เป็นหนังสือภาษาอังกฤษ

รายงานวิจัยฉบับสมบูรณ์

โครงการ
การโคลน cDNA และการศึกษาระดับ transcription ของยีน 1-ดีออกซี-ดี-ไซลูโลส 5-ฟอสเฟต รีดักโตไอโซเมอร์เลส (*dxr*) ในเปล้าน้อย

cDNA cloning and transcription level of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*) gene in *Croton stellatopilosus*

โดย

ผศ.ดร.จุไรทิพย์ หวังสินทวีกุล

ดร.พิมพ์พิมล ตันสกุล

งานวิจัยฉบับนี้ได้รับการสนับสนุนจาก

ทุนอุดหนุนการวิจัยจากเงินรายได้มหาวิทยาลัยสงขลานครินทร์

ประเภททั่วไป ประจำปี 2549

กรกฎาคม พ.ศ. 2550

Acknowledgement

First of all, authors would like to thank the Department of Pharmacognosy and Pharmaceutical Botany for providing facilities in laboratory as well as the Scientific Equipment Center, Prince of Songkla University for services on GC, GC-MS, DNA sequencing and Gel Doc (densitometer).

Acknowledgement also goes to Ms Tanawan Sirisuntipong for her excellent work on this project. Finally grants from Prince of Songkla University and the National Center for Genetic Engineering and Biotechnology (BIOTEC) are acknowledged for their supports.

Juraithip Wungsintaweekul, Dr.rer.nat

July, 2007

ABSTRACT
(Thai)

เอนไซม์ 1-deoxy-D-xylulose 5-phosphate reductoisomerase เป็นเอนไซม์ลำดับที่ 2 ของวิถีชีวสังเคราะห์ชนิด deoxyxylulose phosphate (DXP) ทำหน้าที่เร่งปฏิกิริยาการสร้าง 2C-methyl-D-erythritol 4-phosphate จากสารตั้งต้น DXP ในสภาวะที่มี NADPH และ Mg^{2+} หรือ Mn^{2+} ในการศึกษานี้ยีน dxr จากใบอ่อนของเปล้าน้อยได้ถูกโคลนขึ้นด้วยวิธี homology-based PCR (polymerase chain reaction) และ RACE (rapid amplification of cDNA ends) โดย full-length cDNA ของยีน *dxr* (*CsDxr*) ที่ได้ประกอบด้วยสายนิวคลีโอไทด์ ความยาว 1,404 คู่เบส ที่ถอดรหัสได้สายเปปไทด์ที่มีกรดอะมิโนจำนวน 468 หน่วย มวลโมเลกุล และค่า isoelectric point ถูกทำนายเป็น 50.6 kDa และ 5.64 ตามลำดับ เมื่อวิเคราะห์สายกรดอะมิโนด้วยโปรแกรม TargetP พบว่าปลายด้าน N-terminal ตำแหน่งที่ 1-44 ทำหน้าที่เป็น chloroplast transit peptide ขณะเดียวกันยังพบ NADPH binding motif (GSTGSIGT) อีกด้วย นอกจากนี้ยังพบว่าลำดับกรดอะมิโนของ CsDXr จากใบเปล้าน้อย มีความเหมือนกับลำดับกรดอะมิโนของเอนไซม์ Dxr จากพืชชั้นสูงอื่นๆ มากกว่า 76% identity เมื่อศึกษาระดับการแสดงออกของยีนในส่วนต่างๆ ของต้นเปล้าน้อยด้วยวิธี Semiquantitative RT-PCR และหาปริมาณเปล้าโนทอลด้วยวิธี gas-chromatography ผลจากการทดลองพบว่า การแสดงออกของยีน *dxr* ไม่เปลี่ยนแปลงในใบตำแหน่งที่ 1 ถึง 5 นอกจากนี้ยังพบว่า มีปริมาณน้อยในยอด และไม่พบในลำต้นและราก ในขณะที่พบการสะสมสารเปลาโนทอลเฉพาะที่ใบ โดยพบปริมาณสูงสุดในใบตำแหน่งที่ 1 และ 2 แล้วจึงค่อยๆ ลดลงในใบตำแหน่งที่ 3-5 จากผลการทดลองนี้แสดงให้เห็นว่า CsDxr มีส่วนเกี่ยวข้องในกระบวนการชีวสังเคราะห์ของ isoprene unit ผ่านวิถีชีวสังเคราะห์แบบ DXP อย่างไรก็ตาม CsDxr ไม่ได้ทำหน้าที่เป็น rate-limiting enzyme ในวิถีชีวสังเคราะห์ของสารเปลาโนทอล

Abstract

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR; EC 1.1.1.267) is the second enzyme in the deoxyxylulose phosphate (DXP) pathway. It catalyzes the formation of 2C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate in the presence of NADPH and Mg^{2+} or Mn^{2+}. In this study, the *dxr* gene was cloned from cDNA of *Croton stellatopilosus* young leaves (designated as *CSdxr*) by homology based PCR and rapid amplification of cDNA ends (RACE) methods. The results showed that *CS-dxr* contained an open reading frame (ORF) of 1,404 nucleotides encoding a deduced peptide of 468 amino acid residues. Analyzed data of CSDXR indicated that, CSDXR carried the chloroplast transit peptide at the N-terminal (position 1-44), and contained a proline-rich region and NADPH binding motif. Alignment of CSDXR shared high homology with more than 76% amino acid identity to other known plant DXRs. Expression pattern analysis indicated that *CSdxr* strongly expressed in leaves, rarely in stems and roots. CSDXR was found to be associated with isoprenoid biosynthesis via the DXP pathway, however, did not exhibit as the rate-limiting step in plaunotol biosynthesis.

CONTENT

List of figure

List of table

Abbreviation

AcCoA	=	Acetyl coenzyme A
bp	=	Base pair
BSU	=	Bioservice Unit
°C	=	Degree Celsius
DEPC	=	Diethyl pyrocarbonate
DMAPP	=	Dimethylallyl diphosphate
DX	=	1-deoxy-D-xylulose
DXP	=	Deoxyxylulose phosphate
dxr	=	1-deoxy-D-xylulose 5-phosphate reductoisomerase gene
DNA	=	Deoxyribonucleic acid
Dxr	=	1-deoxy-D-xylulose 5-phosphate reductoisomerase
Dxs	=	1-deoxy-D-xylulose-5-phosphate synthase
dNTP	=	Deoxynucleoside triphosphate
ER	=	Endoplasmic reticulum
FID	=	Flame ionization detector
GAP	=	D-glyceraldehyde 3-phosphate
GC	=	Gas chromatography
GGOH	=	Geranylgeraniol
GGPP	=	Geranylgeranyl diphosphate
HMG-CoA	=	3-hydroxy-3-methylglutaryl-CoA
HMGR	=	3-hydroxy-3-methylglutaryl-CoA reductase
IPTG	=	Isopropyl-β -D-thiogalactopyranoside
IPP	=	Isopentenyl diphosphate
kb	=	Kilobase
kDa	=	Kilodalton
LB	=	Luria Bertaini
M	=	Molar
ME	=	2-*C*-methyl-erythritol
MEOP	=	2-*C*-methylerythrose 4-phosphate
MEP	=	2-*C*-methyl-D-erythritol 4-phosphate
mg	=	Milligram
ml	=	Mililiter
mM	=	Millimolar
mRNA	=	Messenger ribonucleic acid
MVA	=	Mevalonate
µg	=	Microgram
µl	=	Microliter
nm	=	nanometer

OD	=	Optical density
pH	=	-log hydrogen ion concentration
pmol	=	Picomole
Pro (P)	=	Proline
RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
RNA	=	Ribonucleic acid
RNase A	=	Ribonuclease A
rpm	=	Revolutions per minute
Ser (S)	=	Serine
TAE	=	Tris acetate EDTA
TLC	=	Thin-layer chromatography
UV	=	Ultra violet
UV-VIS	=	Ultra violet visible
V	=	Volt
Val (V)	=	Valine
v/v	=	Volume by volume
w/v	=	Weight by volume
w/w	=	Weight by weight
X-GAL	=	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Terpenoids are a structurally diverse group of natural products. More than 25,000 representatives with a variety of biological functions have been reported in the plant kingdom (Sacchiettini and Poulter, 1997). Most of terpenoids play an important role as therapeutic agents such as paclitaxel (Taxol®) from *Taxus brevifolia*, ginkgolides from *Ginkgo biloba*, eleutherobin from a soft coral, etc.

Terpenoids are derived from the unique C 5 atoms, the isoprene unit. It has been currently reported that the origins are provided from two different pathways: the classical mevalonate (MVA) pathway and the recently discovered deoxyxylulose phosphate (DXP) pathway. For the latter DXP pathway, the isoprene unit is formed from the condensation of pyruvate and glyceraldehydes 3-phosphate, catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), yielding the first intermediate 1-deoxy-D-xylulose-5-phosphate (DXP). Further, DXP is intramolecular rearranged to a branch polyol sugar, 2*C*-methyl-D-erythritol 4-phosphate (MEP), catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr). The MEP is then transformed to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) consecutively, catalyzed by IspD, IspE, IspF, IspG and IspH. (for review see Eisenreich *et al.*, 2004). Since the DXP pathway has distributed naturally in eubacteria, malaria parasite and higher plants, therefore, the enzymes involved in the DXP pathway are targets for novel antibacterial, antimalarials and herbicides drug discovery (Lichtenthaler, 1999 and Kaiser *et al.*, 2007).

In higher plants, many genes involved in the DXP pathway have been reported. The benefit of usage the biosynthetic gene is to increase the secondary metabolite production by manipulating the gene either in the intact plant or plant cell cultures. Several studies reported the function and regulatory role of the genes involved the DXP pathway in the terpenoid biosynthesis in higher plant. For example, the investigation of *dxr* gene transcripts from *Lycopersicon esculentum* concluded that *dxr* gene responsible for regulation the carotenoid production, however, non rate-limiting step (Rodriguez-Concepcion *et al.* 2001). In *Coleus forskohlii* cell culture, the *dxr* gene showed to regulate the forskolin biosynthesis and be able to inhibit by fosmidomycin (Engprasert *et al.*, 2005). The transgenic *Lavendula latifolia* by incorporating the *dxs* gene from *Arabidopsis thaliana* enhanced the essential oil production (Munoz-Bertomeu *et al.*, 2006). Those studies are intensifying the regulatory role of on the target genes in terpeniods biosynthesis.

Among Thai medicinal plant, *Croton stellatopilosus* Ohba (formerly name *C. sublyratus* Kurz.) or plau-noi is the only source of plaunotol, which is a potent cytoprotective

antipeptic ulcer (Ogiso *et al.*, 1978). It acts by inhibiting the growth of *Helicobacter pylori* (Koga *et al.*, 1996). Plaunotol is an acyclic diterpene alcohol and composes of four isoprene units attached in head to tail fashion (Ogiso *et al.*, 1978). The isoprene units of plaunotol skeleton have been originated from the DXP pathway (Wungsintaweekul and De-Eknamkul, 2005). The biosynthesis of plaunotol was first studied from the group of De-Eknamkul and his coworkers since 1997. The geranylgeraniol 18-hydroxylase catalyzes the final committed step from the molecule of geranylgeraniol to plaunotol (Tansakul and De-Eknamkul, 1998). Recently, the phosphatase was reported to catalyze the step of dephosphorylation yielding geranylgeraniol from geranylgeranyl diphosphate (Nualkaew *et al.*, 2006). For the early step in the plaunotol biosynthesis, it is unfortunately lack of knowledge that has been reported.

Therefore, the objective of this work aims to study the *dxr* gene in the DXP pathway presence in *C. stellatopilosus* leaves. The *dxr* gene will be cloned by homology base and RACE (rapid amplification of cDNA ends) method and the *dxr* gene will be characterized for the function. The regulatory role of *dxr* gene will be investigated in terms of the transcription level in relation to the amount of plaunotol in various parts of *C. stellatopilosus*. The results obtained from this study will be useful for understanding the plaunotol biosynthetic pathway, which leads to enhance the plaunotol production either in intact plant or plant cell culture by metabolic engineering.

1.2 Review of literature

1.2.1 The biosynthetic pathway of terpenoids

1.2.1.1 The classical mevalonate (MVA) pathway

The classical route for the formation of the C_5 building blocks of terpenoid biosynthesis in plants via the reactions of the mevalonate pathway, first demonstrated in yeast and mammals (for review see Porter and Spurgeon, 1981). This well-characterized sequence (Fig. 1.1) starts from acetyl-CoA as precursor. Sequentially, two molecules of acetyl-CoA are condensed to acetoacetyl CoA by thiolase. The latter compound is then condensed with another molecule of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. Later, HMG-CoA is reduced to mevalonic acid in the presence of NADPH by HMG-CoA reductase. This enzyme attracted and great interest since it catalyzes the rate-limiting step in MVA pathway thus became a key target for interruption of cholesterol biosynthesis. The series of HMG-CoA reductase inhibitors such as mevastatin, lovastatin, simvastatin and pravastatin are highly effective hypochloesterolemic agents (Spurgeon and Porter, 1981). Mevalonic acid is phosphorylated twice to form mevalonic 5-diphosphate via mevalonic monophosphate. Mevalonic 5-diphosphate is then decarboxylated and dehydrated to form isopentenyl diphosphate. Isopentenyl diphosphate is isomerized to dimethylallyl diphosphate by isomerase (Spurgeon and Porter, 1981).

