CHAPTER III

MATERIALS AND METHODS

1. Chemicals

A. Enzymatic preparation of [1-^{3}H]GGDP and [1-^{3}H]GGMP by microsomal fraction of *Nicotiana tabacum*

1. Plant material

Nicotiana tabacum cell suspension culture was mentioned by Dr. G. Herrmann, Institute of Plant Biochemistry, Halle/Saale, Germany. It was grown in LS liquid medium and subcultured every week. The cultured cells were used to prepare microsomes after being subcultured for 4-5 days.

2. Buffer

Homogenized buffer containing 100 mM potassium phosphate buffer pH 7.5, 250 mM sucrose, 4 mM $MgCl_2$, and 5 mM β-mercaptoethanol

Resuspension buffer containing 20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, and 2 mM β-mercaptoethanol

3. Microsome preparation

All of the processes were carried out at 4 ^{O}C using the method described previously with modification (Thai et al., 1999). Cell cultured suspensions of *N. tabacum* grown in 1-litre flask were filtered through Whatman filter paper no.1 by using Büchner funnel. The cells were washed with water 2 times to remove the remaining liquid medium before weighing. The washed cells were homogenized in homogenized buffer in a ratio 1:1.5 (g of cell wet weight : ml of buffer) using mortar and pestle. After being stirred gently for 20 min, the homogenate was filtered through 4-layers of cheese cloth, centrifuged the filtrate for 30 min at 10,000 x *g*. The resulting supernatant was then centrifuged for 1 hour at 100,000 x *g* to obtain a crude microsomal pellet. The pellet was dissolved in resuspension buffer with minimum volume. The protein concentration of the microsomal fraction was determined by Bradford reagent (Bradford, 1976) using a standard curve of BSA.

4. Reaction mixture used for the preparation of [1-^{3}H]GGDP and [1-^{3}H]GGMP.

Enzymatic reaction of 80 µl contained 50 µg microsomal protein of *N. tabacum*, 0.2 µCi [1-^{3}H]GGOH (synthesized as described in **B**), 62.5 mM MOPS buffer pH 6.5, 500 nmol cytidine triphosphate (CTP), 10 mM sodium orthovanadate, and 5 mM $MgCl_2$. The reaction was incubated for one hour at 37 ^{O}C, stopped reaction with 240 µl MeOH, centrifuged at 12,000 rpm for 1 min. The supernatant was collected and the precipitate was washed with 100 µl MeOH:H_2O (4:1) and centrifuged at 12,000 rpm for 1 min. The supernatant was mixed together and concentrated by rotary evaporator at 30 ^{O}C.

5. Purification of [1-^{3}H]GGDP, [1-^{3}H]GGMP, and [1-^{3}H]GGOH

MCI gel CHP20P (Mitsubishi) was packed in a Pasteur pipette. The column was washed with MeOH and equilibrated with 25 mM NH_4HCO_3. The concentrated aqueous residue was applied into the MCI gel column. After washing with 25 mM NH_4HCO_3 to remove water soluble compounds from the reaction mixture, the column was eluted with a stepwise gradient of 25 mM NH_4HCO_3 and MeOH. The amount of MeOH in 25 mM NH_4HCO_3 was 30% , 50% , 70%, 75%, 80%, 85%, 90%, and 100% respectively. Each fraction was applied on to a silica gel TLC precoated plate and the plate was developed using the solvent system of isopropanol : NH_4OH : water (6:3:1). The resulting plate was detected with TLC radioscanner. Under this conditions, the Rfs of [1-^{3}H]GGDP is 0.2, [1-^{3}H]GGMP is 0.6, and [1-^{3}H]GGOH is 0.9.

6. Characterization of ^{3}H-GGDP and ^{3}H-GGMP prepared from the microsomal preparation

[1-^{3}H]GGDP and [1-^{3}H]GGMP eluted from the column at 80% MeOH for [1-^{3}H]GGMP and 90% MeOH for [1-^{3}H]GGDP were characterized by the reaction of dephosphorylation using alkaline phosphatase (Sigma). The reaction mixture contained 30 mM glycine buffer pH 9.4, 0.1 µCi of either of the substrate and 4 units of alkaline phosphatase. The reaction was incubated for overnight (37°C) and stopped by adding MeOH and ethyl acetate. After extraction two times with ethyl acetate and evaporation until dryness with Speed Vac, the residue was redissolved with ethyl acetate, applied onto a silica gel TLC plate and developed using the solvent system of isopropanol : NH_4OH : water (6:3:1). [1-^{3}H]GGOH, the enzymatic product,

was detected by TLC radioscanner comparing with the Rf value of the authentic [1-^{3}H]GGOH.

7. Instruments for radioactive compounds detection

TLC Radioscanner, Automatic TLC-Linear Analyzer, Tracemaster 20

Phosphoimage, Fujifilm BAS-1500

Liquid scintilator, Beckman LS 6000TA

B. Chemical synthesis of [1-^{3}H]GGOH

[1-^{3}H]GGOH was prepared from NaB^3H_4 and geranylgeranial. Geranylgeranial was prepared by using the method of Pattenden and Smithies (1995). GGOH (10 mg, 35 mmol) was added with MnO_2 (30.5 mg, 0.35 mol) (avoid long time exposing air). The reaction mixture was stirred for 24 hr at room temperature. The product was detected by silica gel TLC with solvent system toluene:EtOAc (95:5), sprayed with 2,4-dinitrophenylhydrazine (specific reagent for aldehyde, showed yellow spot) or Iodine vapour.

NaB^3H_4 5 mCi (specific activity 336 mCi/mmol, Perkin Elmer) was mixed with geranylgeranial 15 μmole in 100 μl reaction mixture for 2 hr at room temperature. After that, the reaction was stopped with a mixture of 30 μl MeOH, 15 μl HOAc, and 20 μl water. [1-^{3}H]GGOH was detected by a silica gel TLC plate using the solvent system of benzene:MeOH (95:5). The plate was scanned with TLC radioscanner (Tracemaster 20), and its radioactivity was measured by liquid scintillator (Beckman LS 6000TA). [1-^{3}H]GGOH from the reaction mixture was then purified by a preparative silica gel TLC. Using the solvent system of toluene:EtOAc (95:5) and was eluted for [1-^{3}H]GGOH with CH_2Cl_2, and dried with N_2 gas.

C. Chemical synthesis of dibenzoyl plaunotol

Dibenzoyl plaunotol was the derivative of plaunotol that gave a dark spot under UV254 nm, but plaunotol did not. So it could be used to confirm the enzymatic production of plaunotol from the biosynthetic study of plaunotol. It was prepared from the reaction between plaunotol and benzoyl chloride. Plaunotol was purified from Kelnac^R soft gelatin capsule by silica gel column chromatography

eluting by isopropanol-$CHCl_3$ (96:4, and 90:10). The purified plaunotol was reacted with benzoyl chloride (mole ratio of plaunotol and benzoyl chloride, 1:2.5) in dry pyridine. The reaction mixture was stirred overnight at room temperature. After the reaction was finished, pyridine was removed by N_2 gas, the mixture was then redissolved with EtOAc and applied into silica gel TLC plate, developed with the solvent system of hexane-EtOAc (9:1). Dibenzyl-plaunotol showed dark spot under UV254 nm, its area was eluted with EtOAc and evaporated to dryness with N_2 gas. The chemical structure was confirmed by FTICR (performed by Dr. J. Schmidt, Institute of Plant Biochemistry, Halle/Saale, Germany).

2. Feeding experiments and detection of enzyme activity.

A. Feeding of *Croton stellatopilosus* leaf with [1-^{3}H]GGOH

The seeds of *C. stellatopilosus* were collected from Siri Ruckhachati Medicinal Plant Garden, Mahidol University, Nakorn Pathom. They were germinated by Mr. Wagener, the gardener, and growth in greenhouse of Institute of Plant Biochemistry, Halle/Saale, Germany. The leaves from young plants were used for chloroplast isolation and feeding experiments.

Healthy leaves were cut from the stem and put into a small glass container which contained 230 µl aqueous solution of 10 µCi [1-^{3}H]GGOH for 92 hours. The leaves was cut into small pieces and refluxed with 80% ethanol 20 min for 2 times. The ethanol extract was dried by rotary evaporator, then 10 ml $CHCl_3$ was added, and partitioned with 2N NaOH, followed with 2N HCl. The chloroform extract was applied onto a TLC silica gel plate, developed with solvent system benzene-MeOH (95:5), and detected by TLC radioscanner or phosphoimagery.

In order to confirm that the compound from feeding experiment is plaunotol, the plaunotol area in TLC plate were cut and eluted with EtOAc. The EtOAc extract was mixed with plaunotol 500 µg, and derivatized with benzoyl chloride to obtained dibenzoyl plaunotol, which can be detected under UV 254 nm, and by TLC radioscanner.

