# CHAPTER V

## DISCUSSION

### 1. Preparation of [1-³H]GGMP and [1-³H]GGDP for biosynthetic studies

The aim of synthesizing [1-³H]GGDP and [1-³H]GGMP from [1-³H]GGOH by using microsomal preparations (Thai et al., 1999) was to have both radioactively labeled compounds sufficiently for being used in enzymatic product detection. We found that the microsomal fractions from *Croton stellatopilosus* could convert [1-³H]GGOH into both [1-³H]GGDP and [1-³H]GGMP but in lower rate than using the microsomal fraction from *Nicotiana tabacum* cell suspension cultures. Similar to the case of *N. tabacum* (Thai et al., 1999), CTP was the only phospharyl donor for the microsomal fractions of *C. stellatopilosus*. GGOH kinase has been previously reported in archaebacterium *Sulfolobus acidocaldarius* (Ohnuma et al, 1996). The phosphorylation of GGOH to GGDP in *S. acidocaldarius* was catalyzed by two enzymes of GGOH kinase and GGMP kinase but using ATP rather than CTP as phosphate donor.

In this study, the high activity of conversion of [1-³H]GGOH into [1-³H]GGDP appeared to occure in Tris/HCl pH 7.5, and the formation of [1-³H]GGMP was obtained in MOPS pH 6.5. This suggested that either different kinase enzymes are involved in the reaction or the different pH could affect the catalytic process of the same kinase.

GGDP is the compound that has been used in many studies, especially the biosynthesis study of diterpenoids and carotenoids at the enzyme and molecular levels. Both the non-radioactive GGDP and [1-³H]GGDP are available in commercial, but not for [1-³H]GGMP. There have been many reports describing the synthesis of [¹⁴C]GGDP both enzymatically and chemically. The production of [14,8,12,16-¹⁴C]GGDP by using prenyl transferase enzyme from the mutant stain of *Gibberella fujikuroi*, which is blocked for carotenoid biosynthesis, has been performed from R-[2-¹⁴C]mevalonic acid and the yield of 65% has been obtained (Knoess and Reuter, 1996). [¹⁴C]GGDP has also been reported to be obtained from [¹⁴C]mevalonic acid by using cell-free extract of immature seeds of *Echinocystis lobata* (Sandmann et al, 1980) and pea cotyledon extract (Banthorpe et al, 1992).

Cell-free enzyme extract of *Micrococcus lysodeikticus* has been shown to catalyze the conversion of isopentenyl diphosphate plus farnesyl diphosphate into polyprenyl phosphate from C20-C55 including GGDP (Kurokawa et al, 1971). In 1979, Gafni and Shechter reported the synthesis [$^{14}$C]GGDP from [$^{14}$C]GGOH with 30.9% recovery yield.

In our study, [1-$^3$H]GGDP and [1-$^3$H] GGMP was purified in one step by MCI gel CHP20P column. The advantage of this method is that the aqueous soluble compounds can be easily removed, especially sodium orthovanadate which is the phosphatase inhibitor. In addition, the purification of GGDP from the reaction mixture was simple. Previously, it required several steps of purification until Kenedy and Thompson (1993) has developed the method of prenyl diphosphate purification in single step by preparative TLC or flash chromatography on silica. With this method, 20 μmol FDP can be isolated by TLC and up to 1200 μmol is obtained from flash chromatography. In our case, however, we found that elution of [1-$^3$H]GGPP and [1-$^3$H]GGMP from silica gel TLC by H$_2$O followed by lyophilization led to low yields of both labeled compounds. Most amounts of that of the compounds were changed to other compounds that were fixed at the origin position on the TLC plate. Other separation methods of GGDP have also been reported such as HPLC ion pair (Beyer et al, 1985), reversed-phase HPLC (Zhang and Poulter, 1993), DEAE cellulose (Goldman and Strominger, 1972), silica gel (Gafni an Schechter, 1979), Amberlite (Mackie and Overton, 1977) and cellulose fibre (Davisson, 1986). In this study, although the method of producing GGDP using microsomal protein was time-consuming and resources for management of cell suspension and preparing for microsome, our purification steps were relatively simple. It is, therefore suitable for producing in a small scale which need not much requirement.

## 2. Feeding experiments on *C. stellatopilosus*
### A. Feeding of *C. stellatopilosus* leaves with [1-$^3$H]GGOH

The radiolabeled plaunotol was produced in small amout from feeding with [1-$^3$H]GGOH into the leaves and callus culture with the considerable amount of unknown labeled compounds appearing at the solvent front of TLC plate. This suggested that GGOH can be taken up into the leave, but not being used for

synthesizing plaunotol as main compound. In literature, GGOH has been shown to bind with chlorophyllide a and b to obtain chlorophyll (Shibata et al, 2004), and GGOH was converted into GGMP and GGPP in tobacco (Thai et al, 1998). Since GGDP is the universal substrate of diterpenoids and carotenoids and can be used for prenylating Ras-protein in signal transduction pathway (Christopher and Poulter, 2000) as well as being the component of cell membrane, it is not surprising to detect the unlabeled unknown compounds in our case.

From our preliminary study, we could detect plaunotol in the green parts of stem and leaves of the germinate seedlings. This indicated that the synthesis of plaunotol has started since it is young and takes place in the parts which containing chlorophyll. Our attempt to confirm plaunotol by derivatizing into dibenzoyl plaunotol did not give clear results owing to the small amount of plaunotol forming in the feeding experiments. However, feeding shoot of *C. stellatopilosus* with [U-$^{13}$C]glucose could identify the synthesis of radiolabelled plaunotol by NMR and indicated that it was derived from non-mevalonate pathway (Wungsintaweekul and De-Eknamkul, 2005).