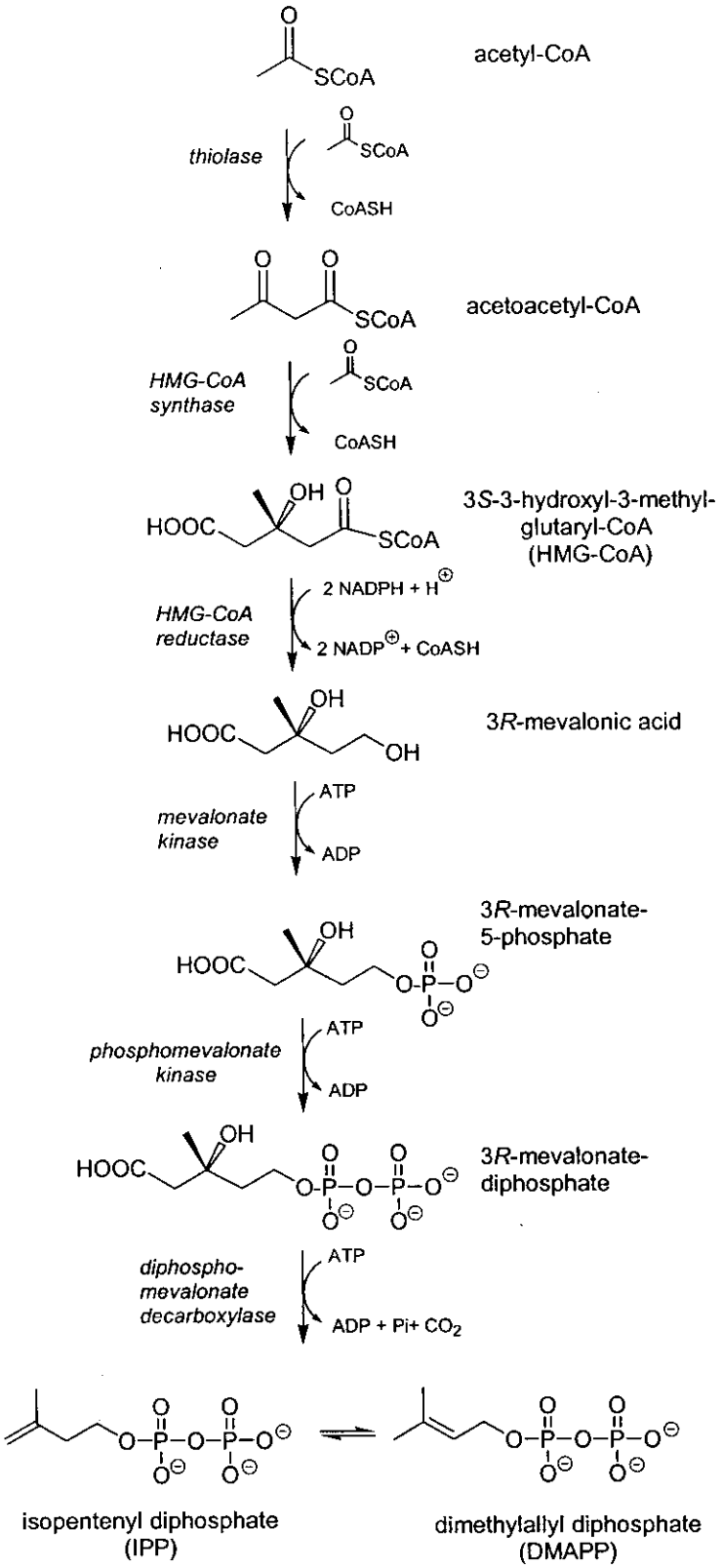

Figure 1.1 The classical mevalonate (MVA) pathway

1.2.1.2 The deoxyxylulose phosphate (DXP) pathway

Recently, the DXP pathway was completely elucidated in *Escherichia coli*. The initial step in the DXP pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and D-glyceraldehyde 3-phosphate, catalyzed by Dxs (Broers, 1994; Schwarz, 1994). The *dxs* gene encoding this enzyme was first cloned from *E. coli* (Lois *et al.*, 1998; Sprenger *et al.*, 1997). This enzyme has a typical thiamine-binding motif and needs both thiamine and a divalent cation such as Mg^{2+} or Mn^{2+} for enzyme activity.

In the second step DXP is transformed into 2*C*-methyl-D-erythritol-4-phosphate (MEP). The formation of MEP from DXP is synthesized in a single step by rearrangement DXP to an intermediate with a branched carbon skeleton, 2*C*-methyl-D-erythrose-4-phosphate (MEOP), followed by reduction using NADPH (Takahashi *et al.*, 1998). The enzyme required Mg^{2+} or Mn^{2+} as cofactor.

The next step of the DXP pathway is the conversion of MEP to 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol (CDP-ME) in a cytidine triphosphate (CTP) dependent reaction catalyzed by enzyme encoded *ygbP* gene (now designated *ispD*), which is present in many bacteria as well as in *Arabidopsis thaliana* (Rohdich *et al.*, 1999).

The further step in the pathway is the ATP-dependent phosphorylation of the 2-hydroxy group of CDP-ME converting into 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol-2-phosphate (CDP-ME2P) by an enzyme namely CDP-ME kinase encoded *ychB* gene (now designated *ispE*) which was identified in the genome of *E. coli* and many other organisms (Lüttgen *et al.*, 2000).

The next step in the pathway was also identified using bioinformatics. Genome analyses had shown that many putative orthologues of the *E. coli ygbB* gene (now designated *ispF*) were linked or fused to putative orthologues of *ispD* (Rohdich *et al.*, 1999 and Herz *et al.*, 2000). Based on these findings, the *E. coli ispF* gene was expressed and the recombinant protein shown to catalyze the conversion of CDP-ME2P into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Herz *et al.*, 2000).

The next enzyme was studied, *IspG* gene of *E. coli* was expressed and this protein was shown to catalyze the conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate. The last step, *IspH* protein catalyzes the subsequent transformation of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into a mixture of IPP and DMAPP (Rohdich *et al.*, 2003). The late steps in the DXP pathway was studied with an IPP isomerase-disruptant of *E. coli* confirmed that IPP isomerase is not essential for growth in this organism. This finding indicates that IPP and DMAPP are synthesized through independent routes in the late steps of the DXP pathway.

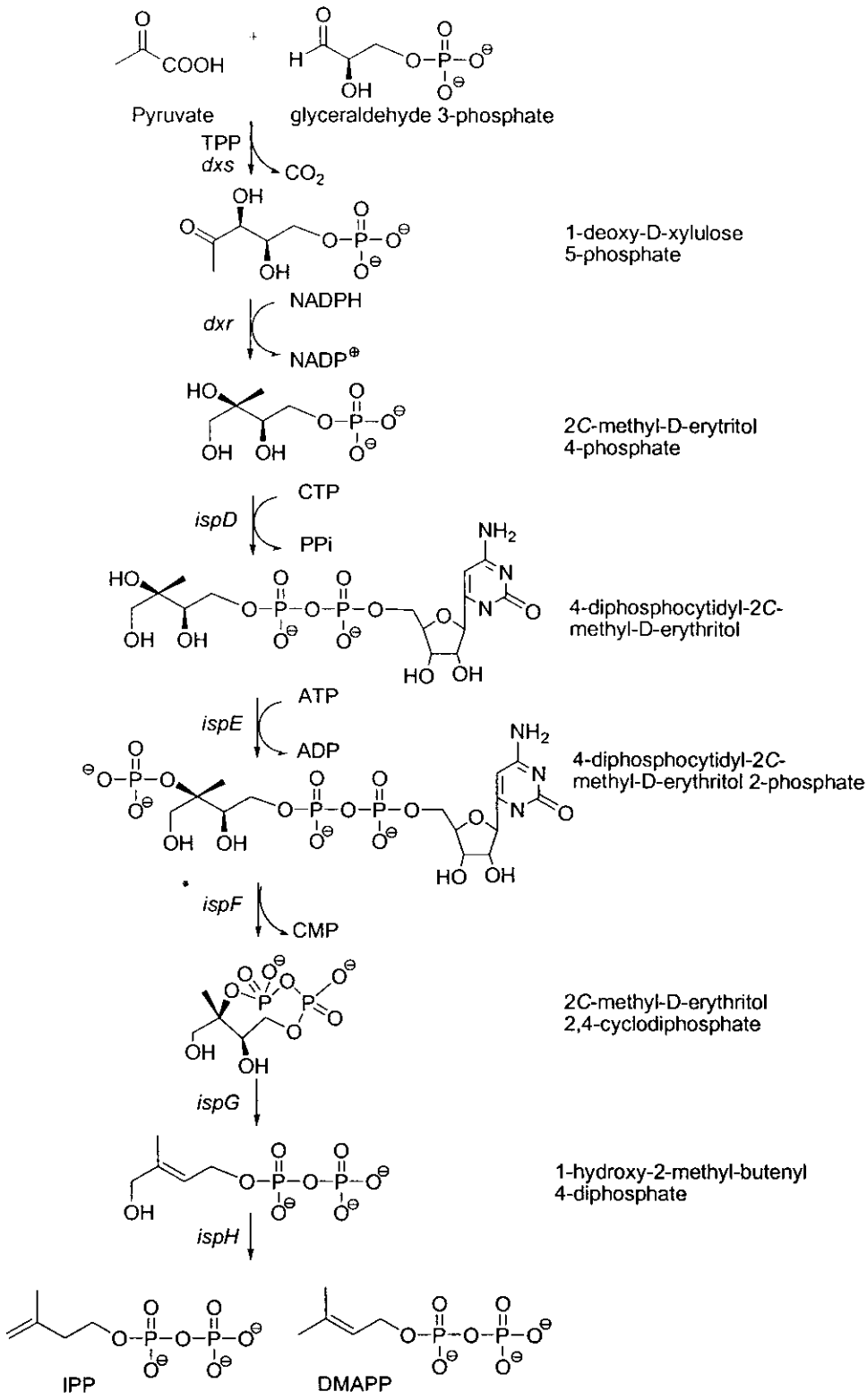

Figure 1.2 The deoxyxylulose phosphate (DXP) pathway

1.2.1.3 Cross-talk between two independent IPP generating pathways in plants

As mentioned earlier, building blocks of terpenoids in higher plants, the isoprene unit can be supplied from MVA pathway and DXP pathway (Arigoni and Schwarz, 1999; Lichtenthaler, 1999; Eisenreich *et al.*, 1998). The MVA route has been reported to operate in the cytoplasm for the formation of sterols, triterpenes and sesquiterpenes whereas the alternate DXP pathway occurs in the chloroplasts for the formation of terpenoids required for photosynthetic machinery (carotenoids, phytol, prenyl side chain of plastoquinone) and various groups of monoterpenoids, diterpnoids, abscisic acid, tocopherol and phylloquinone (Fig. 1.3) (Rohmer, 1999; Bick and Lange, 2003). This compartmentation is, however, not always clear cut based on observations in various labeling experiments (Adam and Zapp, 1998; Itoh *et al.*, 2000; Yang and Orihara, 2002). It can be suggested that exchange of common intermediates such as IPP, DMAPP, GPP and FPP might occur between the two compartments as described by the "Cross-talk theory" (Bick and Lange, 2003). The extent of this crosstalk depends on the species as well as on the presence and concentration of exogenous precursors.

A powerful strategy for quantitative assessment of the differential contribution of the two isoprenoid pathways has been established using the labeled glucose as well as labeled 1-deoxy-D-xylulose and mevalonate as precursors in whole plants, plant tissue cultures or cultured plant cells (Schuhr *et al.*, 2003). For instance, feedings of [1-^{13}C]glucose and [1-^{13}C]1-deoxy-D-xylulose into chamomile flower have been reported. Analysis of the labeling patterns using quantitative ^{13}C-NMR spectroscopy of isolated bisabololoxide-A and chamazulene showed that the two of the isoprene building blocks were predominantly formed via the DXP pathway, whereas the third unit was of mixed origin (Adam and Zapp, 1998 and Adam *et al.*, 1999). Various labeled precursors were also studied in *Scenedesmus obliquus*, *Lemna gibba*, *Hordeum vulgare*, *Daucus carota*, cell cultures of *Taxus chinensis* and *Mentha x piperita* (Schwender *et al.* 1996; Lichtenthaler *et al.* 1997; Eisenreich *et al.*, 1996). Those studies revealed the actual contribution of the MVA and DXP pathway, which in agreement with the explanation of "Cross-talk theory).

1.2.2 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr)

Gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) in the DXP pathway was firstly reported by Seto and his group in 1998. By the mutagenesis approach, genes responsible for the DXP pathway were cloned by using a strategy to prepare *E. coli* mutants with a metabolic block(s) between DXP and MEP (Kuzuyama *et al.*, 1998; Takahashi *et al.*, 1998). The mutant that required 2*C*-methyl-D-erythritol for growth was

screened. By complementation of these mutants, only the *yaeM* gene from *E. coli* was cloned and the gene product was purified and characterized (Takahashi *et al.*, 1998). The *yaeM* gene was later renamed to *dxr* or *ispC* (Takahashi *et al.*, 1998).

1-Deoxy-D-xylulose 5-phosphate reductoisomerase or 2*C*-methyl-D-erythritol 4-phosphate synthase (EC 1.1.1.267) is belonging to the reductoisomerase family. It catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to 2*C*-methyl-D-erythritol 4-phosphate in the presence of NADPH, a co-substrate. It requires a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}) for its activity (Fig. 1.3). Beyond the discovery *dxr* gene and gene product in *E. coli*, homologous proteins were expressed from various bacteria, plants and protozoon (Eisenreich *et al.*, 2004).

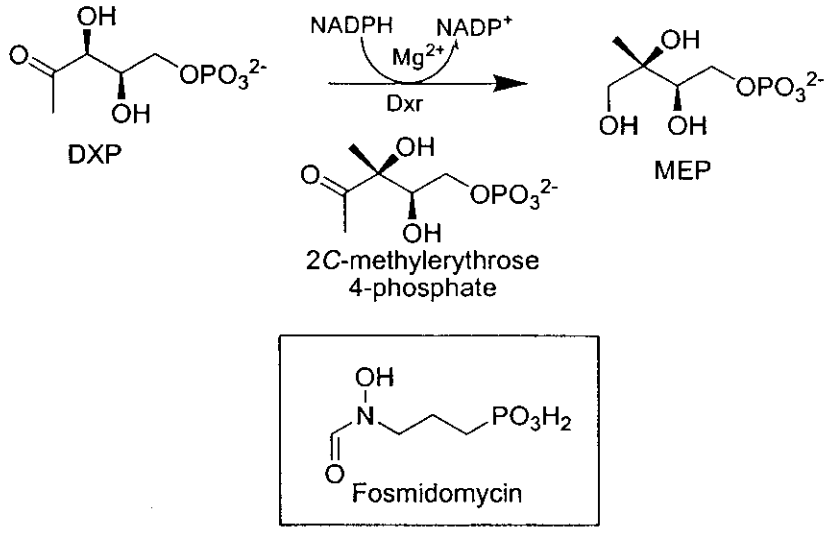

Figure 1.3 The catalytic action of Dxr

Naturally, Dxr protein does not utilize 1-deoxy-D-xylulose. It should be noted that the successful incorporation of 1-deoxy-D-xylulose into terpenoids of various organisms required a phosphorylation step catalyzed by the kinase (Wungsintaweekul *et al.*, 2001). The enzyme prefers to use NADPH rather than NADH. It transfers the pro-s proton from NADPH and is therefore belonging to the class B dehydrogenases (Arigoni *et al.*, 1999; Radykewicz *et al.*; 2000; Proteau *et al.*, 1999). The K_M of DXP and V_{max} values are 171 μM and 18 μmol $min^{-1}mg^{-1}$ (turnover number 13 s^{-1} per subunit). It utilizes NADPH as cofactor with a K_M of 25 μM (Wungsintaweekul, 2001).

Fosmidomycin [3-(N-formyl-N-hydroxyamino) propyl phosphate] inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Kuzuyama *et al.*, 1998). The inhibitory effect of fosmidomycin on 1-deoxy-D-xylulose 5-phosphate reductoisomerase can be explained by a structural similarity of fosmidomycin to 2*C*-methyl-D-erythrose 4-phosphate, a

putative intermediate of the enzyme reaction. Fosmidomycin had been reported as a mixed-type inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* with a *Ki* value of 38 nM (Kuzuyama *et al.*, 1998). For 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Zymomonas mobilis*, fosmidomycin served as a competitive inhibitor with a *Ki* value of 600 nM (Grolle *et al.*, 2000). Currently, 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Arabidopsis thaliana* was inhibited by fosmidomycin with *Ki* value of 85 nM (Rohdich *et al.*, 2006).

