CHAPTER 2
MATERIALS AND METHODS

2.1 Plant Materials

Young leaves of 4 years old of *Croton stellatopilosus* were collected from the horticulture of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus. The leaves were immediately frozen in liquid nitrogen and stored at -80 °C.

2.2 Equipments and Materials

2.2.1 Equipments

Gas Chromatography (GC) system: HP 6850 Series, Hewlett Packard, USA
Column: HP1 Methylsiloxane, Hewlett Packard, USA
Oven: Hewlett Packard, USA
Detector (Flame Ionization Detector, FID): Hewlett Packard, USA
Injector: Agilent technology 7683B Series

Gel documentation	Gel Doc model 1000, BIO-RAD, USA
Gene amplification	GeneAmp, PCR system 9600, Perkin Elmer, USA
Instruments	
autoclave	Model HA-3D, Hirayama, Japan
balances	Explorer, Ohaus, USA
	Avery Berkel, USA
	Sartorius TE 3102S, USA
centrifuges	Hermle Z 323 K, Germany; Kubota 5922, Japan
electrophoresis	Mupidα Mini electrophoresis system, Japan
hot air oven	Memmert, Germany
hot plate and stirrer	Fisher Scientific, USA
incubating block	Thermomixer comfort, Eppendorf, Germany
laminar air flow cabinet	HT-122 ISSCO, Australia
micropipettes	0.1-2.0 µl, 2-20 µl, 20-200 µl, 100-1000 µl, Socorex, Switzerland
microwave oven	LG, Thailand
pH meter	pH meter Model 710A, Benchtop, Germany
refrigerators	For 4°C, Sanden Intercool, Thailand; for -20°C, Whirlpool, Thailand; Deep-freezer (-80°C), Forma Scientific, USA

rotary evaporator	EYELA, Japan
speedvac	SC210A, Savant, USA
UV-Transluminator (312 nm)	Camag, USA
UV-VIS spectrophotometer	Labomed, Inc., USA
vortex	Vortex-Genie 2, USA
waterbath	Memmert, Germany

2.2.2 Chemicals

The authentic plaunotol was kindly provided from Dr. Natsajee Naulkaew, Faculty of Pharmaceutical Sciences, Khon Kaen University. The reagents used for molecular biology were biotechnogical grade. Solvents used in this study were analytical grade. All chemicals and solvents are listed below.

Chemicals

acetic acid	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
agarose	Research Organics,USA
agar (Bacto)	Himedia laboratories, Ltd., India
ampicillin	Bio Basic INC, Canada
casein	Himedia laboratories, Ltd., India
chloroform ($CHCl_3$)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
dimethylformamide	Bio Basic INC, Canada
DNA markers	Sib-enzyme, Russia 2-Log DNA Ladder (0.1-10.0 kb) NEB (New England Biolabs), UK
ethanol (EtOH)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
ethidium bromide	Bio Basic INC, Canada
ethylenediaminetetraacetic acid (EDTA.4Na)	Pharmacia Biotech, Sweden
isoamylalcohol	Merck, Germany
isopropyl-β-D-thiogalactopyranoside (IPTG)	United State Biological, USA
methanol (MeOH)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
sodium chloride (NaCl)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
tris-(hydroxymethyl) amino	Research Organic, Inc., USA
tryptone	Himedia laboratoried Pvt, Ltd., India
yeast extract	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
5-bromo-4-chloro-3-indolyl-β-	

D-galactopyranoside (X-gal)	USB cooperation, USA

2.2.3 Kits

A-addition kit	Qiagen, Germany
Gel extraction kit	Qiagen, Germany
GFX Micro Plasmid Prep Kit	Amersham Biosciences, UK
PCR cloning kit	Qiagen, Germany
PCR purification kit	Qiagen, Germany
RNeasy Plant Mini Kit	Qiagen, Germany
SuperScript™III RT	Invitrogen, USA
taq PCR core kit	Qiagen, Germany

2.2.4 Enzymes

*Eco*RI (EC 3.1.23.13)	TaKaRa, Japan
taq polymerase (EC 2.7.7.7)	Qiagen, Germany

2.2.5 *Escherichia coli* strains

Characteristics of *E.coli* strains were described as followed and used as host for gene cloning.

