CHAPTER III

Material and Methods

3.1 Plant materials

The calli of *Artocarpus lakoocha* Rox. were induced on WPM medium containing 1 mg L^{-1} of 2,4-dichorophenoxyacetic acid (2,4-D) and 1 mgL^{-1} benzyladenine (BA) with 2% sucrose. According to Maneechai, all cultures were grown at 25 ^{o}C in the dark (17 days) to induce the production of secondary metabolites (Maneechai, et al., 2012).

The *Clitoria ternatea* L. leaves were collected from planting area of department of biochemistry and microbiology, faculty of pharmaceutical sciences, Chulalongkorn University.

3.2 Bacterial Strains

Escherichia coli	
Stains	Genotype
DH5α (Invitrogen)	F-, Lambda-, *rec*A1, *end*A1-, *hsd*R17 (rK-, mK+), (*lac*ZYA-*arg*F), *sup*E44, U169, Φ80d*lac*ZΔM15, *thi*-1, *gyr*A96, *rel*A1
One shot TOP10 (Invitrogen)	F_i, mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZM15 ΔlacX74 deoR recA1 araD 139 Δ(ara leu)7697 galU galK rpsL (StrR) endA1 nupG
Agrobacterium tumefaciens	Strain GV3101 (Kan^{R}) carrying the helper plasmid pj19

3.3 Plasmids

pGEM®-T Easy Vector (Promega)	Cloning vector, Amp^R
pENTR™/D-TOPO® (Invitrogen)	Entry vector, Kan^R
pGWB6	Binary vector, Kan^R, GFP

3.4 Total RNA extraction

Total RNAs were purified from plants using the RNeasy Plant Mini Kit (Qiagen), according to the supplier's recommendations. One hundred milligram of plant samples were ground in liquid N_2 with mortar and pestle then homogenized with 450 µl RLT buffer and incubated at 56 °C for 2 – 3 min. The homogenized lysate was transferred into QIAshredder spin column and centrifuged at 13,000 rpm for 2 min. The flow-through was transferred to new tube. After the flow-through was mixed by pipetted with 250 µl of ethanol, that transferred to RNeasy spin column and centrifuged at 12,000 rpm for 15 sec. The RNA was washed with 700 µl RW1 buffer in column and centrifuged in the same condition. The RNA was cleaned again with 500 µl RPE buffer and centrifuged in the same condition for twice times. The RNA was dried by centrifuged at 12,000 rpm for 2 min prior to elute with 50 µl of RNase-free water. Total RNA was treated with DNase I, RNase-free DNase (Fermentas). The following components were added to reaction, 1 ug of total RNA, 1X reaction buffer with $CaCl_2$, 1 U of DNase I, RNase-free DNase and DEPC water up to 10 µl. After the reaction was incubated at 37 °C for 30 min, the 1 µl of 50 mM EDTA was added for inactivation reaction and incubated at 65 °C for 10 min. RNA concentration was calculated following the formula C (µg/µl) = OD_{260nm} × 40 × dilution factor. The ratio of the readings at 260 nm and 280 nm (A260 /A280) ranged from 1.8 to 2.1, indicates

that the purity of RNA. The concentration of RNA was checked by using spectrophotometer.

3.5 Synthesis cDNA

The cDNAs were then prepared using the RevertAid™ H Minus Reverse Transcriptase (Thermo scientific™) in the presence of RiboLock™ RNase Inhibitor (Thermo scientific). The reaction mixture containing 1 ug of total RNA, 5 µg of oligo (dT)18 and DEPC water up to 12.5 µl, was incubated at 65 °C for 5 min. Then the reaction was mixed with 1X reaction buffer, 0.5 µl RiboLock™ RNase Inhibitor, 1 mM dNTP mix (10 mM for each) and 200 U RevertAid™ H Minus Reverse Transcriptase, and incubated at 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 10 min.

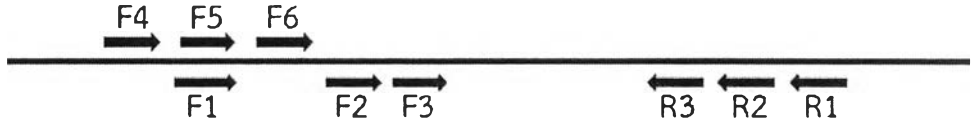
3.6 Determination of core sequences encoding PTases

The primers used in this study (Table 2) were designed based on the conserved regions of the aromatic PTases genes by multiple alignments using ClustalW (Chenna, et al., 2003; Larkin, et al., 2007). These primers were designed as degenerated primers using IUPAC codes to establish the ambiguous nucleotide (Wei, et al., 2003). The position and size of primers were shown in Table 3. The PCR of partial gene was performed with 1 µl cDNA in 50 µl reaction mixture consist of 1X High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM dNTP each, 0.2 µM of forward and reverse degenerate primers (Table 2) and 1U Platinum® Taq DNA polymerase High Fidelity. The reaction was subjected to thermal cycling according to the following PCR program in Table 4.