The structure of the *E. coli* Dxr protein has been published (Reuter *et al.*, 2002; Yajima *et al.*, 2002; Steinbacher *et al.*, 2003; Mac Sweeney *et al.*, 2005). Each subunit of the homodimer consists of an N-terminal dinucleotide binding domain, a connecting domain with the catalytic site and C-terminal helical domain (Fig. 1.4). The structure of a complex with NADPH (Yajima *et al.*, 2002) confirmed the essential role of Gly-14, Glu-231, His-153, His 209 and His-257 in the catalytic process. Glu-231 of the *E. coli* enzyme is important in the conversion of DXP into MEP, and His-153, His-209 and His-257 are part of the binding site of DXP to the enzyme (Kuzuyama *et al.*, 2000).

In plants, two different groups have cloned the genes encoding Dxr from *Arabidopsis thaliana* (Schwender *et al.*, 1999) and *Mentha piperita* (Lange and Croteau, 1999), and expressed those in *E. coli*. Carretero-Paulet and coworkers have cloned a single copy gene (cDNA) from *A. thaliana* encoding the Dxr (Carretero-Paulet *et al.*, 2002). The protein sequence analysis predicted the presence of an N-terminal transit peptide located to plastids, with a conserved cleavage site, and a conserved proline-rich region at the N-terminus of the mature protein (Rodriguez-Concepcion *et al.*, 2001, Carretero-Paulet *et al.*, 2002, Totte *et al.*, 2003, Engprasert *et al.*, 2005; Yao *et al.*, 2006). From *M. piperita* (Lange and Croteau, 1999) have shown that the enzyme can be inhibited by fosmidomycin, as reported in some other plant species (Zeidler *et* al., 1998; Fellermeier *et al.*, 1999; Lichtenthaler, 2000; Engprasert *et al.*, 2005).

In transgenic peppermint (*M. piperita*), overexpressing Dxr led to an increase of essential oil monoterpenes in its leaf tissues compared to the wild type. Gene silencing of partial *dxr* gene of the engineered peppermint plants led to a reduction of essential oil accumulation (Mahmoud and Croteau, 2001). A positive correlation was found between the accumulation of apocarotenoids in mycorrhizal roots from monocots with increase in Dxr transcript (Walter *et al.*, 2000), and similar observation was also reported with indole alkaloids accumulation in *Catharanthus roseus* cell suspension culture (Veau *et al.*, 2000). However, in tomato (*L. esculentum*) fruit during ripening, neither Dxr transcripts nor protein level increased, despite the massive carotenoids accumulation, suggesting a non-limiting role for Dxr in this system (Rodriguez-Concepcion *et al.*, 2001). Later, the expression level of *dxr* gene in relation to terpenoid production were reported from *Gingko biloba* (Gong *et al.*, 2005), *Elaeis guineensis* (Khemvong and Suvachittanont, 2005) and *Camptotheca accuminata* (Yao

et al., 2007). It has been demonstrated that *dxr* gene associated with terpenoid biosynthesis and the gene product does not exhibited as rate-limiting step in the DXP pathway.

1.2.3 *Croton stellatopilosus* Ohba

Figure 1.4 *Croton stellatopilosus* Ohba (Euphorbiaceae)

In 1972, Airy Shaw from Kew garden introduced the name of *C. sublyratus* Kurz. for Plau-noi (Thai name), characterized by distinctly obovate-panduriform leaf shape (Airy Shaw, 1972). This plant belongs to the Euphorbiaceae family. More than 20 years ago, this plant has been reported that it is the only source of antipeptic ulcer (Ogiso *et al.*, 1987). However, in 2001, Esser and Chayamarit has changed plant name from *C. sublyratus* Kurz. to *C. stellatopilosus* Ohba (Esser and Chayamarit, 2001). Because of the characteristic of obovate leaves and scattered hairs containing stellate-dendritic trichomes on the leave blade was strong evidence to other species, not *C. sublyratus* Kurz. (Esser and Chayamarit, 2001). Thus, name of plaunoi has been changed to *C. stellatopilosus* Ohba.

1.2.4 Plaunotol: Structure and chemical properties

The chemical name of plaunotol is (*E, Z, E*)-7-hydroxymethyl-3, 11, 15-trimethyl-2, 6, 10, 14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol. It has a molecular formula of $C_{20}H_{34}O_2$ and molecular mass of 306.2546 (Ogiso *et al.*, 1978). The structure of plaunotol is shown in Figure 1.6.

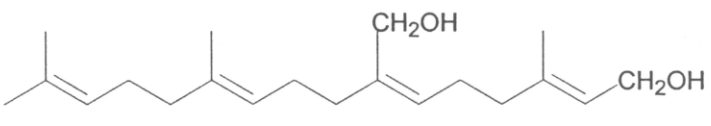

Figure 1.5 The chemical structure of plaunotol

Plaunotol occurs as pale yellow to light brown viscous liquid, having a slightly characteristic odor and a bitter taste. It is soluble in methanol, ethanol, acetone, ethyl acetate, dioxane, ether, chloroform, toluene or vegetable oil, but is practically insoluble in water (Department of Medicinal Information, Sankyo Co., Ltd., 1993).

For physicochemical properties, plaunotol shows its infrared spectrum with absorption band at 3300, 1665, 1440, 1380 and 1000 cm^{-1}. The proton magnetic resonance (PMR) spectrum of plaunotol shows signals due to the vinyl methyl groups at δ 1.9-2.3 (12H, m), two hydroxymethyl groups at δ 1.58 (6H, s) and δ 1.66 (6h, s), six allyl methylene groups at δ 1.9-2.3 (12H, m), two hydroxymethyl groups at δ 3.94 (2H, s) and δ 3.97 (2H, d), and four olefinic protons at δ 5.0-5.3 (4H, m). For mass spectrum, plaunotol shows the molecular ion at *m/e* 306.2585 (M^+, calculated for $C_{20}H_{34}O_2$ 306.256) and also other main peaks at *m/e* 288, 270, 121, 81 and 69 (base) (Ogiso *et al.*, 1978).

1.2.5 Plaunotol: Quantitative analysis

The determination of plaunotol content has been reported that Thin-layer chromatography (TLC) densitometric method (Vongchareonsathit and De-Eknamkul, 1998) and gas chromatography method (Moromoto and Murai, 1989; Ogiso *et al.*, 1981) can be used.

TLC densitometric method was developed for the rapid and precise measurement of plaunotol. This method allows the active constituent to be determined in unpurified plant extracts and therefore allows a large-scale screening program in a plant population to be performed. TLC was performed on silica gel 60 F254 plate using benzene and ethyl acetate (1:1) (Ogiso *et al.*, 1981) or 20% ether in chloroform or chloroform: *n*-propanol (96:4) (Vongchareonsathit and De-Eknamkul, 1998) as developer with 10 cm height of the solvent front. The TLC plate was then scanned by TLC densitometer under the wavelength of 220 nm.

Gas chromatography method can determine the plaunotol content in the microgram range. This method was performed by using a glass column packed with 2% OV17 on 60/80 Supelcoport, flow rate: 30 ml/min N_2, temperature program: from 235 °C (hold 2 min) to 250 °C (temperature rate 15 °C/min) and hold 10 min, injector temperature: 300°C, sample size 2 μl (Vongchareonsathit and De-Eknamkul, 1998).

1.2.6 Plaunotol biosynthesis

Plaunotol biosynthesis has been firstly reported from the group of De-Eknamkul and his coworkers in 1998. Plaunotol is derived from four isoprene units. One molecule of DMAPP and three molecules of IPP were attached by head to tail condensation, catalyzed by prenyltransferases (Fig. 1.6) (Spurgeon and Porter, 1981). Geranylgeranyl diphosphate (GGPP) is formed. GGPP is then dephosphorylated to GGOH by phosphatase enzyme (Nualkaew *et al.*, 2005 and Nualkaew *et al.*, 2006). Finally GGOH is hydroxylated at C-18 position, catalyzed by GGOH 18-hydroxylase (Fig. 1.7) (Tansakul and De-Eknamkul, 1998).

As mentioned earlier, the isoprene unit can be supplied from the MVA and/or DXP pathways. Therefore, the origin of isoprene unit in plaunotol skeleton was investigated. By feeding of isotopic glucoses revealed the isoprene unit was supplied from the DXP pathway dominantly without significant contribution from the MVA pathway. (Wungsintaweekul and De-Eknamkul, 2005).

Recently, localization of plaunotol has been investigated. The results showed that the storage site was in the palisade layer and plaunotol was kept as oil globule (Sitthithaworn *et al.* 2006). This data is also in agreement with the localization of the gene encoding GGPP synthase (Sitthithaworn *et al.* 2001). Evidence of the correlation of plaunotol production with the chlorophyll accumulation was supported that the plaunotol is biosynthesized and storage in the chloroplast (Morimoto and Murai, 1989).

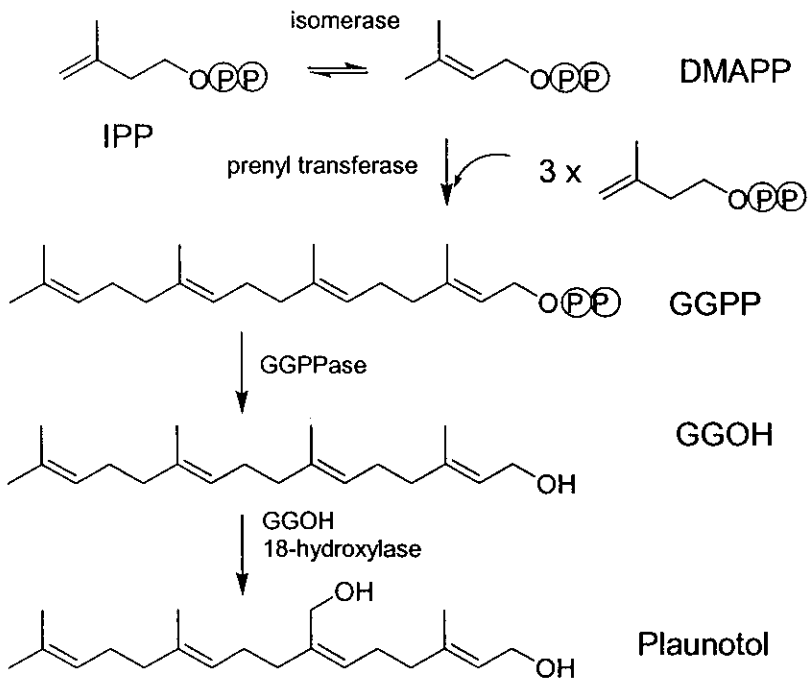

Figure 1.6 Biosynthesis pathway of plaunotol

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant Materials

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

2.2 Equipments and Materials

2.2.1 Equipments

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

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

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

2.2.2 Chemicals

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

Chemicals

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

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

2.2.3 Kits

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

2.2.4 Enzymes

*EcoR*I (EC 3.1.23.13)	TaKaRa, Japan
taq polymerase (EC 2.7.7.7)	Qiagen, Germany

2.2.5 *Escherichia coli* strains

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

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

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

2.2.6 Vectors

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

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

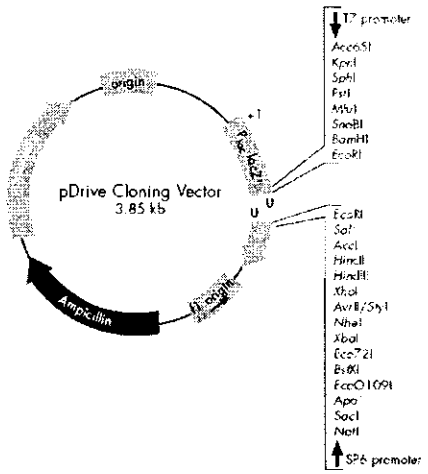

Figure 2.1 Representation of the linearized pDrive Cloning Vector with U overhangs.