Table 2.1 *E.coli* strains used in this study

***E.coli* strains**	**Characteristic**
TOP10	F^-, *mcr*A, Δ(*mrr-hsd* RMS-*mcr*BC), ϕ80*lac*Z, ΔM15, Δ*lac*X74, *rec*A1,
Invitrogens, USA	*ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*G
XL1-Blue MRF'	F', :: Tn10, *proAB*$^+$, *lacI*^{q}, Δ(*lacZ*)M15, *rec*A1, *end*A1, gyrA96, (Nal^r),
Stratagene, USA	*thi*, *hsd*R17(r_k^-, m_k^+), *gln*V44, *rel*A1, *lac*

2.2.6 Vectors

Vectors used in this study were from Qiagen company, Germany. The pDrive vector was used for gene subcloning. The pDrive vector is supplied in a linear form, ready-to-use for direct ligation of PCR products. This vector allows ampicillin and kanamycin selection, as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids. The vector also contains a T7 and SP6 promoter on either side of the cloning site, allowing *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers. In addition, the pDrive cloning vector has a

phage f1 origin to allow preparation of single-stranded DNA. A map of the pDrive vector is provided in Fig. 2.1.

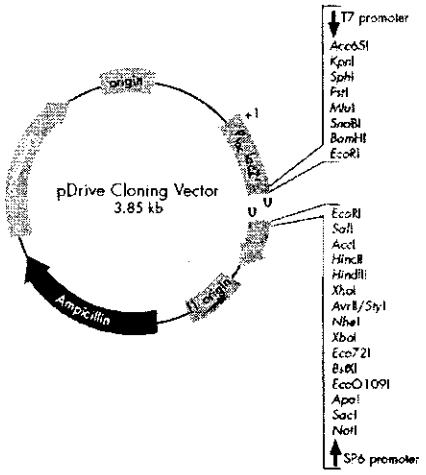

Figure 2.1 Representation of the linearized pDrive Cloning Vector with U overhangs.

2.2.7 Media preparation and solutions

Ampicillin (25 mg/ml)	Ampicillin sodium salt 2.5 g was dissolved in 25 ml distilled water. Fillter-sterilized and stored in aliquots at -20°C.
Ethidium bromide solution	Ethidium bromide 10 µl was dissolved in 100 ml distilled water.
IPTG (1M)	IPTG (isopropyl β-D-thiogalactopyranoside) 0.2 mg was dissolved in 1 ml sterile distilled water and stored in aliquots at -20°C.
LB (Luria-Bertani) medium	Contained 10 g casein hydrolysate, 5 g yeast extract and 5 g NaCl. Adjusted volume with distilled water to 1,000 ml and sterilized using autoclave.
LB agar	Contained 1 g NaCl, 1.5 g agar (Bacto) and 1 g tryptone. Adjusted volume with distilled water to 1,000 ml and sterilized using autoclave. When the temperature of the medium about 50 °C then added 200 µl ampicillin (25 mg/ml), 100 µl X-gal (20 mg/ml) and 10 µl IPTG (1 M). Poured the medium to plate (20 ml per plate) under laminar air flow cabinet.
Running buffer	Contained 20 ml TAE (x50) and adjusted volume with distilled water to 1000 ml
SOB medium	Contained 2 g bacto-tryptone, 0.5 g yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$.

	Adjusted volume with distilled water to 100 ml, mix these components and adjusted pH to 6.7-7.0 with NaOH and sterilized using autoclave.
TAE buffer (x50)	Contained 121 g Tris, 19.7 g EDTA.4Na and 35 ml glacial acetic acid. Adjusted volume with distilled water to 500 ml
TB buffer	Contained 10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl and adjusted pH to 6.7-7.0. Firstly, prepared without $MnCl_2$, sterilized $MnCl_2$ by filtration through filter 0.22 micron.
TE buffer	Contained 5 ml Tris-HCl, pH 8.0 (1 M) (10 mM final) and 1 ml EDTA (0.5 M) (1 mM final). Adjusted volume with distilled water to 500 ml and sterilized using autoclave.
X-gal (20 mg/ml)	X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyra-noside) 200 mg was dissolved in 10 ml dimethylformamide. Protect the solution from light by wrapping in aluminium foil or by using a brown bottle. Store at -20°C.

