CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials

Young leaves of *Alangium lamarckii* were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.2 General chemicals, media and equipments

3.2.1 Agarose (AFFYMETRIX)

3.2.2 DNA markers (GENERULER 1kb, FERMENTAS) (Appendix A)

3.2.3 TAE buffer (AMRESCO)

3.2.4 Ethidium bromide (BIORAD)

3.2.5 TLC plate (TLC silica gel 60 $_{F254}$, MERCK)

3.2.6 Water for PCR (Ultra-pure water grade, conductivity 18 megohms, autoclaved before use)

3.2.7 Enzymes

- 3.2.7.1 Restriction enzymes (FERMENTAS)
- 3.2.7.2 Taq DNA polymerase (PLATINUM *taq*, INVITROGEN)
- 3.2.7.3 Reverse transcriptase (FERMENTAS)
- 3.2.7.4 DNA Ligase (NEB)
- 3.2.7.5 RNase A (AMRESCO)

3.2.8 Authentic standards

- 3.2.8.1 Taraxasterol (CHEM FACES)
- 3.2.8.2 β-amyrin
- 3.2.8.3 ψ-taraxasterol
- 3.2.8.4 Lupeol
- 3.2.8.5 Friedelin

3.2.9 Organic solvent

- 3.2.9.1 Hexane analytical reagent grade (BURDICK&JACKSON)
- 3.2.9.2 Methanol analytical reagent grade (BURDICK&JACKSON
- 3.2.9.3 Acetone analytical reagent grade (CARLOERBA)
- 3.2.9.4 Acetronitrile HPLC grade (BURDICK&JACKSON)
- 3.2.9.5 Ethanol analytical reagent great (RCI LABSCAN)

3.2.10 Other chemicals

- 3.2.10.1 ρ-anisaldehyde (PANREAC SINTESIS)

3.2.10.2 Concentrate sulphuric acid (MERCK)

3.2.10.3 Glacial acetic acid (LABSCAN)

3.2.10.4 Potassium hydroxide (RIEDEL-DE HAEN AG SEELZE-HANNOVER)

3.2.10.5 Sodium hydroxide (MERCK)

3.2.10.6 Potassium dihydrogen phosphate (BAKER ANALYZED)

3.2.10.7 Dipotassium hydrogen phosphate (BAKER ANALYZED)

3.2.10.8 Magnesium chloride

3.2.10.9 Calcium chloride

3.2.11 DNA, strain, oligonucleotide

3.2.11.1 Oligo DNA for PCR primers (FERMENTAS)

3.2.11.2 dNTP (NEB)

3.2.11.3 PYes2 vector (INVITROGEN) (Appendix C)

3.2.11.4 E. coli DH5α (*F endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ*80dlacZ*Δ*M15*Δ*(lacZYA-argF)U169, hsdR17*($r_K^- m_K^+$), λ–)

3.2.11.5 Yeast GIL77 (*gal2 hem3-6 erg7 ura3-167*)

3.2.12 Kits

3.2.12.1 Gel extraction kit (REAL GENOMICS)

3.2.12.2 Plasmid extraction kit (REAL GENOMICS)

3.2.12.3 Frozen-EZ Yeast Transformation II™ (Zymo Research)

3.2.12.4 pGEM-T Easy vector (PROMEGA) (Appendix B)

3.2.13 Equipments

3.2.13.1 Agarose gel electrophoresis (MUPID-exu SUBMARINE ELECTROPHORESIS SYSTEM)

3.2.13.2 Gel documentation system (CHEMIDOC MP SYSTEM FOR IMAGING AND ANALYSIS, BIO-RAD)

3.2.13.3 PCR (MYCYCLE, BIO-RAD)

3.2.13.4 Centrifuges

3.2.13.4.1 High speed refrigerated microcentrifuge (EPPENDORF CENTRIFUGE 5H7R)

3.2.13.4.2 High speed refrigerated centrifuge (ROTOR JA-14 and JA-20, BECKMAN COULTER, AVANTI J-E CENTRIFUGE)

3.2.13.5 Larminar air flow cabinet (ESCD CLASS II BSC)

3.2.13.6 pH meter (EUTECH INSTRUMENTS pH510)

3.2.13.7 Autoclave (TOMY MODEL SX700)

3.2.13.8 Shaker and incubator (MODEL WIS-20R, DAIHAN SCIENTIFIC)

3.2.13.9 Incubator

3.2.13.10 Vertex

3.2.13.11 Rotary evaporator (BUCHI)

3.2.13.12 SUPER-ODS column (4.6 x 200 mm, TOSOH)

3.2.13.13 TLC (CAMAG)

3.2.13.14 HPLC (SHIMADZU)

3.2.13.15 LC-APCIMS (AGILENT 1200 SERIES FOR LC AND MICROTOF, BRUKER FOR MS)

3.2.14 Media

3.2.14.1 LB (AFFYMETRIX)

3.2.14.2 Agar (MERCK)

3.2.14.3 Drop out mixed without uracil and leucine (BIOLOGICAL)

3.2.14.4 Leucine (SIGMA)

3.2.14.5 Yeast nitrogen base without amino acids (DIFCO)

3.2.14.6 Hemin (SIGMA)

3.2.14.7 Tween80 (BIO BASIC)

3.2.14.8 Ergosterol (WAKO)

3.2.14.9 5-bromo, 4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal)