2.2.7 Media preparation and solutions

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

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

2.2.8 Primers

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

Table 2.2 Primers used in this study

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

2.3 Methods

2.3.1 Total RNA extraction

2.3.1.1 Conventional method

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

2.3.1.2 RNeasy Plant Mini Kit

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

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

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

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

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

2.3.2 Synthesis of the first-strand cDNA

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

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

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

2.3.3 Polymerase chain reaction (PCR)

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

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

2.3.3.1 Amplification of the core fragment

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

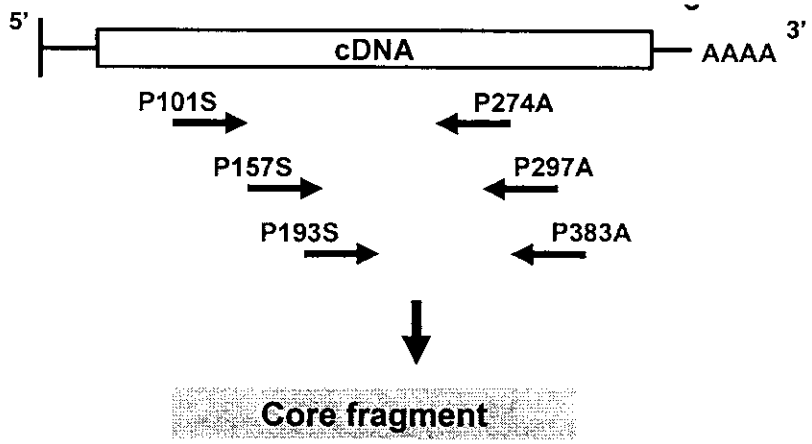

Figure 2.2 The core fragment amplification

PCR parameters for the core fragment amplification

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

2.3.3.2 Amplification of 5'- and 3'- ends

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

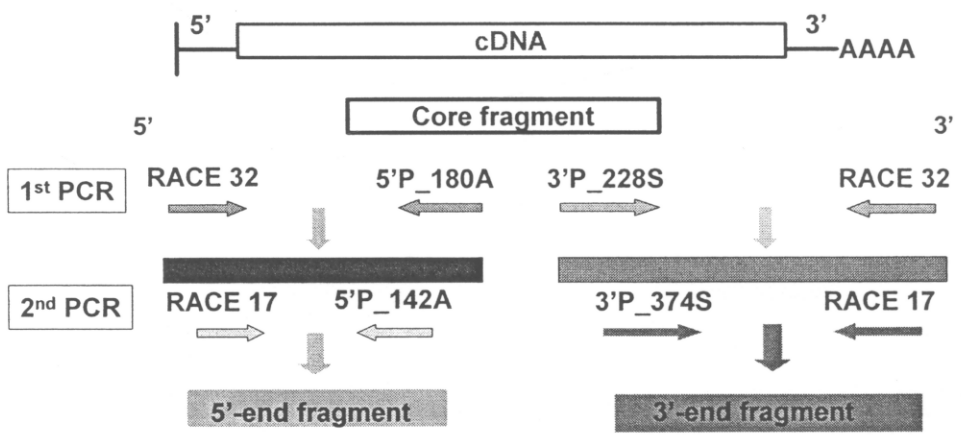

Figure 2.3 The 5'- and 3'- ends amplification

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

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

2.3.3.3 Amplification of the full-length *dxr* gene

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

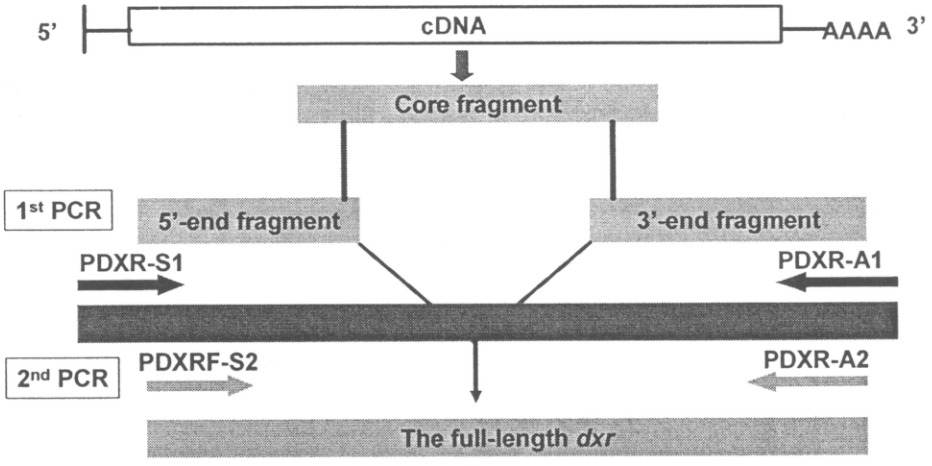

Figure 2.4 The full-length *dxr* gene amplification

PCR parameters for full-length of *dxr* amplification

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

2.3.3.4 Amplification of fragments for mRNA expression

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

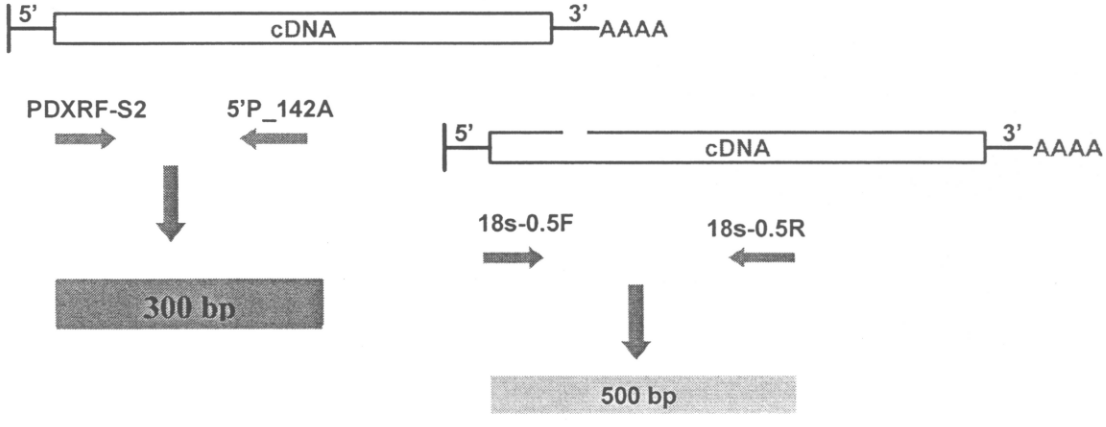

Figure 2.5 The partial DNA and 18S rRNA amplifications

PCR parameters for 18S rRNA amplification

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

PCR parameters for the partial DNA amplification

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

2.3.4 DNA cloning

2.3.4.1 Preparation of ultra-competent cell

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

2.3.4.2 Purification of DNA fragments

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

2.3.4.3 Ligation

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

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

2.3.4.4 Transformation

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

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

2.3.5 Extraction of the recombinant DNA

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

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

2.3.6 Agarose gel electrophoresis

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

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

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

2.3.7 DNA sequencing and sequencing analysis

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

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

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

2.3.8 Gel documentation

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

2.3.9 Extraction and quantitative analysis of plaunotol

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

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

Quantitative determination of plaunotol was performed using the GC method.

Gas chromatographic condition

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

Temperature profile

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

CHAPTER 3
RESULTS

3.1 Cloning and DNA sequencing of a full-length cDNA encoding for *dxr* gene

3.1.1 Degenerated primers design

Degenerated *dxr* oligonucleotides were designed from the highly conserved amino acid sequences among the plants species: *Arabidopsis thaliana* (accession no. NP_201085), *Oryza sativa* (accession no. BAF03694), *Lycopersicon esculentum* (accession no. AAK96063), *Catharantus roseus* (accession no. AAF65145), *Stevia rebaudiana* (accession no. CAD22156), and *Artemesia annua* (accession no. AAW28998). By using BLAST and Gene Doc programs, the alignment of deduced amino acids revealed the highly conserved regions as shown in Fig. 3.1. The degenerated primers were designed from the regions that covered the inner part of the Dxr with the omission of the plastid leader sequences (2.2.5). The sense primers were P101S, P157S, P193S and the antisense primers were P274A, P297A, P383A.

3.1.2 Total RNA from *C. stellatopilosus* young leaves

Total RNA from *C. stellatopilosus* young leaves was isolated according to method described in 2.3.1.1. After precipitation the total RNA with 4 M LiCl, the precipitate was washed with ethanol and dried under vacuum. The RNA was re-dissolved in 100 µl of DEPC water. The concentration of the RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The concentration of total RNA was calculated according to the equation (2.3.1). The concentration of the resulting RNA was 2.25 µg/µl. The purity of the total RNA was measured by means of spectrophotometer analysis (A_{260}/A_{280}) and agarose gel electrophoresis analysis. The ratio of the A_{260}/A_{280} of the isolated RNA was 1.678.

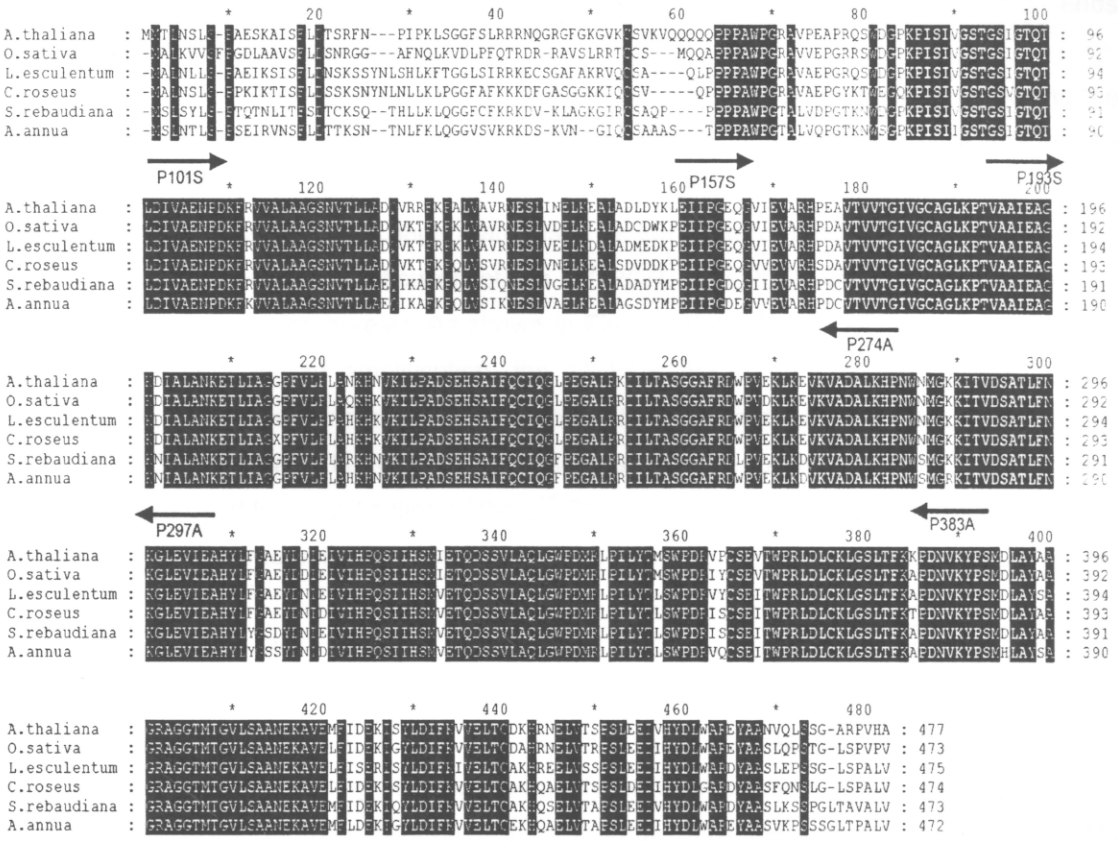

Figure 3.1 Multi-alignment of amino acid sequences of higher plant Dxrs. The identical amino acids were shown in white with black background. The arrows showed the directions of senses and antisense primers.

3.1.3 Cloning of core fragment, 5'- and 3'-ends

For the core fragment amplification (2.3.3.1), the PCR product was successfully obtained from the PCR reaction containing P101S and P297A as primers and cDNA (2.3.2) as template. The resulting PCR product was *ca* 600-bp DNA fragment on agarose gel electrophoresis (Fig. 3.2). The PCR product was purified on agarose gel electrophoresis (2.3.4.1). The purified fragment was ligated with the pDrive vector and transformed into *E. coli* TOP10 competent cells (2.3.4). After selection the white colonies, the recombinant plasmid was isolated and the resulting plasmid was analyzed for restriction site analysis (2.3.5). The double strands DNA were sequenced (2.3.7). The DNA sequence analysis of PCR product revealed the 600-bp DNA fragment.

The 5'- and 3'-ends of *dxr* gene were obtained by RACE method (2.3.3.2). Two gene specific primers for both sides were designed based on the nucleotide

sequence of the core fragment of *dxr* gene (Table 2.1, Scheme 2.2). The 5'- and 3'- ends were amplified using two steps PCR.

For 5'-end amplification, the cDNA template has been modified by adding the Poly-A addition prior using in the PCR reaction. The 1st PCR was performed with RACE32 and 5'P_180A for forward and reverse primers, respectively. The 1st PCR product was purified using the PCR purification kit (Qiagen) and used as template for the 2nd PCR. RACE 17 and 5'P_142A were used for amplification of the 2nd PCR. The PCR product was analyzed by 1.2% agarose gel electrophoresis. As shown in Fig. 3.3 (lane 2), the PCR product of 500-bp was obtained. For 3'-end amplification, the 1st PCR was performed with 3'P_228S and RACE32 for forward and reverse primers, respectively. The 1st PCR product was purified using the PCR purification kit (Qiagen) and used as template for the 2nd PCR. 3'P_374S and RACE17 were used for amplification of the 2nd PCR. The PCR product was analyzed by 1.2% agarose gel electrophoresis. As shown in Fig. 3.3 (lane 3), the PCR product of 1100-bp was obtained.

The PCR products were purified on agarose gel electrophoresis (2.3.4.1). The purified PCR fragments were ligated with the pDrive vector and transformed into *E. coli* TOP10 competent cells (2.3.4). After selection the white colonies, the recombinant plasmid was isolated and the resulting plasmid was analyzed for restriction site analysis (2.3.5). The double strands of DNA fragments of 5'- and 3'- ends were sequenced. The resulting DNA analysis revealed the 500-bp for 5'-end and 1100-bp for 3'-end.

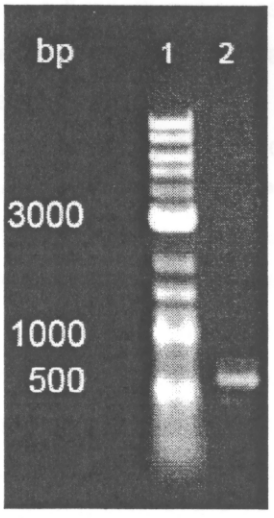

Figure 3.2 PCR product of core fragment was amplified by two steps RT-PCR and analyzed by 1.2% agarose gel electrophoresis

Lane 1: DNA marker

Lane 2: core fragment of *dxr* gene

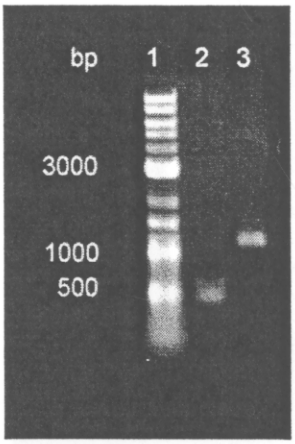

Figure 3.3 1.2% Agarose gel electrophoresis of the PCR products of 5'- and 3'-end fragments.

Lane 1: DNA marker

Lane 2: 5'-end of *dxr* gene

Lane 3: 3'-end of *dxr* gene

3.1.4 Cloning of a full-length cDNA for *dxr* gene (*Csdxr*)

By aligning and assembling the DNA sequences of the core fragment, 5'-end and 3'-end products of *dxr* gene of *C. stellatopilosus*, the two-sets of specific primers for the full-length gene were designed. The full-length gene was amplified using the two-steps PCRs (2.3.3.3). The 1st PCR was performed using PDXR-S1 and PDXR-A1 for forward primer and reverse primers, respectively. The 1st PCR product was purified and used as template for the 2nd PCR. PDXR-S2 and PDXR-A2, a forward primer and a reverse primer, respectively were used for 2nd PCR reaction. Following the 1st and 2nd PCR reactions, the PCR product was obtained as shown in Fig. 3.4. The full- length PCR product was purified, ligated to pDrive and transformed into *E. coli* TOP10 competent cells. The recombinant plasmid was isolated and the double strand DNA was sequenced. The full-length cDNA sequence of *Csdxr* was deduced and subsequently confirmed by sequencing, which was 2055 bp and has an open reading frame of 1404-bp starting with an initiation codon ATG and ending with a termination codon TGA. The protein encoded cDNA of *dxr* gene has 468 amino acid residues (Fig. 3.5) with a predicted molecular mass of 50.6 kDa and a calculated isoelectric point of 5.64. The nucleotide and deduced amino acid sequences of the CsDxr have been submitted to GenBank. The assigned accession numbers are EF451544 and ABO38177, respectively.

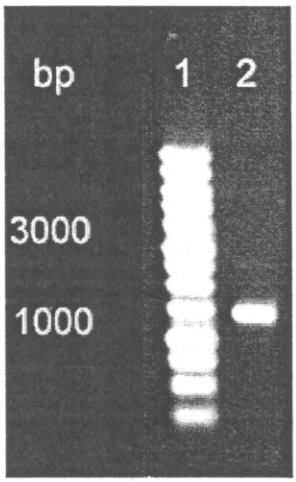

Figure 3.4 1.2% Agarose gel electrophoresis of the PCR products of *Csdxr*
Lane 1 DNA marker
Lane 2 full-length of *dxr* gene

3.2 Comparison of the amino acid sequences of Dxr from higher plants and analysis of conserved residues

The full-length of *dxr* gene from 3.1 was analyzed and translated to the deduced amino acids by using the DNASIS V3.5 software and the CLUSTAL W (1.82). The deduced amino acid sequence of *dxr* from *C. stellatopilosus* (CsDxr) was compared with the amino acid sequence of several representatives Dxr from other plants such as *Hevea brasiliensis, Arabidopsis thaliana, Lycopersicon esculentum, Catharanthus roseus, Stevia rebaudiana and Artemisia annua*. The comparison of sequences was conducted through databases and the alignment was performed using Gene Doc program. The result suggested that CsDxr belongs to the Dxr family as shown the highly conserved to Dxr from other higher plants. The percent identity of the amino acid sequences of CsDxr and Dxr from other plants is ranged from 76-92% (Table 3.2). In contrast, the CsDxr is similar to *Escherichia coli* and *Plasmodium falciparum* with the percent identity of 41 and 34, respectively.