2.2.8 Primers

The oligonucleotides used in this study were supplied from Operon, Germany. The oligonucleotides were designed based on either from the previously reported amino acids homologous or from the DNA sequences as shown in Table 2.1

Table 2.2 Primers used in this study

Designated	T_m (°C)	Sequence
Degenerated primers		
P101S	56.30	5'-GCIGARAAYCCIGAYAARTT-3'
P157S	59.72	5'-ATHATHCCIGGIGARCARGG-3'
P193S	55.96	5'-ATHGARGCIGGIAARGAYAT-3'
P383A	48.75	5'-TAYTTIACRTTRTCIGG-3'
P297A	53.17	5'-TGIGCYTCDATIACYTC-3'
P274A	54.78	5'-GGRTGYTTIARIGCRTC-3'
I: Inosine, D: A+G+T, H: A+C+T, R: A+G, Y: C+T		
Specific primers for 5' and 3'-end cloning		
5'P_142A	58.66	5'-TGCAAGTGCCACCACTTTAAA-3'
5'P_180A	58.66	5'-TTCACCTGATCAGCAAGAAGA-3'
3'P_228S	58.66	5'-TGTTGTTACTGGTATAGTCGG-3'
3'P_374S	58.66	5'-GCTGCAATAGAAGCTGGAAAA-3'
RACE17	64.70	5'-GACTCGAGTCGACATCG-3'
RACE32	58.50	5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3'
Specific primers for full-length cloning		
PDXR-S1	53.07	5'-TTTTCCATTCTATCCCCA-3'
PDXRF-S2	63.08	5'-AATTGGGGTACCATGGCTCTTAATTTG-3'
PDXR-A1	53.07	5'-TATAACACCTATCCTCCA-3'
PDXR-A2	67.45	5'-TTGATGCTGCAGTCATGCAAGAACAGGAC-3'
Specific primers for semi-quantitative RT-PCR		
18s-0.5F	62.45	5'-CAAAGCAAGCCTACGCTCTG-3'
18s-0.5R	62.45	5'-CGCTCCACCAACTAAGAACG-3'
PDXRF-S2	63.08	5'-AATTGGGGTACCATGGCTCTTAATTTG-3'
5'P_142A	58.66	5'-TGCAAGTGCCACCACTTTAAA-3'

2.3 Methods

2.3.1 Total RNA extraction

2.3.1.1 Conventional method

For cDNA cloning, total RNA was isolated from *C. stellatopilosus* young leaves using phenol-chloroform method and lithium chloride (LiCl) precipitation (Sambrook *et al.*, 1989). Batch of total RNA extraction began with fifteen grams of young leaves, which were ground into powder in the presence of liquid N_2. Extraction buffer containing the mixture of 1 M Tris-HCl pH 8.0, 10% (w/v) SDS, PCI (phenol: $CHCl_3$: isoamylalcohol; 25:24:1) in the ratio of 10:9:1 was added. The homogenate was transferred into microcentrifuge tubes, centrifuged at 20000 xg rpm at 4°C for 10 min. The supernatant was deproteinized with 1/5 volume of PCI and extracted with 1/10 of volume CI ($CHCl_3$: isoamylalcohol; 24:1). The nucleic acid was precipitated from the supernatant using 1/10 volume of 3 M sodium acetate and 4 volume of absolute ethanol. The mixture was kept at -80°C for 1 h and then followed by centrifugation at 15,000 rpm for 10 min. The pellets were resuspended in DEPC water in minimum volume. The insoluble matter was got rid of by centrifugation. The total RNA was precipitated with 1 volume of 4 M LiCl and the solution was kept at 4°C for overnight. The total RNA pellets were successively centrifuged and the pellet was washed with 0.5 volume of chilled 70% (v/v) ethanol. After discarding the ethanol and short drying at room temperature under vaccuum, the pellets were resuspended in 100 µl of DEPC water. The total RNA was stored at -20 °C until used.