3.2.14.10 Ampicillin sodium salt (SIGMA)

3.2.14.11 Glucose (BIO BASIC)

3.2.14.12 Galactose (BIO BASIC)

3.3 General techniques

3.3.1 Sterilization

3.3.1.1 The conditions for autoclaving were 121°C with a pressure 15 psi for 20 mins.

3.3.1.2 The sterilization for heat labile drugs or mediums was performing using 0.2 µm filter with aseptic technique.

3.3.2 Media and solution preparations

3.3.2.1 Luria-Bertani (LB)

LB broth was prepared by dissolving 2 g LB powder in 100 ml deionized water. The solid medium was prepared by adding 1.5% w/v bacto-agar. X-gal and ampicillin were added to obtain the final concentration 20 µg/ml and 100 µg/ml respectively before being used.

X-gal stock solution was prepared at 20 mg/ml by dissolving 20 mg X-gal (5-bromo, 4-chloro-3-indolyl-P-n-galactopyranoside) in 100 ml dimethylformamide. Store at -20°C.

Ampicillin stock solution was prepared at 100 mg/ml by dissolving 100 mg ampicillin in 100 ml water and sterilized using 0.2 µm filter with aseptic technique. Store at -20°C.

3.3.2.2 Yeast extract peptone dextrose (YEPD)

YEPD is a complete medium for growing yeast containing of 1 g bacto-yeast extract, 2 g bacto-peptone and 2 g glucose dissolving in deionized water 100 ml and sterilised by autoclaving. Ergo-tween stock solution was added 20 ml to YEPD before used.

3.3.2.3 Synthetic complete minus uracil (SC-U)

SC-U consisted of 0.67 g bacto-yeast nitrogrn base without amino acids, 0.2 g drop-out mix minus uracil and 2 g glucose dissolving in deionized water 100 ml and sterilized by autoclaving. The solid medium was prepared by adding 2% w/v bacto-agar. 2% v/v ergo-Tween and 1% v/v hemin stock solution were added to liquid and agar medium before being used.

Drop-out mix minus uracil contained amino acids following the ingredients [44].

Alanine	2.0 g	Methionine	2.0 g
p-Aminobenzoic acid	0.2 g	Phenylalanine	2.0 g
Arginine	2.0 g	Proline	2.0 g
Asparagine	2.0 g	Serine	2.0 g
Aspartic acid	2.0 g	Threonine	2.0 g
Cysteine	2.0 g	Tryptophan	2.0 g
Glutamic acid	2.0 g	Tyrosine	2.0 g
Glutamine	2.0 g	Valine	2.0 g
Glycine	2.0 g	Adenine	0.5 g
Histidine	2.0 g	Inositol	2.0 g
Isoleucine	2.0 g	Uracil	Absent
Leucine	4.0 g	Lysine	2.0 g

3.3.2.4 Synthetic complete minus uracil minus glucose (SC-U-G)

SC-U consists of 0.67 g bacto-yeast nitrogrn base without amino acids and 0.2 g drop-out mix minus uracil dissolving in deionized water 100 ml and sterilized by autoclaving.

3.3.2.5 Potassium phosphate buffer (KPB)

The buffer KPB was prepared by mixing of 0.1 M potassium dihydrogen phosphate solution (KH_2PO_4) and dipotassium hydrogen phosphate solution (K_2HPO_4) and adjusting the pH to 7.0. The buffer was sterilized by autoclaving.

3.3.2.6 20% galactose and 20% glucose solution

20% galactose and 20% glucose were prepared from 20 g of galactose and 20 g of glucose dissolved in water qs 100 ml and sterilized by using 0.2 µm filter with aseptic technique.

3.3.2.7 Ergo-tween stock solution

Ergo-tween stock solution was prepared by dissolving 20 mg ergosterol and 5 ml Tween80 in 15 ml ethanol.

3.3.2.8 Hemin stock solution

Hemin stock solution was prepared by dissolving 65 mg hemin chloride in 25 ml 0.01 N sodium hydroxide (NaOH) and 25 ml ethanol.

3.3.2.9 Solutions for plasmid extraction using alkaline lysis method

3.3.2.9.1 Solution I

Solution I consisted of 50 mM glucose, 10 mM EDTA and 25 mM Tris. Autoclaving was used for sterilization.

3.3.2.9.2 Solution II

Solution II consisted of 0.2 N NaOH in 1% SDS

3.3.2.9.3 Solution III

Solution III consisted of 50 ml 5 M potassium acetate (KOAc), glacial acetic acid 11.5 ml and water 28.5 ml

3.3.3 Agarose gel electrophoresis

The concentration of agarose gel was up to the size of DNA (0.8-1%). The gel was prepared by dissolving agarose in 1X TAE buffer and heating until all the powder was melt, but avoid boiling over. Then the gel solution was poured in a tray with combs for setting the gel.