Callus and cell suspension cultures of Plau-Noi were fed in our experiments with 10 µCi of [1-$^{3}$H]GGOH. Several attempts were made with various growth stages of both types of *in vitro* culture. However, only the callus cultures gave small amount of the labelled compound with the same Rf value as plaunotol. It was so small amount that cannot be confirmed by derivatizing it to dibenzoyl plaunotol. For the cell suspension, no labeled compound was detected in the plaunotol area. It has been reported that plaunotol can produce in green callus of *C. stellatopilosus* (Morimoto, 1988) with low amount. Therefore, it is likely that the compound detected in the callus culture was plaunotol, whereas the major radioactive products at the solvent front of TLC might be phytosterol, as found previously as the main labeled compound obtained from callus feeding by [$^{13}$C]glucose (Potduang, 2000).

## B. Detection of GGDP phosphatase activity in the cell-free extract of *C. stellatopilosus* leaves

In this study, chloroplast fraction was isolated from the fresh leaf extract of *C. stellatopilosus* by using sucrose gradient technique. The results clearly

showed that the chloroplast preparation could dephosphorylate either GGDP or GGMP into GGOH. GGMP was detected as intermediate of the dephosphorylation from GGDP into GGOH. As shown in Fig. 31, however, high amount of [1-$^3$H]GGDP was still remained in the reaction mixture. This might be due to the enzymatic reaction in the chloroplast was not performed efficiently under the condition. For example, the amount of chloroplast used in the reaction mixture might not be enough. In addition, the chloroplast preparation contained a lot amount of chlorophyll that might be the limitation of increasing amount of chloroplast. This can lead to an overload of concentrated reaction mixture containing chlorophyll onto a TLC plate. Another reason might be that the radiolabeled of GGDP being used in this experiment was contaminated by sodium orthovanadate which is a phosphatase inhibitor and led to the reduction of GGDP phosphatase activity in the reaction mixture.

Diterpenes and carotenoids are the compounds that are known to have their synthetic location in plastid, and plaunotol oil globule was found to contain in the chloroplasts (Potduang, 2000). According to the report of Tansakul and De-Eknamkul (1998), the presence of GGOH 18-hydroxylase in 20,000 x $g$ pellet might compartment might suggest that chloroplast is the biosynthetic location of plaunotol. Therefore, we tried to investigate that GGDP phosphatase is present in chloroplast containing fraction. The results from Figs. 31 and 32 showed clearly the presence of GGDP and GGMP phosphatase activity in the chloroplast preparation of *C. stellatopilosus*. It indicates the occurrence of prenyl phosphatase activity in this compartment. So far, there has been no report on the studying of prenyl phosphatase activity in the chloroplast fraction. Most of works have been on GGDP phosphatase activity present in microsomal fractions, e.g. from in rice seedling (Nah et al., 2001) and rat liver microsome (Bansal and Vaidya, 1994). All of them are membrane-bound enzymes and have not yet been purified. Therefore, there has been no amino acid sequence of GGDP phosphatase in database. In our study, we named our enzyme GGDP phosphatase according to its activity over GGDP.

Our crude GGDP phosphatase which was solubilized by TritonX-100 exhibited its activity in 20,000 x $g$ pellet and Triton X-100 could solubilize most of the membrane-bound GGDP phosphatase. The results from Fig. 35. clearly indicated

that GGDP phosphatase is a membrane-bound enzyme that bound to the chloroplast organelle.

## 3. Purification and characterization of GGDP phosphatase from *C. stellatopilosus*

The results from our enzyme purification suggests the presence of two forms (PI and PII) of membrane-bound phosphatase enzymes that presumably catalyze the dephosphorylation of GGDP to form GGOH in *C. stellatopilosus* leaves. The activity of enzyme in this study was determine by TLC densitometer and calculated the amount of GGOH from peak area. The linearity range of GGOH concentration from standard curve was between 0.5-6.0 μg.

Characterization of both GGDP phosphatase forms revealed that PI has an apparent molecular weight of 58 kD, and that of PII 30 kD on SDS-PAGE gel under denature condition. The native enzyme is also determined for MW by the Superose 6 column gel filtration. It is found that the enzyme activity is detected at a elution volume corresponding to a protein with molecular weight of 232 kD for PI, and molecular weight of 34 kD for PII. The results clearly indicate that PI is a tetrameric enzyme with subunit of 58 kD, whereas PII is a monomeric enzyme of molecular weight 30-34 kD. The optimum pH of PI is 6.0-6.5, and that of PII is 6.5-7.0. Metal ions has less effect on the activity of PI than that of PII (eg. the activity of PI is decreased by $Mn^{2+}$, whereas the activity of PII is inhibited by $Mn^{2+}$, $Zn^{2+}$, and $Co^{2+}$). Both PI and PII activities are completely inhibited by 1 mM $Na_2MoO_4$ which is a general phosphatase inhibitor. For substrate specificity, both PI and PII have specific activity toward GGDP than FDP, and GDP. The $Km$ value of PI appeared to be 0.2 mM, and the $V$max value of PI is 278 pkat/mg. No substrate inhibition was observed. The $Km$ value of PII appeared to be 0.1 mM, and its $V$max value is 7,530 pkat/mg. The velocity of PII showed around 27-fold faster than that of PI. The GGDP phosphatase activity of PII showed the substrate inhibition at concentration of GGDP more than 0.2 mM.

Study on the process of dephosphorylation reaction of $[1-^3H]$GGDP showed the presence of $[1-^3H]$GGMP and $[1-^3H]$GGOH in the reaction mixture with PII preparation, confirming that GGMP is the intermediate of the GGDP phosphatase.

