Molecular Cloning and Characterization of Recombinant 1-Deoxy D-Xylulose 5-Phosphate Synthase of *Croton stellatopilosus* Ohba

Thanaisawan Losuphanporn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2008

Thesis Title	Molecular Cloning and Characterization of Recombinant 1-Deoxy-D-Xylulose 5-Phosphate Synthase of *Croton stellatopilosus* Ohba
Author	Mr. Thanaisawan Losuphanporn
Major Program	Pharmaceutical Sciences

Major Advisor

……

(Assist. Prof. Dr. Juraithip Wungsintaweekul)

Co-advisor

……

(Assist. Prof. Dr. Anan Ounaroon)

Examining Committee :

……..…… Chairperson

(Assist. Prof. Dr. Bhutorn Canyuk)

……..…… Committee

(Dr. Sittiruk Roytrakul)

……..……Committee

(Assist. Prof. Dr. Juraithip Wungsintaweekul)

……..……Committee

(Assist. Prof. Dr. Anan Ounaroon)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

……..

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	การโคลนและศึกษาสมบัติของ Recombinant 1-Deoxy-D-xylulose 5-Phosphate Synthase จากเปล้าน้อย
ผู้เขียน	นายธไนศวรรย์ หล่อสุพรรณพร
สาขาวิชา	เภสัชศาสตร์
ปีการศึกษา	2550

บทคัดย่อ

เอนไซม์ 1-deoxy-D-xylulose5-phosphate synthase (DXS) เร่งปฏิกิริยาการรวมตัวกันระหว่างโมเลกุลของ pyruvate และ glyceraldehydes 3-phosphate ได้เป็นสาร 1-deoxy-D-xylulose 5-phosphate (DXP) ในชีวสังเคราะห์ของสารกลุ่มเทอร์ปีนอยด์ผ่านชีวสังเคราะห์แบบ DXP pathway เอนไซม์เร่งปฏิกริยาในสภาวะที่มี thiamine pyrophosphate (TPP) เป็น co-factor การศึกษาครั้งนี้ได้โคลนยีน 1-deoxy-D-xylulose 5-phosphate synthase (*csdxs*) จากส่วนของใบเปล้าน้อย โดยใช้เทคนิค Rapid Amplification cDNA Ends (RACE) คุณลักษณะของ cDNA ที่ได้ประกอบด้วยลำดับนิวคลีโอไทด์ 2,308 คู่เบส ที่มี open reading frame (ORF) ขนาด 2,163 คู่เบส เมื่อถอดรหัสเป็นสายกรดอะมิโน ได้ลำดับกรดอะมิโนจำนวน 720 หน่วยอะมิโน เมื่อวิเคราะห์สายกรดอะมิโนพบว่าส่วน NH_2-terminus ของ CSDXS จะประกอบด้วย chloroplast transit peptide (cTP) จำนวน 57 หน่วยอะมิโน ลำดับกรดอะมิโนของ CSDXS ยังพบส่วน TPP-binding site ณ ตำแหน่ง 216-246 และตำแหน่งของ transketolase motif ณ ตำแหน่ง 498-532 ของ ORF เมื่อเปรียบเทียบลำดับความเหมือน (identity) กับ DXS ที่ได้จากพืชอื่นๆ พบว่าค่าความเหมือน (% identity) มีค่าระหว่าง 68-89% เมื่อศึกษาระดับการแสดงออกของ mRNA ของ *csdxs* จากส่วนใบ กิ่ง และรากของเปล้าน้อย ด้วยเทคนิค semi-quantitative RT-PCR พบว่าระดับการแสดงออกของยีน *csdxs* มีค่าสูงในใบและกิ่งแต่ตรวจไม่พบในส่วนของราก เมื่อวิเคราะห์ปริมาณสารเปลาโนทอลในส่วนต่างๆ พบว่าสารเปลาโนทอลมีการสะสมในส่วนของใบและกิ่งแต่ไม่พบในรากซึ่งสัมพันธ์กับระดับการแสดงออกของยีน *csdxs* จากผลการทดลองสรุปว่ายีน *csdxs* มีความจำเพาะกับชนิดของเนื้อเยื่อพืช และมีส่วนเกี่ยวข้องในกระบวนการสร้างสารเปลาโนทอลในต้นเปล้าน้อย เมื่อทำการเหนี่ยวนำให้สร้างโปรตีนชนิด full-length และ truncated CSDXS ในเซลล์ *Escherichia coli* พบว่าภายหลังจากการเหนี่ยวนำด้วย 0.5 mM IPTG มีการแสดงออกของโปรตีนทั้งสองชนิด เมื่อวิเคราะห์บน SDS-PAGE ขนาดของโปรตีนประมาณ 62 kDa จากนั้นทำการหาแอคติวิตี้ของเอนไซม์ชนิด truncated CSDXS ด้วยเทคนิค couple assay และวิเคราะห์บนแผ่น TLC พบว่าโปรตีนชนิดที่เป็น truncated CSDXS สามารถเร่งปฏิกิริยาการเกิด 1-deoxy-D-xylulose 5-phosphate เมื่อวิเคราะห์หามวลโมเลกุลด้วยวิธี MALDI-TOF MS ของโปรตีน full-length และ truncated CSDXS พบว่าโปรตีน full-length และ truncated CSDXS มีขนาดเพียง 11 kDa ผลการทดลองสรุปว่าโปรตีน CSDXS ไม่คงตัวในสภาวะทดลองและมีแนวโน้มจะเกิดการสลายตัว (degradation)

