CHAPTER IV
MATERIALS AND METHODS

4.1 Plant collection

Croton stellatopilosus Ohba leaves were collected separately as no. 1, 2, 3, 4, 5 away from the young shoot, including the shoot part (Figure 13) from the plant grown in the open flied of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The location of the plant is 13°44′33″ North latitude, 100°31′49″ East longitude. The voucher specimen of *C. stellatopilosus* (No.184779) was deposited at the Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand. The leaves were collected early in the morning, before starting of the photosynthesis which causes accumulation of carbohydrates and phenolic compounds, which may disturb the isolation of RNA. The collected leaves were immediately kept in zip-lock bags and on ice and transferred to the laboratory.

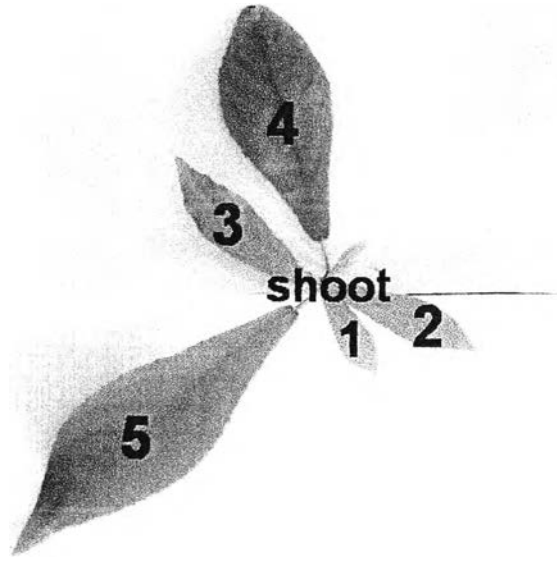

Figure 13 Various position of leaves used in this study.

4.2 Total-RNA preparation

4.2.1 Total-RNA isolation

The leaves no. 2 from the shoot tip were used in the full-length gene experiments as reported previously (Rungrotmongkol et al., 2009; Sitthithaworn et al., 2009), and leaf nos. 1, 2, 3, 4, 5 away from shoot were used in mRNA expression experiments. The collected leaves were washed with distilled water and wiped dried with tissue paper. The total-RNA was isolated based on the guanidine thiocyanate method according to the instruction of RNeasy® Plant Mini kit (QIAGEN). One-hundred milligrams of the leaves were ground under liquid-N_2 using the sterilized ceramic pestle-mortar, then homogenized with 450 µl buffer RLT in a 2-ml micro-tube at 56°C for 3 min. The homogenized lysate was transferred to a QIAshredder spin column and centrifuged at 13,000 rpm at room temperature for 10 min. The flow-through was transferred to a new 2 ml micro-tube and mixed with 500 µl of 95% ethanol prior to transferrged to RNeasy spin column which RNA was allowed to bind to the column membrane. The mixture was then centrifuged at room temperature for 15 sec. To clean RNA, the column was washed with 700 µl buffer RW1 and centrifuged at 10,000 rpm at room temperature for 15 sec. The membrane was washed again with 500 µl buffer RPE and centrifuged at 10,000 rpm at room temperature for 15 sec. The RNA was eluted from the membrane using 50 µl RNase-free water.

4.2.2 DNaseI treatment

Three micrograms of the total-RNA were put into the DNaseI reaction mix (30 µl), containing 1x buffer, 3U DNaseI (Fermentus) and DEPC-water. The mixture was incubated at 37°C for 30 min. To inactivate DNaseI, 2 mM EDTA was added and the reaction was heated at 65°C for 10 min. If the RNA was used in the real-time PCR step the DNaseI treatment was done through the RNeasy spin column (QIAgen). Total-RNA was mixed with 10 µl buffer RDD and 2.5 µl DNase I stock (10 mg/ml), adjusted the volume to 100 µl with RNase-free water. The reaction was then mixed with 350 µl buffer RLT and 250 µl of 96-100% ethanol at 10000 rpm for 15 sec at room temperature. The membrane was washed with 500 µl buffer RPE and centrifuged for 15 sec at 10000 rpm. The RNA was eluted from the membrane using 30 µl RNase-free water

4.2.3 Quality and Quantity of the total-RNA preparation

Quality of RNA was determined by the ratio of absorbance at 260 nm and 280 nm using NanoDrop2000. A ratio of 2 is accepted as pure RNA. The equation calculated for the concentration of ssRNA was;

ssRNA concentration = 40 μg/mL × OD260 × dilution factor

Agarose gel electrophoresis was also applied to confirm the RNA purity. Two microliters of total-RNA were mixed into 1x loading dye (Fermentus) and loaded onto 1% agarose gel in 1xTAE buffer. Electrode running at 70V/1 cm agarose gel length until the dye marker was moved to 1 cm edge of the gel. The gel was then stained in 1 mg/ml EtBr and destain with distilled H20 water

4.3 cDNA synthesis

One microgram total-RNA was added into the cDNA synthesis reaction mix (10 μl) containing 1 mM oligo (dT)18, and DNase-RNase free water, then the reaction was incubated at 72°C for 5 min and immediately placed on ice. The reaction was mixed with 20 Unit of RNase-inhibitor, 1x M-MLV reverse transcriptase buffer, 1 mM dNTPs mix (NEB), and 200 Unit of M-MLV reverse transcriptase (usb) prior to adjusting the final volume to 25 μl by nuclease-RNase free water. Then the reaction mix was incubated at 42°C for 90 min and heat at 95°C for 5 min to inactivated enzyme. The obtained cDNA was kept at -20°C for further use. The equation calculated for the concentration of dsDNA was;

dsDNA concentration = 50 μg/mL × OD260 × dilution factor

4.4 Primer design

The primers used in this study were designed based on several criteria, such as P450 gene localization, and the similar reaction and function in the pathway of plaunotol. Amino acid and nucleotide sequences which fit in the criteria were retrieved from the database. Multiple alignment using ClustalX2 (Thompson et al., 2002) was performed to search for the conserved region which was suitable for the primers.

Primers were designed and evaluated on CloneManager ver.9 before being synthesized by 1stBASE, in 25 nmole DNA oligo and HPLC purification grade.

The primers used in this study were designed based on the strategy obtained the products which were divided into 4 parts: the degenerated primers, 5′-RACE gene specific primers, 3′-RACE gene specific primers, full-length gene primer, and expression hanging the direction enzyme primers (Figure 14).

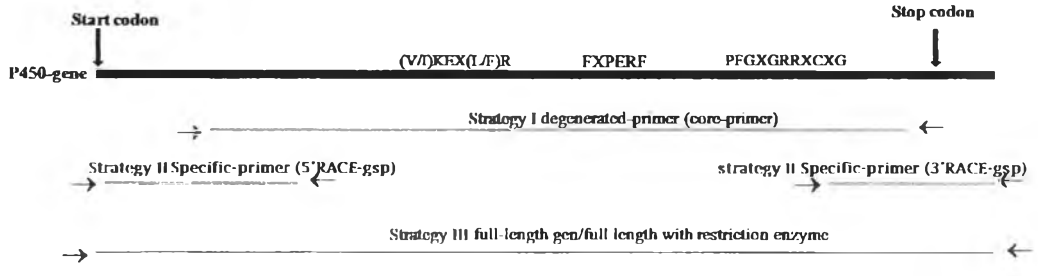

Figure 14 Strategy for primer design. The strategy I is a degenerated primer for core fragment sequence, strategy II is both 5′- and 3′-RACE specific primer for 5′- and 3′-RACE fragment sequences, and the last strategy, the strategy III is for a full length primer for full-length sequence.