Obviously, all of the sequences at N-terminal region were more different than at the C-terminal region. From the amino acid sequence analyzes among the higher plants, the CsDxr can be divided to three domains. The first domain at N-terminal was predicted by TargetP predictor suggested a chloroplast transit peptide at the N-terminal of 44 amino acids. The second domain contained an extended Pro-rich motif at the N terminus of the CsDxr. The other domain was the highly conserved region of NADPH binding motif (GSTGSIGT) (Fig 3.6).

```
gactcgagtcgacatcgatttttttttttttttccattctatccccagctccaggccaca 60
tatttcgtattcctctgagttggaatttttgaattttgattggATGgctcttaatttgc 120
                                                M  A  L  N  L   5
tttctcctgctgaaatcaaatccatttccttcttagactccgccaagtccaaccagcttc 180
L  S  P  A  E  I  K  S  I  S  F  L  D  S  A  K  S  N  Q  L   25
ccaagcttacaggtagtttcagtttgaagagaaaggatttgggcaggaaagtgcaatgtt 240
P  K  L  T  G  S  F  S  L  K  R  K  D  L  G  R  K  V  Q  C   45
ctgttcagtctcctcctccaccagcctggccgggaacagcttttccagaaccaggccgta 300
S  V  Q  S  P  P  P  P  A  W  P  G  T  A  F  P  E  P  G  R   65
agacttgggatggtccaaagcctatttctattgttggatccactggctccattggtactc 360
K  T  W  D  G  P  K  P  I  S  I  V  G  S  T  G  S  I  G  T   85
agacattggatatagtggcagagaatcctgaaaaatttaaagtggtggcacttgcagctg 420
Q  T  L  D  I  V  A  E  N  P  E  K  F  K  V  V  A  L  A  A  105
gttcaaatgtcactcttcttgctgatcaggtgaaaaccttcaaacctcagcttgttgcag 480
G  S  N  V  T  L  L  A  D  Q  V  K  T  F  K  P  Q  L  V  A  125
ttaggaatgagtctttagttgatgaactcaaagaagctttggctgatcttgaagaaaagc 540
V  R  N  E  S  L  V  D  E  L  K  E  A  L  A  D  L  E  E  K  145
ctgagattattcctggggaggaaggagttgttgaggttgcccgccatcctgatgctgcca 600
P  E  I  I  P  G  E  E  G  V  V  E  V  A  R  H  P  D  A  A  165
gtgttgttactggtatagtcggctgtgcaggcttaaggcctacggtggctgcaatagaag 660
S  V  V  T  G  I  V  G  C  A  G  L  R  P  T  V  A  A  I  E  185
ctggaaaagacatatgcttggccaacaaagagactttgattgctggaggtccgtttgtcc 720
A  G  K  D  I  C  L  A  N  K  E  T  L  I  A  G  G  P  F  V  205
ttcctcttgctaacaaatataacgttaaaattcttccagctgattcagaacattctgcta 780
L  P  L  A  N  K  Y  N  V  K  I  L  P  A  D  S  E  H  S  A  225
tatttcagtgtattcaaggcctacctgaaggtgcattgcggcgcattattttaactgctt 840
I  F  Q  C  I  Q  G  L  P  E  G  A  L  R  R  I  I  L  T  A  245
ctggtggggctttcagggattggcctgtagaaaaactgaaagatgttaaggtagctgatg 900
S  G  G  A  F  R  D  W  P  V  E  K  L  K  D  V  K  V  A  D  265
ctttgaagcaccccaactggaatatgggaaaaaagattactgtcgactcagctacccttt 960
A  L  K  H  P  N  W  N  M  G  K  K  I  T  V  D  S  A  T  L  285
tcaacaagggtttagaagttattgaagcccattatctgtttggagctgattatgataata 1020
F  N  K  G  L  E  V  I  E  A  H  Y  L  F  G  A  D  Y  D  N  305
ttgagattgtaattcatccccaatctataatacattcaatggttgaaacacaggattcat 1080
I  E  I  V  I  H  P  Q  S  I  I  H  S  M  V  E  T  Q  D  S  325
ctgttcttgcacaacttgggtggcctgatatgcgcttaccaattctatacaccatgtcat 1140
S  V  L  A  Q  L  G  W  P  D  M  R  L  P  I  L  Y  T  M  S  345
ggcctgacagaatttactgctctgaaataacatggcctcgccttgatctttgcaagcaag 1200
W  P  D  R  I  Y  C  S  E  I  T  W  P  R  L  D  L  C  K  Q  365
gttctctaacatttaaagctcctgataatgtaaaatacccatctatggatcttgcctatg 1260
G  S  L  T  F  K  A  P  D  N  V  K  Y  P  S  M  D  L  A  Y  385
ctgctggaagggctggaggcaccatgacaggagtccttagtgcggctaatgagaaagctg 1320
A  A  G  R  A  G  G  T  M  T  G  V  L  S  A  A  N  E  K  A  405
tggagatgttcatcgatgaaaagataagttatctcgacattttcaagattgtggagctaa 1380
V  E  M  F  I  D  E  K  I  S  Y  L  D  I  F  K  I  V  E  L  425
cctgcaataagcatcgggcggaattggtagccaccccatcgctggaggagattatacact 1440
T  C  N  K  H  R  A  E  L  V  A  T  P  S  L  E  E  I  I  H  445
atgacttgtgggcacgggagtatgctgccagtttgcaaacctctggtcgaagtcctgttc 1500
Y  D  L  W  A  R  E  Y  A  A  S  L  Q  T  S  G  R  S  P  V  465
ttgcaTGAtcgcctatgctgctggaagggctggaggcaccatgacaggagtccttagtgc 1560
L  A  *                                                       468
ggctaatgagaaagctgtggagatgttcatcgatgaaaagataagttatctcgacatttt 1620
caagattgtggagctaacctgcaataagcatcgggcggaattggtagccaccccatcgct 1680
ggaggagattatacactatgacttgtgggcacgggagtatgctgccagtttgcaaacctc 1740
tggtcgaagtcctgttcttgcatgatccagccatcaaccgtggaactaagttttaattct 1800
tttttgaaactggaggataggtgttatactcctgtagaagaagactggcattcttgccgg 1860
ggtttgtggggtcattttgcaaatgtatcatatataaattgtcaatctgacatccgaaac 1920
gtttcccccccatccattttgcagtcattatataattgctgtacttgtgatattcagata 1980
tgaaaattaattttcaaggtgatacaagggaagaatttatattggaaaaaaaaaaaaaaa 2040
aaaaaaaaaaaaaa                                                2055
```

Figure 3.5 Nucleotide and amino acid sequences of *Csdxr*

ATG = Start codon, **TGA** = Stop codon (*).

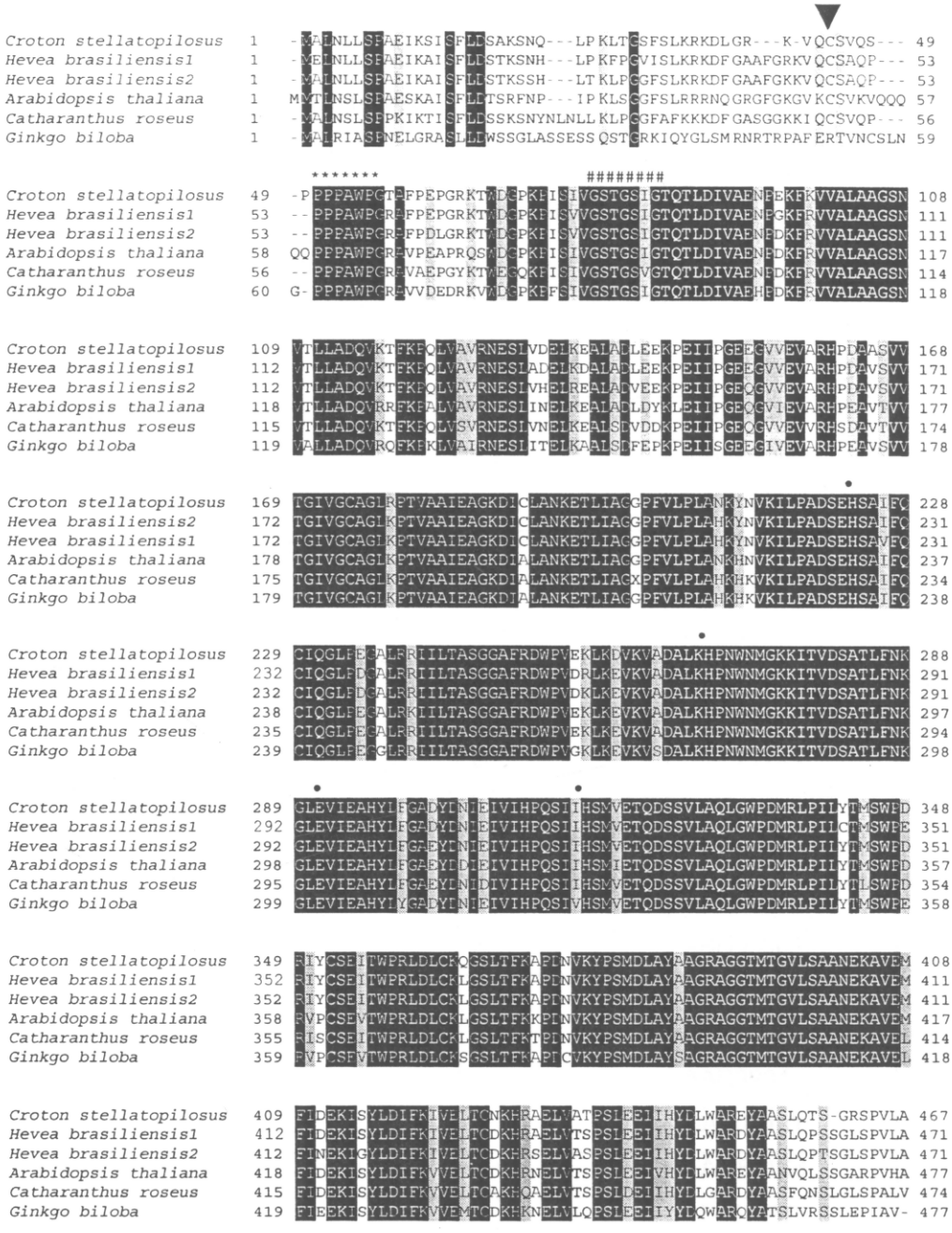

Figure 3.6 Alignment of deduced amino acid sequence of CSDXR and other plant DXRs. Identical residues among six proteins are indicated in inverse contrast. Similarity residues are indicated in shading. Cleavage site of chloroplast transit peptide is indicated by filled triangle. Residues representating a proline-rich motif are indicated by asterisks. Residues representating a putative NADPH-binding site are indicated by #. Putative catalytic residues are indicated by •. GenBank accession numbers as follows: *C. stellatopilosus* (**EF451544**), *H. brasiliensis1* (**DQ437514**), *H. brasiliensis2* (**AY502937**), *A. thaliana* (**NM125674**), *C. roseus* (**AF250235**) and *G. biloba* (**AY443101**).

Table 3.1 List of Dxr from plants, bacteria and malaria parasite from the National Center for Biotechnology Information (NCBI) database

Species	Abbreviations	Accession number
Antirrhinum majus	A.m.	NP_201085
Arabidopsis thaliana	A.t.	CAB43344
Artemisia annua	A.a.	AAW28998
Camptotheca acuminata	C.a.	ABC86579
Catharanthus roseus	C.r.	AAF65154
Chrysanthemum x morifolium	C.m.	BAE79548
Cistus incanus subsp. creticus	C.i.	AAP56260
Croton stellatopilosus	**C.s.**	**ABO38177**
Ginkgo biloba	G.b.	AAR95700
Hevea brasiliensis 1	H.b.1	AAS94121
Hevea brasiliensis 2	H.b.2	ABD92702
Hordeum vulgare subsp. vulgare	H.v.	CAE47438
Linum usitatissimum	L.u.	CAF22092
Lycopersicon esculentum	L.e.	AAK96063
Mentha x piperita	M.p.	AAD24768
Nicotiana tabacum	N.t.	ABH08964
Oryza sativa	O.s.	BAF03694
Picrorhiza kurrooa	P.k.	ABC74566
Plectranthus barbatus	P.b.	AAR9901
Populus alba x Populus tremula	P.p.	CAE00491
Pueraria montana var. lobata	P.m.	AAQ84168
Salvia miltiorrhiza	S.m	ABJ80680
Stevia rebaudiana	S.r.	CAD22156
Taxus cuspidata	T.c.	AAT47184
Taxus x media	T.m.	AAU87836
Zea mays	Z.m.	CAC03581
Plasmodium falciparum	P.f.	AAD03739
Escherichia coli	E.c.	1Q0Q_B

Table 3.2 Percent identity of amino acid sequences of CsDxr and other plant Dxrs, bacteria and malaria parasite.

Sample	C.s.	A.m.	A.t.	A.a.	C.r.	C.a.	C.m.	C.i.	G.b.	H.b. 1	H.b. 2	H.v.	L.u.	L.e.	M.p.	N.r.	O.s.	P.k.	P.b.	P.p.	P.m.	S.m	S.r.	T.c.	T.m.	Z.m.
C.s.	100																									
A.m.	82	100																								
A.t.	84	84	100																							
A.a.	82	79	79	100																						
C.r.	85	87	83	84	100																					
C.a.	82	80	81	76	81	100																				
C.m.	81	78	78	98	81	76	100																			
C.i.	81	78	78	79	77	75	76	100																		
G.b.	78	76	75	74	73	85	73	72	100																	
H.b.1	91	83	84	80	86	83	79	81	78	100																
H.b.2	92	84	85	80	87	83	80	81	78	94	100															
H.v.	79	78	79	76	78	75	74	71	75	77	78	100														
L.u.	85	82	81	81	83	79	80	77	75	83	83	76	100													
L.e.	86	86	84	81	87	83	81	80	79	87	88	79	82	100												
M.p.	81	85	82	80	84	80	79	76	72	83	84	78	79	84	100											
N.t.	85	87	84	80	87	83	79	78	79	85	87	78	84	93	85	100										
O.s.	82	81	83	76	82	78	74	73	76	83	83	82	79	83	80	83	100									
P.k.	83	90	82	80	84	78	79	77	74	83	84	77	81	85	85	86	80	100								
P.b.	80	84	80	80	84	80	79	76	75	81	83	80	81	85	80	85	80	85	100							
P.p.	86	80	82	79	82	79	78	77	74	85	86	74	81	81	79	81	78	79	78	100						
P.m.	76	73	73	74	73	70	73	71	76	75	76	72	73	74	72	74	71	76	73	74	100					
S.m	83	87	82	80	86	81	78	75	75	83	84	77	78	85	94	86	81	85	90	79	73	100				
S.r.	82	80	80	86	81	77	86	77	73	80	81	75	81	81	81	80	77	81	79	78	72	80	100			
T.c.	79	77	75	77	74	83	76	73	84	77	76	76	77	78	73	79	77	75	74	75	76	77	76	100		
T.m.	78	75	74	74	73	81	73	71	85	76	75	75	76	77	72	79	77	74	74	72	74	75	74	97	100	
Z.m.	80	81	84	77	83	79	77	76	75	83	83	88	79	85	81	85	91	79	90	78	73	81	79	77	75	100
E.c.	41	41	41	41	41	40	41	41	40	41	41	41	41	41	40	41	41	40	41	38	41	40	41	41	40	41
P.f.	34	34	34	34	32	33	33	34	33	33	34	32	34	34	34	35	32	33	34	31	35	34	35	34	33	34

3.3 Phylogenetic analysis

Phylogenetic analysis of the Dxr enzymes from different species: higher plants, gymnosperms, eubacteria and malaria parasite was generated with MEGA version 3.1 as shown in Fig. 3.7. Altogether with the percent identity from Table 3.2, the plants Dxr can be divided into two groups, which are gymnosperm (78-79% identity) and angiosperm (80-90% identity). The amino acid sequence of CsDxr revealed high homology throughout the entire coding regions with: *Hevea braziliensis* (91% identity), *Lycopersicon esculentum* (86% identity), *Populus alba* x *Populus tremula* (86% identity), *Catharanthus roseus* (85% identity), *Nicotiana tabacum* (85% identity), *Linum usitatissimum* (85% identity) for instance. From the phylogenetic tree, the CsDxr obtained from this study belongs to angiosperms group and exhibits closely relationship to *Hevea brasiliensis*. The results are in agreement as those plants are belonging to the family of Euphorbiaceae. All the analysis results strongly suggest that CsDxr is a plant Dxr protein involved in the IPP biosynthesis via the DXP pathway.