Thesis Title	Molecular Cloning and Characterization of Recombinant 1-Deoxy-D-Xylulose 5-Phosphate Synthase of *Croton stellatopilosus* Ohba
Author	Mr. Thanaisawan Losuphanporn
Major Program	Pharmaceutical Sciences
Academic Year	2007

ABSTRACT

1-Deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes condensation of pyruvate and glyceraldehydes 3-phosphate to form the first intermediate 1-deoxy-D-xylulose 5-phosphate (DXP) in the terpenoid biosynthesis via the DXP pathway. The DXS requires thiamine pyrophosphate (TPP) as a co-factor. In this study, the *dxs* gene from leaves of *Croton stellatopilosus* Ohba was cloned using the Rapid Amplification cDNA Ends (RACE) technique, designated as *csdxs*. The *csdxs* cDNA composed of 2,308 base pairs and contained the open reading frame (ORF) of 2,163 base pairs, encoding 720 amino acid residues. Analysis of deduced amino acid sequence revealed that the NH_2-terminus of CSDXS carried the chloroplast transit peptide (cTP) at position 57. In addition, CSDXS showed the TPP-binding site at position 216-246 and the transketolase motif at position 498-532 of the ORF. Multiple alignments of CSDXS with others DXSs in higher plants indicated that the CSDXS has identity between 68-89%. The mRNA expression levels of *csdxs* were determined in plant leaf, twig and root using the semi-quantitative RT-PCR technique. The result showed that the transcription of *csdxs* gene was high in leaf and twig, but could not be detected in root. Quantitation of plaunotol content revealed that plaunotol accumulations were found in leaf and twig but not the root, which were related to the *csdxs* gene expressions. This result indicated that the *csdxs* gene is tissue-specific and involved in the plaunotol biosynthesis in *C. stellatopilosus*. Heterologous expression of full-length CSDXS and truncated CSDXS in *Escherichia coli* strains were performed. After induction with 0.5 mM IPTG, *E. coli* cells could produce the soluble proteins of full-length CSDXS and truncated CSDXS of ca. 62 kDa on the SDS-PAGE. Enzyme activity of truncated CSDXS was determined using the couple assay technique and analyzed on TLC plate. The result showed that the truncated CSDXS could catalyze the formation of 1-deoxy-D-xylulose 5-phosphate. Analysis of the native molecular weight with MALDI-TOF MS indicated that the full-length CSDXS and truncated DXS had sizes about 11 kDa. The data indicated that the CSDXS is very unstable and tend to degrade.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Assistant Professor Dr. Juraithip Wungsintaweekul, my advisor, for her concern, guidance and suggestion throughout the research studies.

My sincere acknowledge is expressed to Assistant Professor Dr. Anan Ounaroon, my co-advisor, from Naresuan University for his kindness, encouragement and valuable counsels.

I would like to extend my appreciation to the staffs of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, including the staffs for making this thesis possible. I also wish to thank to the staff of the Scientific Equipment Center (SEC), Prince of Songkla University for their suggestion about gel documentation and DNA sequencing.

This work was supported by grants from the Graduate School and Faculty of Pharmaceutical Sciences, Prince of Songkla University under the Strengthening Program of Pharmaceutical Sciences (FY 2005), the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the International Foundation for Science (IFS, grant no. F/4093-1).

I would like to thank all friends for their supports and friendship. Special thanks to "Losuphanporn family" for inspiring me. Especially, father and mother who have provided me encouragement and endless love.