B. Feeding of cell suspension and callus of *C. stellatopilosus*

Callus culture and cell suspension culture of *C. stellatopilosus* was maintained in MS agar media and MS liquid media, respectively. [1-^{3}H]GGOH 10 μCi was applied directly onto callus, and it was also added into the liquid media of cell suspension culture. After the suitable period of time, the cells were extracted and detected for [1-^{3}H]plaunotol as described in **A**.

C. Detection for geranylgeranyl diphosphate phosphatase activity in the chloroplasts of the Plau-Noi leaves.

1. Buffer

Isolation buffer contained 0.05 mM HEPES-KOH pH 8.0, 1 mM Na-EDTA, 1 mM 1,4-dithioerythritol, and 400 mM sucrose.

Resuspension buffer contained 5 μM HEPES-KOH pH 7.6, and 0.1 mM 1,4-dithioerythritol.

2. Chloroplast preparation

Fresh leaves (10 g) were washed, and cut into small pieces. They were homogeneized with Ultra Turrax^R for 30 seconds at 13,500 rpm. The homogenate was filtered through 4 layers of nylon gauze, and then centrifuged for 10 min, at 5,000 rpm, 4 ^{O}C to obtain a pellet part. The pellet was redissolved with a small volume of isolation buffer centrifuged again for 10 min, at 5,000 rpm, 4 ^{O}C. The pellet of chlorophyll was then resuspended with small amount of resuspension buffer.

3. Determination of the chlorophyll amount

With 2x10 ml 80%(v/v) acetone in centrifuge tubes, 10 μl of chlorophyll suspension was added into each tube followed by centrifugation at 4,500 x *g* for 10 min, at 4 ^{O}C. The absorbance at 652 nm was used to measure chlorophyll content using 80% (v/v) acetone as a blank. The chlorophyll content in the unit of mg/ml was calculated by OD652 x 26.

4. Purification of chloroplast by sucrose gradient

Sucrose solutions (20 ml) in concentrations of 70, 60, 50, 40, 30, and 15% (w/v) was prepared in resuspension buffer. Sucrose gradient tubes were prepared by adding 5.5 ml of 70% (w/v) sucrose to an ultra clear centrifuge tube

(Beckman), followed by 5.5 ml of 60%, 50%, 40%, 30%, and 15% sucrose, respectively. The total volume of sucrose was 33 ml, and the inter-phase between different sucrose concentrations could be seen. The chloroplast suspension was added on top of sucrose, then centrifuged for 60 min, at 50,000 x *g*, 4 ^{O}C. Each band was collected separately in centrifuge tube, diluted to a final sucrose concentration of 15% (v/v) with resuspension buffer, and centrifuged for 10 min at 5,000 x g, 4 ^{O}C. The pellet was resuspended in 200 µl resuspension buffer, and determined for chlorophyll concentration.

5. Assay for geranylgeranyl diphosphate phosphatase activity

The 80-µl reaction mixture contained [1-^{3}H]GGDP 0.07 µCi, 0.5 M MOPS buffer pH 6.5, and chloroplast extract. It was incubated for 1 hr at 37 ^{O}C, and stopped the reaction with EtOAc and MeOH. The mixture was centrifuged for 1 min at 12,000 rpm to remove the precipitate. The supernatant was evaporated to dryness with N_2 gas, redissolved with butanol which was saturated in water and applied to silica gel TLC, developed with solvent system isopropanol:NH_4OH:H_2O (6:3:1). [1-^{3}H]GGOH was detected by TLC radioscanner.

D. Detection of GGDP phosphatase activity from cell-free extract of *C. stellatopilosus* leaves

1. Preparation of 20,000 x *g* fraction from *C. stellatopilosus* leaves

Fresh leaves (10-30 g) of *C. stellatopilosus* were used to prepare for crude GGDP phosphatase as described previously with modification (Tansakul and De-Eknamkul, 1998). The 20,000 x g pellet suspension and the 20,000 x *g* supernatant were desalted by using Sephadex G-25 (PD-10 column, Pharmacia), eluted with resuspension buffer.

2. Detection of enzyme activity

The desalted fractions were incubated for 1 hr at 30 ^{O}C with 67 µM GGDP (Sigma), 100 mM tricine buffer pH 7.8. The reaction mixture was extracted by 300 µl EtOAc for 2 times. The EtOAc layer was dried by vacuum drier (Speed Vac 100, Savant). The dry extract was redissolved with EtOAc and applied on

a silica gel TLC plate, using the solvent system of benzene-EtOAc (9:1). The TLC was detected with TLC densitometer (Shimadzu).

3. Solubilization of membrane-bound enzymes for GGDP phosphatase activity assay

The fraction of membrane-bound enzymes from the 20,000 x *g* leaves pellet was prepared by washing the pellet for 2 times with extraction buffer. The washed pellet was redissolved with resuspension buffer. Triton X-100 was added to the final concentration of 0.1%. The solubilized pellet was mixed well and centrifuged for 1 hour at 100,000 x *g*. The solubilized enzyme and pellet from 100,000 x *g* were desalted by PD-10 column prior to detection for GGDP phosphatase activity.

3. Purification and characterization of GGDP phosphatase

1. Plant material

Fresh leaves of *C. stellatopilosus* 1 kg were collected from the plants growed in the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok.

2. Buffer

Extraction buffer contained 83 mM Tricine/NaOH pH 7.8, 5 mM β-mercaptoethanol, 10 mM EDTA, and 10 mM $MgCl_2$.

Resuspension buffer contained 100 mM Tricine/NaOH pH 7.8, 5 mM β-mercaptoethanol, and 1 mM EDTA.

Buffer C contained 20 mM Tris/HCl pH 7.8, 5 mM β-mercaptoethanol, and 1 mM EDTA.

3. Optimization of enzyme assay condition

The solubilized membrane-bound GGDP phosphatase from 100,000 x *g* supernatant was desalted by PD-10 column. The desalted crude GGDP phosphatase was used to determine time-course of GGDP phosphatase activity, optimum temperature, optimum pH, and enzyme stability.

3.1 Preliminary study for optimum pH of the crude GGDP phosphatase

Various buffers were tested for enzyme activity in different pH, such as Na_2HPO_4/citrate pH 3, 4, 5, 6; sodium phosphate buffer pH 6 and 7; Tris/HCl buffer pH 7, 8, 9; glycine buffer pH 9, 10, 11; and Tricine/NaOH pH 7.8 and 8.5.

3.2 Optimum temperature for the enzyme activity

The enzyme activity was determined for 1 hour at various temperature from 25 ^{O}C to 100 ^{O}C.

3.3 Time course of reaction product formation

The amount of GGOH product in the reaction mixture was detected from the time interval of the reaction mixture from 0, 10, 20, 30, 45, 60, 120, and 180 min. The enzyme reaction contained GGDP, and 0.5 M Tris/HCl pH 7.0 were incubated at 30 ^{O}C and stopped reaction with EtOAc.

3.4 Enzyme stability

The crude enzyme was stored in various conditions (refrigerator, and –20 ^{O}C). The enzyme activity was determined at 0, 1, 3, 7, 10, 14, 17, 25, 32, and 45 days. After incubation for 1 hr at 30 ^{O}C, the GGOH was detected.

4. Enzyme assay

The optimal conditions of GGDP phosphatase activity obtained from optimization study above, was used as the standard method. The 150 µl reaction mixture contained 500 mM Tris/HCl pH 7.0, 67 µM GGDP, and 100 µl enzyme fraction. The reaction mixture was incubated for 60 min at 30 ^{O}C. The reaction was stopped by adding EtOAc. The reaction mixture was partitioned twice by 300 µl EtOAc and vortexed to extract GGOH into EtOAc. The phase was separated by centrifugation in Microcentrifugation (Fisher Model 235C). The EtOAc phase was evaporated to dryness by vacuum drier (Speed Vac 100, Savant). The redissolved GGOH was applied on a silica gel TLC plate (Merck). The TLC system was benzene-EtOAc (9:1). The amount of GGOH was measured by TLC densitometer (Shimadzu Dual-Wavelength Thin Layer Chromo Scan Model CS 930, Shimadzu) at wavelength 210 nm, and calculated from standard curve of GGOH (Sigma).

The activity of enzyme 1 unit (µmol/min) = 16.67 nkat

Specific activity (unit/mg protein) = 16.67 nkat/mg protein

5. Standard curves of GGOH, FOH, and GOH

GGOH (Sigma), FOH (Sigma), and GOH (Sigma) were diluted with 95% ethanol in different concentrations. Each concentration was applied on TLC plate in tripicate, using a TLC system of benzene-EtOAC (9:1), then they were detected at 210 nm by TLC densitometer. The peak area was plotted for standard curve in the range of linearity.