4.4.1 Degenerated primer for amplification of the core fragments

Primers for core fragment were designed as degenerated primers. The IUPAC codes (Table 5) were used to demonstrate the ambiguous nucleotides (Wei et al., 2003). The primers were designed from the conserved region of the multiple alignments from the same family gene. The degenerate primers are listed in Table 6.

Table 5 IUPAC ambiguity code

Code	Description
M	AC
R	AG
W	AT
S	CG
Y	CT
K	GT
V	ACG
H	ACT
D	AGT
B	CGT
N	ACGT

Table 6 Nucleotide degenerate primers

Primer name	Sequence (5' → 3')	PCR expected size (pb)
CPR-de-F	TTTARARTTGTTGATWTGGATGATTATGC	1600
CPR-de-R	GCCATRCCYTTGGCATCACCACASACATA	
GG18H-de-F	GTRTTCAACTAYRAYTTTGA	975
GG18H-de-R	AYSACDGGDGGDTGWGGYTA	

4.4.2 Gene specific primers for 5′-RACE and 3′-RACE

Specific primers for amplification of 5' and 3' ends were obtained based on nucleotide sequences of the core fragments.

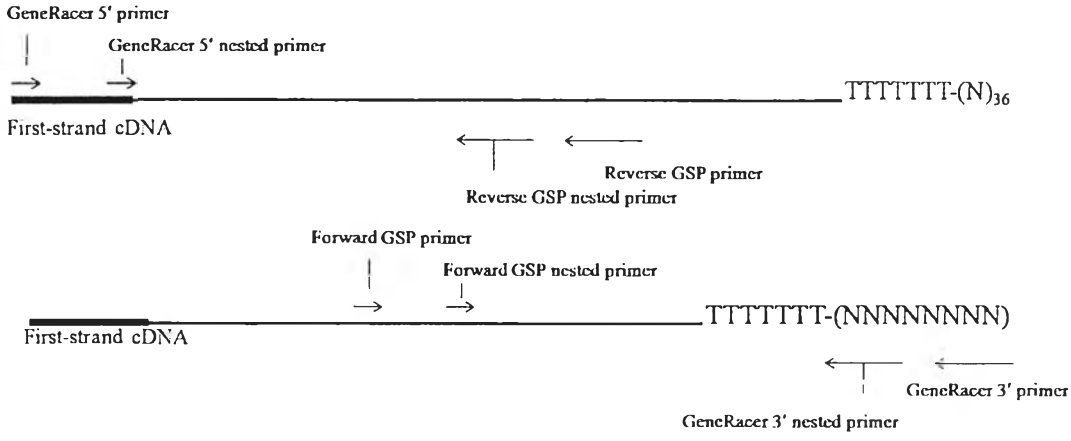

Figure 15 Illustrate the primer annealing position for amplification of 5' and 3' ends.

Table 7 Specific primer for 5′-RACE and 3′-RACE

Primer name	Sequence (5' → 3')	PCR expected size (bp)
CPR-3EACE1143	AACACCAGGAGGACGGCTACACAA	
CPR-3RACEnested1399	GGTTGCAGGAACAGTAAGATGG	685
CPR-5RACE274	CCTGCTCAGCAAGCAACTCATCCAACTC	
CPR-5RACEnested118	CTGTTGGCTCGCCATCTCCATATGT	529
GG18H-3RACE212	AAGTGATTGTGGAGGCCGAAGGTGAA	
GG18H-3RACEnested71	AAGAGGCGGAGGCTCGTTCCACAGATCTT	652
G18H-5RACE379	AACCAGTTGTCTCGTGGCCAGCAACTA	
G18H-5RACEnested298	CGAGCAAGAACCGAAGAATGCTTGGATCAG	470

4.4.3 Specific primer for amplification of the Open reading frame (ORF)

The consensus sequence obtained from the assembly alignment was a template for the full-length primers listed in Table 8.

Table 8 Nucleotide primer for the full-length gene

Primer name	Sequence (5' → 3')	PCR expected size (bp)
CPR-FL-F	ATGGAACCATCATCATCGGCAGGTTCCATC	2300
CPR-FL-R	TCAGTAAAAAAGCTTACCTGCTCTTGG	
GG18H-FL-R	TTAACGCAGACTGCTGCTTGCAAA	1413
GG18H-FL-R	TTACCTTAATGAAGATGAAGCAAATG	

4.4.4 Specific enzyme restriction site adding to the ORF fragment for Ligase Dependent Cloning (LDC)

Restriction sites associated to expression vector were added to the full-length PCR product to facilitate cloning to the expression vector by amplification of the PCR product with primers that possess recognition sites for certain restriction enzymes. The nucleotide sequences of primer were shown in Table 9.

Table 9 Nucleotide primer to obtain the product hanging the 5'-end restriction enzyme for the expression vector

Primer name	Sequence (5' → 3')
CPR-ORF-F-*BamH*I	CTATGGGATCCATGCAATCTTCGACTGGTGC
CPR-ORF-R-*Sal*I	TGATGTCGACTTAGAAATTGCCTGCTTC
GG18H-ORF-F-*Not*I	GCGGCCGCATGGCAACCGCAAAACTGGATGAT
GG18H-ORF-R-*Xho*I	CTCGAGTTAACGCAGACTGCTGCTTGCAAA

4.4.5 Real-time PCR primer

Primer for real-time PCR rationale using the CloneManager 9.1 which the primer length is no more than 250 bp. Primers were blasted using the nucleotide algorithm blast check for the specific to the gene. Real-time primers were listed in Table 10.

Table 10 Nucleotide primer for real-time PCR

Primer name	Sequence (5' → 3')	PCR expected size (bp)
CPR-RT-F	ATGCAACCATCATCATCGGCAGGTTGG	190
CPR-RT-R	GTGCCGTCCTCTTCATCAGTA	190
DXS-RT-R	TATCCCAGTAGCGCCACTCG	212
DXS-RT-F	CCTCCTAACATGGTGGTGATGGCTCCTTC	212
GG18H-RT-F	CTTCGGTTCTTGCTCGCTAGTA	191
GG18H-RT-R	GGCCTTCCTTGTAACACTTGGT	191
GGPPp-RT-F	CCTCCATCATGCCATACTAGGTCTC	299
GGPPp-RT-R	CAACATGGCCTCTGCGGTCAAATGC	299
GGPPs-RT-F	GTCGTGGTAAGCCGACGAATCAT	173
GGPPs-RT-R	TCCTTCAGCACCAATCGCCTTAG	173
MEPS-RT-F	ATAATGCGCCGCAATGCACC	211
MEPs-RT-R	TCGGCTGTGCAGGCTTAAGG	211
18s rRNA-F	CAAAGCAAGCCTACGCTCTG	536
18s rRNA-R	CGCTCCACCAACTAAGAACG	536

4.5 Polymerase chain reaction (PCR)

PCR was done by 3 strategies (Figure 16).