RNA or DNA samples were dyed with gel loading dye before loading to the wells at ratio 1X loading dye:sample 1:5. The dye contained two dyes, xylene cyanol which approximate about 4000 bp and bromophenol blue which approximate about 300 bp, as markers during running the gel and glycerol to form the sample at bottom of the well.

Electrophoresis was carried out in 1X TAE buffer with supplied electric power 100 volt.

After running gel electrophoresis, the gel was stained by using ethidium bromide and visualized with UV light by using gel documentation system (BIO-RAD).

3.4 cDNA cloning of oxidosqualene cyclase gene from *Alangium lamarckii* leaves

3.4.1 RNA preparation

Total RNA was extracted from young leaves powder by using RNeasy Mini Kit (Qiagen) following the protocol.

1) Young leaves of *Alangium lamarckii* about 100 mg were frozen by using liquid nitrogen and ground to powder by using sterile mortar and pestle.
2) The ground plant powder was immediately put into 2 ml microcentrifuge tube and added 450 µl Buffer RLT (added 10µl ß-mercaptoethanol per 1 ml Buffer RLT) then vortex vigorously.
3) The lysate was transferred to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at 13,000 rpm (EPPENDORF CENTRIFUGE 5H7R). The supernatant was transferred

to a new microcentrifuge tube, and then added 0.5 volume of ethanol (95-100%) and mixed by pipetting immediately.

4) The sample was all transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged at 12000 rpm for 15 s. The flow through was discarded.
5) 700 µl of Buffer RW1 was added to the RNeasy spin column to wash the column membrane and was centrifuged at 12,000 rpm for 15 s. The flow through was discarded.
6) 500 µl of Buffer RPE was added to the RNeasy spin column to wash the spin column membrane and was centrifuged at 12,000 rpm for 15 s. The flow through was discarded.
7) 500 µl of Buffer RPE was added to the RNeasy spin column to wash the spin column membrane and was centrifuged at 12,000 rpm for 2 min to dry the column membrane.
8) The RNeasy spin column was then placed in a new 2-ml microcentrifuge tube and was centrifuged for 1 min at 13,000 rpm to eliminate any residual Buffer RPE in the spin column.
9) Place the RNeasy spin colum in a new 2-ml microcentrifuge tube and added 30 µl of RNase-free water before centrifuging for 1 min at 12000 rpm to elute the RNA. The concentration of the extracted RNA was ~1 µg/µl according to the protocol. The extracted RNA was checked by running on gel electrophoresis supplied electric power 70 volt. and visualized by UV exposed EtBr-RNA binding (Appendix D).

The extracted RNA was treated to remove genomic DNA (Fermentas) before doing reverse transcription polymerase chain reaction (RT-PCR) following the protocol. The reaction was composed of RNA 1 µg (1µl), 1 µl 10X reaction buffer with $MgCl_2$, 1 µl Dnase I, RNase-free and DEPC-treated water to 10 µl. The reaction was incubated 30 min at 37°C. The reaction was stopped by adding 3 µl of EDTA and incubated 10 min at 65°C.

3.4.2 First strand cDNA synthesis by RT-PCR

The RNA was used for cDNA synthesis using reverse transcriptase (Fermentas) the following protocol. The reaction was first set in a sterile, nuclease-free tube consisted of 2 µl RNA template, 1 µl 100 µM

oligo$(dT)_{18}$ and DEPC water qs. to 12.5 µl. The reaction was incubated at 65°C for 5 min to and immediately chilled on ice. The following components were added to the tube, 4 µl 5X reaction buffer, 0.5 µl RNase inhibitor (Ribolock™, FERMENTAS) 2 µl 10mM dNTP and 1 µl reverse transcriptase. The reaction was incubated for 60 min at 42°C and inactivated for 10 min at 70°C.

3.4.3 PCR amplification

Full-length AlOSCs were amplified from *Alangium lamarckii* leaves cDNA by using primers, forward primer ALB_f (5'-ATGTGGAGGCTGAAAGTAGCAGAAGG-3') and reverse primer ALB_r (5'-TCAGAGCCTCTGGGGAGGGAACTGAA-3') designed based on ALB, a putative OSC *Alangium lamarckii* leaves [45], by using Clone Manager (Scientific and Education Software, USA). A proper condition for PCR reaction consisted of the following components.

10X PCR Buffer	5	µl
50 mM $MgCl_2$	1.5	µl
10 mM dNTP	1	µl
Forward primer (ALB_f)	1	µl
Reward primer (ALB_r)	1	µl
cDNA template	2	µl
taq DNA Polymerase	1	µl
Ultra-pure water for PCR qs.	50	µl

The mixture was mixed well by gently pipeting. The PCR reaction was performed under cycling condition for 35 cycles:

Predenaturation	94°C	3	min
Denaturation	94°C	30	sec
Annealing	55°C	45	sec
Extention	72°C	2.5	min
Final extention	72°C	10	min

The resulting PCR product was separated on electrophoresis gel. The band at 2.3 kb (Appendix E) was extracted by using gel extraction kit (REAL GENOMICS). The extracted PCR product was cloned into pGEM-T vector (PROMEGA) and transformed into *E. coli* DH5α for plasmid propagation.