6. Enzyme extraction

All steps were performed at 4 ^{O}C. Fresh leaves (1 kg) were washed and ground using pestle and mortar in the presence of liquid nitrogen. The fine powder was homogenized for 20 min by stirring in 2 litres of extraction buffer (83 mM tricine/NaOH pH 7.8 containing 5mM β-mercaptoethanol, 10 mM EDTA, and 10 mM $MgCl_2$). After filtering through 4-layer cheesecloth, the crude homogenate was centrifuged at 20,000 x *g* for 20 min and the green pellet was collected. The pellet was washed with extraction buffer for 2 times. The washed pellet was dissolved with 330 ml extraction buffer and solubilized with final concentration of 0.1% Triton X-100, mixed thoroughly, and centrifuged at 100,000 x *g* for 1 hr to obtain the crude GGDP phosphatase in the green supernatant.

7. Enzyme purification

The column chromatographic techniques were performed with a Biologic HR set (Bio-Rad) in 4 ^{O}C refrigerator. The crude solubilized GGDP phosphatase enzyme from 100,000 x *g* was concentrated by Centriprep-30 (Amicon). It was applied into a BioGelA (Bio-Rad) column which was equilibrated with resuspension buffer. The column was eluted with resuspension buffer at a flow rate 0.5 ml/min and the fractions were collected until no more protein was detected. The fractions showing two peaks of GGDPase phosphatase activity, PI and PII. Each peak fraction was pooled and concentrated by lyophillization. The concentrated fractions of PI and PII were then applied separately into Superose 6 column (Pharmacia) equilibrate with buffer C. The active fractions of PI was pooled and kept at –20 ^{O}C for enzyme characterization, whereas the active fractions of PII from a Superose 6 column were pooled, concentrated by lyophillizatin, and applied to an anion exchange

column (Uno Q, Bio-Rad) equilibrated with buffer C. The column was washed with buffer C and eluted with buffer C containing NaCl gradient from 0 to 1 M to obtain the final enzyme preparation of PII.

8. Protein determination

During chromatographic separation by a Sepharose 6 column and an UNO Q column, protein profiles were monitored by UV detector at 280 nm. For a BioGel A column, the protein concentration of fractions was determined by method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as a protein standard. The pooled active enzyme fractions from each purification step was also determined by the same method. A Sample of protein 160 µl was pipetted into each well of 96-well plate, and mixed with 40 µl of the concentrated dye solution (Bio-Rad Protein Assay, Bio-Rad). The mixture was incubated for 5 min at room temperature and measured at 595 nm by using a Microtiter Plate Reader Model 450 (Bio-Rad).

9. Molecular weight determination

9.1 Determination of the molecular weight by SDS-PAGE

SDS-PAGE was performed on a Mini Protean II Apparatus (Bio-Rad). The protein fractions of PI, PII from the BioGelA column, and the SDS-PAGE protein standard were applied separately into the gel. The protein standard involved proteins with molecular weights of 225, 150, 100, 75, 50, 35, and 25. The gel was stained with Coomasie blue. The dry gel was used to calculate for molecular weight. The standard curve was plotted on the log protein size (Y-axis) of the standard protein versus relative mobility (Rf) (X-axis).

The molecular weights were calculated from the calibration curve of log protein size versus relative mobility (Rf) by the equation of

Log molecular weight = (slope)(Rf of protein) + y-intercept

9.2 Molecular weight determination by gel filtration

PI and PII from the BiogelA column were determined for native molecular weight by a Superose 6 HR 16/50 (1.6 id x 50 cm) column which connected to a Biologic HR Set (Bio-Rad). PI and PII fraction were concentrated by lyophyllization. It was then redissolved with resuspension buffer. The sample 500 µl

was injected to the Superose 6 column equilibrated with resuspension buffer. The column was eluted with the same buffer at the flow rate 0.5 ml/min. The protein was detected by UV detector at 280 nm.

The Superose 6 HR column was calibrated by using the stardard proteins for gel filtration (Bio-Rad). The standard protein included thyroglobulin (670 kD), gammaglobulin (158 kD), ovalbumin (44 kD), myoglobulin (17 kD), vitamin B-12 (1.35 kD). The position of void volume (Vo) was determined by blue dextran. The relative molecular weight of enzyme was calculated from the calibration curve between log molecular weight and Vo/Ve (Ve = elution volume) of standard protein plotted. The equation of molecular weight calibration was;

Log molecular weight = (slope)(Vo/Ve) + y-intercept

10. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis on 12% polyacrylamide gel was performed according to method of Laemmli (Ref). SDS-PAGE was used to determine the purity of protein fraction from column chromatography, and the subunit molecular weight of proteins. It was performed by a Bio-Rad Mini Protean II Apparatus on a Mini-slab gel (70 x 80 x 7.5 mm). The separating gel contained 12% polyacrylamide (prepared from a stock solution of 30% w/v acrylamide (Bio-Rad), and 0.8% w/v N,N'-methylene-bis-acrylamide (Bio-Rad)). The procedure for gel preparation and procedure of SDS-PAGE was described in part **4** (gene cloning).

11. Characterization of GGDPase enzyme

11.1 Enzyme kinetics

The determination of *Km* and *Vmax* values was performed under routine assay conditions with the enzyme fractions of PI and PII from the Superose 6 column. The concentrations of GGDP used for kinetic study of PI are 0.40 mM, 0.25 mM, 0.20 mM, 0.09 mM, and 0.07 mM. The concentrations of GGDP used for kinetic study of PII are 0.40 mM, 0.33 mM, 0.27 mM, 0.20 mM, 0.09 mM, 0.07 mM, 0.03 mM, 0.01 mM, and 0.007 mM. The rates of enzymatic reaction (pkat/mg) were calculated and plotted against various concentrations of GGDP (mM) to obain the

rectangular hyperbolar graph of Michaelis-Menten equation. *Km* and *Vmax* values of PI and PII were determined using Lineweaver-Burk plot.

11.2 Influence of metal ions on the enzyme activity

The effect of metal ions was tested by adding 1 mM of each metal ion solution ($MnSO_4$, $CoCl_2$, Na_2MoO_4, $ZnSO_4$, $MgCl_2$, $MnCl_2$) into the reaction mixture. The enzyme activity assay was done as the standard procedure as described in **4**. PI and PII used in this experiment were obtained from the BioGelA column.

11.3 Substrate specificity

The substrate specificity was determined from PI and PII from the BioGelA column. The standard enzyme activity assay was done by using various substrates included 67 μM isopentenyl diphosphate (IDP) (Sigma), 67 μM geranyl diphosphate (GDP) (Sigma), 67 μM farnesyl diphosphate (FDP) (Sigma), compared with routine substrate concentration of 67 μM GGDP. The boiled control of each alternative substrate was used as the blank of reaction.

11.4 Inhibition of enzyme activity by geraniol, farnesol, and geranylgeraniol

The inhibitory effect of some prenols was determined with PI and PII fractions from the BioGelA column. The reaction mixture contained the substrate of 67 μM GGDP and 200,000 dpm [1-^{3}H]GGDP (Dupont). The 0.1 mM of geraniol (Sigma), farnesol (Sigma), or geranylgeraniol (Sigma) was added into the reaction mixture. The radioactive geranylgeraniol peak was detected by a TLC Radioscanner.

11.5 Optimum pH

The optimum pH for GGDP phosphatase activity was determined with PI and PII fractions from the Superose 6 column. The following buffer was used between the pH range from 4.0-8.5 as follows: 0.5 M citric acid/sodium citrate (pH 4.0, 4.5, 5.0, 6.5); 0.5 M Na_2HPO_4/citric acid (pH 4.0, 5.0, 6.0); 0.5 M MOPS (pH 5.5, 6.0, 6.5. 7.0, 7.5, 8.0), 0.5 M tris/HCl (pH 7.0, 7.5, 8.0, 8.5); 0.5 M glycine (pH 7.5, 8.0, 8.5).

12. Dephosphorylation of [1-^{3}H]GGDP by GGDP phosphatases

PI from the Superose 6 column and PII from the UNO Q column were assayed to detect for intermediate geranylgeranyl monophosphate. The reaction

mixture contained 0.1 μCi [1-^{3}H]GGDP, 15 μM 0.5 M Tris/HCl pH 7.0, and 35 μM enzyme fraction. The mixture was incubated overnight at 30 ^{O}C. The reaction was stopped by adding EtOAc, and partition twice with EtOAc. The EtOAc layer was

separated by centrifugation for 1 min, then it was dried by vacuum drier. The EtOAc fraction was applied on a TLC plate, using the solvent system isopropanol-NH_4OH-H_2O (6:3:1). The radioactive products in TLC plates were detected with the TLC radioscanner.