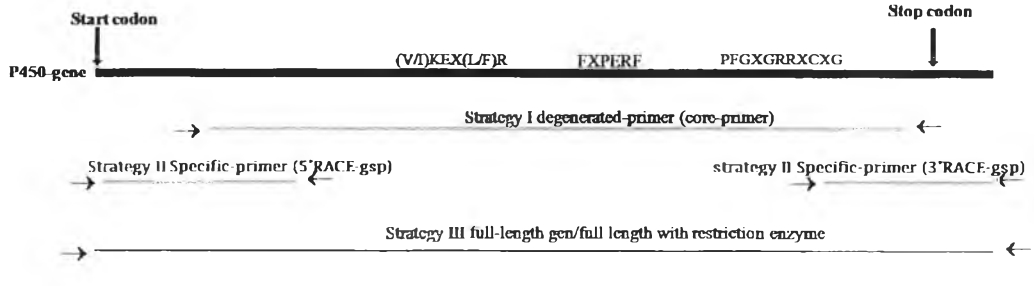

Figure 16 The strategies for the PCR amplification

The PCR reaction mix was done within 25 µl enzymatic reaction, containing template, PCR buffer, primer pair (both forward and reverse) substrate as deoxynucleotides (dNTPs) solution mix (NEB), enzyme: as Platinum® Taq DNA Polymerase High Fidelity (Invitrogen), co-enzyme $MgCl_2$, and make the volume by nuclease-free water (Table 11).

Table 11 PCR reaction

PCR reaction mix	Stock solution	Working solution
PCR buffer	10x	1x
dNTP mix	10 mM each	0.4 mM
$MgCl_2$	50 mM	2 mM
Primer F	10 µM	0,4 µM
Primer R	10 µM	0,4 µM
Template		100 ng
Taq DNA polymerase	5U/µl	0.5U

4.5.1 Amplification of the core fragment

Strategy I was used for amplification of the core fragment. PCR was performed with degenerated primers (Table 6). PCR reaction and PCR cycle were summarized as in Table 11 and Table 12, respectively.

Table 12 Thermal profile used to amplify the core sequence.

Denature step	Amplification step	Last extension
94°C 2 min	94°C 30sec, 50°C 30 sec, 68°C 1 min	68°C 5 min
(1 cycle)	(10 cycles)	(1 cycle)
	94°C 30sec, 55°C 30 sec, 68°C 1 min	
	(25 cycles)	

4.5.2 Amplification of 5′-and 3′- ends

Strategy II was used for amplification of 5′-and 3′- ends as will be describe separately in Section 4.4.2. 5′-RACE gene specific and 3′-RACE gene specific primers were listed in Table 7. PCR reaction and PCR cycle were summarized as in Table 11 and Table 13, respectively.

Table 13 Thermal profile used to amplified RACE sequence

	Denature step	Amplification step	Last extension
First-PCR	94°C 2 min (1 cycle)	94°C 30sec, 72°C 1 min (5 cycles)	72°C 10 min (1 cycle)
		94°C 30sec, 70°C 1 min (5 cycles)	
		94°C 30sec, 55°C 30 sec, 72°C 1 min (25 cycles)	
Nested-PCR	94°C 2 min (1 cycle)	94°C 30sec, 65°C 30 sec, 68°C 2 min (25 cycles)	68°C 10 min (1 cycle)

4.5.3 Amplification of a full-length gene

Strategy III was used for amplification of a full-length gene. Primers were listed on Table 8 and 9. PCR reaction and PCR cycle were summarized as in Table 11 and Table 14, respectively.

Table 14 Thermal profile for amplification

	Denature step	Amplification step	Last extension
GG18H	94°C 2 min (1 cycle)	94°C 30sec, 50°C 30 sec, 68°C 1 min (10 cycles)	68°C 5 min (1 cycle)
		94°C 30sec, 55°C 30 sec, 68°C 1 min (25 cycles)	
CPR	94°C 2 min (1 cycle)	94°C 30sec, 50°C 30 sec, 68°C 1 min (10 cycles)	68°C 10 min (1 cycle)
		94°C 30sec, 55°C 30 sec, 68°C 2 min (25 cycles)	

4.6 Rapid amplification cDNA ends polymerase chain reaction (RACE-PCR)

The total-RNA after DNaseI treatment in section 4.3 was used directly to prepare the cDNA in RACE-PCR for both 5′-RACE and 3′-RACE ends using RNA ligase-mediated (RLM) and oligo-capping method using GeneRacer™ (Invitrogen) according to the manufacturer′s protocol.

4.6.1 RNA Dephosphorylation

Five micrograms of total-RNA were treated with calf intestinal phosphatase (CIP) to remove the 5′ phosphatase (Figure 17) in 10 µl reaction mix, containing 1X CIP buffer, 40 Unit RNaseOut™, 10 Unit CIP. The reaction was incubated at 55°C for 1 hr.

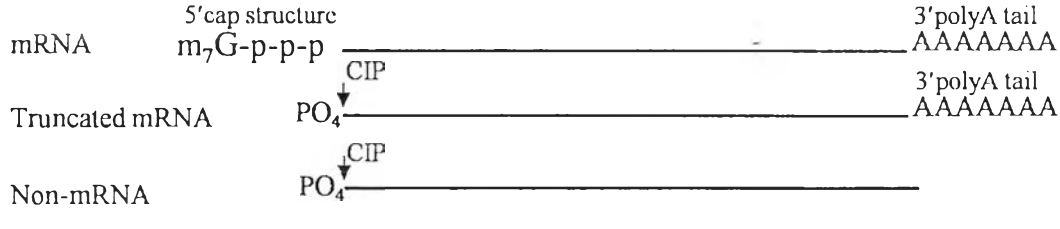

Figure 17 Dephosphorylating RNA with calf intestinal phosphatase

4.6.2 RNA Precipitation

RNA with 100 µl phenol: chloroform, was mixed and transferred into a new 1.5 ml micro-tube, followed by adding 2 µl mussel glycogen, 10 µl 3M sodium acetate, pH5.2, and mixed well. The solution was added with 220 µl 95% ethanol and mixed by vortex briefly. The tube was stored at -20°C for 10 min and centrifuged at 13000 rpm at 4°C for 20 min. The supernatant was removed, then the solution was added 500 µl of 70% ethanol, inverted tube mix and centrifuged at 13000 rpm at 4°C for 2 min, then carefully removed the supernatant. The pellet was air-dry for 2 min at room temperature, and resuspended in 7 µl DEPC water.

4.6.3 mRNA cap structure removing

The dephosphorylated RNA was decapped with tobacco acid pyrophosphatase (TAP) (Figure 18) by mixing in 10 µl reaction, containing 1X TAP, 40 Unit of RNaseOut™, 0.5 Unit of TAP. The reaction was incubated at 37°C for 1 hr and precipitated as described in Section 4.6.2.