Thanaisawan Losuphanporn

CONTENTS

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	degree Celsius
µg	=	microgram
µl	=	microliter
APS	=	ammonium persulfate
bp	=	base pair
BSA	=	bovine serum albumin
BSU	=	Bioservice Unit
cDNA	=	complementary DNA
CSDXS	=	1-deoxy-D-xylulose 5-phosphate synthase of *Croton stellatopilosus*
cTP	=	chloroplast transit peptide
DEPC	=	diethyl pyrocarbonate
DMAPP	=	dimethylallyl diphosphate
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
DTE	=	dithioerythritol
DTT	=	dithithreitol
DX	=	1-deoxy-D-xylulose
DXP	=	1-deoxy-D-xylulose-5-phosphate
dxs	=	1-deoxy-D-xylulose 5-phosphate synthase gene
DXS	=	1-deoxy-D-xylulose-5-phosphate synthase
EDTA	=	ethylenediaminetetraacetic acid
FCSDXS	=	full-length of *C. stellatopilosus* DXS
FID	=	flame ionization detector
Fig.	=	figure
FPLC	=	Fast Protein Liquid Chromatography
FPP	=	farnesyl diphosphate
G3P	=	DL-glyceraldehyde 3-phosphate

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

GC	=	gas chromatography
GGOH	=	geranylgeraniol
GGPP	=	geranylgeranyl diphosphate
GGPPS	=	geranylgeranyl diphosphate synthase
GPP	=	geranylphosphate
h	=	hour
HMG-CoA	=	3-hydroxy-3-methylglutaryl-CoA
IPP	=	isopentenyl diphosphate
IPTG	=	isopropyl-β -D-thiogalactopyranoside
IspC	=	2*C*-methyl-D-erythritol 4-phosphate synthase
IspD	=	4-(diphosphocytidyl)-2*C*-methyl-D-erythritol synthase
IspE	=	4-(diphosphocytidyl)-2*C*-methyl-D-erythritol kinase
IspF	=	2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase
IspG	=	1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase
IspH	=	1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase
kb	=	kilobase pairs
kDa	=	kilodalton
l	=	liter
LB	=	Luria Bertaini
M	=	molar
mA	=	milliampere
MALDI-TOF	=	Matrix-assisted laser desorption/ionization Time-of- flight
ME	=	2*C*-methyl-erythritol
MEP	=	2*C*-methyl-D-erythritol 4-phosphate
MEPS	=	2*C*-methyl-D-erythritol 4-phosphate synthase
mg	=	milligram
min	=	minute

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

ml	=	milliliter
mM	=	millimolar
mRNA	=	messenger ribonucleic acid
MVA	=	mevalonate
MWCO	=	molecular weight cut off
NADPH	=	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	=	nanometer
NMR	=	nuclear magnetic resonance
OD	=	optical density
PCR	=	polymerase chain reaction
pH	=	-log hydrogen ion concentration
RACE	=	rapid amplification cDNA ends
Rf	=	retention factor
RNA	=	ribonucleic acid
RNase H	=	ribonuclease H
rpm	=	round per minute
rRNA	=	ribosomal ribonucleic acid
RT-PCR	=	reverse transcription – polymerase chain reaction
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	=	second
TAE	=	tris acetate EDTA
TCSDXS	=	truncated of *C. stellatopilosus* DXS
TEMED	=	*N,N,N',N'*-tetramethylethylenediamine
TLC	=	thin-layer chromatography
TPP	=	thiamine diphosphate
Tris	=	Tris-(hydroxymethyl)-aminomethane
UV	=	ultraviolet

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

V	=	volt
v/v	=	volume by volume
w/v	=	weight by volume
w/w	=	weight by weight
WHO	=	World Health Organization
wt.	=	weight
X-GAL	=	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Plaunoi (เปล้าน้อย) or *Croton stellatopilosus* Ohba (Euphorbiaceae) is a Thai medicinal plant, formerly well known as *C. sublyratus* (Esser and Chayamarit, 2001). It has been used as anthelmintic and dermatologic agents for skin diseases (Ponglux *et al*., 1987). In addition, Plaunoi and Plauyai (*C. poilanei* Gagnep.) have been used together for various purposes such as stomachic, anthelmintic, digestant, transquilizer and carminative (นันทวัน บุณยะประภัศร, 2532). Since 1978, Ogiso and his co-workers reported that plaunotol isolated from *C. stellatopilosus* leaves acts as a cytoprotective anti-ulcer (Ogiso *et al*., 1978). Later, it was shown that only *C. stellatopilosus* can produce plaunotol not in others species (Vongchareonsathit 1994). Studies on pharmacological effects of plaunotol showed that plaunotol can inhibit the growth of *Helicobacter pylori*, a causative bacterium in gastritis, gastric ulcer and duodenal ulcer (Koga *et al*., 1996), stimulate the secretin production (Shiratori *et al*., 1993), inhibition of the interleukin-8 (IL-8) (an inflammation mediator) (Takagi *et al*., 2000). Using plaunotol in combination with proton pump inhibitors and antibiotics highlights the application of plaunotol in clinic (Koga *et al*., 2002). Therefore, plaunotol is particular interesting. Presently, the leaves of *C. stellatopilosus* are well-known as being raw material for manufacturing an antipeptic-ulcer drug, namely Kelnac® which contains plaunotol as an active ingredient.