Table 2 Specific primers the determination of PTase core sequence.

primer name	Sequence (5'--> 3')
Prenyl-F1	AATCARHTRTNYGAYVTHGAAATAGACAA
Prenyl-F2	GDTTGTHGGTTCDTGGCCRTTRYTKT
Prenyl-F3	TYRATDTRCCNYTDTTGAGATGGAA
Prenyl-F4	GGHDTGAATCARYTRTNTGA
Prenyl-F5	TTGAAATWGACAAGRTHAARAARCC
Prenyl-F6	CTTCCAHTDGCATCTGGRRAAT
Prenyl-R1	AGCTTCCADATAAACATRTARAANG
Prenyl-R2	ATTTCAAAWAGNGHWAYACAARTCCA
Prenyl-R3	CCTTCNAYRTCDGGDATATCCTT

Table 3 The expected size PCR products of each pair of primers.



Primer	F1R1	F1R2	F1R3	F2R1	F2R2	F2R3	F3R1	F3R2	F3R3
Expected Size PCR product (bp)	674	502	418	544	372	283	479	309	224
Primer	F4R1	F4R2	F4R3	F5R1	F5R2	F5R3	F6R1	F6R2	F6R3
Expected Size PCR product (bp)	692	520	437	669	497	414	640	468	414

Table 4 PCR thermal cycling condition for amplification of the core sequence.

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	
Annealing	48 °C	40 sec	35
Extension	72 °C	1 min	
Finale extension	72 °C	5 min	1
Cold	4 °C	infinity	

3.7 Determination of full length gene by rapid amplification of cDNA ends (RACE)

The 5' and 3' end were done by using a 5'-RACE and 3'-RACE system for rapid amplification of cDNA ends, (Invitrogen, USA) according to the manufacturer's instructions (Figure 11). In 5'-RACE end, five microgram of total RNA was removed contaminate by treated with calf intestinal phosphatase (CIP) in the reaction mixture (10 µl) containing 1X CIP buffer, 40 U RnaseOut™, 10 U CIP. After incubated at 50 °C for 1 hour, the mRNA was extracted with 100 µl phenol:chloroform and centrifuged at 13,000 rpm for 5 min. The supernatant was transferred into new tube, and then mixed with 2 µl mussel glycogen, 10 µl 3 M sodium acetate, pH 5.2. The mixture was added 220 µl of 95% ethanol and mixed by vortex and then incubated at -20 °C for 10 minutes. The RNA was precipitated by centrifugation at 13,000 rpm at 4 °C for 20

min. The supernatant was discarded and RNA pellet was washed with 500 µl of 70% ethanol. After centrifuged, the supernatant was removed and the pellet was dry for 2 min prior resuspended in 7 µl DEPC water.

The dephosphorylated RNA was removed 5' cap by tobacco acid pyrophosphatase (TAP) in the reaction mixture containing 1X TAP buffer, 40 U RneaseOut™ and 0.5 U TAP followed by incubated at 37 °C for 1 hour. The recapped RNA was extracted and precipitated same as previous condition. The dephosphorylated and recapped RNA was ligated with the GeneRacer™ RNA oligo to 5'-end using T4 RNA ligase. The ligation reaction started by incubation RNA with 0.25 µg GeneRacer™ RNA oligo at 65 °C for 5 min and 1X ligase buffer, 1 µl of 10 mM ATP, 40 U RneaseOut™ and 5 U T4 RNA ligase were added into the mixture. After incubated at 37 °C for 1 hour, RNA was extracted and precipitated again.

The first stand of cDNA was amplified using ligated RNA as a template and the GeneRacer™ Oligo dT as primer. The reaction mixture (20 µl) containing 1.25 mM dNTP, 1X RT buffer, 15 U Cloned AMV RT and 40 U RneaseOut™ followed by incubated at 45 °C for 1 hour. The reaction was inactivated by heat at 85 °C for 15 min. For investigation full length at 5' and 3' end, the PCR was performed using primers were provided with the kit and gene specific primers (GSP) were designed using the partial gene sequence (Table 5). The RACE PCR reaction was performed follow in Table 6 and thermal cycling according to the following PCR program in Table 7.