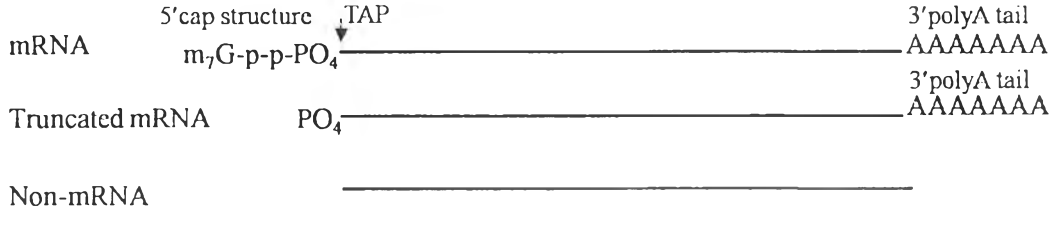

Figure 18 Removing the mRNA cap structure with tobacco acid pyrophosphatase (TAP)

4.6.4 Ligation of RNA oligo to the decapped mRNA

The 5'-end of decapped RNA (25 µg) was ligated to the GeneRacer™ RNA Oligo. Before ligation, the RNA was incubated at 65°C for 5 min to relax the secondary structure and place the tube on ice for 2 min. The RNA was mixed with the reaction (10 µl total volumes), containing 1X Ligase buffer, 1 mM ATP, 40U RNaseOut™ and 5 Unit of T4 RNA ligase and incubated at 37°C for 1 hr, then the RNA was precipitated as described in 4.6.2.

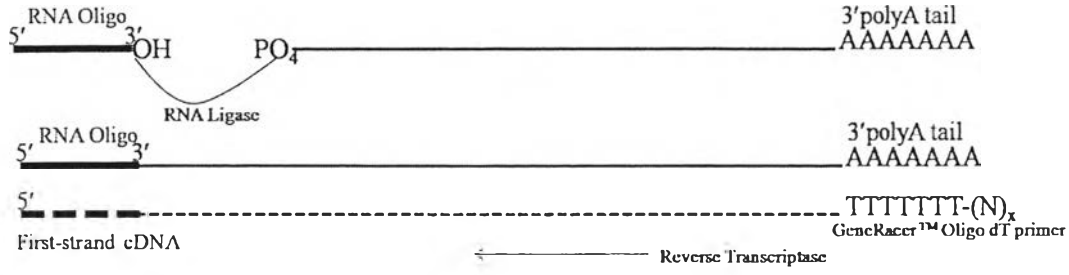

Figure 19 Reverse Transcription of decapped RNA that linked with RNA oligo.

4.6.5 First strand cDNA synthesis

cDNA was synthesized using the RNA that ligated to RNA oligo as a template and either oligo dT_{18} or the random primer (N6) was used as primer. The reaction (20 µl) mixture containing 1.25 mM dNTPmix, 1X RT buffer, 15U Cloned AMV RT, 40 Unit of RNaseOut™ and 0.5 µl of each primer was incubated at 45°C for 1 hr. To stop the reaction, Cloned AMV RT was inactivated at 85°C for 15 min.

4.7 Horizontal agarose gel electrophoresis

PCR-products were subjected to electrophoresis to confirm the expected size of PCR product using 1% agarose gel in 1xTAE buffer (40 mM Tris pH7.6, 20 mM acetic acid, 1 mM EDTA) in the submarine electrophoresis chamber (Mupid®-eXu). The electrod running was 100v for 30 min. The gel then stained within the 1 mg/ml EtBr for 5 min and de-stained with distilled water for 30 min, then visualized under the UV-transluminescent and documented the gel by Chemi Doc XRS (BioRad).

4.8 PCR-product purification

The expected-PCR products were purified using agarose gel electrophoresis and were extracted from the gel using Gel Extraction kit (RBC's bioscience). To extract DNA from gel, the expected DNA band was excised from gel and was put into 2 ml micro-tube, then 500 µl of the DF buffer were added and incubated the tube at 55°C in water-bath to melt the gel for 10 min. The solution was transferred into the DF column and centrifuged at 13000 rpm at room temperature for 30 sec to allowed the DNA to bind to the column and wash with 600 µl wash buffer by before

centrifugation at 13000 rpm at room temperature for 30 sec. The DNA was eluted from the gel with 30 µl pre-warmed at nuclease free water (55°C)

4.9 Ligation reaction

4.9.1 TA cloning ligation

Fifty nanograms of the purified-DNA were ligated to the pGEM-T easy vector (Promega). The 10 µl ligation reaction consisted of; 150 ng PCR-product, 50 ng pGEM-Teasy vector, 1x T4 ligation buffer, and 3 Unit of T4 DNA ligase. The ligation reaction was incubated at 4°C for 18 hr and was used to transform *E. coli* strain TOP10 to propagate the recombinant genes.

4.9.2 Ligase Dependent Cloning (LDC)

4.9.2.1 Preparation of expression vector and inserts

Plasmid (pGEM-T easy and pET32a, Table 15, Figure 20) and PCR products were cut with the corresponding restriction enzymes. The restriction reaction (50 µl) consist of 1 µg of PCR-product or expression vector, 1 Unit of each enzyme and 1x BSA. The reaction was incubated at 37°C for 1 hr and was inactivated by heating at 65°C for 15 min. Plasmid vector and PCR products that has been cut were purified using agarose gel electrophoresis and were extracted from the gel using Gel Extraction kit.

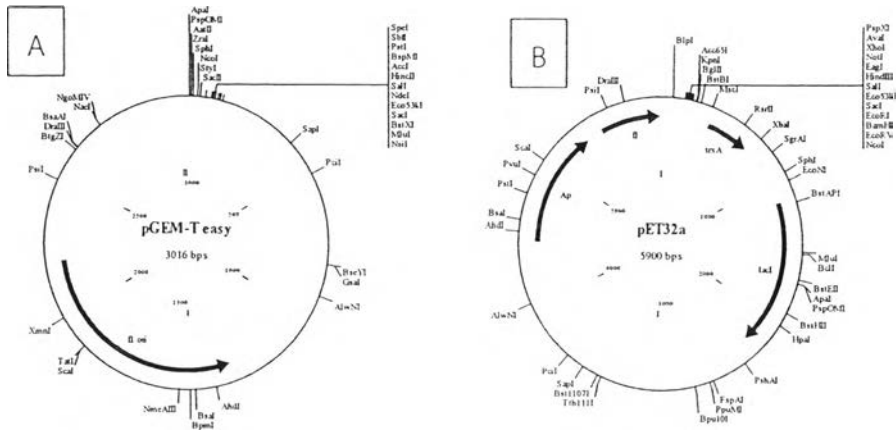

Figure 20 Plasmids, pGEM-T easy (A) and pET32a (B). The multiple cloning sites were labeled in a black alphabet group. Drawing-plasmid was done in CloneManager program.

Table 15 Vectors used in this study

Plasmid name	Antibiotic	Restriction site used to checked for inserted gene
pGEM-T easy	100 mg/ml ampicillin	*EcoR*I (Fermentus)
pET32a	100 mg/ml ampicillin	G10H: *Not*I (NEB) /*Xho*I (NEB)
		CPR: *BamH*I (NEB) /*Sal*I (NEB)
		GG18H: *Xho*I (NEB) /*Not*I (NEB)

4.9.2.2 Ligation of the inserted gene into the expression vector

PCR product was inserted to expression vector and the ligation reaction was performed with T4 DNA ligase (NEB). The reaction consist of 1X T4 DNA ligation buffer, 1 Unit of T4 DNA ligase, PCR product and expression vector (ratio 3:1). The reaction was incubated at 16°C for 18 hr and terminated by heating at 65°C for 10 min to inactivated the enzyme.

4.9.2.3 Gene transformation into Escherichia coli

The ligation product was transformed *E. coli* strain BL21(DE3). The *E. coli* culture (100 µl) was spread onto the LB agar plate containing 100 mg/ml ampicillin as described in Section 4.10.