There are numerous enzymes in chloroplast such as enzymes involved in photosynthetic system and glucose synthesis. Monoterpenes and diterpenes also believed to be synthesized in this compartment. The enzymes with phosphatase activity in the chloroplast from Swiss-Prot database include fructose-1,6-bisphosphate phosphatase, lipid phosphate phosphatase, ADP-glucose pyrophosphorylase, etc. Based on the information on molecular weight and subunit of these enzymes, the protein of PI was tetramer with subunit of 58 kD seems to that of ADP-glucose pyrophosphatase from various plants, i.e. *Zea mays*, *Arabidopsis thaliana*, *Oryza sativum*, etc. This enzyme played a role in starch biosynthesis. It catalyzes the synthesis of the activated glycosyl donor (ADP-glucose) from glucose-1-P and ATP. On the other hand, the molecular weight of PII seems to correspond to that of *C. stellatopilosus* prenyl diphosphate phosphatase which was obtained from gene cloning in this study.

The inhibition by higher concentration of pyrophosphate substrates has been also found in the other works (Scher and Waechter, 1984; Perez et al, 1980). Excess of dolichyl pyrophosphate (Dol-PP) for 2-fold led to an induction of Dol-PP phosphatase 52%. Other than this, some properties of PI and PII were compared with those of previously reports of prenyl phosphatases and are summarized in Table 13.

**Table 13** Comparison of some properties of prenyl phosphate phosphatases obtained from this study and those reported previously.

| Prenyl phosphatases | Km ($\mu$M) | Vmax (nmol/min/mg) | Optimum pH | Inhibitor | Increasing of activity by Mg2+ | Reference |
|---|---|---|---|---|---|---|
| PI | 200 | 16.6 | 6.0-6.5 | $Mo^{2+}$ | slightly | This study |
| PII | 100 | 454.5 | 6.5-7.0 | $Mo^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$ | No | This study |
| Dol-PPase (calf) | 20 | 3.1 | 7.0 | $Mn^{2+}$ | Yes | Scher and Waechter, 1984 |
| Dol-Pase (pig) | 45 | n.d. | 7.2 | orthovanadate, $F^{-}$, $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$ | No | Frank and Waechter, 1998 |
| FDPase (rat) | 7 | 6.8 | 5.5 | GGDP | No effect | Bansal and Vaidya, 1994 |
| GGDPase (rat) | 12 | 14 | 6.0 | $Mn^{2+}$, $Zn^{2+}$ | No effect | Bansal and Vaidya, 1994 |
| FDPase (rice) | n.d. | n.d. | 6.3 | GGDP | No effect | Nah et al, 2001 |
| GGDPase (rice) | n.d. | n.d. | 7.9 | $Mn^{2+}$, $Zn^{2+}$ | No effect | Nah et al, 2001 |
| GGDPase (orange) | n.d. | 69 | 5.5 | $Mn^{2+}$ | No effect | Perez et al, 1980 |

The *C. stellatopilosus* GGDP phosphatase seems to be closely related to the enzyme group of EC 3.1.7.1 that catalyzes the hydrolysis of prenyl phosphoric ester to form prenol. In literature, this group of plant phosphatases has been called both prenyl diphosphatases such as GGPPase from rice seedlings (Nah et al., 2001) and prenyl phophatase such as those from *Citrus sinensis* (Perez et al., 1980). The latter has been shown clearly the involvement of the reaction sequence prenyl-PP $\rightarrow$ prenyl-P $\rightarrow$ prenol. Interstingly, there has been a report on this reverse sequence of the conversion of farnesol or GGOH to its monophosphate and diphosphate products by *Nicotiana tabacum* cell cultures (Thai et al, 1999). These enzymes involving either the prenyl dephosphorylation or prenyl phosphorylation have mostly been shown to be membrane-bound enzymes of microsomal fractions (Nah et al, 2001; Bansal and Vaidya, 1994; Thai et al, 1999)

## 4. Gene cloning of prenyl diphosphate phosphatase from *C. stellatopilosus*

### 1. Full-length gene of prenyl diphosphate phosphatase

The amino acid sequences for reported prenyl diphosphate phosphatases were searched from database. The degenerated primers were designed from the consensus among protein prenyldiphosphate phosphatase from plants.

Total RNA from young leaves of *C. stellatopilosus* was extracted by phenol-chloroform method, and synthesized for single-strand cDNA by RT-PCR principle. The single cDNA was used as template with degenerated primer of sense and antisense direction to obtain cDNA fragment of 512 bp by Nested PCR. The amplicon was then sequenced for nucleotides and determined by program TBLASTX 2.2.9 of the NCBI. By this program, the nucleotide sequence was translated into amino acid sequence and compared with protein sequences in database. The output is shown in the comparison of amino acid sequences of the significant alignment. The results showed homology to phosphatidic acid phosphatases and prenyl diphosphate phosphatases.

Full-length gene was obtained by 5′-RACE and 3′-RACE PCR using SMART kit. The 5′-end of gene was determined by 5′-RACE PCR which amplified nucleotides upstream in antisense direction from 5′-RACE primer. The PCR reaction mixture contained UPM (universal primer) and gene specific primer of prenyl diphosphate phosphatase. 5′-RACE product contained nucleotide sequence of UPM following with nucleotide sequence of our gene in 5′-end until reaching the nucleotide sequence of antisense primer. So it was clearly that the 5′-end of prenyl diphosphate phosphatase was obtained as shown in diagram in Fig. 63. 3′-end of gene was determined by 3′-RACE PCR product. The nucleotide sequence from 3′-end primer to poly-A tail were obtained. Due to the 3′-RACE and 5′-RACE fragments were designed to have an overlapping area, so the fragments can be combined to get full-length gene nucleotide sequence. The open reading frame (ORF) was determined from the start codon of ATG to the stop codon of TAA.
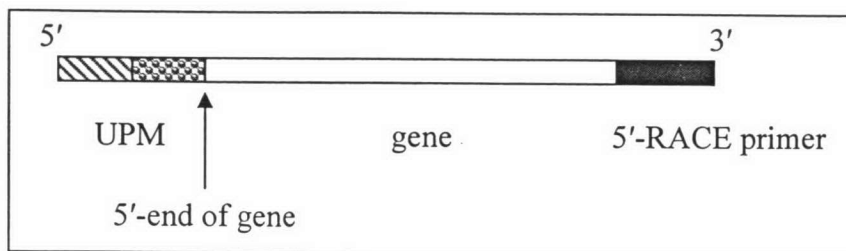
**Figure 63**    Diagram of synthesizing 5′-end of gene by 5′-RACE PCR using SMART kit.