3.4.4 Plasmid ligation

The total reaction 10 µl was mixed of 3µl PCR product, 1 µl T-vector 5 µl, ligation buffer and 1 µl ligase enzyme (PROMEGA). The reaction was incubated at 4 °C overnight.

3.4.5 *E. coli* competent cell preparation and plasmid transformation

Fresh competent *E. coli* preparation using calcium chloride and plasmid transformation using heat shock was applied from the standard protocol [46].

1) A single colony of E. coli was picked from a subculture plate (LB agar plate) and grown in 3 ml LB-broth.
2) The 500 µL overnight culture was taken and transferred into 50 ml LB-broth in 250-ml erlenmeyer flask. The flask was incubated at 37°C with shaking at 250 rpm until the cell concentration reached OD_{600} 0.6 that would take approximate 2.5 hr.
3) The contents were transferred to a sterile, precooled 50-mL tube, and placed on ice for 30 min.
4) The cells were collected by spinning at 5,000 rpm, 4°C for 7 min, carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
5) The cells were suspended in 25 ml of cold 0.1 M $MgCl_2$, collected by spinning at 5,000 rpm, 4°C for 7 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
6) The cells were suspended in 25 ml of cold 0.1 M $CaCl_2$, collected by spinning at 5,000 rpm, 4°C for 7 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
7) The cells were suspended in 25 ml of cold 0.1 M $CaCl_2$ placed on ice for 60 min, collected by spinning at 5,000 rpm, 4 °C for 7 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
8) The cells were suspended in 1 ml of 0.1 M $CaCl_2$ in 15% glycerol of each tube for this modified protocol. The cells were then

dispensed aliquot 109 µl into 1.5-ml sterile, precooled microcentrifuge tubes.

9) Ligated plasmids were added 5 µl to each tube, swirl gently and stored on ice at least 30 min.
10) Heat shock transformation was performed by transferring the tubes on the rack into a water bath at 42°C for 90 sec then rapidly moved into the ice and stored for 5 min.
11) For cells recovering period, 900 µl LB-broth was added to each tube. The cells were then incubated and shaken at 37°C, 250 rpm for 60 min.
12) Each entire transformation cells were spread all on each LB-ampicill-X-gal agar plate by centrifuging to collect the cells at 10,000 rpm for 1 min and discarded the broth, the trace amount of broth at the buttom of the tube would be approximate 100 µl to resuspend and spread all on the agar plate. Each plate was spreaded until the medium was dry and inverted the plate for incubating at 37°C for 12-16 hr.

3.4.6 Plasmid extraction and sequencing

The transformed plasmids were extracted from *E. coli* cells following the applied standard method and purified by using plasmid extraction kit (REAL GENOMICS) following the protocol. The purified plasmids were sent for sequencing at 1st Base Company, Singapore. The sequencing was carried out with pGEM-T sequencing primers (M13 Forward Sequence – 5'-CACGACGTTGTAAAACGAC-3', M13 Reverse Sequence 5'-GGATAACAATTTCACACAGG-3') and seq_ALB_f forward primer - 5' GTATTCTGGGCCGATCACA 3'

Alkaline lysis standard method was applied for plasmid extraction.

1. White colonies were picked individually and grown into separate sterile tubes containing 5 ml ampicillin added LB medium. The picked colonies were shaken at 37°C overnight.
2. The cells from each sample were transferred in a 2-ml microcentrifuge tube for collecting the cell by centrifuging at 10,000 rpm for 1 min and discarded the medium.
3. 100 µl of solution I was added and resuspended the cells by

vertex.

4. 200 µl of solution II was added, and the tubes were inverted gently 5 times and stand the tubes on ice.
5. 150 µl of solution III was added and inverted the tubes immediately. A white precipitate from bacteria was formed in this step.
6. The tubes were centrifuged at 10,000 rpm for 10 min.
7. The supernatants were transferred to new 1.5-ml microcentrifuge tubes.
8. 1 µl of 10 mg/ml RNase was added to each tube and incubated at 37°C for 10 min.
9. Isopropanol was added for 2 volumes and mixed well by inverting the tubes.
10. The tubes were frozen at -20°C for 20 min and then centrifuged at 4°C, 12,000 rpm for 30 min to precipitate the plasmids. Discarded the isopropanol.
11. The pellets were washed for twice by adding of 800 ml 70% ethanol, gently inverted the tube, and discarded the ethanol.
12. 900 ml absolute ethanol (95-100%) was added for the last wash, carefully inverted the tube not to let the plasmid slip from the bottom of the tube. The ethanol was discarded. The tubes were respinned at 12,000 rpm for 1 min to collect the remaining ethanol at the bottom of the tubes. The remaining ethanol was discarded by pipeting the ethanol out, and the tubes were dried in incubator at 40°C for 5-10 min.

After plasmid extraction, the extracted plasmid was checked for insertion by using *EcoR*I restriction enzyme and gel electrophoresis.

The reaction for enzyme digestion was consisted of 2 µl 1X FastDigest Green buffer, 2 µl plasmid in nuclease free water, nuclease free water to 20 µl and 1 µl *EcoR*I for the last. The reaction was incubated at 37°C for 30 min. The reaction was then run on gel electrophoresis checking for insertion that should have a band near 2.3 kb (Appendix F).