As mentioned earlier that *C. stellatopilosus* is the only source of plaunotol. Plaunotol is an acyclic diterpene and composes of four isoprene units, which attach via head-to-tail fashion (Fig. 1.1) (Ogiso *et al*., 1978). By plant tissue culture, attempts to obtain cell lines producing plaunotol have been unsuccessful. To clarify why *C. stellatopilosus* callus culture could not

produce plaunotol, study on genes and enzymes involved in the plaunotol biosynthesis is necessary.

For the biosynthetic point of view, plaunotol biosynthesis was first studied from the group of De-Eknamkul and his co-workers since 1997. The first enzyme names 18-geranylgeraniol hydroxylase was reported in 1998 to catalyze the final committed step in plaunotol biosynthesis. It hydroxylates the geranylgeraniol at position 18, yielding the 18-hydroxygeranylgeraniol or plaunotol (Tansakul and De-Eknamkul, 1998). Recently, the geranylgeranyl diphosphate phosphatases were reported to cleave phosphate moieties from geranylgeranyl diphosphate yielding geranylgeraniol (Nualkaew *et al*., 2006). For the early step of plaunotol biosynthesis, there is still lack of knowledge that has been reported from this plant.

Originally, plaunotol is biosynthesized from 3 molecules of IPP and 1 molecule of DMAPP via an intermediate, geranylgeranyl diphosphate (Sitthithaworn *et al*., 2001). Currently, many research groups demonstrated that at least two different pathways for the biosynthesis of IPP exist, namely the mevalonate (MVA) pathway and the alternative deoxyxylulose phosphate (DXP) pathway. Interestingly, distribution of both pathways in nature is different in microorganisms, plants and bacteria (Eisenreich *et al*., 2001). The MVA pathway performed in archea bacteria, fungi, animals, and plants (cytoplasm), whereas the alternative DXP pathway appeared in eubacteria, malaria parasite and plants (plastid). Therefore, study on inhibitors in the DXP pathway may serve the target-specific for novel antibacterials, antimalarials and herbicides, which had no affect to human (Lichtenthaler, 1999). In higher plants, it has been proposed that the DXP pathway is generally operative for formation of monoterpenoids, diterpenoids, phytols and carotenoids (Hirai *et al*., 2000; Umlauf *et al*., 2004; Bouvier *et al*., 2005) whereas the MVA pathway served for the skeletons of phytosterols, sesquiterpenes, triterpenes and polyterpenes. The two pathways leading to IPP are thus compartmentalized in the cytosol and the plastid, respectively.

In *C. stellatopilosus*, the origin of IPP in the skeleton of plaunotol has been studied using the feeding experiment of isotopic glucoses. Feeding of [U-^{13}C]glucose and [1-^{13}C]glucose into young shoots of *C. stellatopilosus* revealed that the IPP moieties in plaunotol are originated from the DXP pathway dominantly (Wungsintaweekul and De-Eknamkul, 2005).