4.10 Gene transformation

Ten microliters of the ligation reaction transformed into 100 µl of competent cell (*E. coli*, strain TOP10 or BL21(DE3)) (Section 4.11) in a 1.5 ml micro-tube. Transformation reaction was performed by incubation of mixture of E. coli cells and ligation product on ice for 30 min, and then heat shocked the cell at 42°C in water-bath for 1.30 min before immediately place the tube on ice for 3 min. Prior to grow *E. coli* cells 200 µl of LB broth was added and the culture was incubated at 37°C with 250 rpm shaking for 1 hr. The cells collected by centrifugation at 6000 rpm for 3 min at room temperature. Two hundred microlitres of supernatant discarded and 100 µl remains dissolved the pellet and spread onto 100 mg/ml ampicillin LB agar plate containing 40 mg/ml Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The plate incubated at 37°C for 18 hr.

4.11 Preparation of competent cells using chemical reaction

The competent cells were streaked on the LB agar plate without antibiotic and incubated at 37°C for 18 hr. The white separated single colony was then selected and inoculated as starter culture. The starter culture was inoculated in 5 ml LB broth without antibiotic and was incubated at 37°C for 18 hr with 250 rpm shaking. The *E. coli* culture was added into 50 ml LB broth with the ratio as 1:100 (starter cultured cell: medium) in 250 ml flask and incubated at 37°C with 250 rpm shaking until the OD600 equal to 0.4. The cells were collected by centrifugation at 3500 rpm at 4°C for 15 min. The pellet was washed twice with 100 mM $CaCl_2$ for a half volume and a quarter volume of the starter cell, respectively. The pellet was resuspended with 2 ml of 100 mM $CaCl_2$ containing 15% glycerol and kept at -80°C for further use.

Table 16 Characteristic of competent cells

Bacterial strain	Genotype	Company
TOP10	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galKrpsL (StrR) endA1 nupG	Invitrogen
BL21(DE3)	F– ompT hsdSB(rB–, mB–) gal dcm (DE3)	Novagen

4.12 Screening the recombinant clones

4.12.1 Screening the recombinant clones obtained from TA cloning

The white separating single colony was picked up by sterilized 200 µl tip and inoculated into 3 ml LB broth containing 100 mg/ml ampicillin. The inoculated colony was incubated at 37°C with 250 rpm shaking for 18 hr. The inoculated colony was examined for DNA insertion by sampling one point five milliliters of the culture suspension and the plasmid was extracted using plasmid mini-prep kit (see Section 4.13). Colony PCR was performed with corresponding primers to confirm the DNA insertion in the plasmid as described in Section 4.13.

4.12.2 Screening the recombinant clones obtained from Ligase Dependent Cloning

The separated single colony was picked up with sterilized 200 µL tip and streaked onto the new 100 mg/ml ampicillin LB agar plate (incubated at 37°C for 8 hr), the remaining cells that stick on the tip was inoculated put into the 4 ml LB broth containing 100 mg/ml ampicillin in 15 ml tube. The tube inoculated colony was incubated at 37°C with 250 rpm shaking for 18 hr. The cell was collected by centrifugation at 10000 rpm for 2 min at room temperature and the recombinant plasmid was obtained from the cells using the Presto™ Mini Plasmid kit (cat. No. PDH100, Gene-Aid) as described in Section 4.13.2. The recombinant plasmids were examined for the DNA insertion into the expression vector by setting up the restriction enzyme digestion. The reaction (20 µl) consisted of 1 µg of the purified-recombinant plasmid 1x enzyme buffer, 1 Unit of restriction enzyme. The reaction was incubated at 37°C for 1 hr.

4.13 Extraction of plasmid from *E. coli* cells

Routine plasmid extraction was performed by alkaline lysis. Commercial mini-preparation when the plasmid was used for DNA sequencing or ligation.

4.13.1 The alkaline lysis method

The pre-cultured cell was transferred into a 2 ml micro tube. The cell pellet was collected by centrifugation at 10000 rpm for 2 min at room temperature. The cell

was resuspended by 0.1 ml of the alkaline lysis buffer solution I. Alkaline lysis buffer solution II (0.2 ml) was added and mixed by inverted tube for 7 times. The cell lysis solution was then mixed with 0.15 ml of the alkaline lysis buffer solution III, The supernatant containing the plasmid was collected by centrifugation at 13000 rpm for 10 min at room temperature, then transferred into new 2 ml micro-tube and the equal volume of the phenol: chloroform: isoamyl alcohol (25:24:1) was added to get rid of the protein contamination. The upper layer aqueous plasmid DNA was collected by centrifugation at 13000 rpm for 5 min at room temperature and transferred into new 2 ml micro-tube. To precipitate the plasmid, 2 volumes of absolute ethanol and that previously frozen at -20°C for 20 min was added. The plasmid pellet was collected by centrifugation at 13000 rpm for 20 min at 4°C and the pellet was washed with 70% ethanol and was centrifuged at 13000 rpm for 2 min, 4°C. The pellet was dry by opened lid in 65°C oven for 10 min to get rid of the assess ethanol and was reconstituted by 30 µl nuclease free water.

Table 17 Combination of Buffer used in plasmid miniprep based on the Alkaline Lysis Method

Alkaline lysis buffer	
Solution I	1% glucose, 10mM EDTA pH 8.0, 50mM Tris pH 7.5
Solution II	1% SDS, 0.2M NaOH
Solution III	5M potassium acetate, 11% glacial acitic acid

4.13.2 Presto™ Mini Plasmid Kit (GeneAid)

The plasmid was extracted and purified according to the manufacturer's protocol (GeneAid). The cells were harvested by transferred 1.5 ml of cultured bacterial cell into a 2 ml micro tube and centrifuged at 10000 rpm at room temperature for 1 min. The pellet was resuspended into 200 µl PD1 buffer by vortex mixer. The cell pellet was then lysis by adding 200 µl PD2 buffer and neutralized in 300 µl PD3 buffer. The cell was transferred to PD column and let the plasmid DNA bind to the membrane by centrifuged at 13000 rpm at room temperature for 30 sec. The membrane was washed by 600 µl of washing buffer. After centrifugation the column at 13000 rpm at

room temperature for 2 min for drying the membrane, the plasmid DNA was eluted with 30 μl nuclease-free water. The plasmid DNA was ready to use in the next step.

4.14 Sequencing and sequences analysis

The plasmid was diluted to 200 ng with nuclease-free water and was sent for DNA sequencing at sequencing company service (1st BASE, Malaysia). Sequencing reaction was performed using the liquid polymer POP-7 on an ABI3730xl (96 Capillary) DNA analyzer (Applied Biosystems). The sequencing primers were listed in Table 18. The sequences were analyzed by Sequencer Scanner v1.1 program. The nucleotide fragments were aligned assembly and deduced to amino acid sequence using CloneManager 9.2 Program. The sequence was search for nucleotide and protein similarity with the nucleotide and protein sequences established in NCBI database using BLAST tools (Altschul et al., 1990; Altschul and Lipman, 1990; Altschul et al., 1997). Molecular mass and pI was calculated by a protein calculate tool access from web.expasy.org/translate/ and the protein localization was predicted by TargetP v1.1 program (www.cbs.dtu.dk/services/TargetP/). The deduced amino acid of each gene was submitted into the NCBI database.