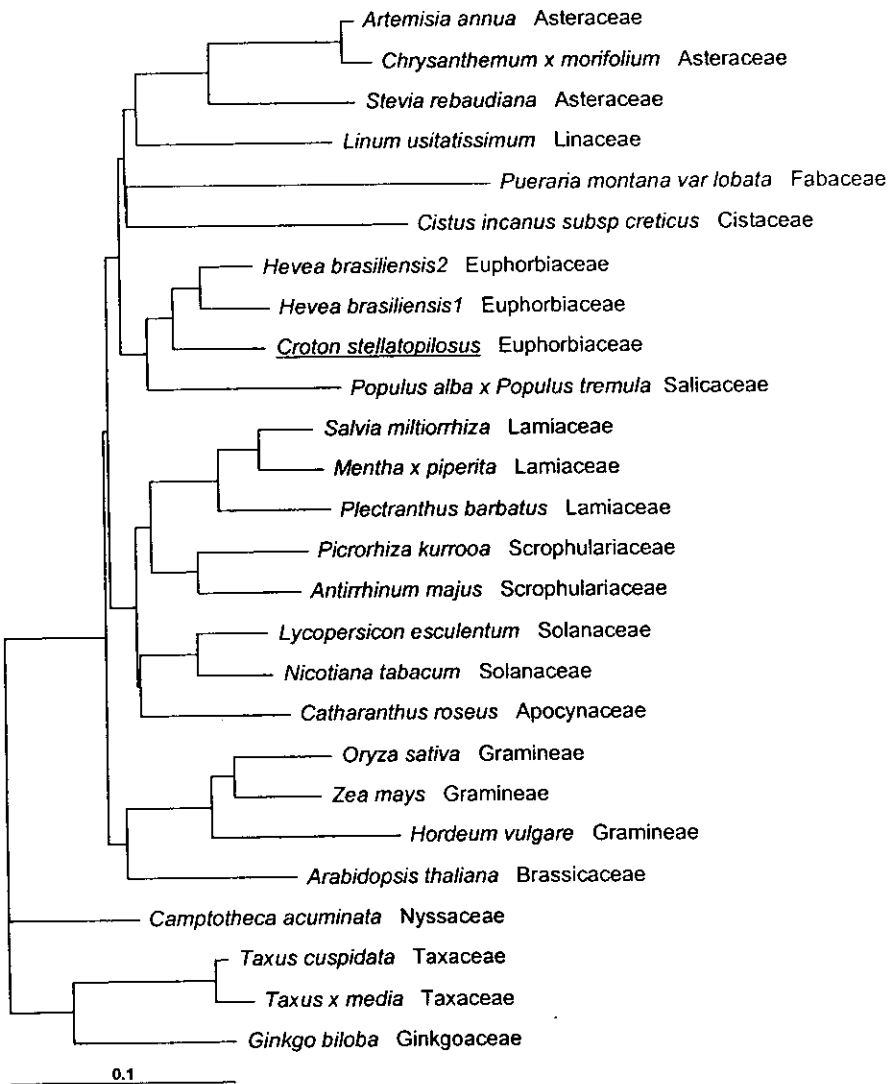

Figure 3.7 A molecular phylogenetic tree of the deduced amino acid sequences of the plant DXRs. The tree was constructed by CLUSTALW-XXL and viewed with Tree View version 1.6.6. The DXRs mentioned in the tree are including: *A. majus* (GenBank accession no. **AY770406**), *A. thaliana* (GenBank accession no. **NM125674**), *A. annua* (GenBank accession no. **AF182287**), *C. acuminata* (GenBank accession no. **DQ355159**), *C. roseus* (GenBank accession no. **AF250235**), *C. morifolium* (GenBank accession no. **AB205045**), *C. incanus*(GenBank accession no. **AY315651**), *C. stellatopilosus* (GenBank accession no. **EF451544**), *G. biloba* (GenBank accession no. **AY443101**), *H. brasiliensis1* (GenBank accession no. **DQ437514**), *H. brasiliensis2* (GenBank accession no. **AY502937**), *H. vulgare* (GenBank accession no. **AJ583446**), *L. usitatissimum* (GenBank accession no. **AJ623266**), *L. esculentum* (GenBank accession no. **AF331705**), *M. piperita* (GenBank accession no. **AF116825**), *N. tabacum* (GenBank accession no. **DQ839130**), *O. sativa* (GenBank accession no. **AK099702**), *P. kurrooa* (GenBank accession no. **DQ347963**), *P. barbatus* (GenBank accession no. **AY515699**), *P. alba x P. tremula* (GenBank accession no. **AJ574852**), *P. montana var. Lobata* (GenBank accession no. **AY315651**), *S. miltiorrhiza* (GenBank accession no. **DQ437514**), *S. rebaudiana* (GenBank accession no. **AY834755**), *T. cuspidata* (GenBank accession no. **AY575140**), *T. media* (GenBank accession no. **AY588482**) *and Z. Mays* (GenBank accession no. **AJ297566**).

3.4 Expression of the *dxr* gene in organs of *C. stellatopilosus*

3.4.1 Determination of cycling parameter for RT-PCR

The protocol of polymerase chain reaction for study on the expression profile was optimized in term of the cycling parameter. It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified by gel documentation. The total RNA of the young leave was used as material for the cycling number determination. The PCR reaction was performed using PDXRF-S2 for forward primer and 5'P_142A for reverse primer. The numbers of cycles were varied from 20, 25, 30, 35, 40, 45 and 50 cycles under the same thermal profile. The amplification products were analyzed by 1.2% agarose gel electrophoresis and the band intensity was measured as a relative intensity by densitometric analysis using gel documentation (2.3.8). The relationship between the relative intensity and number of cycles was plotted. The result showed that the intensity of the amplification product was related to the cycling number like sigmoid curve, which composed of initial phase (20-25 cycles), exponential phase (30-40 cycles) and plateau phase (45-50 cycles). The optimal cycling parameter should be the number that is in the exponential phase. Thus, the cycling number of 40 cycles was chosen for the investigation of the *dxr* gene expression profile in *C. stellatopilosus*.

3.4.2 Semiquantitative RT-PCR

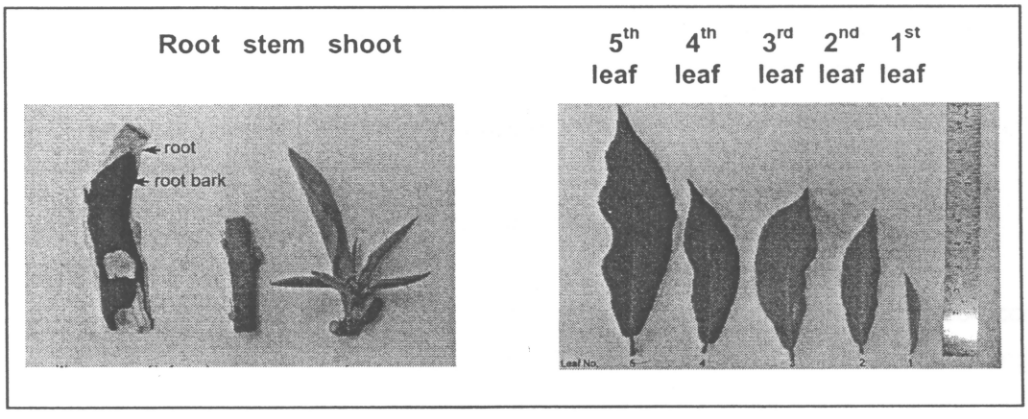

Figure 3.8 Various parts of *C. stellatopilosus*

Semiquantitative RT-PCR was carried out to investigate the expression profiles of *dxr* in various parts of *C. stellatopilosus* including shoot, 1st-5th leaf, stem and root (Fig. 3.8). Aliquots of 500 ng total RNA extracted from each sample was used as templates in two-steps RT-PCR reaction with the forward primer PDXRF-S2 and the reverse primer 5'P_142A specific to the coding sequence of part of full-length of *dxr*. The specific primers are PDXRF-S2 forward primer and 5'P_142A reverse primer were used to amplify 312 base pair fragment of *dxr* cDNA. For the

control gene, a house-keeping gene (18S rRNA) was amplified, 300 base pair fragment using 18s-0.5F for forward primer and 18s-0.5R for reverse primer.

All PCR products containing samples and the house keeping gene were separated by 1.2 % agarose gel electrophoresis and band intensity was measured by densitometric analysis using gel documentation. mRNA expression of *dxr* transcripts at various parts of *C. stellatopilosus* were evaluated by using the intensity ratio of the *dxr* band with 18S rRNA. The expression pattern analysis revealed that *dxr* mRNA were detected only in shoot and leaves with at similar expression levels, not in stem and root as shown in Table 3.3.

Table 3.3 The mRNA expression of *dxr* gene in various parts of *C. stellatopilosus*

sample	Intensity		Relative intensity (*dxr*/18S rRNA)
	dxr	18S rRNA	
1st leaf	10.80	13.20	0.82 ± 0.08
2nd leaf	10.90	13.30	0.82 ± 0.10
3rd leaf	10.60	13.00	0.82 ± 0.06
4th leaf	10.50	12.90	0.81 ± 0.09
5th leaf	11.00	12.90	0.85 ± 0.05
shoot	4.50	13.00	0.35 ± 0.04
stem	0.20	12.80	0.02 ± 0.00
root	0.10	12.80	0.01 ± 0.00

3.5 Quantitative determination of plaunotol

3.5.1 Calibration curve of plaunotol

The calibration curve of the authentic plaunotol was established according to the method 2.3.9. The linearity of the plaunotol calibration curve was observed in the range from 0.01-0.50 μg/μl with R^2 of 0.9984. Each calibration point was carried out in triplicates. The calibration curve is shown in Fig. 3.9. The detection limit was the concentration less than 0.4 μg.

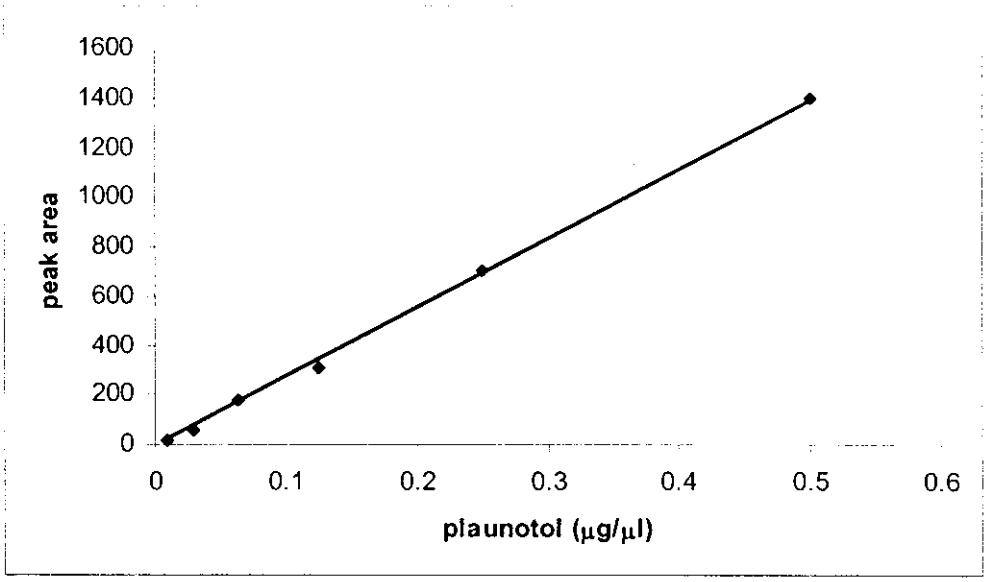

Figure 3.9 Calibration curve of plaunotol

3.5.2 Determination of plaunotol content in organs of *C. stellatopilosus*

The plant materials, which were used for total RNA extraction, were also used for determination of plaunotol content. The extraction of each sample was performed and plaunotol content was determined using gas chromatography (2.3.9). As shown in Table 3.4, the plaunotol was accumulated dominantly in plant leaves, especially the 1^{st}-2^{nd} leaves and declined in the 3^{rd}-5^{th} and stem. However, the plaunotol could not be detected in shoot and root under tested condition.