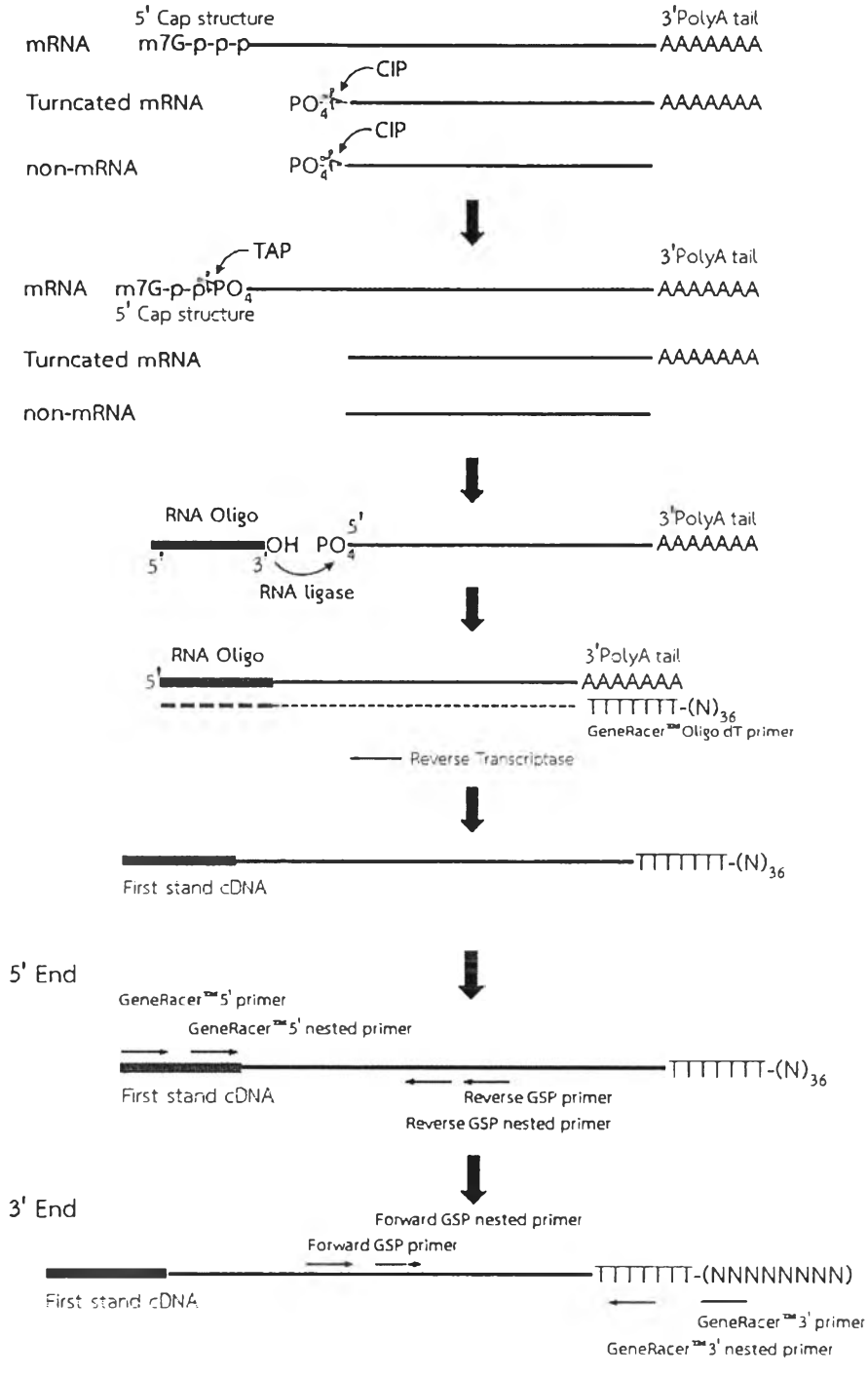


Figure 11 The strategies for RACE PCR.

Table 5 The specific primers for RACE PCR technique.

Primer name	Sequence (5'--> 3')
5' RACE	
CTin-R**	TGTACACATGGGTCTGCATGTGCAGGAAA
CTout-R*	GAGAAGAAGCTCATAAATGCAGTAGCAAAA
ALRin-R**	GACACCCGCTCGAATCGGAGCCTGGA
ALRout-R*	TAAAGAGGGCAGCAACCACAGCCTCCAG
3' RACE	
RACEin-F**	TTTCCTGCACATGCAGACCCATGTGTACAA
RACEout-F*	AGTTTTGTACTAGGAACTGCTTATTCAAT

* Primer for first RACE PCR and ** Primer for second RACE PCR

Table 6 The components of RACE PCR reaction.

First PCR		Second PCR	
Reagents		Reagents	
10 µM GeneRacer™ 5' or 3' primer	4.5 µl	10 µM GeneRacer™ 5' or 3' Nested primer	1 µl
10 µM Reverse or Forward GSP primer	1.5 µl	10 µM Reverse or Forward Nested GSP primer	1 µl
cDNA	1 µl	First PCR (dil 10X)	1 µl
10X High Fidelity PCR buffer	5 µl	10X High Fidelity PCR buffer	5 µl
dNTP Solution (10 mM each)	1.5 µl	dNTP Solution (10 mM each)	1 µl
Platinum® Taq DNA polymerase High Fidelity (5U/µl)	0.5 µl	Platinum® Taq DNA polymerase High Fidelity (5U/µl)	0.5 µl
50 mM $MgSO_4$	1 µl	50 mM $MgSO_4$	1 µl
water	35 µl	water	39.5 µl

Table 7 RACE PCR thermal cycling condition.