2.3.1.2 RNeasy Plant Mini Kit

For the study on mRNA expression of *dxr* gene using semiquantitative RT-PCR, total RNA was prepared from RNeasy Plant Mini Kit (2.2.3). According to manufacturer instruction, the plants tissue was ground into powder in the presence of liquid N_2. The powder was transferred to an RNase-free microcentrifuge tube, 450 µl buffer RLT was added, vortex vigorously. The lysate was transferred to QIAshredder spin column and centrifuge at 20,000 xg for 2 min. The supernatant of the flow-through was transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. A half volume of absolute ethanol was added to the clear lysate, mixed by pipetting and transferred to an RNeasy spin column, and centrifuged at 20,000 xg for 15 s. The flow-through discarded, 700 µl of buffer RW1 was added onto the RNeasy spin column and centrifuged at 20,000 xg for 15 s to wash the spin column membrane. 500 µl Buffer RPE was added and centrifuged at 14,000 rpm for 15 s. After drying the membrane by centrifuged at 20,000 xg for 1 min, the RNeasy spin column was removed and placed on a new microcentrifuge tube and 30 (l RNase-free water

was added. The total RNA was eluted after centrifuge at 20,000 xg for 1 min. The total RNA solution was stored at -20°C until used.

The concentration and purity of the total RNA (2.3.1.1-2.3.1.2) were measured by a spectrophotometer. The concentration of the total RNA was analyzed by dilution of the total RNA solution with distilled water to 30-50 times and determined the concentration using the A260 nm with equation as follow.

Concentration of total RNA (μg/μl) = (A_{260})(dilution factor)(40 μg/μl)

The purity of total RNA was analyzed by two methods: spectrophotometer analysis (A_{260}/A_{280}) and electrophoretic analysis. By spectrophotometer, the purity of total RNA was judged by the ratio of A_{260}/A_{280}, of which has a ratio of 1.6-1.8. The pattern of intact RNA was evaluated by agarose gel electrophoresis.

2.3.2 Synthesis of the first-strand cDNA

The first-strand cDNA of *C. stellatopilosus* was synthesized using the Superscript™III reverse transcriptase (2.2.3) and RACE32 primer (2.2.5). According to manufacturer instruction, the cDNA synthesis mixture contained solutions as followed.

	Volume	Final concentration
Total RNA	varied	10 pg-5 μg
RACE32 primer, 50 μM	1 μl	2.5 μM
dNTP mix, 10 mM each	1 μl	0.5 mM
Sterile distilled water	varied	to 14 μl

The solution was incubated at 65°C for 5 min then quickly chilled on ice for 1 min. 4 μl of 5x First strand buffer, 1 μl of 0.1 M DTT and 1 μl of Superscript™III RT were added into the solution, incubated at 50°C for 1 h. The reaction was inactivated by heating at 70°C for 15 min, afforded the cDNA solution. The resulting cDNA was stored at -20°C until used.

2.3.3 Polymerase chain reaction (PCR)

The DNA fragment was amplified using Taq PCR core kit (2.2.3). The standard mixture composed of primers (2.2.5) and cDNA template (2.3.2) according to the instruction manual.

Component (Master mix)	Volume/reaction	Final concentration
10x QIAGEN PCR Buffer	5 µl	1x
dNTP mix, 10 mM each	1 µl	200 µM of each dNTP
Primer A	varied	0.1-0.5 µM
Primer B	varied	0.1-0.5 µM
Taq DNA Polymerase	0.25 µl	2.5 units/reaction
Distilled water	varied	
Template cDNA	varied	≤1 µg/reaction
Total volume	50 µl	

2.3.3.1 Amplification of the core fragment

The core fragments were amplified by varied the pair of degenerated primers (2.2.5) and annealing temperature. The scheme of amplification and the thermal profile were performed as shown below.

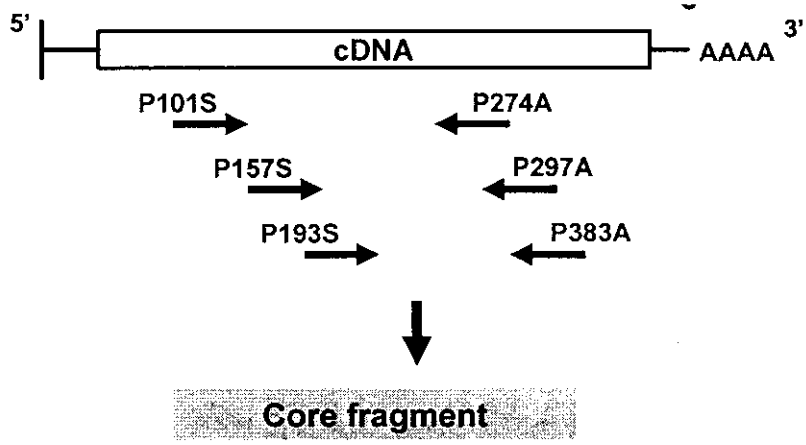

Figure 2.2 The core fragment amplification

PCR parameters for the core fragment amplification

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	95	3	1
2	Denaturing	95	1	35
	Annealing	50	2	
	Extension	72	3	
3	Extension	72	10	1
	Holding	4	∞	