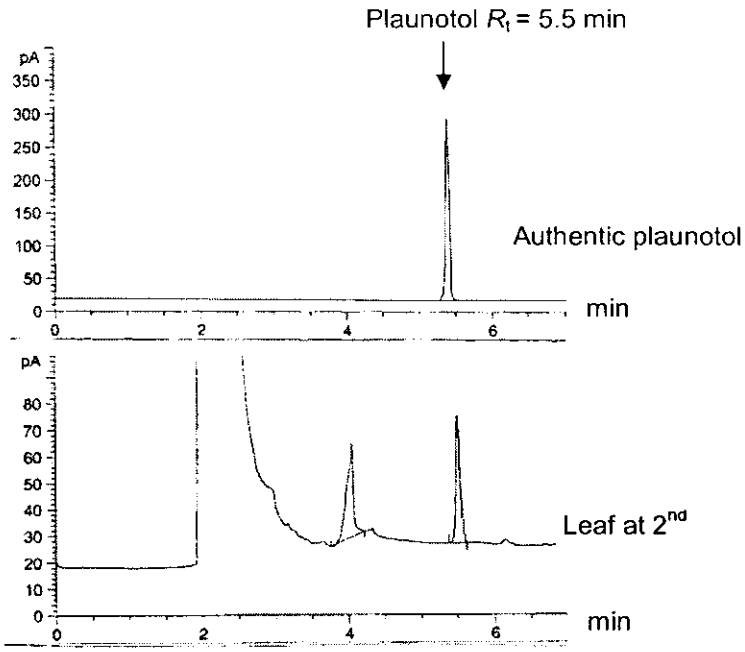

Figure 3.10 Example GC chromatograms of authentic plaunotol and at 2nd position leaf

Table 3.4 Plaunotol content from various parts of *C. stellatopilosus*

Sample	Weight (mg)	Plaunotol content (μg)	Plaunotol content (%w/w) ± SD (n = 3)
1st leaf	0.6	8.92	1.48 ± 0.08
2nd leaf	1.1	18.51	1.68 ± 0.16
3rd leaf	0.9	8.33	0.92 ± 0.17
4th leaf	5.8	4.33	0.07 ± 0.01
5th leaf	0.5	2.56	0.51 ± 0.04
shoot	5.7	0	n.d.
stem	1.7	4.01	0.24 ± 0.04
root	2.7	0	n.d.

n.d. = not determined (≤ 0.4 μg)

3.6 Correlation of *dxr* transcript levels and plaunotol biosynthesis

The correlation between the patterns of *dxr* expressions and plaunotol accumulation suggested the functional of the gene in the plaunotol biosynthesis. As shown in Fig. 3.13, the *dxr* gene transcripts were affected by the various organs in *C. stellatopilosus*. The results showed that *dxr* transcripts were detected in leaves, albeit low amount in shoot. The profile of the plaunotol accumulation was found to increase from 1st leaf to 2nd leaf reach to maximum and then declined gradually from 3rd -5th leaves. From this data, it can be concluded that the *dxr* gene was associated with the plaunotol production in leaves. However, the rarely *dxr* transcripts were found in stem and root, which was correlated to the absence or low amount of plaunotol content.

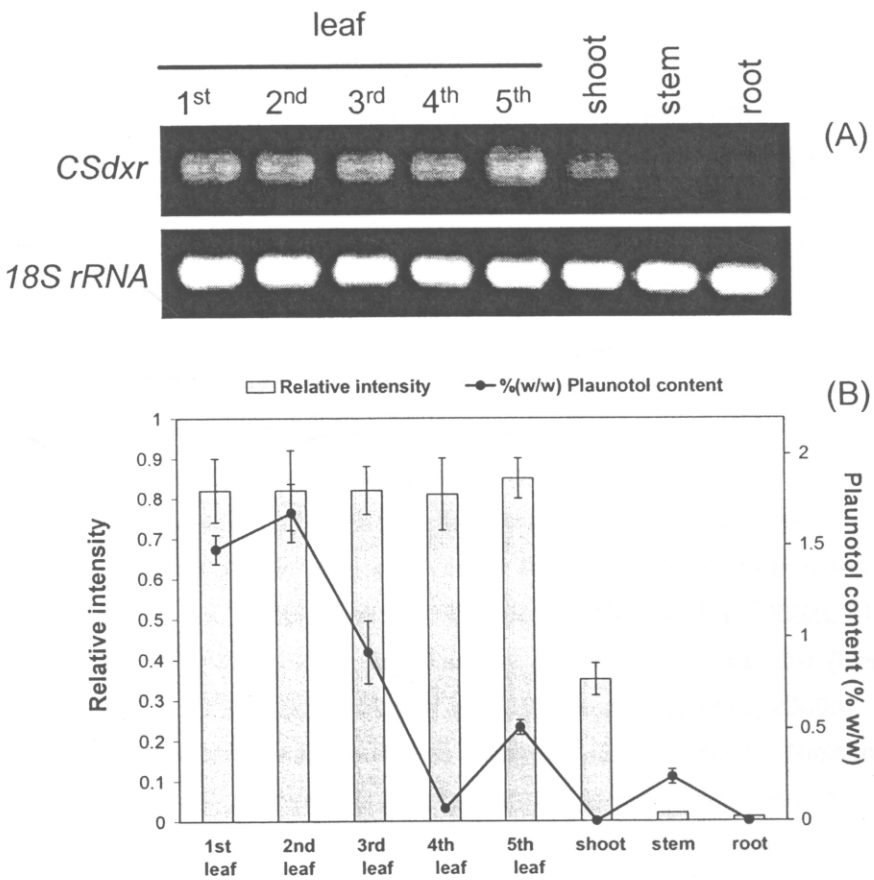

Figure 3.11 Semiquantitative RT-PCR analysis of *CSdxr* expression in various tissues of *Croton stellatopilosus*. (A). Pattern of *CSdxr* transcripts in comparison to *18S rRNA* as a loading control. (B).Correlation of relative intensity of each transcript and plaunotol content.

CHAPTER 4
DISCUSSIONS

4.1 1-Deoxy-D-xylulose 5-phosphate reductoisomerase from *C. stellatopilosus* Ohba

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267) catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to 2*C*-methyl-D-erythritol 4-phosphate in the presence of NADPH and Mg^{2+} (Takahashi *et al.*, 1998). In this study, the gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase was cloned and functionally characterized from *C. stellatopilosus* Ohba young leaves. The full-length of *dxr* gene had an open reading frame of 1,404 base pairs, starting with an initiation codon ATG and ending with a termination codon TGA. The deduced amino acid encoding by this cDNA has 468 amino acid residues with a predicted molecular mass of 50.6 kDa and isoelectric point of 5.64.

The *dxr* gene have been firstly cloned and characterized from *E.coli* (Takahashi *et al.*, 1998; Kuzuyama *et al.*, 2000a). In higher plants, *dxr* genes have been reported and the mRNA sequence available at National Center of Bioinformatic Information Center (NCBI) more than 20 plant species. Only 9 plants were characterized in detail such as *Arabidopsis thaliana* (Schwender *et al.*, 1999; Rohdich *et al.*, 2006), *Mentha piperita* (Lange and Croteau, 1999), *Catharanthus roseus* (Veau *et al.*, 2000), *Lycopersicon esculentum* (Rodriguez-Concepcion *et al.*, 2001), *Stevia rebaudiana* (Totte *et al.*, 2003), *Zea mays* (Hans *et al.*, 2004), *Coleus forskohlii* (Engprasert *et al.*, 2005), *Gingko biloba* (Kim, *et al.*, 2006) and *Campthotheca acuminata* (Yao *et al.*, 2006). All the *dxr* nucleotide sequences were about 1,242-1,434 base pairs encoded 406-486 amino acid residues and three domains were found in all plants *dxr* sequence: domain I was chloroplast transit peptide in N-terminal of Dxr, which directed the enzyme to plastids where the DXP pathway operated in plants, domain II was proline-rich motif P(P/Q)PAWPG(R/T) in N-terminal region of Dxr, which was not present in prokaryotic Dxrs (Carretero-Paulet *et al.*, 2002), The last domain was NADPH binding site (GSTGSIGT) in N-terminal region of all plants Dxr (Rane and Calvo, 1997; Lange and Croteau, 1999; Schwender *et al.*, 1999; Kuzuyama *et al.*, 2000b).

The deduced amino acid was analyzed using TargetP (Nielsen *et al.*, 1997; Nielsen *et al.*, 1990). Three domains: chloroplast transit peptide, proline-rich motif and NADPH binding site were found in CsDxr sequence. The premature CsDxr contained the chloroplast transit peptide at the N-terminal of 44 amino acid sequences. Thus, the mature CsDxr after cleave the leader sequence consisted of 424 amino acids. Alignment of the deduced amino acid sequence of CsDxr obtained from this study with the amino acid sequence of several representative Dxrs from other plants, malaria parasite and *E. coli* revealed high identity more than 76% to higher plants, especially *Hevea braziliensis* (90% identity). But it exhibited low similarity to Dxr protein of malaria parasite and *E. coli*. Therefore, *dxr* gene obtained from this study, is belonging to the reductoisomerase family. It can be noted that *dxr* gene of *C. stellatopilosus* located in chloroplast indicated by the chloroplast transit peptide. The results is in line with the investigation of the isoprenoid biosynthesis (Wungsintaweekul and De-Eknamkul,

2005) in *C. stellatopilosus* young shoots and localization of plaunotol in its leaves (Sitthithaworn *et al.*, 2006). Moreover, gene encoding geranylgeranyl diphosphate synthase was shown to be located in the chloroplast (Sitthithaworn *et al.*, 2001).

The catalytic residues of Dxr protein were reported from the *E. coli dxr*-gene deficient mutants (Kuzuyama *et al.*, 2000b). CsDxr amino acid sequence has Glu-231 that important in the conversion of 1-deoxy-D-xylulose 5-phosphate into 2*C*-methyl-D-erythritol 4-phosphate (Kuzuyama *et al.*, 2000b). The amino acid residues of His-153, His-209 and His-257 were shown as part of the binding sites of 1-deoxy-D-xylulose 5-phosphate (Kuzuyama *et al.*, 2000b). Fig. 4.1 showed the location of the catalytic residues, also present in the deduced amino acid of CsDxr.

4.2 1-Deoxy-D-xylulose 5-phosphate reductoisomerase in *C. stellatopilosus* is not rate-limiting step enzyme in plaunotol biosynthesis

The mRNA transcription level of *dxr* gene from *C. stellatopilosus* was determined using semiquantitative RT-PCR. This technique had high reproducibility and reflected trends in gene expression as observed by Northern blot (Burleigh, 2001). Comparison to household gene (18S rRNA) indicated that the *dxr* transcripts in leaves were found in relation to plaunotol accumulation. The result showed that *dxr* mRNA were detected only in shoot and leaves, with the strong expression in leaves and weak expression in shoot. That was found to be associated with plaunotol production. However, the stem *dxr* mRNA was not detected but the plaunotol accumulation was found. It is probably because of two specific primers that used to amplify the part of *dxr* gene were designed from CsDxr from leaves. The mRNA expression in leaves indicated that chloroplast organelle plays an important role in plaunotol biosynthesis. In addition, none of plaunotol was found in root as well as absence of *dxr* gene transcript. From the accumulation curve of plaunotol indicated that plaunotol is not the end product of terpenoid biosynthesis in *C. stellatopilosus*. Plaunotol is accumulated highly in young leaves and decreased gradually. Probably, downstream intermediates such as GGPP and GGOH, serve as substrate for cyclic diterpenoid compounds such as plaunolide, plaunol A, B, etc. and latex.

Previously, several studies demonstrated that gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase play a role in terpenoid biosynthesis (Veau *et al.*, 2000; Walter *et al.*, 2000; Mahmoud and Croteau, 2001). Tomato cDNA encoding a Dxr protein was cloned and the levels of mRNA related the plastid isoprenoid biosynthesis during tomato fruit ripening (Rodriguez-Concepcion *et al.*, 2001). Metabolic engineering of 1-deoxy-D-xylulose 5-phosphate reductoisomerase and menthofuran synthase in *Mentha piperita* increase the essential oil yield (Mahmoud and Croteau, 2001).

Study on β-carotene production in *Elaeis guineensis* demonstrated that gene encoding 1-deoxy-D-xylulose 5-phosphate synthase has different transcription levels during oil palm fruits ripening following weeks after fertilization. However, 1-deoxy-D-xylulose 5-phosphate reductoisomerase gene transcript occurs in all stages after fertilization (Khemvong and

Suvachittanont, 2005). This data indicated that *dxr* gene is not rate limiting step in β-carotene biosynthesis (Khemvong and Suvachittanont, 2005).

From this study, expression of *dxr* mRNA were constant in 1^{st}-5^{th} leaf and decreased in shoot while the amounts of plaunotol were varied, therefore *dxr* gene of *C. stellatopilosus* is involved in the IPP biosynthesis via the DXP pathway. Moreover, Dxr protein is not rate-limiting step in plaunotol biosynthesis.

REFERENCES

Adam, K.P. and Zapp, J. 1998. Biosynthesis of the isoprene units of chamomile sesqiterpenes. *Phytochemistry*. 48, 953-959.

Adam, K.P., Thiel, R.J. and Zapp, J. 1999. Incorporation of [1-^{13}C] 1-deoxy-D-xylulose in chamomile sesquiterpenes. *Arch. Biochem. Biophys*. 369, 127-132.

Airy Shaw, H.K. 1972. The Euphorbiaceae of Siam. *Kew Bull*. 26, 191-363.

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A. and Zenk, M.H. 1997. Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proc. Natl Acad. Sci. USA*. 94, 10600-10605.

Arigoni, D. and Schwarz, M.K. 1999. Ginkgolide biosynthesis. In *Comprehensive Natural Product Chemistry* (Vol. 2) (Cane, D., ed.), pp. 367-399, Pergamon.

Bick, J.A. and Lange, B.M. 2003. Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Biochem. Biophys*. 415, 146-154.

Broers, S.T.J. 1994. Uber die fruhen Stufen der Biosynthese von Isoprenoiden in *Escherichia coli*. [On the early stages of isoprenoid biosynthesis in *E. coli*]. Thesis Nr. 10978, ETH Zurich, Schweiz.

Burleigh, S.H. 2001. Relative quantitative RT-PCR to study the expression of plant nutrient transporters in arbuscular mycorrhizas. *Plant Sci*. 160(5), 899-904.

Cane, D.E., Chow, C. Lillo, A. and Kang, I. 2001. Molecular cloning, expression and characterization of the first three genes in the mevalonate-independent isoprenoid pathway in *Streptomyces coelicolor. Bioorg. Med. Chem*. 9, 1467-1477.

Carretero-Paulet, L., Ahumada, I., Cunillera, N., Rodriguez-Concepcion, M., Ferrer, A., Boronat, A. and Campos, N. 2002. Expression and molecular analysis of the *Arabidopsis DXR* gene encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase, the first committed enzyme of the 2*C*-methyl-D-erythritol-4-phosphate pathway. *Plant Physiol*. 129, 1581-1591.

Department of medical information, Sankyo Co., Ltd. 1993. Mucosal protective antiulcer drug: Kelnac capsules Kelnac fine granules. Tokyo: Sankyo Co., Ltd.

Eisenreich, W., Menhard, B., Hylands, P., Zenk, M.H. and Bacher, A. 1996. Studies on the biosynthesis of taxol: The taxane carbon skeleton in not of mevalonoid origin. *Proc. Natl. Acad. Sci. USA*. 93, 6431-6436.

Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M.H. and Bacher, A. 1998. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. Chem. & Biol. 5, R221-233.

Eisenreich, W., Bacher, A., Arigoni, D. and Rohdich, F. 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell. Mol. Life Sci*. 61, 1401-1426.

Engprasert, S., Taura, F. and Shoyama, Y. 2005. Molecular cloning, expression and characterization of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Coleus forskohlii* Briq. *BMC Plant Biol.* 169, 287-294.

Esser, H.J. and Chayamarit, K. 2001. Two new species and a new name in Thai *Croton* (Euphorbiaceae). *Thai. For. Bull.* 29, 51-57.

Fellermeier, M. A., Maier, U. H., Sagner, S., Bacher, A. and Zenk, M. H. 1998. (-)-2 C-methyl-D-erythrono-1, 4-lactone is formed after application of the terpenoid precursor 1-deoxy-D-xylulose. *FEBS Lett.* 437, 278-280.