Table 18 Sequence primers

vector	Sequencing primer name/sequence (5' → 3')
pGEM-T easy	T7-promoter-TAATACGACTCACTATAGGG
	SP6-terminator-GCTAGTTATTGCTCAGCGG
pET32a	T7-promoter-TAATACGACTCACTATAGGG
	T7-terminater- ATTTAGGTGACACTATAG

4.15 Phylogenetic analysis

Phylogenetic tree was performed by multiple alignment of the obtained sequences in this study with the sequences retrieved from NCBI database using ClustralX and the tree was constructed using the computer program, MAGA6 (Tamura et al., 2011; Tamura et al., 2013) based on the distance algolithimic neighbor-joining (NJ) method

(Saitou and Nei, 1987). Tree was evaluated using the bootstrap analysis with 1000 replicates (Felsenstein, 1992; Kuhner and Felsenstein, 1994).

4.16 IPTG induction of Protein Expression in *E. coli*

The optimal condition for recombinant protein expression is in LB broth. Separated colony was selected and inoculated into 5 ml LB broth containing 100 mg/ml ampicillin. The cell was incubated for 18 hr with 250 rpm shaking at 37°C. In the next day, the pre-cultured cell was transferred to 50 ml LB broth containing 100 mg/ml ampicillin (ratio 1:100 pre-cultured-cell: LB broth) in 250 ml flask. The flask was incubated at 37°C with 250 rpm shaking until the OD600 value reached to 1.

4.16.1 Cytochrome P450

One ml of cell suspension culture was transferred to a 1.5 ml microtube and labeled as the cell at hour 0 (H0). Isopropyl β-D-1-thiogalactopyranoside (IPTG) at the concentration of 1 mM, 0.5 mM, and 0.1 mM was added to induce protein induction. Cells were incubate at 30°C with 250 rpm shaking for the next hour and took 1 ml and labeled as H2, H3, H4, H5, H6, and overnight in 1.5 ml micro-tube. The optimal condition of the culture was used as the pre-culture from single colony was grow at 37°C for overnight and then inoculated with 0.5% v/v into 1 liter LB broth contained 100 µg/ml ampicillin at 37°C with 250 rpm shaking speed. Once, cell density at OD600 reach to 0.6, the bacteria cultures were induced by adding 1 mM IPTG and 0.005 µM Delta-Aminolevulinic Acid Stimulates (δ-ALA) for heme-binding protein expression, and continuous shaking at 37°C with 180 rpm until the OD600 equal to 1.0. The cultures were incubated at 4°C for 18 hours. After the incubation at 4°C, cell cultures were incubated and shake at 37°C with 180 rpm for 3 hr before cell harvesting and kept at -20°C until uses.

4.16.2 Cytochrome P450 Reductase

One ml of cell suspension culture was transferred to a 1.5 ml microtube and labeled as the cell at hour 0 (H0). Isopropyl β-D-1-thiogalactopyranoside (IPTG) at the concentration of 1 mM, 0.5 mM, and 0.1 mM was added to induce protein induction. Cells were incubate at 30°C with 250 rpm shaking for the next hour and took 1 ml and labeled as H2, H3, H4, H5, H6, and overnight in 1.5 ml micro-tube. The optimal condition of the culture was used as the pre-culture from single colony was grow at

37°C for overnight and then inoculated with 0.1% (v/v) into 1 liter LB broth contained 100 µg/ml ampicillin at 37°C with 250 rpm shaking speed until OD600 reach to 1. The cells were induced by 0.1 mM IPTG and grown at 30°C with 200 rpm shaking for over two nights (40 hr).

4.17 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cell suspension was centrifuged at 10000 rpm at room temperature for 3 min to remove supernatant. The cell pellets were resuspended in 100 µl of 2x loading and was boiled in boiling water for 5 min to make protein denatured then let the protein cooled down at room temperature. Ten microliters of the boiled protein were loaded onto 12% polyacrylamide gel against the pre-stained protein marker (cat. No. N7633, ThermoScience). The vertical electrophoresis running was at 120v for 1.30 hr in running buffer tank (BioRad). The gel was stained with 10 ml of Coomassie® Brilliant Blue G-250 (usb) (40% methanol, 10% acetic acid, 50% distilled water, 0.1% (w/v) Coomassie® Brilliant Blue G-250 (usb) dye for 3 min and de-stained with dH2O by gently shaking for 1 hr. the presented protein bands were analyzed and documentation using Chemi Doc XRS, BioRad.

4.18 Western blot

The proteins from SDS-PAGE gel were transferred to the polyvinylidene difluoride (PVDF) transfer membrane (PALL). The SDS-PAGE gel was equilibrated in transfer buffer for 10 min and also for PVDF membrane was pre-cultured in 100% methanol for 5 min and then equilibrated in transfer buffer for 10 min. the proteins were transferred from gel to PVDF membrane by electroblotting apparatus (BioRad Mini Protein II transfer apparatus) containing transfer buffer at 4°C and 100v for 1.30 hr as shown in the Figure 21.

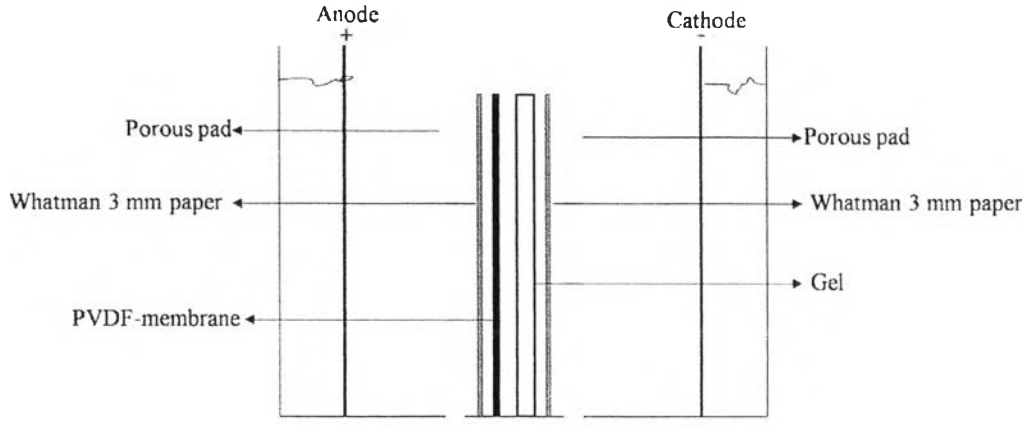

Figure 21 The alignment of the blot

The blot was blocked with 10 ml blocking buffer for 1 hr with gently agitate using a rocker platform. The membrane was washed with 10 ml TTBS buffer with gently agitation for 5 min for 3 times. The membrane was transferred into a tray containing 10 ml of 1:2000 AP-conjugated antibody (Alkaline Phospatase (AP)-conjugated Antibodies, Invitrogen) in dilution buffer and was incubated with gently agitation for 2 hr at room temperature. The membrane was washed three times with 20 ml of TTBS buffer and washed once with TBS buffer. The membrane was rinsed twice with the alkaline phosphatase buffer, then the 10 ml of the substrate solution was added and the reaction was incubate with gently agitation at room temperature for 10 min. The color development was stopped by washed the membrane in distilled water for 10 min, then air-dry membrane on filter paper.