2.3.3.2 Amplification of 5'- and 3'- ends

The fragment of 5'- and 3'-ends were amplified from the sets of specific primers (2.2.5). The scheme of amplifications and the thermal profile were performed as shown below. The A-addition (2.2.3) was required for 5'-end amplification.

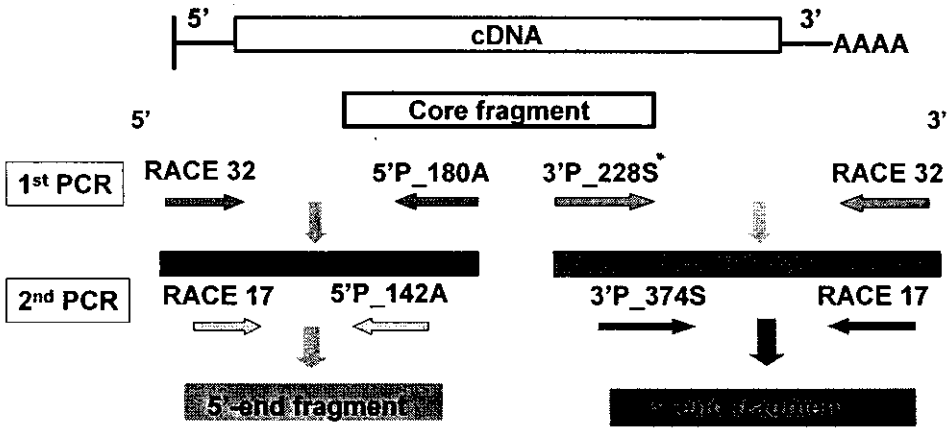

Figure 2.3 The 5'- and 3'- ends amplification

PCR parameters for the 5'- and 3'-end amplification

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	95	3	1
2	Denaturing	95	1	30
	Annealing	52 (1^{st})	2	
	Annealing	48 (2^{nd})		
	Extension	72	3	
3	Extension	72	10	1
	Holding	4	∞	

2.3.3.3 Amplification of the full-length *dxr* gene

By aligning and assembling the nucleotide sequences of the core fragment, 5'-end and 3'-end products, four specific primers for full-length gene amplified were designed (2.2.5). The scheme of amplifications and the thermal profile were performed as shown below.

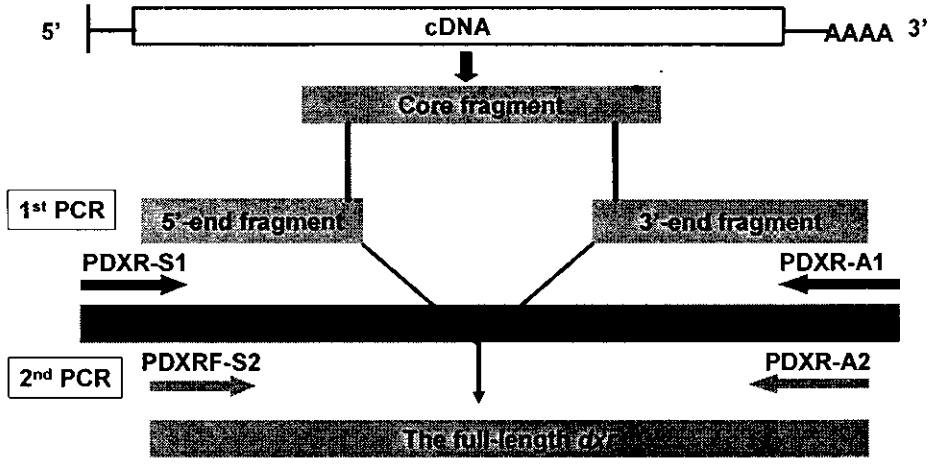

Figure 2.4 The full-length *dxr* gene amplification

PCR parameters for full-length of *dxr* amplification

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	95	3	1
2	Denaturing	95	1	40
	Annealing	45 (1^{st})	2	
	Annealing	58 (2^{nd})		
	Extension	72	3	
3	Extension	72	10	1
	Holding	4	∞	