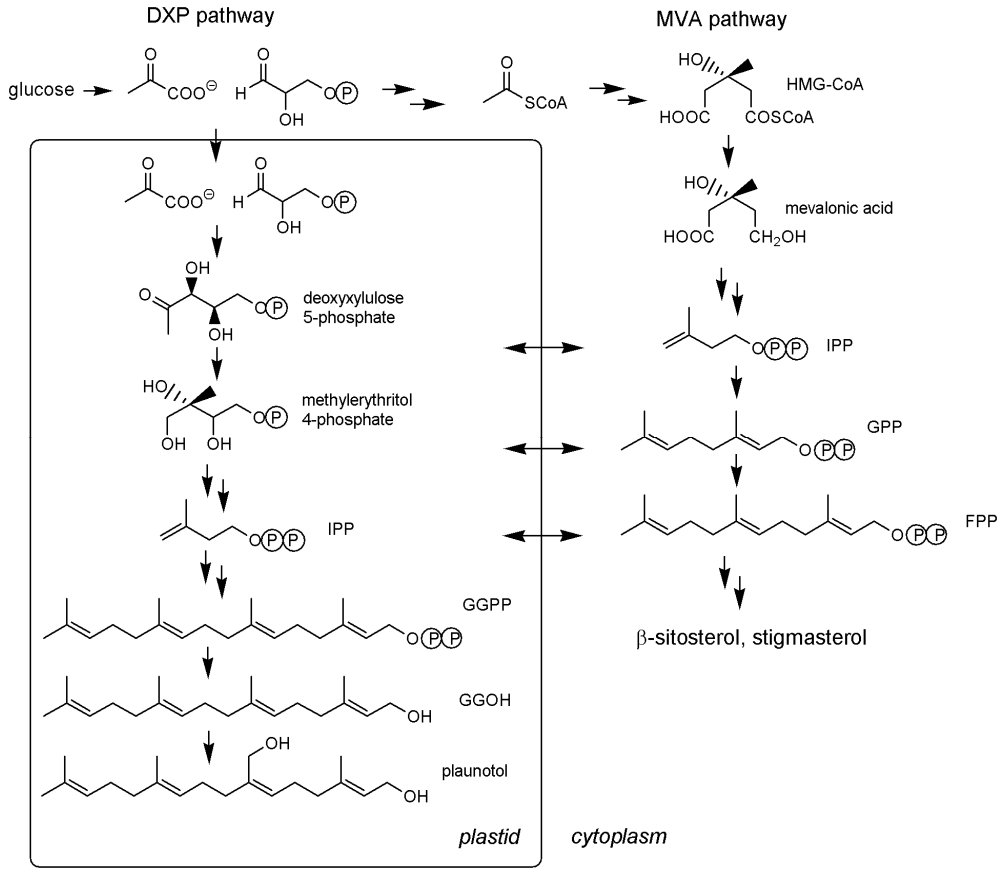


Figure 1.1 Crosstalk between the cytosolic mevalonate (MVA) pathway and plastidic deoxyxylulose phosphate (DXP) pathway in IPP biosynthesis in *Croton stellatopilosus*.

Based on the crosstalk theory suggested by Schwarz and Arigoni, the IPP biosynthesis in *C. stellatopilosus* via the DXP pathway should occurred in plastid (Fig. 1.1). They mentioned that intermediates such as IPP, GPP and FPP could contribute in between two compartments (Arigoni and Schwarz, 1999). This evidence was observed in the green callus culture of *C. stellatopilosus.* Phytosterols biosynthesis in green callus suggested that the IPP moieties were supplied from

mixed origins of the MVA and DXP pathways in the ratio 1:1 (De-Eknamkul and Potduang, 2003). It can be proposed that the DXP pathway appeared in relation to plastid compartment. This hypothesis is supported by the finding of plaunotol localization in chloroplast in palisade layers (Sitthithaworn *et al*., 2006). To confirm that hypothesis, genes and enzymes involved in the DXP pathway are needed to explain the regulation in the plaunotol biosynthesis.

During the past decades, information on the alternative DXP pathway has been discovered in *Escherichia coli* (for detail see 1.2.3). Briefly, 1-deoxy-D-xylulose 5-phosphate obtained by condensation of pyruvate and glyceraldehydes 3-phosphate undergoes a rearrangement coupled to a reduction step. The resulting 2*C*-methyl-D-erythritol 4-phosphate is converted into its cyclic diphosphate by the sequential actions of IspD, IspE and IspF (Rohdich *et al*., 1999; Lüttgen *et al*., 2000; Herz *et al*., 2000). The 2*C*-Methyl-D-erythritol 2, 4-cyclodiphosphate is transformed into IPP and DMAPP via 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate by the sequential actions of IspG and IspH (Hecht *et al*., 2001; Altincicek *et al*., 2001).

In higher plants, many genes involved in the DXP pathway have been reported. Several studies reported the function and regulatory role of the genes involved in the DXP pathway through the terpenoid biosynthesis in higher plant. The first enzyme in the DXP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) was shown to be a rate-limiting step of the DXP pathway and is an attractive target for the development of novel antibiotics, antimalarials, and herbicides. The first *dxs* gene, encoding the DXS protein, was first identified in *E.coli* (Sprenger *et al*., 1997). The cognate *dxs* gene was cloned and the corresponding gene product was hyperexpressed, purified and characterized. The enzyme belongs to transketolase enzymes and requires thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors (Sprenger *et al*., 1997). Up to date, the *dxs* genes have been cloned from several plants such as *Mentha piperita* (Lange *et al*., 1998), *Capsicum annuum* (Bouvier *et al*., 1998), *Arabidopsis thaliana* (Estévez *et al*., 2000), *Catharanthus roseus* (Chahed *et al*., 2000), *Elaeis guineensis* (Khemvong and Suvachittanont,

2005) and *Ginkgo biloba* (Gong *et al*., 2006). Characteristic of the DXS protein carrying plastid targeting sequences suggested that this protein is located in chloroplast compartment. The DXSs from *A. thaliana* and *Lycopersicon esculentum* have been reported the translocation in chloroplast (Estevez *et al*., 2000; Lois *et al*., 2000). The DXS enzyme plays a role in chloroplast formation in higher plants (Lois *et al*., 2000). Molecular cloning of *dxs* gene from *E. guineensis* demonstrated that the transcription level of *dxs* correlated with β-carotene accumulation during oil palm fruit ripening (Khemvong and Suvachittanont, 2005).

