated CnFatB genes were ligated into pMD18-T and cloned in *E.coli* DH5α. After successful cloning, recombinant pMD18-T-FatB was digested with BglII and BstEII and CnFatB genes were purified after enzyme digestion. pCAMBIA1302 was also digested with the same enzymes and CnFatB genes were ligated into linearized pCAMBIA1302 and cloned in *E.coli* DH5α. It was further validated by enzyme digestion. CnFatB genes were seen in expected size after enzyme digestion.

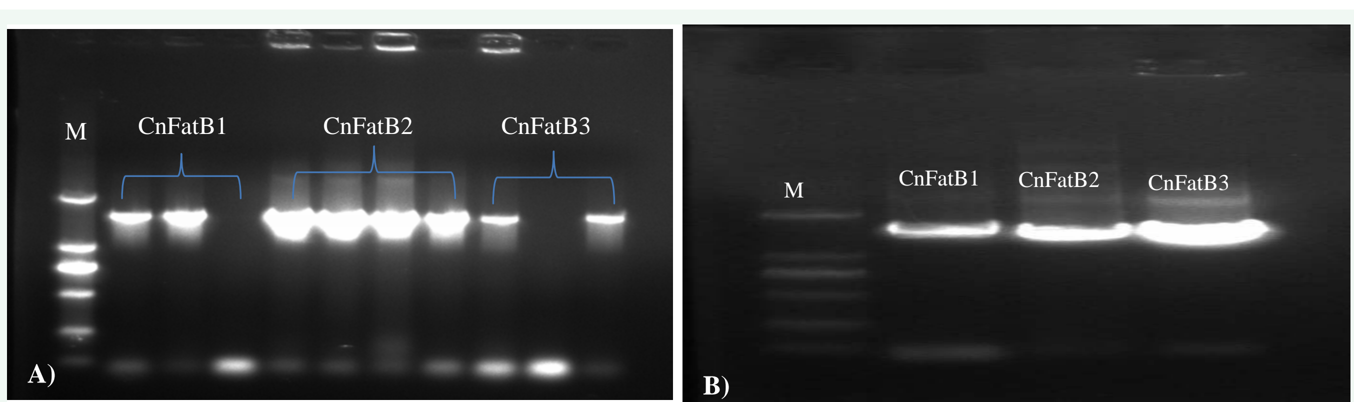



**Figure 3** **(a)** Isolated CnFatB1, CnFatB2 and CnFatB3 were ligated into pMD18-T and cloned in *E.coli* DH5α. Some colonies of *E.coli* DH5α appeared on LB media containing ampicillin were selected and confirmed the presence of pMD18-T harboring FatB genes (pMD18-T-FatB) by colony PCR. **(b)** After identifying positive colonies of *E.coli* DH5α after transformation with pMD18-T-FatB, pMD18-T-FatB was extracted from *E.coli* DH5α and used as template for validation of FatB gene of pMD18-T-FatB by PCR. Same primers for CnFatB1, CnFatB2 and CnFatB3 were used for amplification of PCR. CnFatB1, CnFatB2 and CnFatB3 were seen in expected size when checked by 1% agarose gel.

**Figure 4** After ligation of CnFatB1, CnFatB2 and CnFatB3 to pMD18-T and cloning in *E.coli* DH5α, pMD18-T-FatB was extracted from *E.coli* and used as template for validation of pMD18-T-FatB harboring FatB gene by PCR using same respective primers for three genes. pMD18-T-FatB was digested with BglII and BstEII and the products were visualized by 1% agarose gel. After enzyme digestion of pMD-18-T with two enzymes, the size of CnFatB1, CnFatB2 and CnFatB3 was consistent with the expected size; M- Marker,Lane 1,3,5- By using pMD18-T-FatB as Template and Lane 2,4,6- After Enzyme Digestion.

**Figure 5** Neighbor-joining tree showed the position of FatB family gene of Coconut (*Cocos nucifera*) to a selected number of FatA and FatB of some plant species. After computing the lowest BIC value, K2+G parameter was 59 with 500 of Bootstrap replications. Values are the branch lengths reflecting the actual distances between the sequences. The analysis indicated that CnFatB1 was highly related to CnFatB1 (JX275886.1 and JF338903.1) of *Cocos nucifera*, CnFatB2 was highly related to CnFatB2 (JF338904.1) of *Cocos nucifera*, and CnFatB3 was highly related to CnFatB3 (JF338905.1) of *Cocos nucifera* Figure 5- pMD18-T-FatB Containing CnFatB1, CnFatB2, and CnFatB3 and pCAMBIA1302 after Digestion of BglII and BstEII.