13. Dephosphorylation of [1-^{3}H]GGMP by GGDP phosphatases

PII from the BioGelA was used to dephosphorylate [1-^{3}H]GGMP. The reaction mixture contained 0.07 μCi [1-^{3}H]GGMP, 0.5 M Tris/HCl pH 7.0, and 35 μM enzyme fraction. The mixture was incubated for 1 hr at 30 ^{O}C. The reaction was stopped by adding EtOAc and MeOH, and centrifuged at 12,000 rpm for 1 min. The supernatant was collected and evaporated to dryness with N_2 gas. It was redissolved with butanol and applied on a silica gel TLC plate using the solvent system of isopropanol-NH_4OH-H_2O (6:3:1). The radioactive products in TLC plates were detected with the TLC radioscanner.

4. Gene cloning of *Croton stellatopilosus* prenyl diphosphate phosphatase

1. Organisms

1.1 Plant material

Young leaves of *C. stellatopilosus* Ohba was obtained from open field, Faculty of Pharmaceutical Sciences, Srinakarindwirot University, Ongkarak, Nakorn Nayok, Thailand.

1.2 Microorganisms

Table 4 Microorganisms used in this study.

Microorganisms	Genotype
E. coli DH5α (Clontech)	F^- *ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1hsdR17* (r_K^-,m_K^+) *phoA supE44λ⁻thi-1 gyrA96 relA1*
E.coli BL21 codon plus (DE3) RIL strain (Stratagene)	B F^- *ompT hsdS($r_B^-m_B^-$) dcm^+ Tet^r gal endA Hte [argU ileY leuW Cam^r]*
One Shot® TOP 10 (Invitrogen)	F^- *mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*

2. Enzymes

Tag DNA-Polymerase (prepared by Dr. J. Ziegler, Institute of Plant Biochemistry, Halle/Saale, Germany)

Pfu DNA-Polymerase (Promega)

Advantage 2 Polymerase mix

Lysozyme (Sigma)

Other enzymes were included in the kits

3. Kits

Superscript First Strand system (BD Biosciences)

QIA quick gel extraction kit (QIAGEN)

QIAprep Spin Miniprep kit (QIAGEN)

SMART RACE cDNA Amplification kit (BD Biosciences)

pET directional TOPO Expression kit, Invitrogen

4. Nucleic acids

5.1 DNA

5.1.1 1 kb DNA Ladder (New England Biolabs)

5.1.2 Deoxynucleotides (dATP, dCTP, dGTP, dTTP) (Life Techno - logies)

5.1.3 Nucleotide primers specific for gene target were synthesized by MWG-Biotech AG

5.1.4 Nucleotide primers for routine experiments:

T7 TOPO primer was designed by Mr. N. Guennewich (Instutute of Plant Biochemistry, Halle/Saale, Germany), the others were common primers of the kits.

- Nucleotide sequencing of plasmid from pGEM-T Easy vector
  T7 primer 5' GAA TTG TAA TAC GAC TCA CTA TAG 3'
  SP6 primer 5' GAT TTA GGT GAC ACT ATA GAA TAC 3'
- Nucleotide sequencing of plasmid from pET 101/D-TOPO vector
  T7 TOPO primer 5' CGA AAT TAA TAC GAC TCA CTA TAG 3'
  T7 Reverse primer 5' TAG TTA TTG CTC AGC GGT GG 3'

5.2 Plasmids:

pGEM-T Easy Vector System (Promega)

pET 101/D-TOPO vector (Invitrogen)

5. Chemicals

6.1 Common chemicals

All chemicals used were standard commercial products of analytical grade from the companies: Biomol, Boehringer Mannheim, Fluka, Boehringer Ingelheim, Merck, Roth and Sigma.

6.2 Specific chemicals

5-Bromo-4-chloro-3-indolyl-β-D-galctopyranoside (X-gal) (Biomol)

Isopropyl-β-D-thiogalactoside (IPTG) (Biomol)

Big Dye^{R} Terminator v1.1 Cycle sequencing RR-100 (Applied Biosystem)

Amplicillin (Sigma)

Chloramphenicol (Sigma)

6.3 Substrate

[1-^{3}H]GGDP (specific activity 16 Ci/mmol) (Amersham)

6. Instruments

Gel document (Gel Doc 1000, BioRad)

PCR machine (GeneAmp^{R} PCR System 9700, Applied Biosystem)

UV/Visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech)

Nucleotide sequencer (ABI PRISM 3100-Avant Genetic analyzer, Hitachi)

Refrigerator centrifuge (Sorvall RC 26 plus, DuPont)

Bench-top refrigerator centrifuge (Centrifuge 5810 R, Eppendorf)

Bench-top centrifuge (Centrifuge 5810 R, Eppendorf)

Vertical gel electrophoresis apparatus (BioRad, Hercules)

Power supply Phero-stap 500 (Biotec Fischer, Reiskirchen)

Western Blot (Techware, Sigma-Aldrich)

Heat Block (Thermostat plus, Eppendorf)

Shaker (Duomax 2030, Heidolph)

Laminar Hood for microbial (HERA Safe, Heraeus Instruments)

TLC radioscanner (Rita, Raytest)

7. Software

DNASTAR

-EditSeq Windows 32 3.9.10 1989-1998, DNASTAR, Inc.

-SeqManII Window 32 SeqMan 3.6.1 1989-1998

-MegAlign Window 32 3.18 1993-1998

GeneDoc version 2.6.002, 2000

Clustal W v1.82 (www.ebi.ac.uk/clustalw/)

ChloroP v1.1 (Emanuelsson et al., 2000) (www.cbs.dtu.dk/services/ChloroP)

Sosui v1.0 (Hirokawa et al., 1998) (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.htm)

Primer design (www.basic.nwu.edu/biotools/oligocalc.html)

8. Media (Sambrooke, 1989)

SOB medium

20% (w/v) Bacto-tryptone

5% (w/v) Bacto-yeast extract

0.5% (w/v) NaCl

The solultion was added with 10 ml of 250 mM KCl, adjusted to pH 7.0 with NaOH, and then adjusted volume to 1 l. After the mixture was autoclaved, 5 ml steriled 2 M $MgCl_2$ was added (19 g $MgCl_2$ in 100 ml H_2O).

SOC medium

Added the filtered sterile 1 M glucose into SOB medium

LB medium

1.0 % (w/v) tryptone

0.5 % (w/v) yeast extract

1.0 % (w/v) NaCl

Adjusted to pH 7.0 with 1 M NaOH

LB agar medium

Added 1.5% (w/v) agar into LB medium, autoclaved, and stored at 4 ^{O}C

9. Electrophoresis

9.1 Agarose gel electrophoresis

Agarose gels are for DNA size of 0.5-25 kb. The 1.2%-1.5% agarose is used for small DNA fragments. The 0.6 g agarose gel was dissolved with 50 ml 1xTAE buffer using microwave. The gel solution was added with 4 µl of 0.4 µg/ml ethidium bromide, and then poured in the agarose gel apparatus. DNA sample was mixed with 5x probe buffer to the ratio 5:1 (buffer:DNA solution), centrifuged shortly, and pipetted into the well of the gel. The loading gel apparatus was connected to the power supply, and run at 75 volts.

50x TAE buffer

Tris-base	242.0	g
Glacial acetic acid	57.1	ml
0.5 M EDTA	100	ml

Adjusted to pH 8.0, and adjusted volume to 1 l.

5x probe buffer for DNA agarose gel

40%(W/V) Saccharose

0.001%(W/V) Orange G

9.2 SDS-PAGE

The sample was added with the sample buffer and heated for 5 min at 95 ^{O}C. The sample preparation was then centrifuged for 1 min at 13,000 rpm to precipitate the insoluble matters, 10-20 ml of protein sample was loaded into the gel well.

The glass cases was prepared by making the space between two pieces of glasses with the rubber, and clamped tightly together with clippers at both sides of the glass case. The separating gel was poured into the space between glasses around 7.5 cm height, and applied carefully with BuOH-water on the top, and of the separating gel. When the gel was hardened for 1.5 hr, BuOH was discarded and the gel was washed with water. The stacking gel was poured on the top of separating gel, and the comb was put inside to make the wells. After leaving the gel to be hardened for 0.5 hr, it was ready to use. The glasses with gel, which were already removed the clippers and the rubber, was placed into the electrode chamber. The glass case was clipped tightly with the chamber to make a reservoir for electrode buffer. The electrode buffer was poured inside and outside the chamber, the samples were loaded into the well. The power supply of 100 volts was applied until the blue dye almost reaching the bottom edge, and then the gel was removed for staining.