3.5 Functional expression of OSC genes from *A. larmarckii* in yeast

3.5.1 Preparation of PCR products for p-YES2 ligation

Each plasmid-insertion was used as the templates for PCR amplification by using modified forward and reverse primers. Forward primers, ALB-Hin-F-TGG2 (5'-GTACTGAAGCTTHAMAMAATGTGG-3') and ALB-Hin-F-TCC2 (5'- GTACTGAAGCTTHAMAMAATGTCS-3'), were introduced 6 extra bases, a restriction enzyme site and a yeast consensus sequence following protocol suggestion (INVITROGEN), and reward primer, ALB-Xba-R2 (5'-GATCTATCTAGAGAGCCTCTGGGGAGGGAACTGAA-3') was introduced restriction enzyme site and 6 extra bases. The expected PCR product would contain of the 6 extra bases for complete digestion at 5' and 3' sites, enzyme restriction sites at 5' and 3' sites and yeast consensus sequence at 5' site to get a suitable sequence for pYES2 ligation and expression in yeast. During the PCR product sequencing, the primers were designed because of there are no *Hind*III restriction site on the multiple cloning site of pGEM-T vector and just in case of AlOSC sequences allowed to be prepared sticky end by using *Hind*III. By the way, the sequencing results showed that AlOSCs contained *Hind*III restriction site on the sequences. However, there are *Not*I restriction site on the multiple cloning site of pGEM vector, and it was not found on ALOSC sequences.

PCR product preparation for ligation with pYES2 vector was set as the following condition.

10X PCR Buffer	5	µl
50 mM $MgCl_2$	1.5	µl
10 mM dNTP	1	µl
Forward primer	1	µl
Reverse primer	1	µl
pGEM-ALOSC	1	µl
taq DNA Polymerase	1	µl
Ultra-pure water for PCR qs.	50	µl

The mixture was mixed well by gently pipeting. The PCR reaction was performed under cycling condition for 35 cycles:

Predenaturation	94°C	3	min

Denaturation	94°C	30	sec
Annealing	55°C	40	sec
Extention	72°C	3	min
Final extention	72°C	10	min

The resulting PCR product was separated on electrophoresis gel. The band at 2.3 kb was extracted and purified by using a gel extraction kit (REAL GENOMICS). The extracted PCR product was cloned into pGEM-T vector (PROMEGA) and transformed into *E. coli* DH5α for plasmid propagation and for complete restriction enzyme digestion. The extracted plasmids were cut for insertion checking with *Hind*III and *Xba*I restriction enzymes. The plasmids that contained the expected insert at about 2.3 kb size and pYES2 vector were digested with *Not*I and *Xba*I restriction enzymes to get the suitable stricky end for ligation. (Figure III-1).

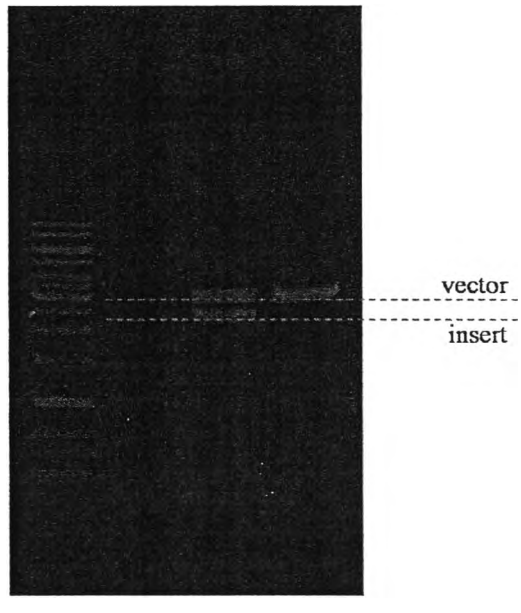

Figure III-1 electrophoresis gel under UV light of the plasmid that contained expected insert after cut-checking with *Hind*III and *Xba*I restriction enzymes was cut with *Not*I and *Xba*I restriction enzymes to get the suitable stricky end and sequence to ligate with pYES2. The size of p-GEM was 3015 bp. The band of insert was cut from the gel and purified by using gel extraction kit (REAL GENOMICS).

3.5.2 Ligation of pYES2-AlOSC plasmid and plasmid preparation for yeast transformation

The known sequence AlOSC-pGEM plasmids were double digested with *Not*I and *Xba*I restriction enzymes (FERMENTAS) and ligated with pYES2 vector (INVITROGEN) that was also cut by the same restriction enzymes.

Double digestion with restriction enzymes was set as the following protocol. 2 µl 1X FastDigest Green buffer, 2 µl plasmid in nuclease free water, nuclease free water to 20 µl and enzymes for the last (each 0.5 µl of *Not*I and *Xba*I) were subsequently mixed. The reaction was incubated at 37°C for 30 min and inactivated at 80°C for 5 min by using a PCR machine. The reaction was then run on gel electrophoresis for separating the digested insert The separating insert DNA and digested p-YES2 vector were extracted and purified by using a gel extraction kit (REAL GENOMICS).