Gong, Y., Liao, Z., Chen, M., Zuo, K., Guo, L., Tan, Q., *et al.* 2005. Molecular cloning and characterization of a 1-deoxy-D- xylulose 5-phosphate reductoisomerase gene from *Ginkgo biloba*. DNA Sequence. 16:111-120.

Grolle, S., Bringer-Meyer, S., Sahm, H. 2000. Isolation of the *dxr* gene of *Zymomonas mobilis* and characterization of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase. *FEMS Microbiol Lett.* 191, 131-137.

Herz, S., Wungsintaweekul, J., Schuhr, C.A., Hecht, S., Lüttgen, H., Sagner, S., Fellermeier, M., Eisenreich, W., Zenk, M.H., Bacher, A. and Rohdich, F. 2000. Biosynthesis of terpenoids: YgbB protein converts 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate to 2*C*-methyl-D-erythritol 2, 4-cyclodiphosphate. *Proc. Natl. Acad. Sci. USA.* 97, 2486-2490.

Inoue, Y., Shiraishi, A., Hada, T., Hirose, K., Hamashima, H. and Shimada, J. 2004. The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. *FEMS Microbiol Lett.* 237(2), 325-331.

Itoh, D., Karunagoda, R.P., Fushie, T., Katoh, K. and Nabeta, K. 2000. Nonequivalent labeling of the phytyl side chain of chlorophyll a in callus of the hornwort, *Anthoceros punctatus*. *J. Nat. Prod.* 63, 1090-1093.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., *et al.* 1999. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science*. 285, 1573-1576.

Kaiser, J., Yassin, M., Prakash, S., Safi, N., Agami, M., Lauw, S., Ostrozhenkova, E., Bacher, A., Rohdich, F., Eisenreich, W., *et al.* 2007. Anti-malarial drug targets: Screening for inhibitors of 2C-methyl-d-erythritol 4-phosphate synthase (IspC protein) in Mediterranean plants. *Phytomedicine*. 14, 242-249.

Khemvong, S. and Suvachittanont, W. 2005. Molecular cloning and expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate synthase from oil palm *Elaeis guineensis* Jacq. *Plant Science*. 169, 571-578.

Kim, S.M., Kuzuyama, T., Chang, Y.J., Song, K.S., Kim, S.U. 2006. Identification of class 2 1-deoxy-D-xylulose 5-phosphate synthase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase genes from *Ginkgo biloba* and their transcription in embryo culture with respect to ginkgolide biosynthesis. *Planta Med.* 72, 234-240.

Kumar, S.K., Tamura, K., Jakobsen, I.B., Nei, M. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*. 17, 1244-1245.

Kuzuyama, T., Shimizu, T., Takahashi, S. and Seto, H. 1998. Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett.* 39, 7913-7916.

Kuzuyama, T., Takagi, M., Takahashi, S. and Seto, H. 2000a. Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from *Streptomyces* sp. strain CL190, which uses both the mevalonate and nonmevalonate pathways for isopentenyl diphosphate biosynthesis. *J. Bacteriol.* 182, 891-897.

Kuzuyama, T., Takahashi, S., Takagi, M., Seto, H. 2000b. Characterization of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic amino acid residues. *J. Biol. Chem.* 275, 19928-19932.

Lange, B.M. and Croteau, R. 1999. Isoprenoid biosynthesis via the mevalonate independent pathway in plants: cloning and heterologous expression of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from peppermint. *Arch. Biochem. Biophys.* 365, 170-174.

Lichtenthaler, H.K. 1999. The 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 47-65.

Lichtenthaler, H.K., Schwender, J., Disch, A. and Rohmer, M. 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Lett.* 400, 271-274.

Lois, L.M., Campos, N., Putra, S.R., Danielsen, K., Rohmer, M. and Boronat, A. 1998. Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyses the synthesis of 1-deoxy-D-xylulose 5-phosphate, a common precursur for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proc. Natl. Acad. Sci. USA.* 95, 2105-2110.

Lüttgen, H., Rohdich, F., Herz, S., Wungsintaweekul, J., Hecht, S., Schuhr, C.A., Fellermeier, M., Sagner, S., Zenk, M.H., Bacher, A. and Eisenreich, W. 2000. Biosynthesis of terpenoids: YchB protein of *Escherichia coli* phosphorylates the 2-hydroxy group of 4-diphosphocytidyl-2*C*-methyl-D-erythritol. *Proc. Natl. Acad. Sci. USA.* 97, 1062-1067.

Mahmoud, S.S. and Croteau, R. 2001. Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proc. Natl. Acad. Sci. USA.* 98, 8915-8920.

Mac Sweeney, A., Lange, R., Fernandes, R.P.M., Schulz, H., Dale, G.E., Douangamath, A., Proteau, P.J. and Oefner, C. 2005. The crystal structure of *E. coli* 1-deoxy-D-xylulose-5-phosphate reductoisomerase in a ternary complex with the antimalarial compound fosmidomycin and NADPH reveals a tight-binding closed enzyme conformation. J. mol. Biol. 345, 115-127.

Munoz-Bertomeu, J., Arrillaga, I., Ros, R. and Segura, J. 2006. Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant Physiol.* 142, 890-900.

Morimoto, H. and Murai, F. 1989. The effect of gelling agents on plaunotol accumulation in callus cultures of *Croton sublyratus* Kurz. *Plant Cell Rep.* 8, 210-213.

Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997. GeneDoc: Analysis and Visualization of Genetic Variation. *EMBNEW. NEWS.* 4, 14.

Nielsen, H, Engelbrecht, J. and Brunak, S. 1997. A neural network method for identification of prokaryotic and eukaryotic signal peptidase and prediction of their cleavage site. *Internation. J. Neur. Syst.* 8(5), 581-591.

Nielsen, H, Brunak, S and Heijne, G.V. 1999. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Prot. Engineer.* 12(1), 3-9.

Nualkaew, N., De-Eknamkul, W., Kutchan, T.M. and Zenk, M.H. 2006. Membrane-bound geranylgeranyl diphosphatases: purification and characterization from *Croton stellatopilosus* leaves. *Phytochemistry.* 67(15), 1613-1620.

Ogiso, A., Kitazawa, K., Kurabayashi, M., Sato, A., Takahashi, M., Noguchi, H., Kuwano, H., Kobayashi, S. and Mishima, H. 1978. Isolation and structure of antipeptic ulcer diterpene from Thai medicinal plant. *Chem. Pharm. Bull.* 26(10), 3117-3123.

Ogiso, A., Kitazawa, E., Mikuriya, I. and Promdej, C. 1981. Original plant of Thai crude drug, Plau-noi. *Shoyakugaku Zasshi.* 35(4), 287-290.

Proteau, P. J., Woo, Y. H., Williamson, R. T., and Phaosiri, C. 1999. Stereochemistry of the reduction step mediated by recombinant 1-deoxy-D-xylulose 5-phosphate isomeroreductase. *Organic Lett.* 1, 921-923.

Radykewicz, T., Rohdich, F., Wungsintaweekul, J., Herz, S., Kis, K., Eisenreich, W., Bacher, A., Zenk, M.H. and Arigoni, D. 2000. Biosynthesis of terpenoids: 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *Escherichia coli* is a class B dehydrogenase. *FEBS Lett.* 465, 157-160.

Rane M.J. and Calvo K.C. 1997. Reversal of the nucleotide specificity of ketol acid reductoisomerase by site-directed mutagenesis identifies the NADPH binding site. *Arch. Biochem. Biophys.* 338, 83-89.

Reuter, K., Sanderbrand, S., Jomaa, H., Wiesner, J., Steinbacher, I., Beck, E., Hintz, M., Klebe, G. and Stubbs, M.T. 2002. Crystal structure of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a crucial enzyme in the non-mevalonate pathway of isoprenoid biosynthesisthesis. *J. Biol. Chem.* 277, 5378-5384.

Rodriguez-Concepcion, M. Ahumada, I., Diez-Juez, E., Sauret-Gue-to, S. Lois, L.M., Gallego, F., Carretero-Paulet, L. Campos, N. And Boranat, A. 2001. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tamato fruit ripening. Plant J. 27, 213-222.

Rohdich, F., Wungsintaweekul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A., and Zenk, M.H. 1999. Cytidine 5'-triphosphate-dependent biosynthesis of isoprenoids: YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2-*C*-methylerythritol. *Proc. Natl. Acad. Sci. USA.* 96, 11758-11763.

Rohdich, F., Zepeck, F., Adam, P., Hecht, S., Kaiser, J., Laupitz, R., Grawert, T., Amslinger, S., Eisenreich, W., Bacher, A. and Arigoni, D. 2003. The deoxyxylulose phosphate pathway

of isoprenoid biosynthesis: studies on the mechanisms of the reactions catalyzed by IspG and IspH protein. *Proc. Natl. Acad. Sci. USA*. 100(4), 1586-1591.

Rohdich, F., Lauw, S., Kaiser, J., Feicht, R., Kohler, P., Bacher, A. and Eisenreich, W. 2006. Isoprenoid biosynthesis in plants-2*C*-methyl-D-erythritol-4-phosphate synthase (IspC protein) of *Arabidopsis thaliana*. *FEBS J*. 273, 4446-4458.

Sacchettini, J.C. and Poulter, C.D. 1997. Creating isoprenoid diversity. *Science*. 277, 1788-1789

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4, 406-125.

Sakai, T., Yamashita, Y., Maekawa, T., Shirakusa, T. and Okabe, N. 2000. Effect of plaunotol on bacterial translocation in the rat amall intestine. *Int. Surg*. 85(1), 27-29.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd edition. New york: Cold Spring Harbor Laboratory Press. pp. 7.9-7.12.

Schuhr, C.A., Radykewicz, T., Sagner, S., Latzel, C., Zenk, M.H., Arigoni, D., Bacher, A., Rohdich, F. and Eisenreich, W. 2003. Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. *Phytochem. Rev.* 2, 3-16.

Schwender, J., Seemann, M., Lichtenthaler, H.K. and Rohmer, M. 1996. Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glaceraldehyde-3-phosphate non-mevalonate pathway in the green algae *Scenedesmus obliquus*. *Biochemical J*., 316, 73-80.

Schwender, J., Muller, C., Zeidler, J. and Lichtenthaler, H.K. 1999. Cloning and heterologous expression of a cDNA encoding 1- deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana*. *FEBS Lett*. 455, 140-144.

Schwarz, M.K. 1994. Terpen-Biosynthese in *Ginkgo biloba*: Eine überraschende Geschichte. [Terpene biosynthesis in *Ginkgo biloba*: a surprising story] Thesis Nr. 10951, ETH Ziirich, Schweiz.

Sitthithaworn, W., Kojima, N., Viroonchatapan, E., Suh, D.Y., Iwanami, N., Hayashi, T., Noji, M., Saito, K, Niwa, Y. and Sankawa, U. 2001. Geranylgeranyl diphosphate synthase from *Scoparia dulcis* and *Croton sublyratus* plastid localization and conversion to a farnesyl diphosphate synthase by mutagenesis. *Chem. Pharm. Bull*. 49(2), 197-202.

Sitthithaworn, W., Potduang, B. and De-Eknamkul, W. 2006. Localization of plaunotol in the leaf of *Croton stellatopilosus* Ohba. *Sci. Asia*. 32, 17-20.

Shiratori, K., Watanabe, S. and Takeuchi, T. 1993. Role of endogenouse prostaglandins in secretin and plaunotol-induced inhibition of gastric acid secretion in the rat. *Am. J. Gastroenterol*. 88, 84-89.

Sprenger, G.A., Schorken, U., Wiegert, T., Grolle, S., Degraaf, A.A., Taylor, S.V., Begley, T.P., Bringermeyer, S. and Sahm, H. 1997. Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-Dxylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. *Proc. Natl. Acad. Sci. USA*. 94, 12857-12862.

Spurgeon, S.L. and Porter, J.W., in Biosynthesis of isoprenoid compounds, eds. Porter, J.W. and Spurgeon, S.L., Wiley, New York, 1981, vol. 1, 5-38.

Steinbacher, S., Kaiser, J., Eisenreich, W., Huber, R., Bacher, A., Rohdich, F. 2003. Structural basis of fosmidomycin action revealed by the complex with 2-C-methyl-D-erythritol 4-phosphate synthase (IspC). *J. Biol. Chem.* 278, 18401-18407.

Takahashi, S., Kurabayashi, M., Kitazawa, E., Haruyama, H. and Ogiso, A. 1983. Plaunolide, a furanoid diterpene from *Croton sublyratus*. *Phytochemistry*. 22(1), 302-303.

Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H. 1998. A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalysing the formation of 2*C*-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl. Acad. Sci. USA*. 95, 9879.

Tansakul, P. and De-Eknamkul, W. 1998. Geranylgeraniol-18-hydroxylase: The last enzyme on the plaunotol biosynthetic pathway in *Croton sublyratus*. *Phytochemistry*. 47, 1241-1246.

Totte, N., Ende, W.V.D., Damme, E.J.M.V., Compernolle, F., Baboeuf, L. and Geuns, J.M.C. 2003. Cloning and heterologous expression of early genes in gibberellin and steviol biosynthesis via the methylerythritol phosphate pathway in *Stevia rebaudiana*. *Can. J. Bot.* 81, 517-522.

Veau, B., Courtois, M., Oudin, A., Chenieux, J.C., Rideau, M. and Clastre, M. 2000. Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*. *Biochim. Biophys. Acta*. 1517, 159-163.

Vongchareonsathit, A. and De-Eknamkul, W. 1998. Rapid TLC-densitometric analysis of plaunotol from *Croton sublyratus* leaves. *Planta. Med.* 64, 279-280.

Walter, M.H., Fester, T. and Strack, D. 2000. Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. *Plant J.* 21, 571-578.

Wungsintaweekul, J. 2001. Enzymes of the alternative terpenoid pathway in *Escherichia coli*. *Ph. D. thesis*. Technischen Universität München, Germany.

Wungsintaweekul, J., Herz, S., Hecht, S., Eisenreich, W., Feicht, R., Rohdich, F., Bacher, A. and Zenk, M.H. 2001. Phosphorylation of 1-deoxy-D-xylulose by D-xylulokinase of *Escherichia coli*. *Eur. J. Biochem.* 268, 310-316.

Wungsintaweekul, J. and De-Eknamkul, W. 2005. Biosynthesis of plaunotol in *Croton stellatopilosus* proceeds via the deoxyxylulose phosphate pathway. *Tetrahedron. Lett.* 46, 2125-2128.

Yao, H., Gong, Y., Zuo, K., Ling, H., Qiu, C., Zhang, F., Wang, F., Pi, Y., Liu, X. Sun, X. and Tang, K. 2006. Molecular cloning, expression profiling and functional analysis of a *DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Camptotheca acuminata*. *J. Plant Physiol.* in press.

Yang, J.W. and Orihara, Y. 2002. Biosynthesis of abietane diterpenoids in cultured cells of *Torreya nucifera* var. *radicans*: Biosynthetic inequality of the FPP part and the terminal IPP. *Tetrahedron*. 58, 1265-1270.