Coomasie Blue stain

The gel was immersed into the coomasie blue staining buffer for one hour, then it was destained with the destaining buffer with gently shaking.

Separating gel 12%

Milli Q water	3.4	ml
1.5 M Tris/HCl, pH 8.8	2.5	ml
20% SDS (w/v)	50	µl
Acrylamide/Bis-acrylamide (Rotiphorese^{R} gel 30, Roth)	4	ml
10% Ammonium persulfate (Serva, analytical grade)	50	µl
TEMED (Roth)	5	µl

Stacking gel 4% gel

Milli Q water	3.075	ml
0.5 M Tris/HCl, pH 6.8	1.25	ml
20% SDS (w/v)	25	µl
Acrylamide/Bis-acrylamide	670	µl
10% Ammonium persulfate	25	µl
TEMED	5	µl

5x Running buffer, pH 8.8 1 liter

Tris-Base (Ultra quality, Roth)	15	g
Glycine	72	g
SDS	5	g
Milli Q water q.s.	1	lt

1xSample buffer 8 ml

milli Q water	4	ml
0.5 M Tris/HCl, pH 6.8	1	ml
Glycerol	800	µl
10% SDS	1.6	ml
β-mercaptoethanol (Roth)	400	µl

0.05% Bromphenol Blue (Sigma)	200	µl

Coomasie Blue stain solution

HOAc	100	ml
MeOH	300	ml
Water	600	ml
Coomasie Brilliant Blue G-250 (Roth)	1	g

Destain solution

HOAc	100	ml
MeOH	300	ml
Water	600	ml

Storage gel in the solution containing1%HOAc and 3% glycerine.

Silver stain

The gel can be stained after coomasie blue staining by washing out the destaining buffer with water. Or the silver stain can be done immediately after removing gel from the glasses plate, then it was immersed into the fixing buffer and followed with silver stain protocol below.

First, the gel was immersed into the fixer agent containing 40% EtOH and 10% HOAc for 3 x 45 min. Then, it was rinsed by water to remove fixer for 3 x 30 min, after that the gel was placed overnight into the sensitizer containing 0.5% (w/v) glutaraldehyde, 30% EtOH, 2g/l sodium thiosulfate.$5H_2O$, and 68 g/l sodium acetate. The gel was rinsed by water for 4 x 30 min, and stained for 30-120 min with silver stain reagent containing 1 g/l silver nitrate, and 250 µl/l 37% (w/v) formaldehyde. After being washed rapidly (10-20 seconds) with water, the gel was developed until the protein bands appeared (5-15 min) with developer consisting of 25 g/l sodium carbonate, and 100 µl/l 37% (w/v) formaldehyde. The reaction was terminated by immersion in 14.6 g/l EDTA for 5-10 min. The staining gel was stored in solution consisted of 3% glycerol, and 1% HOAc.

Protein molecular weight marker (Fermentas SM0431) consisted of β-galactosidase (116 kD); bovine serum albumin (66.2 kD); ovalbumin (45.0 kD); lactate dehydrogenase (35.0 kD); Rease BspSS1 (25.0 kD); β-lactoglobulin (18.4 kD); and lysozyme (14.4 kD).

11. Determination of nucleic acids

The concentration of nucleic acids was determined by a UV spectro photometer at the wavelength of 260 nm. Depending on the type of nucleic acid, OD_{260}= 1.0 represents the following concentrations :

double-stranded DNA	50 μg/ml
RNA	40 μg/ml

The 2 μl of DNA sample was diluted with 48 μl of water (dilution factor of 25), measured for the absorbance at OD_{260} by UV-spectrophotometer, and then calculated into the concentration of DNA by the following equation:

$$\text{DNA concentration } (\mu g/\mu l) = \frac{(OD_{260} \times \text{dilution factor} \times 50\ \mu g)}{1000\ \mu l}$$

$$\text{RNA concentration } (\mu g/\mu l) = \frac{(OD_{260} \times \text{dilution factor} \times 40\ \mu g)}{1000\ \mu l}$$

12. Nucleotide sequencing

The plasmid with the insertion of DNA was further sequenced for nucleotides as follows:

12.1 Big Dye PCR

The PCR was performed in a volume of 10 μl containing 4 μl Big Dye diluted mix (DNA polymerase,dNTPs, ddNTPs labeled and Big Dye buffer); 1 μl of 10 mM primer (T7 or SP6 primer for pGEM-T Easy plasmid;T7 TOPO or T7 reverse primer for pET 101/D-TOPO vector); and plasmid (400-600 ng). The PCR amplification was carried out by the following program: 96 ^{O}C for 2 sec, and 25 cycles consisted of 96 ^{O}C for 10 sec, 50 ^{O}C for 5 sec, and 60 ^{O}C for 4 min.

After finishing, the 10 μl of water was added into the PCR reaction mixture.

12.2 Purification of Big-Dye PCR reaction mixture

The PCR product of Big-Dye PCR was purified from the reaction mixture prior to sequencing for nucleotide. The Sephadex G50-50 Superfine (Pharmacia) was loaded into the wells of MultiScreen^{R} HV plate by using the column loader, then the gel was swollen with 300 µl Milli Q water in each well. The plate was covered and placed at 4 ^{O}C for more than 3 hr. The water was removed from the column by centrifugation for 5 min at 910 x *g*. Then the sample from Big-Dye PCR was loaded drop by drop into the center of each column. The plate of gel filtration column was then placed on the top of 96-well microtiter plate and centrifuged for 5 min at 910 x *g*. The cleaned DNA sample was obtained and ready to put into the sequencer machine (ABI PRISM 3100-Avant Genetic analyzer, Hitachi).

Dilution of Big Dye v1.1 concentrated

Big Dye v1.1 concentrated (Applied Biosystem)	50 µl
Fresh Milli Q water	75 µl
Big Dye 5x Sequencing buffer (Applied Biosystem)	75 µl

13. DNA concentration

The DNA solution was added with 0.1 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol, then it was kept in –20 ^{O}C for 1 hr. After that, the reaction mixture was centrifuged for 20 min at 13,000 rpm, discarded supernatant, and washed pellet with 70% ethanol. Ethanol was removed by centrifugation for 10 min at 13,000 rpm. DNA pellet was redissolved with 5 µl of water, and measured for DNA concentration by a UV spectrophotometer.

14. Total RNA extraction by Phenol-Chloroform nucleic acid extraction method

(Performed in room temperature, use free RNAse water, and wear gloves in every step)

Fresh, young leaves of *Croton stellatopilosus* (1 g) were frozen with liquid nitrogen, grounded into powder by using mortar and pestle, and then transferred into a falcon tube. It was added with 3.5 ml of cooled lysis buffer and 3.5 ml of phenol/chloroform (Roti^{R}- Phenol:chloroform:Isoamyl alcohol 25:25:1, Roth), and

shaked for 30 min. The extract was centrifuged for 10 min at 5,000 rpm. The aqueous layer was collected. The organic phase was extracted again for 2 times with phenol/chloroform (add 3.5 ml, and 3 ml phenol-chloroform, respectively). The aqueous fraction was combined together and precipitated by 0.1 volume of 3 M sodium acetate, pH 5.2 and 1 volume of isopropanol. The mixture was placed at –20 ^{O}C for 1-2 hr, then centrifuged for 20 min at 5,000 rpm. The obtained pellet was washed with 500 µl of 70% EtOH (without redissolved the pellet) and centrifuged again for 10 min at 5,000 rpm. The pellet was dried, and then redissolved with 300 µl of TE buffer gently (do not vortex). The RNA was precipitated from the dissolved pellet by adding 300 µl of 6 M LiCl, and then left at 4 ^{O}C overnight. The pellet of RNA was obtained by centrifugation at 14,000 rpm for 15 min at 4 ^{O}C, then it was washed with 500 µl of 70% EtOH, followed by centrifugation at 5,000 rpm 10 min at 4 ^{O}C, and leaved it dried for a moment. The dried pellet was redissolved with 50 µl of TE buffer by vortexing to obtain the total RNA. The amount of RNA was quantitated by measuring the absorbance at 260 nm of the dilution of 1:50.

Lysis buffer

10 mM Tris/HCl pH 7.5
50 mM NaCl
1% SDS
4% PVPP (polyvinylpolypyrrolidone)
1 mM EDTA pH 8.0
14 mM β-mercaptoethanol
Autoclaved at 121 ^{O}C for 20 min.

TE buffer

10 mM Tris/HCl pH 8.0
1 mM EDTA pH 8.0
Sterile filtered.