The plasmid construction was performed by using ligase enzyme (NEB) following the protocol. The reaction was composed of 2 µl 10X T4 DNA Ligase Buffer, 3 µl pYES2 vector, 1 µl insert DNA, nuclease free water to 20 µl and 1 µl T4 DNA Ligase for the last. The reaction was mix by pipetting and spinned down shortly. The reaction was incubated at 16 °C for 19 hr and heated to inactivate at 65 °C for 10 min using a PCR machine (MYCYCLE, BIO-RAD). After overnight incubating, 10 µl of the reaction was transformed into 109 µl *E. coli* DH5α competent cells for propagation. 4 white bacterial colonies were picked from each plate due to the various yeast consensus sequences. The plasmids were grown at 37°C overnight. The plasmids were then extracted with alkaline lysis method and checked for insertion using *Hind*III and *Xba*I restriction enzymes (Figure III-2). The plasmids that contained the expected inserts were purified using a plasmid extraction kit (REAL GENOMICS) for transforming into yeast. All the picked samples were labeled as mentioned in Table III-1. The group of samples was divided to 2 groups according to yeast consensus sequence that introduced to forward primers, ALB-Hin-F-TGG2 and ALB-Hin-F-TCC2. The amino acid after start codon, TGG, encode for aromatic amino acid tryptophan. Changing the TGG to other codon would effect to protein structure and expression.

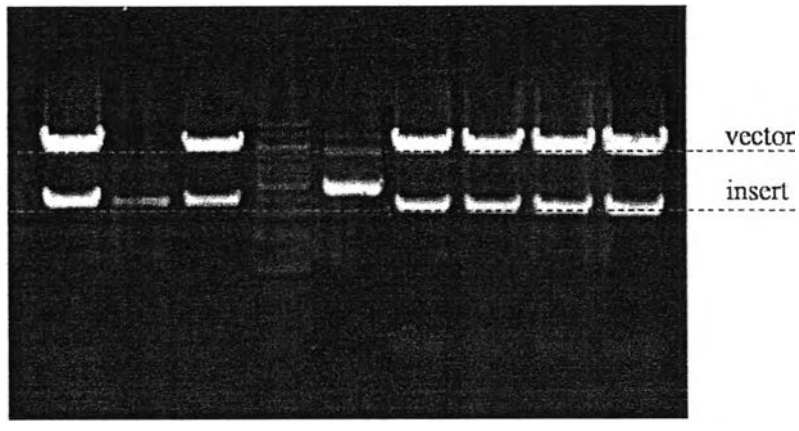

Figure III-2 insertion check of pYES2-AlOSC plasmid using *Hind*III and *Xba*I restriction enzymes. pYES2 vector size was 5.9 kb and AlOSC size was 2.3 kb.

Table III-1 Number labeled samples that separated to codon changing group

	Codon changing							
	TCC				TGG			
AlOSC1	1	11	16	21	6	26	31	36
AlOSC3	2	12	17	22	7	27	32	37
AlOSC4	3	13	18	23	8	28	33	38
AlOSC5	4	14	19	24	9	29	34	39
AlOSC6	5	15	20	25	10	30	35	40

E. coli competent cells preparation for pYES2-AlOSC transformation was applied from the routine standard method for a proper transformation condition.

1. A single colony of *E. coli* was picked from a subculture plate (LB agar plate) and grown in 3 ml LB-broth.
2. The overnight culture was taken 500 µL and transferred into 50 ml LB-broth in a 250-ml erlenmeyer flask.
3. The flask was incubated at 37°C with shaking at 250 rpm until the cell concentration reached OD_{600} 0.6 that would take approximate 2 hr. The contents were transferred to a sterile, precooled 50-mL tube, and place on ice for 30 min.
4. The cells were collected by spinning at 3,500 rpm, 4°C for 15 min, carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
5. The cells were suspended in 30 ml of cold O.1 M $MgCl_2$, placed on ice for 30 min, collected by spinning at 3,500 rpm, 4°C for 15 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
6. The cells were suspended in 25 ml of cold 0.1 M $CaCl_2$, placed on ice for 30 min, collected by spinning at 3500 rpm, 4°C for 15 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
7. The cells were suspended in 12.5 ml of cold 0.1 M $CaCl_2$, collected by spinning at 3500 rpm, 4 °C for 15 min, and carefully poured off the broth and inverted the tube on tissue paper to drain the last traces.
8. The cells were suspended in 1 ml 0.1 M $CaCl_2$ and 1ml 30% glycerol of each tube and placed on ice at least 20 min before used.
9. The cells were dispensed aliquot 109 µl into a 1.5-ml sterile, precooled microcentrifuge tubes. Ligated plasmids were added 10 µl to each tube, swirl gently and stored on ice at least 30 min.
10. Heat shock transformation was performed by transferring the tubes on the rack into a water bath at 42°C for 90 sec then rapidly moved into the ice and stored for 5 min.

11. For cells recovering period, 100 µl LB-broth was added to each tube. The cells were incubated and shaken at 37°C, 250 rpm for 60 min.
12. Each entire transformation cells were spread all on each LB-ampicill-X-gal agar plate. Each plate was spread until the medium was dry and inverted the plate to incubate at 37°C for 12-16 hr.