## 2. Sequence analysis

The alignment of deduced amino acid sequence of prenyl diphosphate phosphatase from *C. stellatopilosus* with the sequences of prenyl diphosphate phosphatase and phosphatidic acid phosphatase (PAP) from Swiss-Prot database by ClustalW revealed high homology (52-71%) to phosphatidic acid phosphatase as shown in Table 14.    The protein that exhibited the most similarity to prenyl diphosphate phosphatase from *C. stellatopilosus* was PAP from *Vigna unguiculata* (cowpea) (71%).

The phylogenetic tree was predicted by ClustalW as shown in Fig. 64.
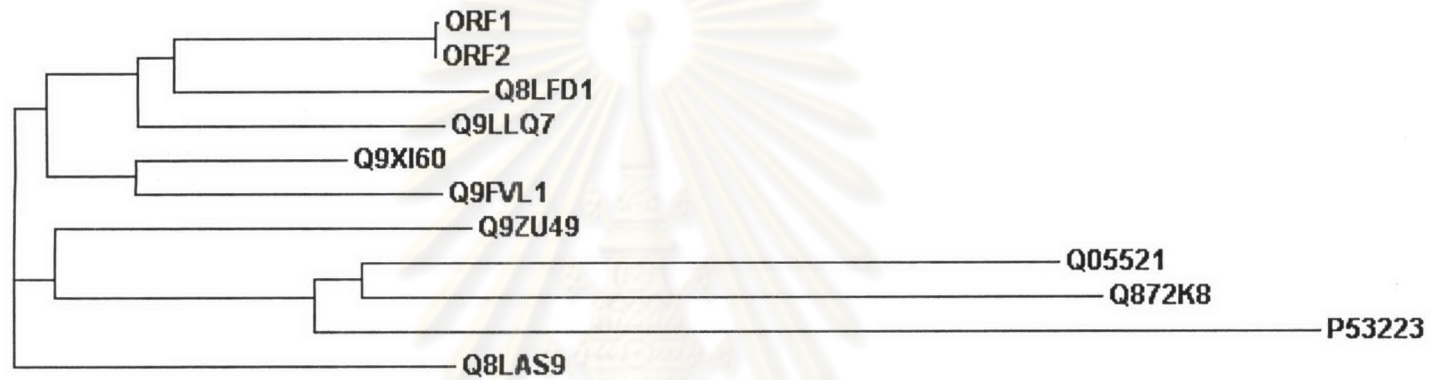
**Figure 65**  phylogenetic tree of prenyl diphosphate phosphatase, phosphatidic acid phosphatase, diacylglycerol diphosphate phosphatase, and dolichyl diphosphate phosphatase. The protein names according to accession no. was shown below table 14.

**Table 14** Percent (%) homology of prenyl diphosphate phosphatase, phosphatidic acid phosphatase, diacylglycerol diphosphate phosphatase, and dolichyl diphosphate phosphatase

|  | ORF1 | ORF2 | Q9 ZU49 | Q9 XI60 | Q8 LFD1 | Q9 LLQ7 | Q9 FVL1 | Q8 LAS9 | Q0 5521 | Q8 72K8 |
|---|---|---|---|---|---|---|---|---|---|---|
| ORF2 | 100 | | | | | | | | | |
| Q9 ZU49 | 56 | 57 | | | | | | | | |
| Q9 XI60 | 62 | 60 | 61 | | | | | | | |
| Q8 LFD1 | 69 | 70 | 55 | 63 | | | | | | |
| Q9 LLQ7 | 71 | 72 | 54 | 65 | 63 | | | | | |
| Q9 FVL1 | 58 | 58 | 51 | 73 | 56 | 59 | | | | |
| Q8 LAS9 | 51 | 52 | 52 | 61 | 51 | 54 | 57 | | | |
| Q0 5521 | 26 | 26 | 23 | 25 | 25 | 25 | 25 | 23 | | |
| Q8 72K8 | 25 | 27 | 25 | 22 | 20 | 21 | 23 | 20 | 25 | |
| P5 3223 | 14 | 12 | 15 | 8 | 11 | 11 | 10 | 12 | 10 | 7 |

Accession no.: Q9ZU49: Lipid phosphate phosphatase 1 (AtLPP1) (Phosphatidic acid phosphatase 1) (AtPAP1) (Prenyl diphosphate phosphatase) from *Arabidopsis thaliana* (*At*); Q9XI60: Lipid phosphate phosphatase 2 (AtLPP2) (Phosphatidic acid phosphatase 2) (AtPAP2) (Prenyl diphosphate phosphatase) from *At*; Q8LFD1: Putative lipid phosphate phosphatase 3, chloroplast precursor (AtLPP3) (Phosphatidic acid phosphatase 3) (Phosphatidate phosphohydrolase 3 from *At*; Q9LLQ7: phosphatidic acid phosphatase (PAP) alpha from *Vigna unguiculata* (*Vu*); Q9FVL1: PAP beta from *Vu*; Q8LAS9: DGPP phosphatase from *At*; Q05521: DGPP phosphatase from *S. cerevisiae* (yeast); Q872K8: diacylglycerol pyrophosphate phosphatase (DGPP phosphatase) from *Neurospora crassa*; P53223: dolichyl diphosphate phosphatase from yeast.