2.3.3.4 Amplification of fragments for mRNA expression

Determination of the mRNA transcription levels using semiquantitative RT-PCR techniques. This was performed by conducting parallel reactions on each RNA sample: one using specific primers for *dxr* and the other using primer for a house-keeping gene (18S rRNA) (2.2.5). The scheme of amplifications and the thermal profile were performed as shown below.

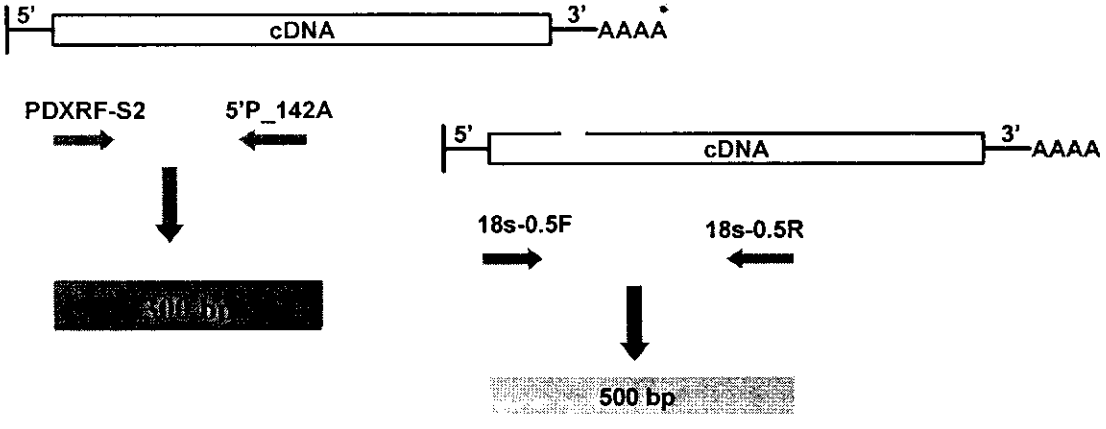

Figure 2.5 The partial DNA and 18S rRNA amplifications

PCR parameters for 18S rRNA amplification

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	94	3	1
2	Denaturing	94	0.5	35
	Annealing	58	0.5	
	Extension	72	0.5	
3	Extension	72	5	1
	Holding	4	∞	

PCR parameters for the partial DNA amplification

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	95	3	1
2	Denaturing	95	1	40
	Annealing	48	2	
	Extension	72	3	
3	Extension	72	10	1
	Holding	4	∞	

2.3.4 DNA cloning

2.3.4.1 Preparation of ultra-competent cell

Cells of *E.coli*, strain TOP-10 and XL1-Blue MRF' were streaked onto a LB agar plate containing 25 mg/ml ampicillin. The plate was incubated at 37°C overnight. A single colony of *E.coli* was picked up from this plate into 5 ml of LB medium in a 50 ml flask and shaken overnight at 37°C. This culture was then added to 250 ml SOB in the ratio of 1:50 and incubated at 25°C until the OD_{600} reached 0.4-0.6. The suspension 50 ml was transferred into two centrifuge tubes. After that two tubes were incubated on ice for 10 min. The cell pellet was harvested by centrifugation at 5,000 xg for 10 min at 4°C and washed with 10 ml of ice-cold TB and stored on ice for 10 min and centrifuge at 5,000 xg for 10 min at 4°C. The pellets were resuspended in 2 ml of ice-cold TB and DMSO. The cell suspension was aliquoted in a volume of 50 µl per tube and kept frozen at -80°C

2.3.4.2 Purification of DNA fragments

DNA fragments (2.3.3.1-2.3.3.3) were separated on 1.2% (w/v) agarose gel electrophoresis, excised the expected bands and purified on the gel purification kit (2.2.3). According to manual protocol, 3 volumes of buffer QG were added to 1 volume of the gel. The mixture was incubated at 50°C until the gel slice had completely dissolved. The sample was then applied to silica-gel membrane column, allowed to stand at room temperature for 1 min and centrifuged at 20,000 xg for 1 min. The column was washed by adding 0.75 ml of buffer PE to the column, left at room temperature for 5 min, and then centrifuged at 20,000 xg for 1 min. After drying the column, the DNA fragment was eluted with 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5), stand for 1 min and centrifuged at 20,000 xg for 1 min. For the 1st PCR fragment purification, the DNA fragment was purified directly using PCR purification kit (2.2.3) without gel separation.