Recently, cDNA encoding the *csdxr* gene, the second gene in the DXP pathway, was cloned from *C. stellatopilosus* leaves. It was proposed to locate in the chloroplast compartment as it carries the chloroplast transit peptide. Study on physiological role of *csdxr* gene in plaunotol biosynthesis suggested that the *csdxr* gene is related to the plaunotol production, however, it does not regulates a rate-limiting step in plaunotol biosynthesis (Sirisuntipong 2007; Wungsintaweekul *et al*., 2008). As we focus on the IPP biosynthesis via the DXP pathway, in this study, we aim to clone the *csdxs* gene, the first committed step of IPP biosynthesis from *C. stellatopilosus* leaves. The regulatory role of *csdxs* gene is investigated in terms of the transcription level in relation to the amount of plaunotol in leaf, twig and root of *C. stellatopilosus*. The deduced amino acid (CSDXS) is then functional and analyzed and predicted to its subcellular localization. The full-length and truncated genes are then heterologous expressed in *E. coli*. The proteins are further purified by affinity chromatography. Enzymes activities are determined using the couple assay and analyzed using thin layer chromatography technique. The molecular weights of proteins are determined using SDS-PAGE and MALDI-TOF MS. The regulatory role of *csdxs* gene is investigated in terms of the transcriptional level in relation to the amount of plaunotol in leaf, twig and root of *C. stellatopilosus*. The results obtained from this study will fulfill the knowledge of IPP biosynthesis in *C. stellatopilosus*. It will be useful for understanding the plaunotol biosynthetic pathway, which may lead to enhance the plaunotol production either in intact plant or plant cell culture by metabolic engineering.

1.2 Review literatures

1.2.1 *Croton stellatopilosus* Ohba

1.2.1.1 Botanical aspects of *Croton stellatopilosus* Ohba

Croton stellatopilosus Ohba formerly known as *Croton sublyratus* Kurz (Fig. 1.2) is a tropical plant belonging to the family Euphorbiaceae. This plant has been called in Thai as "Plaunoi". It is a kind of shrub to 6 m tall, branching from base; younger parts distinctly pubescent; flowering with mature leaves. *Indumentum* consisting of stellate-dendritic, cream-yellowish-brown hairs with a slightly darker center, not flat, 0.2-0.8 mm in diameter, with 10-15 (on leaves 6-10) free radii. *Stipules* 3-4 mm long, densely pubescent. *Leaves* crowded but not pseudo-verticillate; petiole 0.5-2.5 cm long, distinctly but not densely pubescent at least on younger leaves; blade obovate and sometimes constricted near base, rarely nearly elliptic, 6-18 by 3-7 cm, index 2.0-4.0, chartaceous, base attenuate but very base always rounded, margin serrate (teeth c. 5 mm apart), apex acute to short-acuminate, glabrous above, with scattered and distinct but not dense hairs below, brighter below; basal glands sessile, flat, 0.8-1 mm in diameter and often elongate-narrowed, partly to completely on the blade very close to the petiole rather than on the petiole, few marginal glands sometimes present, small (ca. 0.3 mm diameter), sessile; side veins 8-14 pairs, the basal pair slightly different (angle more acute), tertiary veins visible. *Inflorescences* whitish, 7-10 cm long, most often collected as stiff and dense, erect buds sometimes on a bent stalk of 1-2.5 cm long, distinctly proterogynous and often the apical, shorter staminate part still in bud when the lower pistillate part is already in flower or even in fruit (precocious fruiting), with 4-10 pistillate flowers, without bisexual bracts; with scattered and distinct indumentum on all parts, slowly glabrescent; bracts 1.5-2 mm long, glandular, pubescent, caducous. *Staminate flowers* densely pubescent throughout; pedicel 2-6 mm long; sepals and petals both 2.5 by 1 mm; stamens 10, glabrous or with few hairs at base. *Pistillate flower* densely

pubescent throughout; pedicel 4-6 mm long (-8 mm in fruit); sepals 3 by 1.5 mm, as long as the ovary; petals not seen; stigmas 3 mm long, free, undivided in the lower half and once divided apically. *Fruits* 5 by 6 mm, smooth to very slightly muriculate, sulcate, quite thin-walled, sparsely pubescent to subglabrous. *Seeds* 4 by 2.5 mm, with a very small caruncle. Flowering and fruiting on December to February. Distribution areas of this plant in Thailand appear to be in Lop Buri (Khao Thungna), Sa Kaeo, Prachin Buri (Kabin buri), Chachoengsao, Chon Buri (Khao Khieo). This information was cited in the web site of Flora of Thailand Euphorbiaceae (http://www.nationaalherbarium.nl/ thaieuph/ThSearchSc/ThSearchScT.htm)