Step	Temperature	Time	Number of cycles
First PCR			
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	30 sec	5
Annealing	72 °C	30 sec	
Denaturation	94 °C	30 sec	5
Annealing	70 °C	30 sec	
Denaturation	94 °C	30 sec	
Annealing	60 °C	30 sec	25
Extension	68 °C	1 min	
Finale extension	68 °C	10 min	1
Step	**Temperature**	**Time**	**Number of cycles**
Second PCR			
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	30 sec	
Annealing	60 °C	30 sec	25
Extension	68 °C	1 min	
Finale extension	68 °C	10 min	1

3.8 Determination of full length gene for prenyltransferases genes

In order to confirmed the sequence of genes that obtained from RACE PCR. The sequence of contigs at 5' and 3' ends were aligned with partial gene and observed start codon at 5'-end and stop codon at 3'-end. Then the primers were designed from sequence of start codon and stop codon as showed in Table 8 and

also PCR reactions were performed in reaction mixture containing 1 µl cDNA, 1X High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM dNTP, 0.4 µM of forward and reverse primer and 1 U Platinum® Taq DNA polymerase High Fidelity. The reaction was subjected to thermal cycling according to the following PCR program in Table 9.

Table 8 The specific primers of full length gene.

Primer name	Sequence (5'--> 3')
CTFull-Fw	ATGGATTCGGTGCTCTATGGATCTT
CTFull-Rv	TCATCTAACATAAGGGAGGAGCAGGT
ALRFull-Fw	ATGGATTCTTTTCTTCTGGGTTCATTGAA
ALRFull-Rv	TCACCTAACGAACGGTATAAGTAGATACT

Table 9 PCR thermal cycling condition of full length gene.

Step	Temperature	Time	Number of cycles
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	30 sec	30
Annealing	60 °C	30 sec	
Extension	68 °C	1.30 min	
Finale extension	68 °C	10 min	1

3.9 Cloning of gene in pGEM®-T Easy Vector for sequencing

The PCR products from every process were ligated into pGEM®-T easy vector for checked the sequence. The cloning was carried out by set up a ligation reaction following in Table 10 and incubation overnight at 4 °C. The ligation product was

transformed into *E. coli* DH5α competent cell and screening by blue-white colonies selection on LB agar containing 50 μg ml^{-1} ampicillin, 40 μg ml^{-1} x-gal and 0.1 mM IPTG. A group of white colonies were picked and grown overnight in 5 ml LB broth containing 50 μg ml^{-1} ampicillin. The mixture was incubated overnight at 37 °C with shaking at 250 rpm. These white colonies were confirmed the insert gene by extraction plasmid using alkaline lysis method and restriction digestion analysis. In addition to sequencing, the plasmid was extracted with Presto™ Mini Plasmid Kit (GeneAid) and sequencing by universal primer in pGEM®-T easy vector (M13 Forward: 5' GTAAAACGACGGCCAGT 3' and M13 reverse: 5' GCGGATAACAATTTCACACAGG 3').

Table 10 The components of ligation reaction for pGEM®-T Easy Vector.

Reaction Component	Standard reaction
pGEM®-T Easy Vector (50ng)	1 μl
PCR product*	x μl
2X Rapid Ligation Buffer	5 μl
T4 DNA Ligase	1 μl
Nuclease-free water	up to 10 μl

* Insert:Vector Molar Ratios = 1:1 - 3:1

3.10 Bioinformatics analysis

The sequences were compared with nucleotide and protein sequence in NCBI (http://www.ncbi.nlm.nih.gov) database using tblastx tool. Molecular mass and pI value was calculated by ExPASy Proteomic tools (http://web.expasy.org/compute_pi/). Protein localization and signal peptide were predicted by TargetP v1.1 (http://www.cbs.dtu.dk/services/TargetP/), SignalP v4.1 (http://www.cbs.dtu.dk/services/SignalP/), WoLF PSORT

(http://www.genscript.com/psort/wolf_psort.html) and Protcomp 9.0 (http://www.softberry.com/berry.phtml?topic=protcomppl). The phylogenic tree was carried out with MEGA6 program (Tamura, et al., 2013) base on the distance algorithmic neighbor-joining method. The transmembrane (TM) domains were predicted by TMHMM program (http://www.cbs.dtu.dk).

3.11 Alkaline lysis method for plasmid extraction (Sambrook, et al., 1989)

The overnight grown *E. coli* culture was collected by centrifugation at 12,000 rpm for 1 min. The obtained pellet was resuspended with 200 µl of the alkaline lysis solution I (25 mM Tris-HCl pH 8, 10 mM EDTA pH 7 and 50 mM glucose). Adding 200 µl of alkaline lysis solution II (0.2 N NaOH, 1 % SDS) for lysis cells and mixed by inversion and incubation for 2 min at room temperature. The mixture was renatured by adding 300 ml solution III (3 M potassium acetate, 11.5 % glacial acetic acid) and remove a contaminate by centrifugation at 13,000 rpm for 5 min at 4 °C. The supernatant was obtained and mixed with isopropanol. After incubation for 30 min at -20 °C, the pellet was obtained by centrifugation at 13,000 rpm for 10 min at 4 °C and wash pellet with 70% ethanol (twice). After remove alcohol, the pellet was resuspended with 50 µl nuclease-free water and keep at -20 °C.