3.5.3 Transformation and functional expression of cDNA in yeast

Yeast competent cell preparation and yeast transformation were performed by using Frozen-EZ Yeast Transformation II™ (ZYMO RESEARCH).

1. Yeast culture in YEPD was taken at 200 µl to 20 ml YEPD supplemeted with 400 µl ergosterol-tween stock solution and incubated with shaking at 30°C, 220 rpm for 2 days as the starter.
2. 100 µl of the starter was taken per one sample preparation. The cells were collected by centrifuging at 2,500 rpm for 4 min using a high speed microcentrifuge (EPPENDORF CENTRIFUGE 5H7R) and discarded the medium.
3. 100 µl EZ1 solution was added to each tube and mixed gently. The cells were collected by centrifuging at 2,500 rpm for 4 min and discared the supernatant.
4. 10 µl EZ2 solution were added to each tube and mixed gently to make competent cells. The vector transformation was set by following components, 1 µl transform vector, 10 µl competent cell and 100 µl EZ3 solution, then the transformed cells were incubated and shaked at 30°C, 90 rpm for 90 min and mixed vigorously every 30 min by lightly flicking the tubes.
5. The mixture was spread all on SC-U agar plate supplemented with hemin and ergosterol-tween stock solution. The plates were incubated at 30°C for 2 days.

Small scaled of yeast culture for expression screening was performed as the following protocol [32].

1. One colony of yeast was picked up and cultured as a stock culture at 3 ml SC-U supplemented with 200 µl hemin and

400 µl ergosterol-tween stock solution and incubated with shaking at 30°C, 220 rpm for 2 days.

2. 200 µl from stock culture was taken and transferred to 20 ml SC-U supplemented with 200 µl hemin (13 µg/ml) and 400 µl ergosterol-tween stock solution (20 µg/ml) and incubated with shaking at 30°C, 220 rpm for 2 days.
3. The cells were collected by centrifuging at 3000 rpm for 6 min, and the medium was discarded. 5 ml SC-U without glucose was added to resuspend the cells for washing. The cells were centrifuged at 3,000 rpm for 6 min and discarded the medium.
4. 18 ml SC-U without glucose was added supplemented with 2 ml 20% galactose for inducing, 200 µl hemin and 400 µl ergosterol-tween stock solution. The cells were incubated with shaking at 37°C, 220 rpm for 1 day.
5. The cells were collected by centrifuging at 3,000 rpm for 6 min, and the medium was discarded.
6. 5 ml potassium phosphate buffer pH 7.0 (KPB) was added to resuspend the cells for washing. The cells were centrifuged at 3,000 rpm for 6 min and discarded the medium.
7. 10 ml KPB was added supplemented with 1.76 ml 20% glucose and 111 µl hemin. The cells were incubated with shaking at 37°C, 220 rpm for 1 day.
8. Yeast extraction was carried out after cell culture and inducing by collecting the cells at 3,000 rpm for 6 min and discarding the medium.
9. 1 ml 20% potassium hydroxide (KOH) in 50% ethanol was added to resuspend the cells (the cells changed to be very viscous) and transfer to a new test tube, and 1 ml 20% KOH was added again to rinse and transfer all the cells to the test tube.
10. The cells were boiled at 100°C for 5 min.
11. 2 ml hexane was added for partition. The hexane yeast extracts were concentrated and checked for triterpene production on TLC plate.

3.5.4 OSC product analysis

The crude yeast extracts were spotted on a TLC plate and double developed using hexane:acetone 19:1 as a mobile phase compared to the triterpene mono-alcohol standards, β-amyrin, and triterpene ketone standard, friedelin. The TLC plate was then visualized by spraying with anisaldehyde-sulphuric acid reagent (AS) and heated at 100°C for 5 min. The interesting yeast samples were chosen to scale up for product detection. The crude extract of scaled up samples were separated on TLC glass plates (Figure III-3). The band of the large scaled yeast extracts that has corresponding Rf to the standard β-amyrin was scratched as fraction F1 and to the standard friedelin was scratched as fraction F2. The scratched silica F1 and F2 of each yeast sample were eluted by using acetone. The eluates were evaporated and redissolved with hexane. The fractions were applied to LC-APCIMS (Agilent 1200 series, Bruker MicrOTOF) using a SUPER-ODS column (4.6 x 200 mm, Tosoh) eluted with 95% acetronitrile as solvent (flow rate 1 ml/min, temperature 40°C) compare to the standards. All triterpene mono-alcohols (MW=426) gave peak ion at *m/z* 409 $[M+H-H_2O]^+$ in APCI mode due to loss of H_2O at A ring. Retention time and LC-APCIMS extracted ion chromatogram (EIC) at *m/z* = 409-411 were used to compare triterpene mono-alcohol product and authentic triterpene mono-alcohols. Retention time and LC-APCIMS EIC at *m/z* = 425-430 were used to compare triterpene ketone product and and authentic triterpene ketone.