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ORF1     ------------------------------------------------------------
ORF2     ------------------------------------------------------------
Q8LFD1   -MARFSFPCFPNFGGFN---QAVTNRGFEISETADNWVSPSDIPLI-EPNSKEHRMREAQ 55
Q9LLQ7   MASWWDLRRLFGFQSIRTRFQDLSSRRLGISAVSGAHFSSINFPLIDKKNEEDFRTREVQ 60
Q9XI60   ----------------------------------------------------MPEIH 5
Q9FVL1   ----------------------------------------------------MPEIQ 5
Q8LAS9   ----------------------------------------------------MAKIM 5
Q9ZU49   ------------------------------MTIGSFFSSLLFWRNSQDQEAQRGRMQEID 30
Q05521   ----------------------------------------------------MNRVS 5
Q872K8   --------------------------MGFFNRQPRAADGAAPPVAANGTTRNEKRARRHGFE 36
P53223   ------------------------------------------------------------
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ORF1     ------------------MHDWLIFLLLVVIEVILYVIHPFKR-------YAGKDMMTDLR 36
ORF2     ---------------------------------------MMTDLR 6
Q8LFD1   LGGHTLRSHGMTVARTHMHDWIILVLLVILECVLLIIHPFYR-------FVGKDMMTDLS 108
Q9LLQ7   LGSHTVSSHGYAVARTHKHDWLILLLLVLVAIGLYVVHPFHR-------FVGKDMMTDLR 113
Q9XI60   LGAHTIRSHGVTVARFHMHDWLILLLLIVIEIVLNVIEPFHR-------FVGEDMLTDLR 58
Q9FVL1   LGMHTIRSHGTRVARIHMHDWLILLLLVIIDAVLNIIEPFHR-------FVGEGMMTDLR 58
Q8LAS9   LGSHSVKSHGWKVAREHLCDWLILVVLGLIDIVLNVIEPFHR-------YIGPDMLTDLT 58
Q9ZU49   LSVHTIKSHGGRVASKHKHDWIILVLIAIEIGLNLISPFYR-------YVGKDMMTDLK 83
Q05521   FIKTPFNIG----AKWRLEDVFLLIIMILLNYPVYYQQPFER-------QFYINDLTISH 54
Q872K8   PYTMTTRPTFGQWLKYTWLDILTMAALGAIGLGVYYAHPAPSRSFAVQFSDGEVVYPQFA 96
P53223   ----------------------------MNSTAAAINPNPN-----------VIPFDDT 20
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ORF1     YPLQSNTVPVWAVPMYAILLP----MLVFLVVYSRRRDIYDLHHAILGLFYSVLVTAVIT 92
ORF2     YPLQSNTVPVWAVPMYAILLP----MLVFLVVYSRRRDIYDLHHAILGLFYSVLVTAVIT 62
Q8LFD1   YPLKSNTVPIWSVPVYAMLLP----LVIFIFIYFRRRDVYDLHHAVLGLLYSVLVTAVIT 164
Q9LLQ7   YPLKSNTVPVWSIPIYAVLLP----IVIFLVVYIRRRDVYDLHHAVLGLLFSLLITAVIT 169
Q9XI60   YPLQDNTIPFWAVPLIAVVLP----FAVICVYYFIRNDVYDLHHAILGLLFSVLITGVIT 114
Q9FVL1   YPLKGNTIPFWAVPIVAILLP----LAVFLVYYFIRKDVYDFHHAILGLLFSVLITAVIT 114
Q8LAS9   FPFYEDTIPMWAVPIICILVP----ICIFIVYYYYRRDVYDLHHAILGIGFSCLVTGVTT 114
Q9ZU49   YPFKDNTVPIWSVPVYAVLLP----IIVFVCFYLKRTCVYDLHHSILGLLFAVLITGVIT 139
Q05521   PYATTERVNNNMLFVYSFVVPSLTILIIGSILADRRHLIFILYTSLLGLSLAWFSTSFFT 114
Q872K8   YPMRKEIIPIWLAAFLASIIP----IFIILCMQIRIRSFWDVNNGVLGLLYSLITAAVFQ 152
P53223   YILYDSHDFLSFLSAYFSLMP------ILVLAFYLSWFIITRELEACIVAFGQLMNEIFN 74
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ORF1     DSIKNAVGRPRFDFFWRCFP-DGKDVYDQLG---------------NVICHGD-KNVIKE 135
ORF2     DSIKNAVGRPRFDFFWRCFP-DGKDVYDQLG---------------NVICHGD-KNVIKE 105
Q8LFD1   DAIKNAVGRPRFDFFWRCFP-DGKALYDSLG---------------DVICHGD-KSVIRE 207
Q9LLQ7   EAIKNGVGRPRFDFFWRCFP-DGKDVYDKLG---------------DVICHGE-KGVVKE 212
Q9XI60   DAIKDAVGRPRFDFFWRCFP-DGIGIFHNVTK--------------NVLCTGA-KDVVKE 158
Q9FVL1   DAIKDGVGRPRFDFFWRCFP-DGKGVFDPVTS--------------DVRCTGD-KGVIKE 158
Q8LAS9   DSIKDAVGRPRFNFFYRCFP-NGKPKFHPDTK--------------DVVCHGV-KKIIKE 158
Q9ZU49   DSIKVATGRPRFNFYWRCFP-DGKELYDALG---------------GVVCHGK-AAEVKE 182
Q05521   NFIKNWIGRLRFDFLDRCQPVEGLPLDTLFTA--------------KDVCTTKNHERLLD 160
Q872K8   VFIKWLIGGLRFHFLTVCKPDITRATNTQIAEKGYSAQGFAEIYYTKDICTGD-PNEIDD 211
P53223   NVIKNIIKQPRFVSFGASFQ---------------------------NDTIRS 100
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            **   I      **          ·                    :