3.12 Presto™ Mini Plasmid Kit for plasmid extraction

The extraction plasmid for sequencing was done by Presto™ Mini Plasmid Kit (GeneAid). After harvested the overnight grown *E. coli* cells, the pellet cells were resuspended with 200 µl PD1 buffer. The cell mixture was lysis with 200 µl PD2 buffer and neutralized in 300 µl PD3 buffer. The cell lysated was transferred to PD column and centrifuged at 13,000 rpm for 30 sec. The plasmid was washed two times with 400 µl W1 buffer and 600 µl washing buffer, respectively. After dry by

centrifugation at 13,000 rpm for 2 min, plasmid was eluted with 30 – 45 µl nuclease-free water.

3.13 Preparation of competent *E. coli* cells

Bacterial strain *E. coli* DH5α was used as a host cells for all intermediate cloning constructs. Competent cells of *E. coli* were prepared modified from Nakata's protocol (Nakata, et al., 1997). *E. coli* from glycerol stock was streaked on LB agar plate and incubated at 37 °C for overnight. Single colony from the plate was transferred to 5 ml LB broth for overnight. The culture was subculture to grown at 37 °C with vigorous shaking (250 rpm) until OD_{600} = 0.3 - 0.5. The culture was transferred to centrifuge tubes under sterile condition and incubated on ice for 30 min. The culture was centrifuged at 3,500 rpm at 4 °C for 10 min. The pellet was gently washed twice time with 20 ml and 10 ml of pre-chilled 100 mM $CaCl_2$ followed by centrifugation using same conditions. The new pellet was dissolved with 2 ml pre-chilled 100 mM $CaCl_2$ and 15 % glycerol with gentle swirling on ice for 30 min. Dissolved competent cells were aliquoted (100 µl) into eppendorf tubes and incubated in ice for 5 min and then frozen in liquid nitrogen before storing at -80 °C.

3.14 Transformation by heat-shock

Transformation into *E. coli* were performed according to previous protocol (Sambrook, et al., 1989). The 100 µl competent cells were thawed on ice and 2-5 µl plasmid DNA (up to 100 ng) or 3 – 5 µl ligation products was mixed gently and incubated on ice for 30 minutes. The cells were then incubated at 42 °C for 30 - 45 sec and placed on ice for 2 min. 500 µl of LB broth were added and gently mixed by inverting the tube. The transformation was achieved by shaking (200 rpm) for 1 hour at 37 °C. Selection to appropriate antibiotic was conducted by spreading 100 µl of

the transformed competent cells onto LB plate containing antibiotic. After overnight incubation at 37 °C, resistant colonies were observed on next day.

3.15 Construction of expression vector

3.15.1 pENTR™/D-TOPO® vector

The full-length genes from *A. lakoocha* and *C. ternatea* were amplified by *alrc*2-pENTR Fw: 5'-CACCATGGATTCTTTTCTTCTG-3', *alrc*2-pENTR Rv: 5'-TCACCTAACGAACGGTATAAG-3', *clt*-pENTR Fw: 5'-CACCATGGATTCGGTGCTCTATGGATCT-3' and *clt*-pENTR Rv: 5'-TCATCTAACATAAGGGAGGAGCAG-3' (Fig12: I and II). The full-length gene was clone into pENTR™/D-TOPO® vector (Invitrogen, USA) by following the manufacturer's protocol (Figure 12: III and IV). The components of cloning reaction consist of 5 µg fresh PCR product, 1 µl salt solution, 1 µl pENTR/D-TOPO vector and water to final volume 6 µl. The reaction was incubated at room temperature for 5 minutes. The pENTR-*alrc2* and pENTR-*clt* plasmid (Entry clone) constructs were individually transformed into Oneshot® TOP10 competent cells. Successful cloning of genes was confirmed by PCR, restriction digestion and sequencing.