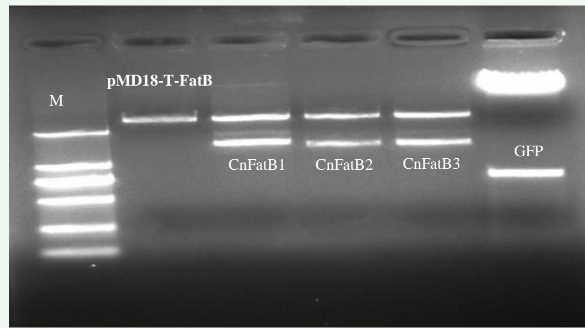



**Figure 6** pMD18-T-FatB was extracted from *E.coli* DH5α and digested with BglII and BstEII to ligate to pCAMBIA1302. CnFatB1, CnFatB2 and CnFatB3 and other parts of plasmid were obtained. pCAMBIA1302 was also digested with the same two enzymes and two bands, GFP in ~538bp of size and other parts of pCAMBIA1302 was obtained. GFP was deleted in this study. After agarose gel electrophoresis, CnFatB1, CnFatB2, CnFatB3 and pCAMBIA1302 in which GFP was deleted were purified for ligation.

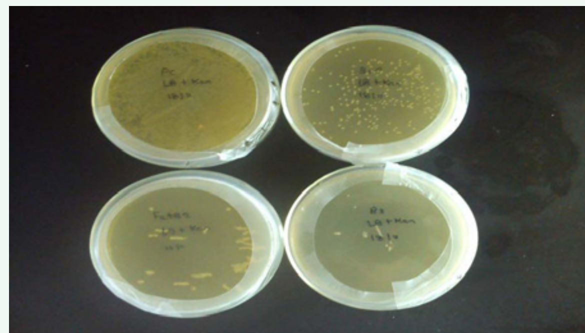


**Figure 7** After construction of expression vector- ligation of FatB gene to linearized pCAMBIA1302, it was transformed into competent *E.coli* DH5α. pCAMBIA1302 contains kanamycin resistant gene. Positive colonies were grown on LB media containing kanamycin as shown in figure after overnight incubation. In positive control, more colonies appeared on LB media containing kanamycin, but in other three media, a few colonies were appeared. It might be due to the fact that ligation efficiency of CnFatB genes to linearized pCAMBIA1302 was low.

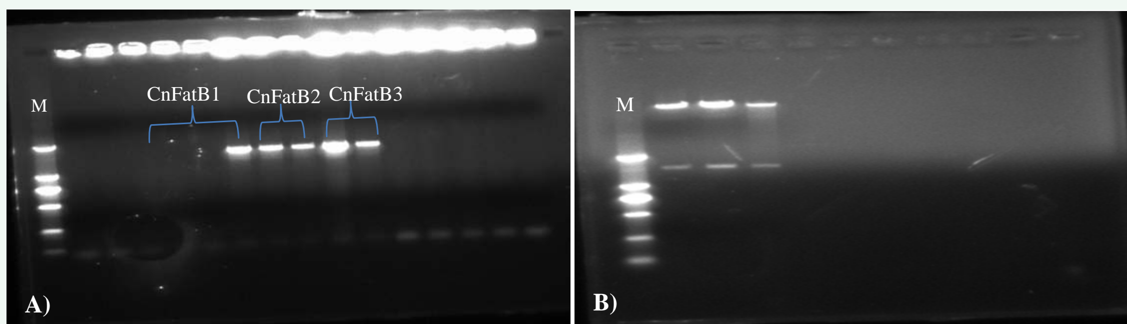



**Figure 8** **(a):** After construction of expression vector, it was transformed into competent *E.coli* DH5α. Positive colonies were validated by colony PCR. Single colony was picked up by tooth pitch and inoculated in 20μ PCR reaction mixture. PCR reaction was performed using same respective primers for three genes. CnFatB1, CnFatB2 and CnFatB3 in expected size were seen from positive colonies. **(b)** Expression vector was extracted from *E.coli* and digested with BglII and BstEII. CnFatB1, CnFatB2 and CnFatB3 were seen in expected size.

Growth of colonies on LB media containing Kanamycin was shown in (Figure 7). Potential colonies harboring FatB genes were screened by colony PCR and expression vector was digested with BglII and BstEII. CnFatB1, CnFatB2 and CnFatB3 were seen in expected size from both methods. It was shown in (Figure 8ab).