ORF1     GHKSFPSGHTSWSFAGLGFLSLYLSGKLKAFDRRG-HVAKLCIIFLPLLVACLVGISRVD 194
ORF2     GHKSFPSGHTSWSFAGLGFLSLYLSGKLKAFDRRG-HVAKLCIIFLPLLVACLVGISRVD 164
Q8LFD1   GHKSFPSGHTSWSFSGLGFLSLYLSGKIQAFDGKG-HVAKLCIVILPLLFAALVGISRVD 266
Q9LLQ7   GYKSFPSGHTSWSFSGLGFLSLYLSGKKAFDRRG-HVAKLCIVFLPLLFASLVGISRVD 271
Q9XI60   GHKSFPSGHTSWSFAGLGFLSLYLSGKIRVFDQRG-HVAKLCIVILPLLVAALVGVSRVD 217
Q9FVL1   GTKVSPSGHTSWSFAGLVYLSWKLSGKIRVFDRRG-HVAKLCLVFLPLLVAAMIAGSRVD 217
Q8LAS9   GYKSFPSGHTSWSFAGLTFLAWYLSGKIKVFDRRG-HVAKLCLVFLPILISILIGISRVD 217
Q9ZU49   GHKSFPSGHTSWSFAGLTFLSLYLSGKIKAFNNEG-HVAKLCLVIFPLLAACLVGISRVD 241
Q05521   GFRTTPSGHSSESFAGLGYLYFWLCGQLLTESPLM-PLWRKMVAFLPLLGAALIALSRTQ 219
Q872K8   SLESMPSGHBTAAFAGFIFLALYLNAKLKVFSNYHPALWKLAAVYAPVLGACLIAGALTI 271
P53223   GYG-MPSAHB--QFMGFCFT--YNSLKIYTSWKNLNFLEKCIFSGALALLSFCVCFSRVY 155
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            III