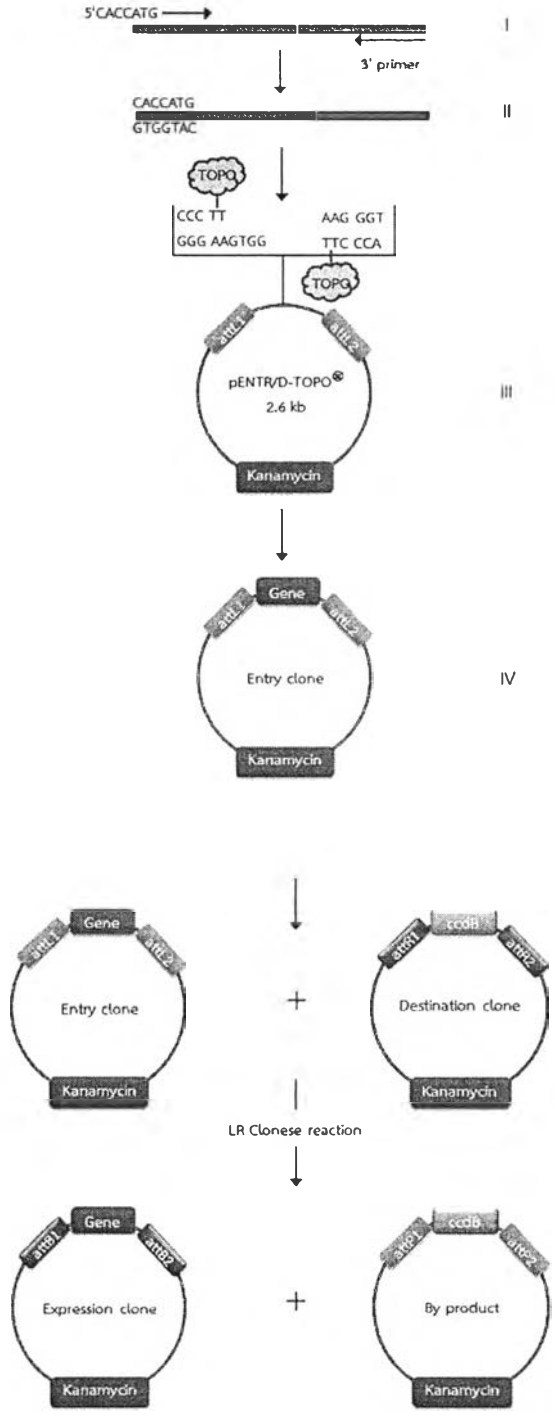


Figure 12 The strategies for construction of expression vector.

3.15.2 Binary vector

In order to construction of plant expression vector was be done by Gateway® Technology (Invitrogen, USA) following the manufacturer's protocol. The entry vector allowed recombining the desired sequences into the destination vector (pGWB6) by LR recombination events using Gateway® LR CLonase enzyme kit (Invitrogen, USA). The LR reactions were carried out by added 100 – 300 ng μl^{-1} entry clone with the gene of interest, 150 ng μl^{-1} destination vector, 2 µl 5X LR Clonase reaction buffer and TE buffer, pH8 to make volume to 8 µl. Start reaction by added 2 µl LR Clonase enzyme mix and mixed reaction mixture by vortex for 2 sec (two times). After incubated the reaction mixture at room temperature for 1 hour, one microliter of protease K was added into reaction and incubated at 37 °C for 10 min for stop enzyme activity. The ligation reaction was transformed into E. coli and checked correction by PCR.

3.16 Preparation of competent *Agrobacterium* cells

The preparation of competent cells were performed according to previous protocol (Lin, 1995). *A. tumifaciens* GV3101 cells were streaked on YEB agar plates containing antibiotics (25 µg ml^{-1} Rifampicin) and incubated at 28 °C for 2 days. A single colony was inoculated in 10 ml of LB medium containing antibiotic and incubated at 28 °C for overnight with shaking (200 rpm). A fresh overnight culture was inoculated in 50 ml of LB medium containing antibiotic until OD_{600} is 0.6 was reached. The cells were chilled on ice for 15 min and spin down by centrifugation at 5,000 xg for 10 min at 4 °C. The culture medium was discarded and pellet was washed in 25 ml and 10 ml of cold water and followed by centrifugation in the same condition. The pellet was resuspended in 2 -5 ml of cold water containing 10 % (v/v)

glycerol. 50 µl aliquots of the suspension were dispensed into prechilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80 °C.

3.17 Transformation of *A. tumefaciens* by electroporation

The transformation of *A. tumefaciens* by electroporation was performed according to previous protocol (Lin, 1995). The 50 µl competent cells *A. tumifaciens* GV3101 were thawed on ice and mixed with 1-2 µl of DNA sample (10 – 50 ng). The mixture was transferred into pre-chilled electroporation cuvette and then placed the cuvette in the holder. The electroporation was performed at 2.5 kV, 200 Ω and 25 µF. Immediately 500 µl of SOC medium was added and whole mixture was transferred into new sterile eppendorf and incubated at 28 °C for 2 hours. One hundred microliters of culture was spread on YEB agar containing 25 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin and incubated at 28 °C for 2 days. Then successful cloning was check by colony PCR.