4.19 Extraction of the expression protein

The cells were grown as described in Section 4.16.1 and 4.16.2 with the optimal condition of both cytochrome P450 and cytochrome P450 reductase.

4.19.1 Extraction for enzymatic reaction

Cells were collected by centrifugation at 3500 rpm, 4^{o}C for 15 min and washed by deionized water for 2 times before centrifugation at 20000 rpm at 4^{o}C for 20 min. The cell was resuspened in 50 mM Tris-HCl pH7.5 and 0.1 M NaCl and ready to use for enzymatic reaction.

4.19.2 Extraction for protein characterization

Cells were collected by centrifugation at 7000 rpm at 4°C for 20 min and washed by washing buffer (50 mM Tris-HCl pH7.4) and centrifuged at 4000 rpm at 4°C for 30 min. the cells were then lysated by re-suspended in breaking buffer (containing; 50 mM Tris-HCl pH7.5, 20 mM NaCl, 0.1% TritonX-100, 10% glycerol, and 0.2 mM PMSF) and disrupted by sonication (Sonic Vibra Cell™). the sonication condition was done for 6 cycles of 30 sec of 40% amplitude (5 sec pulse on and 9 sec pulse off) sonic probe. The sonicated-solution was incubated on ice for 30 min with 0.1% TritonX-100 (buffer: cell ; 3:1) to increase the soluble of the protein. The microsomes were collected by 17000 rpm at 4°C for 1 hr centrifugation. The microsomes were in the supernatant and ready for enzyme characterization in Section 4.22.3.

4.19.3 Purification of protein

The cells were transferred into a centrifuge tube and collected by centrifugation (5000 rpm at 4°C for 10 min). and was incubated on ice for 30 min. The cells were disrupted using the sonic probe (Sonic Vibra Cell™) with 6 cycles for 30 sec of probe pulse on for 5 sec and pulse off for 9 sec with 40% amplitute in the lysis buffer (Table 19) and was collected by centrifugation at 12000 rpm, 4°C for 20 min. The supernatant obtained after centrifugation was collected and designated as S1. The cell pellet was denatured with 2 ml of the denaturing buffer (Table 19) and was gently agitated on ice for 2 hr. The supernatant and pellet were collected as S2 and P, respectively, by centrifugation (12000 rpm, 4°C, 20 min). The supernatant containing solubilized protein was incubated with 1 ml Ni^{2+}-NTA raisin or 2 hr with the rotary mix at 4°C, then cells were applied into 3 ml column. The column was washed with wash buffer for 3 times, the protein then was eluted by elution buffer containing imidazole. The protein was determined for the protein content by Bradford method (BioRad) as will be described in Section 4.20 and pooled to dialysis. The protein was dialyzed in dialysis buffer for overnight, then the 25 volumes of the refolding buffer were added in the dialyzed protein and incubated at 4°C with shaking for 2 hr. Protein was concentrated by ultracentrifugation using the a Ultracel®-30K regenerated Cellulose 30000 NMWL (Amicon®Ultra centrifuge Filters).

Table 19 Lysis buffer, denaturing buffer, wash buffer, elute buffer, dialysis buffer, refolding buffer

Buffer	Lysis buffer	Denaturing buffer
Buffer I	40 mM Tris pH7.5, 120 mM NaCl, 0.5% TritonX-100	1%CHAPS, 40 mM Tris pH7.5, 120 mM NaCl, 0.5% TritonX-100
Buffer II	100 mM MOPS, 10% glycerol, 0.2 mM DTT, 1 mM EDTA	1%CHAPS, 100 mM MOPS, 10% glycerol, 0.2 mM DTT, 1 mM EDTA
Buffer III	50 mM Tris pH7.5, 1 mM PMSF, 5 mM β -ME, 1 mM EDTA	5M Urea, 50mM Tris-HCl pH7.5, 100mM EDTA
Buffer IV	20 mM sodium phosphate buffer pH7.4, 10% glycerol, 10 mM imidazole, 5 mM β -ME, 0.5M NaCl, 0.1% tritonX-100	1%CHAPS, 20 mM sodium phosphate buffer pH7.4, 10% glycerol, 5 mM β -ME, 0.5M NaCl, 0.1% tritonX-100
Buffer V	50 mM Tris-HCl pH7.6, 1 mM PMSF, 1mM EDTA, 10% glycerol, 0.5M NaCl, 5mM β -ME, 10 mM Lysozyme	1%CHAPS, 50 mM Tris-HCl pH7.6, 1mM EDTA, 10% glycerol, 0.5M NaCl, 5mM β -ME
Buffer VI	20 mM Tris-HCl pH7.6, 0.3M NaCl, 5 mM imidazole	1%CHAPS, 20 mM Tris-HCl pH7.6, 0.3M NaCl

4.20 Protein assay using Bradford method (BioRad)

The assay was done in micro-titer 96-well plate. BSA (bovine serum albumin) protein standard was prepared in five dilutions which range from 0.5, 0.25, 0.125, 0.0625, to 0.03125 mg/ml in triplicate. Ten micro-liters of each standard and sample solution were separately added in micro-titer plate. The diluted dye reagent (200 µl, prepare by 1 part of Dye Reagent with 4 parts of distilled water, filtered through a Whatman#1 filter to remove particles) was added to each well and mix. The micro-titer plate was incubated for 15 min at room temperature and measured for the absorbance at 595 nm. The linear equation was used to calculate the concentration of samples proteins.

4.21 Real-time Polymerase Chain Reaction (real-time PCR)

Total RNA was extracted by grinding the leaf under the liquid-nitrogen using the sterilized ceramic pestle-mortar and used a sterilized spatula was used to transfer the powder to the homogenized column. The homogenized leaf powder was then used for RNA isolation according to the Plant Mini RNase kit (QIAGEN) manufacturers' instruments (Section 4.1). Total-RNA was checked for quality and quantity as described in Section 4.2.3 and cDNA synthesis was performed as described in Section 4.3. The cDNA was diluted to 20 ng for the relative quantitative real-time PCR. The real-time PCR reaction contained 1X LightCycler480 SYBR Green I Master mix, 20 ng of the cDNA, and 1 mM of primer pair. The reactions were set in the LightCycler480 multiwell plate (Roche) with triplicate. The PCR condition was; one cycle: pre-incubation at 95°C 1 min, 44 cycles: amplification at 95°C 10sec, 55°C 30s, 72°C 5 sec, one cycle: melting curve at 65°C 5 sec, 95°C 5 sec. The relative mRNA expression was done with 8-target genes, namely, the 1-Deoxy-D-xylulose 5-phosphatesynthase (DXS), 2C-methyl-D-erythritol4-phosphate (MEPS), geranylgeranyl diphosphate synthase (GGPPS), geranylgeranyl diphosphate phosphatases (GGPPP), cytochrome P450-reductase (CPR), gernylgeraniol-18-hydroxylase (GG18H), and 18S rRNA as a reference gene and gDNA as a positive. The experiment was done in triplicate for each gene. The reaction was done in the real-time thermal cycler (BioRad). The relative expression fold was analyzed by using CFX manager program (BioRad).