15. First-strand cDNA synthesis

First-strand cDNA was synthesized according to protocol of Superscript ™ First Strand system (BD Biosciences). This First-strand cDNA kit was used to convert 1 ng to 5 μg of total RNA into the first strand cDNA by the principle of RT-PCR.

The reaction mixture was performed in the 500-μl eppendorf tube in the following steps:

1. Denature:

Total RNA (4.5 μg RNA per reaction)	1	μl
10 mM dNTP mix	1	μl
2 μM of antisense Primer	1	μl
water q.s.	10	μl

The reaction mixture was incubated in water bath at 65 ^{O}C for 5 min, then placed on ice at least one min.

2. Annealing:

The second reaction mixture below was prepared, and then added into the first reaction mixture. The second reaction mixture contained:

10X RT buffer	2 μl
25 mM $MgCl_2$	4 μl
0.1 M DTT	2 μl
Rnase OUT ™ Recombinant Rnase Inhibitor	1 μl

The mixture of reactions from 1 and 2 were incubated for 2 min at 42 ^{O}C.

3. cDNA synthesis:

Enzyme SuperScript ™ II RT (50 units) was added and incubated for 50 min at 42 ^{O}C.

4. Termination

The reaction was terminated at 70 ^{O}C for 15 min., chilled on ice, and then stored at –20 ^{O}C until use.

16. Amplification of prenyl diphosphate phosphatase fragment of *C. stellatopilosus* by PCR.

Oligonucleotides were designed from the high homology region of known prenyl diphosphate phosphatase amino acid sequences. Six degenerated primers were used to perform PCR with the template of single-stranded cDNA (see section **15**) in a volume of 50 µl as follow;

10x PCR buffer (Tag buffer) + 15 mM $MgCl_2$	5 µl
10 mM dNTP mix	1 µl
10 µM sense primer	1 µl
10 µM antisense primer	1 µl
Tag Polymerase (5 unit/µl)	0.5 µl
First strand cDNA of the antisense primer	2 µl
Water q.s.	50 µl

Touchdown PCR of the template of first strand cDNA and the degenerated primer was carried out in all combination of sense and antisense primers. The PCR conditions was composed of pre-denaturing 2 min at 95 ^{O}C; denaturing 1 min at 95 ^{O}C; annealing 1 min touch-down from 65 ^{O}C to 47 ^{O}C in 2 ^{O}C step of 2 cycles, followed by 10 cycles at 45 ^{O}C; extension 1 min at 72 ^{O}C; and addition 7 min extension at 72 ^{O}C. The PCR cycle can be written as follow:

95 ^{O}C	2 min	
95 ^{O}C	1 min	
65 ^{O}C	1 min	x 2
72 ^{O}C	1 min	
95 ^{O}C	1 min	
63 ^{O}C	1 min	x 2
72 ^{O}C	1 min	

↓

↓

95 ^{O}C	1 min	
47 ^{O}C	1 min	x 2
72 ^{O}C	1 min	
95 ^{O}C	1 min	
45 ^{O}C	1 min	x 10
72 ^{O}C	1 min	
72 ^{O}C	7 min	
4 ^{O}C	infinity	

The resulting PCR products were verified on agarose gel containing ethidium bromide as described in **9.1**. The specificity of touchdown PCR products was indicated by Nested PCR in different temperature from 54-60 ^{O}C with the gradient PCR machine.

The amplicon from Nested PCR was purified as follow. The 40 µl of reaction mixture was mixed with 8 µl of DNA loading buffer, and loaded into the wells of agarose gel. The gel was detected under UV light. The band around 500 bp in size was cut under UV light, and extracted for DNA by QIAquick^{R} gel extraction kit (QIAGEN).

17. Ligation

The gel extract containing an amplicon was ligated into pGEM-T Easy vector system I (Promega). The reaction mixture containing:

2x Rapid ligation buffer	5 µl
50 ng/µl pGEM-T Easy vector system I	1 µl
Gel extraction of the PCR product	3 µl
3 unit/µl T4 DNA ligase	1 µl

The reaction mixture was incubated for overnight at 4 ^{O}C. The amount of inserted DNA in pGEM-T Easy vector with 3:1 molar ratio (insert:vector) was calculated as follow:

$$\text{Amount of DNA (ng)} = \frac{(50 \text{ ng vector} \times \text{kb insert}) \times 3}{\text{Size of vector (kb)}}$$

18. Transformation

The 100 µl of competent cells was pipetted into a falcon tube for more oxygen, and then it was mixed with 2 µl of ligated reaction mixture. The tube was swirled, incubated for 30 min on ice, immersed in water bath for 25 sec at 42 ^{O}C, and then placed immediately on ice for 2 min. The 900 µl of SOC medium was added into the tube, and shaked at 200 rpm 37 ^{O}C for 1 hr. The cell culture was then inoculated onto LB agar plates containing 100 µg/ml ampicillin, 80 µg/ml X-gal, and 0.5 mM IPTG. The plates were incubated for overnight at 37 ^{O}C. There were blue and white colonies on the agar plate. The white colonies were selected and detected for gene insertion by colony PCR, using T7 primer as sense primer and SP6 primer as antisense primer.

19. Detection for gene insertion into vector

19.1 Colony PCR

Of all positive colonies on each agar plate, eight colonies were picked and inoculated into PCR tubes containing 50 µl of LB medium with 1 µg/ml ampicillin (performed in laminar hood) to make miniculture. Then the PCR tubes were shaked in the shaking incubator at 180-200 rpm, 37 ^{O}C for 3 hr. After incubation, the PCR was performed in the reaction volume of 30 µl as follow:

10x Tag buffer	3	µl
10 mM dNTPs	0.6	µl
10 µM sense primer	0.6	µl
10 µM antisense primer	0.6	µl
Tag polymerase	0.3	µl
Water	23.9	µl
Miniculture	1	µl

PCR conditions were 30 sec at 94 ^{O}C, followed by 30 cycles of 30 sec at 94 ^{O}C, 30 sec at 50^{O}C, 45 sec at 72 ^{O}C, and ending with a 5-min final extension at 72 ^{O}C.

19.2 Double digestion with restriction enzyme

The other method to check for the gene insertion is the double digestion. The pGEM-T vector has the restriction site for *Eco*R I at the end of both sites of gene insertion, therefore the cleavage of the vector with *Eco*R I yielded the gene fragment that can be detected its size by agarose gel electrophoresis. The positive colonies were picked from agar plate and directly cultivated in the 6 ml of LB medium with ampicillin. Or pipette 1 µl of miniculture into 6 ml of LB medium with ampicillin, and incubated at 37 ^{O}C for overnight. The plasmid was extracted using QIAprep^{TM} spin miniprep kit (QIAGEN), and then the concentration was measured by UV spectrophotometer as mentioned in section **11**.

The reaction mixture for double digestion in the total volume of 20 µl, which was performed in PCR tube, contained 0.3 µl of *EcoR* I, 1 µg of plasmid, and 2 µl of 10x *EcoR* I-buffer. The reaction mixture was incubated for 1 hr at 37 ^{O}C, and detected for the required cDNA size by agarose gel electrophoresis.

20. Plasmid extraction from culture of *E.coli.*

The 1 µl of miniculture was pipetted into 6 ml of LB medium with ampicillin, and then incubated at 37 ^{O}C for overnight. The *E. coli* culture was harvested by centrifuged 4,000 rpm, at 4^{O}C for 5 min. The plasmid was extracted from the cell pellet by using QIAprep^{TM} spin miniprep kit (QIAGEN), and the concentration was measured by UV spectrophotometer as mentioned in section **11**.

21. Full-length gene synthesis by 5'RACE and 3'RACE PCR (SMART^{TM} RACE cDNA Amplification kit, BD Biosciences)

SMART (Switching Mechanism at 5' end of RNA Transcript) is a method for performing both 5'-and 3' rapid amplification of cDNA ends (RACE). This kit can utilize first strand cDNA directly in RACE PCR without the need of secondary strand synthesis and adaptor ligation. The total RNA can be used as starting material for constructing the full-length cDNA. The procedure according to the manual included:

1. First strand DNA synthesis; the reaction mixtures were shown in Table 5.

Table 5 Reaction mixtures of first strand DNA synthesis using for RACE PCR.

5'-RACE-Ready-cDNA		3'-RACE-Ready cDNA	
Total RNA (50 ng-1 ng total RNA)	3 µl	Total RNA (50 ng-1 ng total RNA)	3 µl
5'-CDS primer	1 µl	3'-CDS primer	1 µl
SMART IIA Oligonucleotides	1 µl		
Water q.s.	5 µl	Water q.s.	5 µl

SMART IIA Oligonucleotides contain terminal G residues that anneal to dC rich cDNA tail and serve as extended template for enzyme reverse transcriptase.