3.18 Agrobacterium infiltration into tomato leaves

The transient expression was performed modified previous descripted protocol (Mangano, et al., 2014). Overnight-grown Agrobacterium cultures in YEB medium (50 µg mL^{-1} kanamycin and 25 µg mL^{-1} rifampicin) carrying expression vector an optical density (OD_{600}) of 1 were pelleted by centrifugation at 3000 xg for 5 min. The pellets were resuspended in induction solution (10 mM 2-N-morpholino-ethanesulfonic acid (MES) pH 5.5, 10 mM $MgCl_2$, and 200 µM acetosyringone). After 2 h incubations at room temperature with shaking at 50 rpm, Agrobacterium cultures carrying each expression vector were collected by centrifugation in the same condition. The pellets were washed with infiltration buffer (10 mM 2-N-morpholino-ethanesulfonic acid (MES) pH 5.5 and 10 mM $MgCl_2$) two times and resuspened in

infiltration buffer until OD_{600} is 0.5. The mature leaves of 5–8 weeks old tomato plants (*Lycopersicon esculentum* Mill.) were co-infiltrated using a 5 mL syringe without a needle from underneath of the leaves. Harvested leaf materials at 1, 3, 6 and 9 day post agroinfiltration (dpa) and stored at -20 ^{0}C until further use.

3.19 Gene expression analysis

3.19.1 RT-PCR

RNA was extracted from the infiltration tomato leaves (100 mg) at different dpi using RNeasy Plant Mini Kit (Qiagen). The first stand cDNA was synthesized by RevertAid™ H Minus Reverse Transcriptase (Thermo scientific) with 1 µg total RNA and 5 µg oligo dT. PCR was performed using specific primers ALR1_Fw: 5'-ATGGATTCTTTTCTTCTGGGTTCATTGAAAGG-3', ALR250_Rv: 5'-GTCTTTTGTTTTGTCCATGGTAAAATGTAG-3', CT34_Fw: 5'-GCCTCTTCACTAACCACTGGTGCC-3' and CT494_Rv: 5'-TAAATATTCATAAACAGGGCAGCAACC-3' also using β-tubulin as a house keeping gene (TUB_Fw: 5'-TGAGCACCAAGGAAGTTGATGA-3' and TUB_Rv: 5'-CCATTCCTTCACCTGTGTACCA-3'). The PCR reactions were conducted in final volume in 25 µl using 0.6 µl first stand cDNA, 1X One *Taq* standard reaction buffer, 0.2 mM dNTP mix, 0.2 µM each specific primers, 0.625 U One *Taq*® DNA polymerase (NEB) and water. Cycling conditions were following in Table 11.

Table 11 PCR thermal cycling condition of gene expression analysis.

Step	Temperature	Time	Number of cycles
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	30 sec	
Annealing	50 °C	40 sec	30
Extension	68 °C	40 sec	
Finale extention	68 °C	1 min	1

3.20 Extraction of recombinant protein from tomato leaves

The total proteins were obtained from modified previous descripted protocol (Liu, et al., 2007). Total proteins were prepared from 1 g of infiltration leaves. It was ground into powder with liquid nitrogen and resuspended in extraction buffer, pH 7.8 (50 mM Tris-HCl, pH 7.8,150 mM NaCl, 1mM PMSF, 0.5 mM DTT, 0.5% TritonX-100 and 10% glycerol). After stirred at 4 ^{0}C for 30 min, the homogenize was centrifuged at 12,000 xg for 15 min (4 ^{0}C). The supernatant was collected and measured protein concentration using Bradford method.

3.21 Protein analysis

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

One hundred microgram of total proteins from infiltration leaves were mixed with 2X loading dye and boiled in boiling water for 5 min to denatured protein then cooled down protein on ices for 2 min. Twenty microliters of denatured protein were loaded into 10% polyacrylamide gel against PageRuler™ plus pre-stained protein marker (Thermo Scientific™). The SDS-PAGE was running at 110 V for 1.20 hour.

3.21.2 Western blot

After separated protein on SDS-PAGE, proteins were transferred to PVDF membrane. The PVDF was soaked in methanol for 1 minute and equilibrated in transfer buffer, pH 8.3 (25 mM Tris-HCl, 190 mM Glycine and 20% methanol) for 10 min before transfer protein also gel was equilibrated in transfer buffer, pH 8.3 for 10 min. The proteins were transfer to PVDF membrane by electroblotting apparatus (BioRad) was assembled followed as Figure 13 and transferred at constant current of 100 V for 1.30 hour (4 °C). After transferred, the blot was rinsed in water and blocked with blocking buffer (5% skim milk in TBST buffer, pH 7.5) at room temperature for 1 hour with gentle agitates. Then blotted membrane was washed with TBST buffer, pH 7.5 (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20) for 5 min for tree times and incubated with 1:2000 in dilution buffer (1% skim milk in TBST buffer, pH 7.5) of anti-GFP antibody conjugated HRP (Thermo Scientific™) for 2 hours with gentle agitate. After washed with TBST buffer, pH 7.5 for 5 min for tree times, the blot was treated with chemiluminescent HRP substrate (Luminata™ Crescendo, Merck) and captured the chemiluminescent signal by CCD camera (Gel Doc™ XR, Bio-Rad)