ORF1     DYWHHWQDVFAGGLLGLTVSTFCYLQFFP---------PPYHPQGWGPYAYFRVLEESHG 245
ORF2     DYWHHWQDVFAGGLLGLTVSTFCYLQFFP---------PPYHPQGWGPYAYFRVLEESHG 215
Q8LFD1   DYWHHWQDVFAGGLLGLAISTICYLQFFP---------PPYHTEGWGPYAYFQVLEAARV 317
Q9LLQ7   DYWHHWQDVFAGGLLGLTVSTFCYLQFFP---------PPYHSEGWGPYAYFRMLEESRQ 322
Q9XI60   DYWHHWQDVFGGAIIGLTVATFCYLQFFP---------PPYDPDGWGPHAYFQMLADSRN 268
Q9FVL1   DYWHHWQDVFAGGLIGTTIASFCYLQFYP---------PPYDLDGWGPHAYFQMLAESRN 268
Q8LAS9   DYWHHWTDVFAGAIIGFVASFSYLHFFP---------YPYDENGWAPHAYFRMLAERST 268
Q9ZU49   DYWHHWQDVFAGALIGTLVAAFCYRQFYP---------NPYHEEGWGPYAYFKAAQERGV 292
Q05521   DYRHHFVDVILGSMLGYIMAHFFYRRIFP---------PIDD-----PLPFKPLMDDSDV 265
Q872K8   DEFHHWYDVLAGAVIGTIMAFSAYRMVYASIWDWRYNHIPLNRSNPFPFGFSRDDMELGGA 331
P53223   LHYHNLDQVIVGFSVGALTGSLYFFIVGIIR------------ELGLINWFLKLRIVRL 202
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ORF1     NTQASSATN----LLNSESRVGEE-EESNVFMGLHLARNSSLPMESHQDVERGPK----- 295
ORF2     NTQASSATN----LLNSESRVGEE-EESNVFMGLHLARNSSLPMESHQDVERGPK----- 265
Q8LFD1   QGAANGAVQ----QPPPQVNNGEE-EDG-GFMGLHLVDNPTMRREEDVETGRG------- 364
Q9LLQ7   MTQVPNVPNSGHAQLTEVQAEGEEGQGCHGCMGLSLSRDRNATLN-DIESGRG------- 374
Q9XI60   DVQDSAGMNHLS-VRQTELESVR----------------------------------- 290
Q9FVL1   GSQPSTVNNEIHHVQSSELQAVSVYIPPQHDADTRVNSWDSSPMLGASQNVRTH----- 322
Q8LAS9   GRATTMTRTGSRGMLDNDVEPGNS--------------ASSPHDRHRESTDSDF----- 308
Q9ZU49   PVTSSQNGD--ALRAMSLQMDST--------------SLENMESGTSTAPR------- 327
Q05521   TLEEAVTHQR---IPDEELHPLSD--------------EGM------------- 289
Q872K8   TFTRQVGWGTSGAGFPFDDKHGYAGGGYGGYGGYGGGPAINRKPVAGNGVGGPFHSHGAR 391
P53223   FYMTDSYNLAPLTLKENYEAYWKR---------------INQRSFNDKSKRD-------- 239
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ORF1     -----
ORF2     -----
Q8LFD1   -----
Q9LLQ7   -----
Q9XI60   -----
Q9FVL1   -----
Q8LAS9   -----
Q9ZU49   -----
Q05521   -----
Q872K8   GEQMV 396
P53223   -----
```

**Figure 65**    Multiple alignment of prenyl diphosphate phosphatase, phosphatidic acid phosphatase, diacylglycerol diphosphate phosphatase, and dolichyl diphosphate phosphatase. The protein names according to accession no. was shown below table 14. (I, II, III = phosphatase motif of KX$_6$RP, PSGH, and SRX$_5$HX$_3$D, respectively)

As shown in the Table 14, there is a 51 % homology between amino acid of *A. thaliana* DGPP phosphatase but it is less observed when compares between that of phosphatase from yeast and *Neurospora* (26% homology).

The phosphatase motif consisting of three consensus (Stukey and Carman, 1997) was found among all 13 amino acid sequences of prenyl diphosphate phosphatases, phosphatidic acid phosphatases, diacylglycerol pyrophosphate phosphatases, dolichyl phosphate phosphatase, ORF1 and ORF2 as shown in the alignment by CLUSTAL W in Fig. 65. The phosphatase motifs consisting of $KX_6RP$, PSGH, and $SRX_5HX_3D$.

Moreover, the amino acid of ORF1 was aligned to those of terpene synthase which were the enzyme using the substrate with pyrophosphate group. There is a very low level of homology (3-9%) as shown in Table 15. And those sequences have no phosphatase motif.

**Table 15** Percent (%) homology of *C. stellatopilosus* prenyl diphosphate phosphatase, terpene synthases, and terpene cyclases.

|  | ORF1 | ORF2 | Q8L5K3 | Q38710 | P80042 |
|---|---|---|---|---|---|
| ORF2 | 100 |  |  |  |  |
| Q8L5K3 | 8 | 9 |  |  |  |
| Q38710 | 8 | 9 | 26 |  |  |
| P80042 | 5 | 5 | 6 | 7 |  |
| Q9SSU0 | 3 | 3 | 6 | 9 | 61 |

Accession no.: Q8L5K3: (+)-limonene synthase 1 from *Citrus limon* (Lemon); Q38710 abietadiene synthase, (abietadiene cyclase) from *Abies grandis*; P80042: geranylgeranyl pyrophosphate synthetase from *Capsicum*; Q9SSU0: geranylgeranyl pyrophosphate synthase from *Croton sublyratus*.

Mutation of phosphate motif were studies in DGPP phosphatase from yeast, there were evidences that mutation of arginine at motif I and histidine at motif III of phosphaptase motif caused completely loss of DGPP phosphatase activites. And mutation of histidine at motif II also led to 91% decreasing of DGPP phophatase activity (Toke et al, 1999).

The model for the topology of the deduced amino acid sequences of ORF1 and ORF2 were predicted by program Sousi v1.0 (Hirokawa et al., 1998) (http://sosui.proteome. bio.tuat.ac.jp/sosuiframe0.htm). The prediction indicated that both of them were transmembrane protein with 6 and 5 transmembranes regions, respectively as shown in Fig. 66.