4.22 Enzymatic reaction

4.22.1 Geranylgeraiol-18-hydroxylase (GG18H)

The crude-recombinant protein was tested the *in vitro* activity. One hundred microliters (50-250 µg) of the crude recombinant protein were added in the plaunotol enzymatic reaction assay mixture (300µl) containing: 83 mM tricine pH7.8, 0.8 mM NADPH, and 57 mM GGOH as substrate. The reaction was incubated at 30°C for 18 hr (Figure 22) and extracted with ethyl acetate. The extraction was dried under vacuum at 37°C for overnight and then resuspended in 5 µl ethyl acetate and then spotted onto the TLC plate.

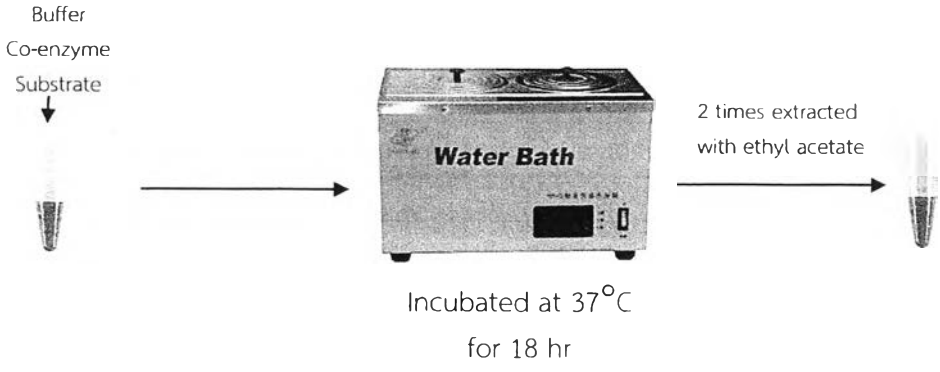

Figure 22 Enzymatic reaction

4.22.2 Cytochrome P450 Characterization

The characterization of the enzyme, CsGG18H, was done based on the method described by Omura group (Omura and Sato, 1963; Omura and Sato, 1964a; Omura and Sato, 1964b) and Guengerich group (Guengerich et al., 2009). Crude cytochrome p450 enzymes (from Section 4.19.2) were diluted into 1 ml with 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 20% glycerol, 0.5 % sodium cholate, and Triton-X100 in a 2 ml microtube, mix by inverting the tube for several times as a control. Other tube the same mixture as a control added 1 mg dithionite, mix by inverting tube and the measured the spectrum for the 420 nm spectrum of the cytrochrome P450. The tube was slowly bubble carbon monoxide (CO) gas through sample from a clean Pasteur pipette as shown in Figure 23. Immediately place the sample into the spectrophotometer and record for the spectrum between 400-500 nm.

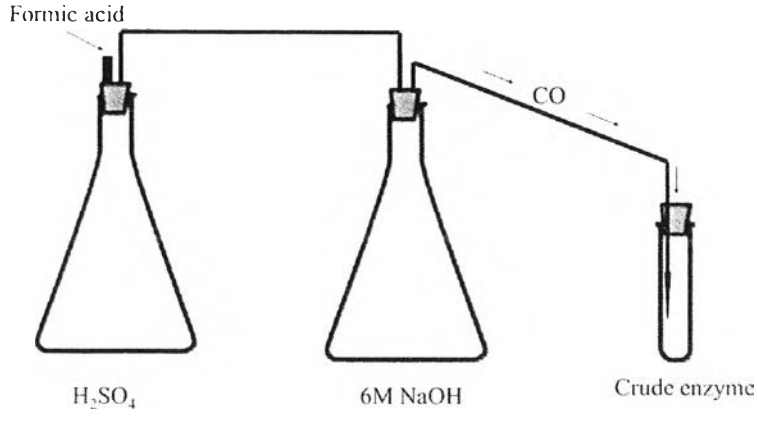

Figure 23 Carbon monoxide (CO) generated apparatus.

4.22.3 Cytochrome P450 reductase (CPR)

The characterization of the enzyme, CsCPR, was done based on the method described in Guengerich et. al. (Guengerich et al., 2009). Crude CPR enzyme (38 ng) (from Section 4.19.2) was used in the 250 µl enzymatic reaction containing 300 mM potassium phosphate buffer pH7.7 and NADPH with various concentrations. For steady stage of NADPH, the concentration of NADPH varies from 20, 40, 80, 100, and 120 µM and fixed the cytochrome c at 50 µM. For Steady stage of cytochrome c, the concentration of cytochrome c varies from 1.875, 3.75, 7.5, 15, 20, 30 µM and fixed NADPH at 120 µM. The reaction was done in the 96-well plate and monitored by increase of the absorbance at 550 nm at 25°C. The absorbance was measured in spectrophotometer (AD200, BeckmanCoulter). The reduction of the cytochrome c (Equine Heart, Calbiochem) was evaluated by measuring the absorbance at 550 nm. Rate of the reduction was calculated by extinction co-efficeincy 28000 $M^{-1}cm^{-1}$ of cytochrome c and 6,220 $M^{-1}cm^{-1}$ of NADPH. The kinetic constants of the Km and Vmax were calculated with non-linear least square algorithm in KaleidaGraph® software.

4.23 Thin layer chromatography (TLC)

The spotted TLC plate, then developed into the saturated TLC twin-trough chamber, which pre-equilibrated with a mobile phase. The plate allowed the mobile phase reached to the 90 mm developing distance, then removed from the chamber and immediately dried to stop the chromatography process. Visualized and documentation of the chromatogram was done under 254 nm UV light. Densitometry scanning at a specific spectrum scanning was used to identify characterization (Table 20).

Table 20 TLC mobile phase

Mobile phase system	Derivatized	Spectrum characterization (nm)
Toluene:Acetonitite:Ethyl acetate:gracial acetic acid (35:5:15:0.15)	I_2	210

4.24 Enzymatic reaction product identification

Products of the enzymatic reaction removed from the TLC plate into the 10 ml of methanol in vial to dissolve the product from the silica gel. The products were then passed through the Whatman No.1 (0.45 μM) filter paper and vacuum dried. Products of the reaction were identified using the Liquid Chromatography-Electron Spray Ionixation–Mass Spectrometry (LC-ESI-MS). Product from the reaction was analyzed by liquid chromatography coupled mass spectrometry. LC-MS analysis was performed on Agilent1200 series (Bucker Micro-TOF) in positive ionization mode with turbo ion spray interface. The product was analyzed in an isocratic elution system with methanol: water (8:2 for diterpenoid and 6:4 for monoterpenoic). The assay product dissolved in methanol was separated on gel with TSKgel® Super-ODS column (TOSOH, Japan) using helium as carrier gas at flow rate of 0.8 ml/min for diterpenoid and 0.4 ml/min for monoterpenoid. The separation conditions were

followed: injection split ratio, 20 µl; injection temperature, 35°C; and hold for 20 min. The MS date was collected from 0-500 m/z.

4.25 Plaunotol contents

The leaves of the *C. stellatopilosus* plant were ground under liquid nitrogen using sterilized ceramic pestle-mortar into powder. The powdery were homogenized with the 10 ml methanol into the glass tube and refluxed at 80°C for 1 hr (Figure 24). The refluxed solution was filtered through 1 mm Whatman filter paper into new glass tube and adjusted the volume back to the 10 ml. The solution was dried with the speed vacuum and recovered into 1 ml with methanol. Ten microliters of solution were spotted onto the TLC silica gel 60 F254 aluminium plate and done as described in Section 4.23.

Figure 24 Pluanotol extract by reflux at 80°C