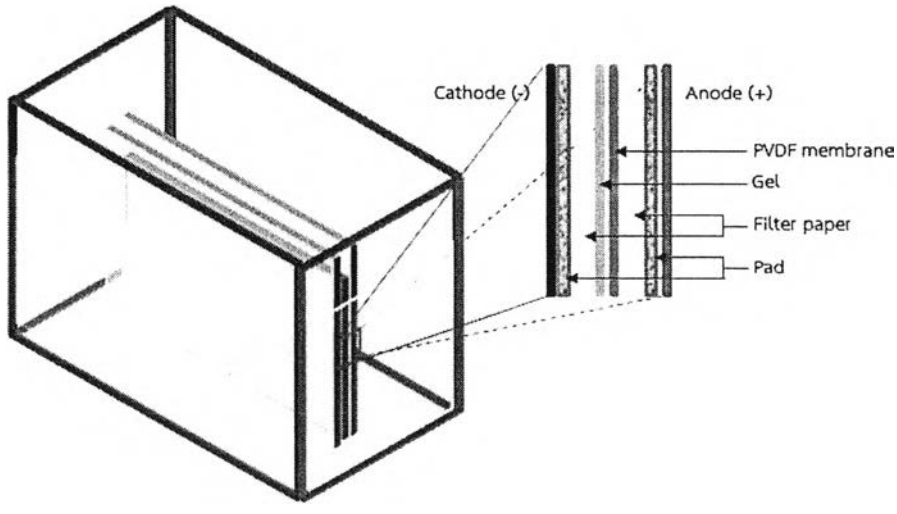


Figure 13 The alignment of western blot setup.

3.22 Tocopherol extraction

α-Tocopherol was extracted from agroinfitration tomato leaves according to the protocol described previously (Wilmoth, 2002). One gram of leaves was grounded in liquid N_2. Leaves powder was resuspened with 0.5 mM EDTA containing 20 mg ascorbic acid (pH 3.1) and mixed with vortex. After added 2 ml 100 mM SDS, the extract was incubated on ices for 6 min. And then 5 ml cooled-ethanol and 3 ml of hexane containing 0.2 % BHT (w/w) were added to an extracted. The extracted was mixed with vortex for 6 min and centrifuged for 3 min at 1,200 xg at 18 ^{o}C. The upper organic phase was kept. After evaporated to dryness under N_2 gas, an extracted was redissolved in 300 µl hexane containing 0.2 % BHT (w/w).

3.23 Chlorophyll analysis

Chlorophylls content from transient tomato leaves were determined according to method validated previously (Lima, et al., 2014), based on the molar

coefficient of chlorophyll in acetone:hexane (4:6 v/v). The samples were measured total chlorophyll at 663 and 645 nm and conversed to chlorophyll (mg/100 ml) following equations:

Total chlorophylls = Chlorophyll a + Chlorophyll b

Chlorophyll a = $0.999(A_{663nm}) - 0.0989(A_{645nm})$ (1)

Chlorophyll b = $-0.328(A_{663nm}) + 1.77(A_{645nm})$ (2)

3.24 Tocopherol analysis

3.24.1 Thin Layer Chromatography (TLC)

The tocopherols were analyzed by TLC technique. Five microliters of each extracted and α-tocopherol standard was loaded on TLC plate using Limonat IV (CAMAG). After TLC plate was developed with chloroform/cyclohexane (11:9) (Pyka, et al., 2011), the tocopherol was detected by scanned at 292 nm with TLC scanner 3 (CAMAG). Visualization and documentation of TLC was done under 254 nm after derivatized by iodine vapor. For quantitative analysis α-tocopherol, the various concentration of α-tocopherol standard was prepared by dissolved in 95% ethanol and spotted on TLC in range 0.05 – 1 µg. The calibration curve was calculated using relationship between peak area (AU) and concentration of α-tocopherol standard.

3.24.2 Gas Chromatography-Mass Spectrophotometer (GC-MS)

Eighty microliters of leaves extracted were evaporated in nitrogen gas stream and resuspended with 40 µl pyridine. Then the extracted was derivatized by silylation (Kobayashi and DellaPenna, 2008), added 100 µl BSTFA + 1% TMCS (Fluka) and incubated at 50 °C for 45 min. GC-MS was performed on an Agilent 7890B GC system with Agilent 7000C GC/MS Triple Quad mass detector using an HP5 column

(30 m x 0.25 mm, 0.25 μm) and the oven temperature was programed from initial 150 °C for 1 min to 260 °C at a rate of 25 °C min^{-1} and up to 300 °C (20 min hold) at a rate of 5 °C min^{-1}. Flow rate was 1.2 ml min^{-1}. The injection volume was 0.5 μl, split ratio 10:1 with MS detection in electron ionization mode at 70 eV. Full-scan spectra were recorded over a mass range of 33–650.