The enzyme involved in the biosynthetic pathway of plaunotol which is diterpene should be taken place in chloroplast. So we expected the available of the chloroplast transit peptide (cTP) in the amino acid sequences of ORF1 and ORF2. By using ChloroP program, no area of cTP was predicted. There was no exactly characteristic homology of cTP and its cleavage site. The program available at present could not 100% exactly predict, therefore we designed the truncate proteins of ORF1 and ORF2 by trial and error method. We constructed the truncate gene from the basis of inputting nucleotide sequences into ChloroP program, the cleavage site of cTP from nucleotide data was obtained as the output of the program. The RR motif (twin-arginine motif) was reported as the cleavage sites of monoterpene synthase and diterpene synthase, so the truncate protein after this motif of ORF1 was also designed. The 5 clones of ORF1, truncate ORF1, ORF2, truncate ORF2, and truncate after RR motif were constructed, in addition with a clone of empty vector as a control. All genes were expressed in *E. coli*

| No. | N terminal | transmembrane region | C terminal | type | length | A |
|---|---|---|---|---|---|---|
| 1 | 2 | HDWLIFLLLVVIEVILYVIHPF | 23 | PRIMARY | 22 | |
| 2 | 43 | TVPVWAVPMYAILLPMLVFLVVY | 65 | PRIMARY | 23 | |
| 3 | 74 | LHHAILGLFYSVLVTAVITDSIK | 96 | SECONDARY | 23 | |
| 4 | 139 | SFPSGHTSWSFAGLGFLSLYLSG | 161 | SECONDARY | 23 | |
| 5 | 171 | HVAKLCIIFLPLLVACLVGISRV | 193 | PRIMARY | 23 | |
| 6 | 203 | VFAGGLLGLTVSTFCYLQFFP | 223 | SECONDARY | 21 | |

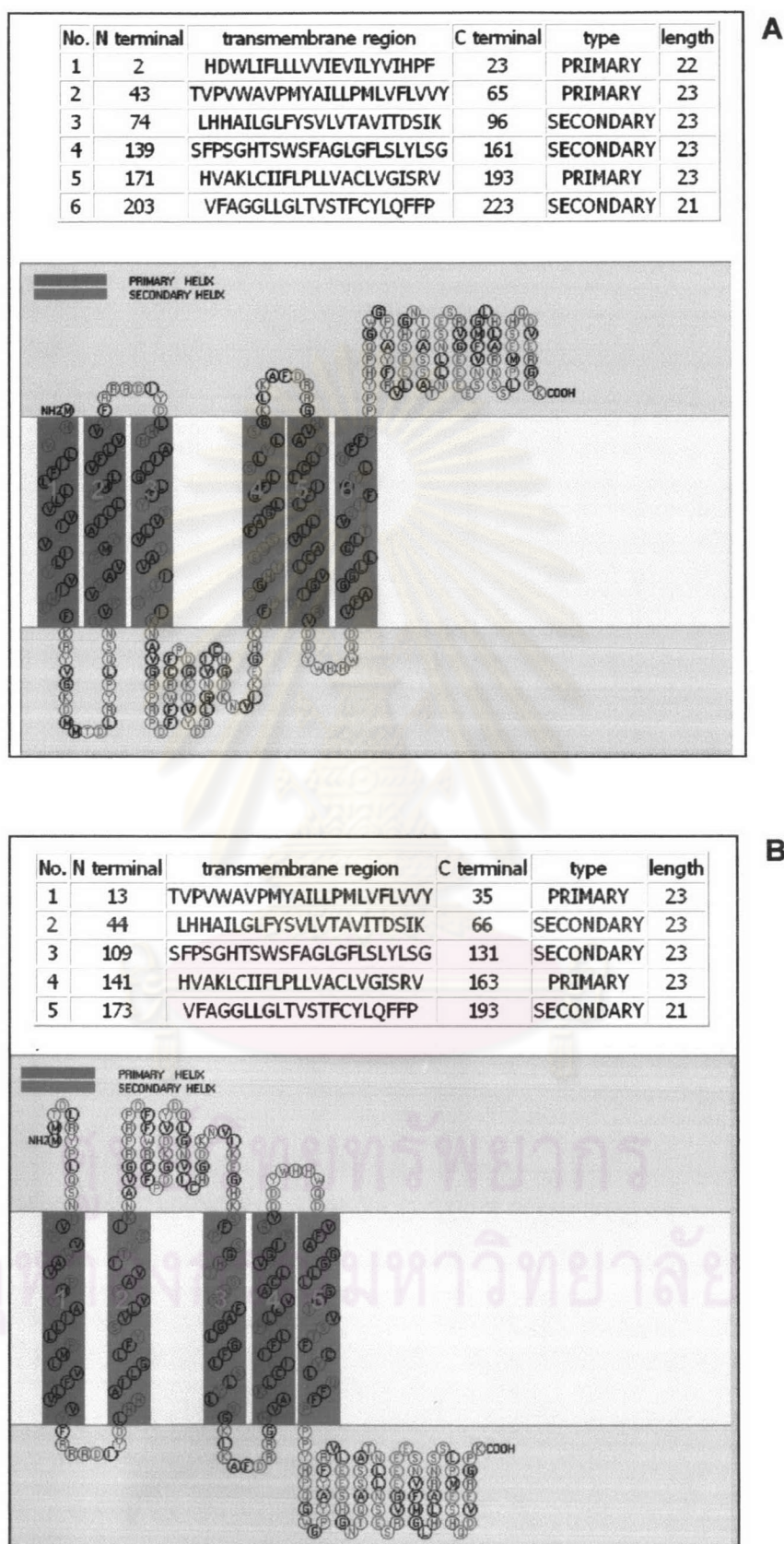| No. | N terminal | transmembrane region | C terminal | type | length | B |
|---|---|---|---|---|---|---|
| 1 | 13 | TVPVWAVPMYAILLPMLVFLVVY | 35 | PRIMARY | 23 | |
| 2 | 44 | LHHAILGLFYSVLVTAVITDSIK | 66 | SECONDARY | 23 | |
| 3 | 109 | SFPSGHTSWSFAGLGFLSLYLSG | 131 | SECONDARY | 23 | |
| 4 | 141 | HVAKLCIIFLPLLVACLVGISRV | 163 | PRIMARY | 23 | |
| 5 | 173 | VFAGGLLGLTVSTFCYLQFFP | 193 | SECONDARY | 21 | |

**Figure 66** Proposed model for the topography of deduced amino acid sequence of ORF1 (A); and ORF2 (B) in the organelle membrane using program Sousi v1.0.

### 3. Expression of *C. stellatopilosus* prenyl diphosphate phosphatase in *E. coli.*

Gene was constructed and cloned into the plasmid pET 101/D-TOPO vector. The fusion protein with His-tag at C-terminal of ORF1, truncate ORF1, ORF2, truncate ORF2, and gene after RR motif were obtained from the expression in *E. coli*. As many reports of gene cloning in enzyme involve in terpenoid pathway using *E. coli* expression system, so we chose this system as a first tool for expression of gene. Because of several number of arginine, isoleucine and leucine contained in the amino acid sequence of *C. stellatopilosus* prenyl diphosphate phosphatase, so the expression was performed in *E. coli* strain BL21 Codon Plus (DE3)-RIL.

### 4. Purification of *C. stellatopilosus* prenyl diphosphate phosphatase by affinity column

The protein expression pattern from crude lysate cell showed two major bands that seem to be the recombinant proteins because of the appearance after induction by IPTG. However, the results from Western Blot showed clearly that the major bands were not the his-tag protein. The protein ORF1, truncate ORF1, ORF2, and truncate ORF2 showed obvious bands of interaction with His-tag Mab around 30 kDa position. Protein after RR motif showed no band at that area, but the band below the membrane. Since *E. coli* also have phosphatase enzymes, the empty vector control was performed using the similar steps of recombinant protein preparation in the expression and purification procedures.

### 5. GGDP dephosphorylation of recombinant *C. stellatopilosus* prenyl diphosphate phosphatase

The fraction from Talon resin column, which was desalted by PD-10 column and concentrated by using Centricon-10, was used to determine for GGDP phosphatase activity. Protein ORF1 showed the main enzymatic product of GGOH, whereas the others showed the main product of GGMP. Another proteins that yielded GGOH were truncate protein ORF1, protein ORF2, and truncate protein ORF2 (Fig. 61 and 62).

The result indicated that dephosphorylation of prenyl diphosphate phosphatase consisting of 2 steps. First, GGDP was cleaved one group of phosphate to obtain GGMP and then the second phosphate group was cleaved to yield GGOH.

The deduced amino acid of *C. stellatopilosus* prenyl diphosphate phosphatase which were obtained from this study showed highly identity to that of phosphatidic acid phosphatase and diacylglycerol pyrophosphate phosphatase as shown in the alignment. These enzymes were transmembrane protein. DGPP phosphatase and lipid phosphate phosphatase from yeast were reported for the dephosphorylation activity to Dol-PP, FDP, and GGDP (Faulkner et al, 1999). They showed that the production of GGMP from GGDP by DGPP phosphatase was linear for 30 min, after that, the apparent production of GGMP was reduced, whereas production of GGOH increased. The optimum pH was 5.0-5.5. Furthermore, both DGPP and GGDP were equally good substrates for DGPP phosphatase (Faulkner et al, 1999).

It can be noted that there are relationship between prenyl diphosphate phosphatase, phosphatidic acid phosphatase, diacylglycerol pyrophosphate phosphatase and lipid phosphate phosphatase that they have phosphatase motif, high homology between amino acid sequences, and the broad specificity among their substrate.