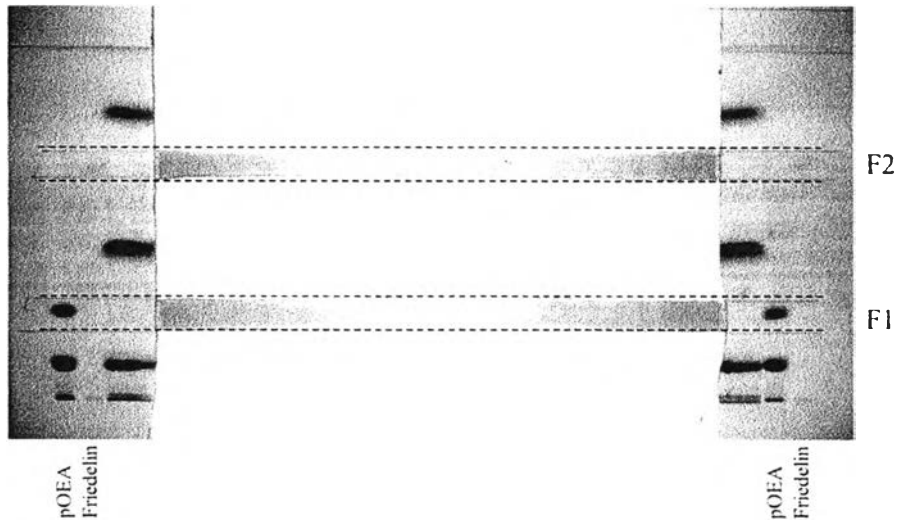

Figure III-3 The crude yeast extract was separated by double developing on a TLC glass plate. The corresponding Rf to the standards, β-amyrin and friedelin, after developing were scratched as F1 and F2, respectively.

3.6 Triterpenoid analysis in *Alangium larmarckii* leaves

3.6.1 Sample preparation

The leaves of *A. lamarckii* were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The leaves were dried at 50°C and grounded to powder for extraction.

3.6.2 Compound isolation and detection

The dried and powdered leaves 9.78 g were macerated with methanol 200 ml, 2 days, 3 times. The macerate was then evaporated to give the crude methanolic extract 958.7 mg. The crude methanolic extract was redissolved in aqueous methanol and partitioned with hexane to give the extract 584 mg. The hexane extract was redissolved with chloroform and spotted on TLC glass plate and double developed using hexane:acetone 19:1 as a mobile phase compared to the standards β-amyrin and friedelin. The TLC glass plate was visualized by spraying with anisaldehyde-sulphuric acid reagent (AS) and heated at 100°C for 5 mins. The band of the leaves extract that has corresponding Rf to the standard β-amyrin was scratched as f1 and to the standard friedelin was scratched as f2 (Figure III-4). The scratched silica f1 and f2 were eluted by

using acetone. The eluates were evaporated and redissolved with hexane for checking on TLC after elution by using the same condition. The TLC plate was visualized by spraying anisaldehyde-sulphuric acid reagent (AS). The fractions were applied to LC-APCIMS (Agilent 1200 series, Bruker MicrOTOF) using a SUPER-ODS column (4.6 x 200 mm, Tosoh) eluted with 95% acetronitrile as solvent (flow rate 1 ml/min, temperature 40°C). Retention time and LC-APCIMS EIC at *m/z* = 409-411 were used to compare triterpene mono-alcohol product and authentic triterpene mono-alcohols. Retention time and LC-APCIMS EIC at *m/z* = 425-430 were used to compare triterpene ketone product and authentic triterpene ketone.

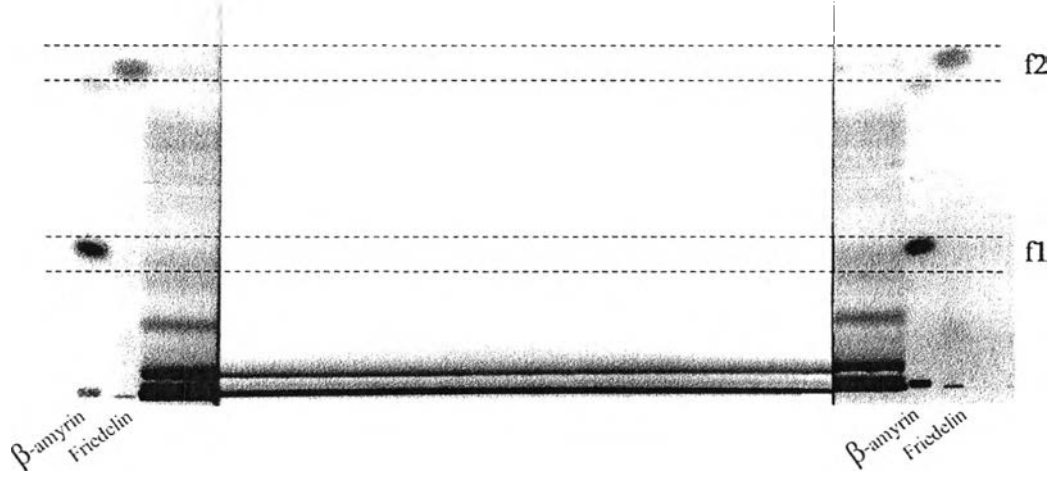

Figure III-4 The crude leaves extract from hexane part was separated by double developing on a TLC glass plate. The corresponding Rf to the standards, β-amyrin and friedelin, after developing were scratched as f1 and f2 respectively.