5'-CDS primer = 5'-$(T)_{25}$VN-3'

3'-CDS primer = 5'-AAG CAG TGG TAT CAA CGC AGA GTA C T_{30}VN-3'

The reaction mixture showed in the Table 5 was mixed, placed in 70 ^{O}C for 2 min, and placed on ice for 2 min, then mixed with the reaction mixture containing:

5x First-Strand buffer	2 µl
20 mM DTT	1 µl
10 mM dNTP mix	1 µl
PowerScript RT	1 µl

The reaction mixture was placed in 42 ^{O}C for 1.5 hr. Then it was diluted with 100 µl Tricine-EDTA buffer, and placed in 70 ^{O}C for 7 min. It can be stored at –20 ^{O}C until used.

PowerScript RT when functioned until reached the end of an RNA template, it exhibited terminal transferase activity by adding 3-5 dC to3'-end of the first strand cDNA. At the same time, PowerScript RT switched template from mRNA to SMART Oligo in order to generate a complete first strand cDNA copy of the original RNA with the additional SMART sequences at the end.

5x First-Strand buffer contained 250 mM Tris-HCl pH 8.3, 375 mM KCl, and 30 mM $MgCl_2$.

Tricine-EDTA buffer contained 10 mM Tricine-KOH pH 8.5, and 1mM EDTA.

2. Designed primer for RACE PCR

The primer should be designed with 23-28 nt, 50-70% GC, and Tm >65 ^{O}C. The best result would obtained if Tm >70 ^{O}C (enable to use Touchdown PCR), and avoid using self-complementary primer.

Primer for 5'-RACE PCR (antisense):

5'GGC CTC TGC GGT CAA ATG CTT TCA ACT TCC CAG3'

Primer for 3'- RACE PCR (sense):

3R1: 5' GCA CTT GGC CGG CCT AGG CCT GAT TTC TT TGG CGC 3'

3R2: 5' TGG CAG GAT GTA TTT GCT GGA GGT CTC TTA GGG 3'

3. RACE PCR to generate 5' and 3' cDNA fragments

Amplification was performed in a 50-ml volume reaction mixture as shown in Table 6:

Table 6 Reaction mixtures of 3' and 5'-RACE PCR.

5'-RACE PCR reaction mixture			3'-RACE PCR reaction mixture		
Water	34.5	µl	Water	34.5	µl
10x Advantage 2 PCR buffer	5	µl	10x Advantage 2 PCR buffer	5	µl
10 mM dNTP mix	1	µl	10 mM dNTP mix	1	µl
50x Advantage 2 Polymerase mix	1	µl	50x Advantage 2 Polymerase mix	1	µl
5'-RACE-Ready cDNA	2.5	µl	3'-RACE-Ready cDNA	2.5	µl
UPM 10x (Universal Primer A mix)	5	µl	UPM 10x (Universal Primer A mix)	5	µl
10 µM 5'-RACE primer	1	µl	10 µM 3'-RACE primer	1	µl
total volume	50	µl	total volume	50	µl

Advantage 2 polymerase mix composed of BD TITANIUM ^{TM} Tag DNA polymerase and BD Tagstart^{TM} Antibody to provide automatic hot-start PCR and a minor amount of a proof reading polymerase.

UPM = 5' CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAA CGCAGAGTACT$_{30}$VN 3'

Touchdown PCR for 5'-RACE PCR; annealing temperature touchdown from 72 ^{O}C to 60 ^{O}C, 2 ^{O}C step decrease. The PCR cycles were shown as followed:

94 ^{O}C	10 sec	
94 ^{O}C	5 sec	
72 ^{O}C	3 min	x 5
94 ^{O}C	5 sec	
70 ^{O}C	10 sec	x 5
72 ^{O}C	3 min	

94 ^{O}C	5 sec	
60 ^{O}C	10 sec	x 20
72 ^{O}C	1 min	
72 ^{O}C	7 min	
4 ^{O}C	infinity	

Touchdown PCR for 3'-RACE PCR; annealing temperature touchdown from 72 ^{O}C to 68 ^{O}C, 2 ^{O}C step decrease.

94 ^{O}C	10 sec	
94 ^{O}C	5 sec	
72 ^{O}C	3 min	x 5
94 ^{O}C	5 sec	
70 ^{O}C	10 sec	x 5
72 ^{O}C	2 min	
94 ^{O}C	5 sec	
68 ^{O}C	10 sec	x 25
72 ^{O}C	2 min	
72 ^{O}C	7 min	
4 ^{O}C	infinity	

4. Nucleotide sequencing of RACE products and full-length gene

The amplicons from 3'- and 5'-RACE PCR were detected by agarose gel electrophoresis. Then they were extracted from gel by QIA quick gel extraction

kit. The purified cDNA fragment was ligated into pGEM-T Easy vector and transformed into *E. coli* DH5α. The transformant was cultured into LB agar plate containing X-gal, IPTG, and ampicillin as described in Section **18**. The white colonies were collected and checked for gene insertion by colony PCR. The positive clones were inoculated into 6 ml of LB medium containing ampicillin, incubated at 200 rpm, 37 ^{O}C, overnight. Then, the cells were harvested, and extracted the plasmid by QIAprep Spin Miniprep kit. The plasmid was measured for the concentration. Nucleotide sequencing of plasmid was done by BigDye PCR using T7 primer for sense direction, and SP6 primer for antisense direction. The nucleotide sequences of each fragment were aligned by program SegMan (DNASTAR). The whole nucleotide sequences derived from 3'-and 5'-RACE fragments were searched for open reading frame (ORF) by program EditSeq (DNASTAR).

22. cDNA sequence analysis

Full-length gene within ORF included start codon (ATG) and stop codon (TAA) were translated into amino acid sequences by program EditSeq (DNASTAR). It was multiple aligned with amino acid sequences of prenyl diphosphate phosphatase and phosphatidic acid phosphatase from various plants in database by program Clustal W or MagAlign (DNASTAR), and reported for the homology and phylogenetic tree.

The chloroplast transit peptide and its cleavage site was detected by program ChloroP.

23. Heterologous expression in *E. coli*

The cDNA with and without sequence upstream to the cleavage site, and the cDNA sequence downstream to the RR motif, were generated by PCR. The PCR were performed in a 50-μl volume containing

10x *Pfu* reaction buffer	5 μl
10 μM sense primer	1 μl
10 μM antisense primer	1 μl
10 μM dNTPs	1 μl
Single strand cDNA	1 μl

Pfu polymerase	1 µl
Water	40 µl

The single stranded cDNA was prepared from total RNA by Superscript First Strand system kit as described in Section **14** with primer Oligo dT. The sense primers and antisense primers were designed following the manufacturer's instructions of pET directional TOPO Expression kit (Invitrogen):

Sense primer for ORF1

5' CACC ATG CAT GAT TGG CTT ATC TTT CTG CTT 3'

Sense primer for ORF2

5' CACC ATG ATG ACA GAT CTC AGA TAC CCT 3'

Sense primer for truncate ORF1

5' CACC ATG AAA CGA TAT GCT GGG AAA GAC A 3'

Sense primer for truncate ORF2

5' CACC ATG AGT AAT ACG GTA CCC GTT TGG GCA 3'

Sense primer after RR motif

5' CACC ATG GAC ATC TAT GAC CTC CAT CAT G 3'

Antisense primer without stop codon

5' TTT TGG TCC CCT TTC AAC GTC CTG GTG GCT 3'

Touchdown PCR cycles with the reaction mixture above were performed. The PCR conditions were used: 3 cycles of 94 ^{O}C 2 min, followed by 3 cycles of 94 ^{O}C 1 min, annealing temperature touchdown from 64 ^{O}C-54 ^{O}C, 2 ^{O}C step decrease, and 72 ^{O}C 2 min, and the final condition of touchdown annealing temperature 20 cycles with the 52^{O}C, and ending with a 10-min final extension at 72 ^{O}C.

Amplicons were analyzed by agarose gel electrophoresis, bands were excised from the gel and the DNA was extracted from the agarose by using gel extraction kit. The DNA concentration was measured by UV spectrophotometer. The DNA in gel extraction was then inserted into vector (pET 101/D-TOPO vector, Invitrogen) in the ratio of PCR product: TOPO vector (5:1 molar ratio)

$$\text{PCR product inserted into vector (ng)} = \frac{\text{vector (ng)} \times \text{kb insert)}}{\text{vector size (kb)}}$$

Reaction mixture of TOPO cloning reaction

PCR product	1.5	µl
Salt (1.2 M NaCl + 0.06 M $MgCl_2$)	1	µl
TOPO vector (pET101/D-TOPO)	1	µl
Milli Q water	2.5	µl

The reaction mixture was vortexed, placed at room temperature (22-23 ^{O}C) for 30 min, and then placed on ice until doing transformation.