1.2.1.2 The evidence of changing name from *Croton sublyratus* Kurz to *Croton stellatopilosus* Ohba

The botanical name of "Plaunoi" has been changed recently based on its taxonomic characteristic. It was originally identified as *C. sublyratus* by Airy Shaw (1972) by comparing with the collections from Andaman Island of India. However, the samples of Thai collections differ in at least two characters from the Andaman plants. First, the basal leaf glands are sessile and flat (distinctly protruding to nearly stipitate in *C. sublyratus*), and many plants have been collected peculiarly cone-like, dense, pyramidal inflorescence buds (not found on the Andaman Island). Second, the leaves are generally slightly smaller in Thailand. These differences have been used as the major points to separate plants from the two countries. *C. stellatopilosus* has, therefore, been described from south-eastern Thailand and is the collect name for the source plant of plaunotol in the strictest sense as described by Esser and Chayamarit, 2001. In 1972, Airy Shaw from Kew garden introduced the name of *C. sublyratus* Kurz. for Plaunoi (Thai name), characterized by distinctly obovate-panduriform leaf shape (Airy Shaw, 1972). 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 obviate leaves and scattered

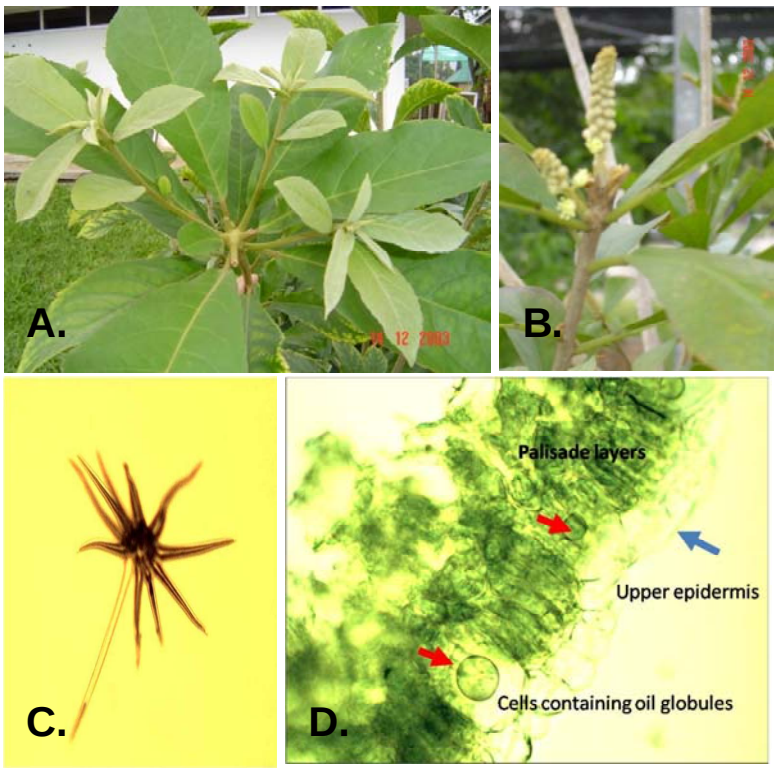


Figure 1.2 Botanical aspects of *C. stellatopilosus* Ohba (Euphorbiaceae). A, pubescent leaves in young plant; B, inflorescences; C, stellate dendritic hair, a major characteristic of plaunoi; D, transverse section of mature leaf (x400): red arrows indicate oil cells containing plaunotol in palisade layer.

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.