2.3.4.3 Ligation

The purified DNA fragments were ligated to the vector using the PCR cloning kit (2.2.3). The ligation mixture contained the molar ratio of 5-10 times of the DNA fragment than the vector as follow. The ligation mixture was incubated at 16°C for 2 h and ready for transformation.

	Volume (µl)
DNA fragment	4
Vector	1
2x ligation master mix	5
Total volume	10

2.3.4.4 Transformation

The ligation mixture or plasmid DNA was transformed into the *E. coli* host (2.2.6) (Sambrook *et al.* 1989). A volume of 50 µl of competent cells was mixed gently with 5 µl of the ligation mixture or plasmid DNA. The mixture was left on ice for 30 min, placed at 42°C for 30 s and put on ice. The transformed cells were mixed with 250 µl of SOC medium and incubated at 37°C for 1 h with constant shaking. Finally, 150 µl of transformed culture was spread onto LB agar plate supplemented with 0.05 mg/ml ampicillin, 0.02 mg/ml X-gal and 0.1 mM IPTG. The transformed agar plate was incubated at 37°C for 16 h. In case of using the pDrive as vector, the transformant was selected by ampicillin LB-agar plate, and the

presence of the pDrive containing the insert was determined by screening of blue/white colonies using IPTG and X-gal.

2.3.5 Extraction of the recombinant DNA

The transformant was selected from agar plate and prepared for the overnight culture. A single bacterial colony was inoculated into 3 ml of LB medium containing 6 µl of 25 µg/ml ampicillin in 15 ml falcon tube and incubated at 37°C with vigorous shaking 200 rpm for 16 h. A plasmid DNA was isolated from 1.5 ml of overnight *E.coli* cells culture using GFX *Micro* Plasmid Prep Kit (2.2.3). According to manufacturer protocol, cell culture was transferred to a 1.5 ml microcentrifuge tube and centrifuge at 20,000 xg for 30 s to pellet the cells. The pellet was resuspended in 150 µl of solution I with vigorous vortexting and then 150 µl of solution II was added and mixed by inverting the tube 10-15 times. The protein was precipitated by adding 300 µl of solution III, mixed by inverting the tube until a flocculent precipitate appeared. The mixture was centrifuged at 20,000 xg for 5 min to precipitate cell debris and proteins. The supernatant was transferred to the GFX mini column (glass fiber matrix), incubated for 1 minute and centrifuged at 20,000 xg for 1 min. The column was washed by adding 400 µl of washing buffer and centrifuged at 20,000 xg for 1 min. The matrix was dried prior elution. Finally, the mini column was transferred to a fresh microcentrifuge tube and 100 µl of TE buffer was added directly to the top of the glass fiber matrix. After incubation for 1 min, the purified DNA was eluted with 100 µl of TE buffer by centrifuge at 20,000 xg for 1 min. The resulting DNA was stored at -20°C until use.

For restriction site analysis of pDrive containing the insert, the enzymatic mixture contained 5 µl of the recombinant DNA (2.3.5), 1 µl of 10x H buffer (TaKaRa), 0.5 µl of *Eco*RI, and adjusted volume to 10 µl. The solution was incubated at 37 °C for 2 h. The solution was loaded into 1.2% (w/v) agarose gel electrophoresis to analyze DNA fragments.

2.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze the PCR products. The 1.2% (w/v) of agarose gel was prepared (2.2.8). The mixture was boiled using microwave oven until cleared solution was obtained. The solution was poured into the tray and comb was placed in the agarose gel. The gel was placed at room temperature for 1 h for gel setting. The agarose gel tray was carefully removed and placed on the platform in the electrophoresis tank containing 1x TAE buffer. The RNA or DNA sample was mixed with loading buffer and slowly loaded into the slots of the submerged gel using the micropipette. Electrophoresis was carried out at a constant 50 V for 45 min. The gel was stained with ethidium bromide solution for 10

min. The resulting RNA or DNA pattern was observed under UV transluminator (312 nm) and the picture was developed.