Transforming competent cell

3-µl of TOPO cloning reaction was added into vial containing *E. coli* cell (One Shot TOP10, Invitrogen), mixed gently without pipetted up and down, incubated on ice for 30 min, put in a water bath at 42 ^{O}C for exactly 30 sec without shaking, then put on ice 2 min and added 250 µl of SOC medium. The mixture was shaked at 200 rpm, at 37 ^{O}C for 30 min. Then it was inoculated on agar plate of LB medium containing 100 µg/ml ampicillin, and incubated at 37 ^{O}C overnight.

The colonies were picked and inoculated into 6 ml of LB medium plus ampicillin. Then the cells were harvested and extracted for the recombinant plasmid by using plasmid extraction kit. The plasmids were sequenced for nucleotide sequences. The exact nucleotide sequence in the correct direction will be further used for expression.

The 10 ng of desired recombinant plasmid was transformed into *E. coli* BL21 (DE3) Codon plus RIL with the same method described above, and inoculated into LB medium containing ampicillin. It was then incubated at 37 ^{O}C overnight. Positive clones were screened by PCR (same method as Section **19**) using T7 forward and T7 reverse vector-based primers (T7 TOPO and T7 reverse primer). The colony with insertion was inoculated into 50 ml of LB medium with 100 µl /ml ampicillin and 50 µg/ml chloramphenicol, grown overnight at 200 rpm, 37 ^{O}C. Aliquots of 15 ml were used to inoculate 1 l Erlenmeyer flasks containing 500 ml of LB medium with ampicillin and chloramphenicol. These were grown at 37 ^{O}C 180 rpm to OD_{600} = 0.5-0.7. The cell suspension was placed on ice and started to induce for expression by adding IPTG with the final concentration of 1 mM, shaked at 22 ^{O}C, 180 rpm overnight.

Cells were harvested by centrifugation at 8,000 rpm for 10 min. The pellet was redissolved with His-Tag lysis buffer containing 750 μg/ml lysozyme and placed on ice for 1 hr. Then the suspension was sonicated for 30 sec twice to break cell. The homogenized was centrifuged at 10,000 x *g* for 30 min. The supernatant was used for purification.

24. Purification of recombinant proteins by affinity column chromatography

24.1 Talon resin (BD Biosciences Clontech)

Talon resin is an immobilized metal affinity chromatography (IMAC) using Co^{2+} which can bind to his-tag protein. The 2 ml of Talon resin suspension (equivalent with 1 ml of Talon resin) was transferred into a 15 ml Greiner tube, and washed with HWB buffer twice by centrifugation at 700 x g for 2 min. The resin pellet was resuspended in 10 ml of HWB. After that, it was resuspended in 10 ml of crude bacterial lysate and gently agitated on a platform shaker for 20-60 min to allow the his-tag binding to the resin. The resin plus the lysate was transferred to 15 ml-Greiner tubes, and centrifuged at 700 x *g* for 5 min. The supernatant was kept at –20 ^{O}C for later SDS-PAGE analysis of the unbound his-tag fraction. The resin was washed twice by resuspending the resin in 12-14 ml of HWB and agitating on platform shaker for 10 min, and then centrifuged at 700 x *g*. After the supernatant was discarded, the resin was resuspended with 1 ml HWB. The resin was transferred to a 2-ml gravity flow column, and was washed with 10 ml of HWB. Then, the protein was eluted by HEB. The first 3-ml eluant was combined and desalted with a PD-10 column that has been equilibrated in 100 mM Tricine buffer, pH 7.8. The recombinant protein was stored at –20 ^{O}C. The Talon resin was recovered with 10-column volumes of 20 mM MES pH 5.0 to remove bound imidazole and β-mercaptoethanol. The column should be kept at 4 ^{O}C. Talon resin can be re-used for 3-4 times.

24.2 Hi-Trap HP affinity column (Amersham)

HiTrap HP is a prepacked column for purification of his-tag recombinant protein by immobilized metal affinity chromatography (IMAC) using Ni^{2+}. It was operated with liquid chromatography system (Aekta, Amersham). The

crude lysate of *E.coli* was loaded onto the column. After the column was washed with 10 ml HWB, it was eluted with linear gradient of 0-500 mM imidazole in HWB at a flow rate of 0.5 ml/min for 20 ml. The fractions of 1 ml were collected and detected for the his-tag protein by Western Blot with his tag monoclonal antibody.

His-Tag lysis buffer (HLB)

50 mM Sodium phosphate buffer pH 7.0
500 mM NaCl
2.5 mM imidazole
10% glycerol
10 mM β-mercaptoethanol
1% Tween 20
750 μg/ml lysozyme (Sigma)

His-tag wash buffer (HWB)

50 mM Sodium phosphate buffer pH 7.0
500 mM NaCl
2.5 mM imidazole
10% glycerol
10 mM β-mercaptoethanol

His-tag elution buffer (HEB)

50 mM Sodium phosphate buffer pH 7.0
500 mM NaCl
250 mM imidazole
10% glycerol
10 mM β -mercaptoethanol

The desalted recombinant protein from a PD-10 column was concentrated by Centricon-10 (Amicon). The concentrated protein was detected with 12%SDS-PAGE and assayed for GGDP phosphatase activity. The recombinant protein was identified by his-tag monoclonal antibody.

25. Detection for gene expression by Hig-Tag monoclonal antibody.

1. Electroblotting from SDS-PAGE

The Whatman filter paper size 7x9 cm, Nitrocellulose membrane 7x9 cm (Sartorius), and SDS-PAGE gel were placed into blotting buffer for 15-30 min. The electrode plate was moistured with water. The Whatman filter paper was placed one by one onto the electrode plate for 3 layers, and air bubbles was removed by rolling the stirring rod over each layer. Then the nitrocellulose membrane was placed, and followed with SDS-PAGE gel. Finally, the filter paper was placed one by one for 3 layers. The lid was covered onto electrode plate, and then connected to power supply of 0.8 mA/cm^2 for 1.5 hr twice (7 cm x 9 cm x 0.8 mA/cm^2 = 50 mA). After blotting was done, protein on the nitrocellulose was stained with Fast Green Dye, marked for interesting positions by pencil, and then destained the dye by water.

2. Western hybridization

The 10 ml of TBST buffer with 5% Milk powder (Roth) was added into glass petri dish. Then the nitrocellulose membrane which has been blotted already was applied and shaked for 1 hr. After that, the membrane was transferred into the new petri dish containing 7.5 ml of TBST with 5% Milk powder and 2.5 µl of His-Tag monoclonal antibody, Novagen (3 µg His-Tag Ab dissolved in 15 µl water) and shaked for 1 hr. The membrane was washed with TBST for 4 x 10 min. Then it was transferred into new petri dish with 10 ml of TBST with 3% milk powder and 2 µl of alkaline phosphatase secondary antibody, and shaked for 1 hr. The membrane was washed with TBST for 4 x 10 min. Then it was stained with 10 ml TBS buffer, pH 9.5 contained 45 µl of Nitro Blue Tetrazolium (NBT, 75 mg/ml in dimethylformamide), 35 µl of 5-bromo-4-chloro-3-indolylphosphate (50 µg/l in dimethylformamide), and 5 mM $MgCl_2$. The protein with his-tag will showed red band within 5-60 min. The reaction was stopped with 20 mM EDTA in TBS buffer.

Blotting buffer for Electroblotting (Western blot) pH 9.1

48 mM Tris

39 mM Glycine

20 vol % MeOH

0.037% SDS

Fast Green Dye

1% glacial acetic acid

0.1% Fast green

Tris-buffered saline/Tween 20 (TBST)

20 mM Tris/HCl pH 7.5

150 mM NaCl

0.05% Tween 20

Tris-buffered saline (TBS)

20 mM Tris/HCl pH 9.5

150 mM NaCl

26. Geranylgeranyl diphosphate phosphatase enzyme assays.

The reaction mixture contained 35 µl of concentrated recombinant protein, 0.1 µCi [1-^{3}H]GGDP (Amersham), and 15 µl of 0.5 M Tris/HCl pH 7.0 buffer. Assay mixture was incubated at 30 ^{O}C for overnight. Products were collected with three times ethyl acetate extraction (3 x 150 µl). The EtOAc layer were combined together and dried with Speed Vac. They were applied into a silica gel TLC plate with the solvent system of isopropanol-NH_4OH-H_2O (6:3:1), and detected for GGMP and GGOH with a TLC radioscanner.