Jing et al., (2011) cloned Acyl-ACP TE cDNAs from endosperm of nearly mature coconuts, constructed expression vector pUC57 and transformed into *E.coli* strain K27. Each TE expressed in *E.coli* strain K27 and free fatty acids accumulated in the medium were extracted and analyzed *in vitro*. It was also reported that it appears that CnFatB1, CnFatB2, and CnFatB3 might work together to determine the fatty acid composition of coconut oil, which contains primarily 12:0 (43-50%) and 14:0 (16-22%) and small amounts of 6:0, 8:0, and 10:0 fatty acids [9-11].

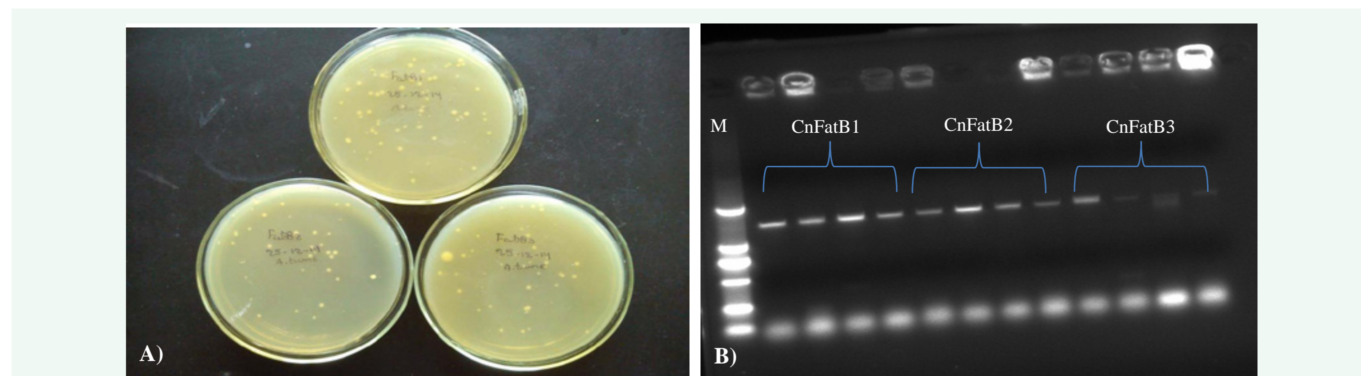



**Figure 9 (a):** Expression was also transformed into *A.tumefaciens* GV3103. Expression vector contains kanamycin resistant gene and *A.tumefaciens* GV3103 is resistant to gentamycin. So positive colonies were selected on LB media containing kanamycin and gentamycin after transformation with expression vector. Growth of transformed *A.tumefaciens* GV3103 on LB media containing Kanamycin and Gentamicin and **(b)** Colony PCR Results of CnFatB1, CnFatB2 and CnFatB3 from transformed *A.tumefaciens* GV3103 with expression vector.

CocoFatB1 was isolated from coconut endosperm during fruit development and transformed into Tobacco by *A.tumefaciens*. Fatty acid composition of transgenic tobacco seeds was increased [28].

### Transformation of expression vector into *A. tumefaciens* GV3103

FatB genes are important gene family that can determine the chain length of fatty acid. Therefore, many researchers studied the role of FatB genes by transforming into other species using different transformation methods. In this study, it was also studied the cloning ofCnFatB gene family in *A.tumefaciens* and validated their cloning by PCR for the purpose of transformation to other species. Expression vector was transformed into *A.tumefaciens* Gv3103 and growth of *A.tumefaciens* on LB media containing Kanamycin and Gentamycin was shown in (Figure 9a). Potential colonies harboring FatB genes were checked by colony PCR. FatB1, FatB2 and FatB3 in expected size were seen in (Figure 9b).

## CONCLUSION

CnFatB1, CnFatB2 and CnFatB3 were isolated and cloned in *E.coli* DH5α. After sequencing, isolated CnFatB1, CnFatB2 and CnFatB3 were highly related to CnFatB1 (JX275886.1 and JF338903.1) of *Cocos nucifera*, CnFatB2 was to CnFatB2 (JF338904.1) of *Cocos nucifera*, and CnFatB3 was highly related to CnFatB3 (JF338905.1) of *Cocos nucifera*). For construction of expression vector, pCAMBIA1302 was used after deletion of GFP. After transformation of expression vector in *E.coli* DH5α, some positive colonies were identified by colony PCR. In addition, it was confirmed that expression vector harboring FatB genes by using expression vector as template for PCR and enzyme digestion by BglII and BstEII. All results showed that CnFatB1, CnFatB2 and CnFatB3 were consistent in size with expected size.

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