Composition per one gel	For agarose gel (1.2% w/v)
Agarose	0.24 g
TAE (x50)	0.4 ml
dH_2O .	20 ml
Total volume	20 ml

2.3.7 DNA sequencing and sequencing analysis

The nucleotide sequence was analyzed at Bioservice Unit (BSU, Ratchathewi, Bangkok) using Dye Terminator Version 3.1 cycle sequencing kit. After amplification using the M13-forward and M13-reverse primers, the samples were precipitated with 75% (v/v) isopropanol and samples were separated in the Genetic Analyzer equipped with computer workstation Model 3100, Version 3.7 (ABI PRISM, Applied Biosystems 3730 DNA analyzer).

The nucleotide sequence analyses were carried out using DNASIS V3.5 software (Hitachi software engineering) and CLUSTAL W (1.82) (http://www.ebi.ac.uk/clustalw/). Comparative analyses of nucleotide sequences and deduced amino acid sequences were analyzed using BLAST programs at the websites: http:// www.ncbi.nlm.nih.gov and http://cn.expasy.org. The comparison of sequences was conducted through databases and alignment by using Gene Doc program (Nicholas *et al.*, 1997).

TargetP program: http://www.cbs.dtu.dk/services/TargetP was used to predict for the chloroplast transit leader sequence (Nielsen *et al.*, 1997; Nielsen *et al.*, 1999). The phylogenetic analysis was performed with CLUSTAL W (1.82) using default parameters. A phylogenetic tree was constructed using MEGA version 3.1 (Kumar *et al.*, 2001). The neighbor-joining method was used to construct the tree (Saitou and Nei, 1987).

2.3.8 Gel documentation

The %volume of band intensity on agarose gel was determined by gel documentation. After staining agarose gel with ethidium Bromide solution, picture was developed and band intensity was measured. For blank, empty gel was integrated and substracted to the sample. The relative intensity was calculated as a ratio of intensity of sample and intensity of standard DNA.

2.3.9 Extraction and quantitative analysis of plaunotol

The plaunotol content was determined according to Vongchareonsathit 1998. Samples including shoots, 1^{st}-5^{th} of leaves, stems and roots were dried at 50°C for overnight in a hot air oven and ground into powder. The sample, 200 mg, was refluxed in 10 ml of absolute ethanol for 1.5 h, filtered. The filtrate, 2 ml, was evaporated to dryness using the Speedvac. The residue was dissolved in 2 ml of 50% (v/v) ethanol/water in the presence of 0.4 ml of 10% (w/v) NaOH solution, gentle heated for 30 min. The solution was partitioned with 3 ml of *n*-hexane for 3 times. The *n*-hexane fractions were pooled and evaporated to dryness. The dried residue was re-dissolved in 500 µl of *n*-hexane and centrifuged at 20,000 xg for 1 min. The clear solution was ready to analyze the plaunotol content by gas chromatography (GC) (Vongchareonsathit, 1998).

The calibration curve was constructed using the authentic plaunotol (2.2.2). The stock solution of the authentic plaunotol was prepared in volumetric flask to obtain 3 mg/ml stock solution (30 mg of plaunotol in 10 ml of *n*-hexane). The stock solution was diluted by half-dilution technique and the concentration range of 0.01-0.5 µg/µl was constructing the calibration curve of plaunotol.

Quantitative determination of plaunotol was performed using the GC method.

Gas chromatographic condition

Column	HP1 Methylsiloxane size 30 m, 0.32 mm x 0.25 µm fused silica capillary HP-6850 GC-Hewlett Packard, USA
Detector	Flame Ionization Detector (FID), Hewlett Packard, USA
Inlet temperature	Splitless, 220°C
Oven temperature	235-280°C, gradient 15°C/min
Injector temperature	220°C
Nitrogen carrier gas	15 ml/min
Hydrogen supply	30 ml/min
Air supply	300 ml/min
Sample size	1 µl

Temperature profile

Temperature (°C)	Time (min)
235	1
235-280	2.33
280	2.5