1.2.1.3 Chemical constituents of *C. stellatopilosus*

Since 1978, Ogiso and co-workers have isolated and identified plaunotol as an antipeptic ulcer substance from the acetone extract of stem of *C. stellatopilosus* (Ogiso *et al*., 1978). Other compounds with antiulcer activity were tested by Kitazawa and his co-worker (1980), they are diterpenelactone, namely, plaunol B, plaunol C, plaunol D and plaunol E. This made it interests to continue research on the isolation of chemical constituents from *C. stellatopilosus*. In addition, furanoid diterpene: plaunolide (Takahashi *et al*., 1983), diterpene alcohols: *ent*-13α-hydroxy-13-epimanool, *ent*-16β-hydroxykaurane (Kitazawa and Ogiso, 1981), ester of 18-hydroxygeranylgeraniol: octadecanoic acid, 2-(6-hydroxy-4-methyl-4-hexenylidene)-6,10-dimethyl-5,9-undecadienyl ester, (*Z*, *E*, *E*), 9-octadecanoic acid (9*Z*)-(2*Z*, 5*E*)-2-[(4*E*)-6-hydroxy-4-methyl-4-hexanylidene]-6,10-dimethyl-5,9-undecadienyl ester, hexadecanoic acid, ester with 2-(4,8-dimethyl-3,7-nonadienyl)-6-methyl-2,6-octadiene-1,8-diol monodecanoate, (*Z*, *E*, *E*). Structures of compounds, which possess the antiulcer activity, are listed in Fig. 1.3.

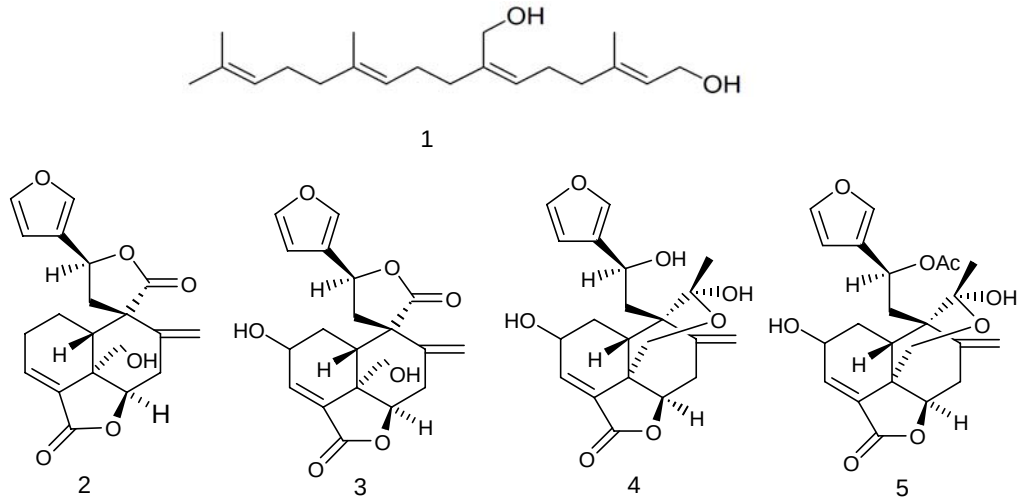


Figure 1.3 Chemical structures of plaunotol [1], plaunol B [2], plaunol C [3], plaunol D [4], plaunol E [5].

1.2.2 Plaunotol

1.2.2.1 Structure and chemical properties

In 1978, Ogiso and his co-workers isolated plaunotol from acetone extract of *C. stellatopilosus*. Using techniques of partition with *n*-hexane and benzene, together with silica gel column chromatography afforded plaunotol and furanoditerpenes (Ogiso *et al*., 1978). The oily compound, plaunotol possesses a molecular composition of $C_{20}H_{34}O_2$ (molecular mass of 306.2546) by high resolution mass spectrometry. The infrared spectrum of plaunotol exhibits absorption bands at 3300, 1665 and 1100 cm^{-1}. The proton magnetic resonance (PMR) spectrum shows signals due to four vinyl methyl 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 four olefinic protons at δ 5.0-5.3 (4H, m). In addition to the above spectral data, the results of giving a diacetate and a bis-3,5-dinitrobenzoate suggest that plaunotol possesses an acyclic tetraprenyl structure having two hydroxyl groups. Chemical reactions of using tris(dipivalomethano)europium as shift reagent supports that the extra hydroxyl group must be at C-18 position (Fig.1.4). Configuration of plaunotol was determined and shown to bear (*E, Z, E*)-configuration. Therefore, plaunotol is named as (*E, Z, E*)-7-hydroxymethyl-3, 11, 15-trimethyl-2, 6, 10, 14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol.

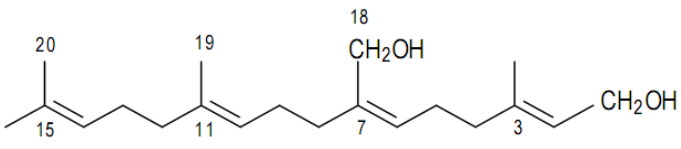


Figure 1.4 The chemical structure of plaunotol ((*E, Z, E*)-7-hydroxymethyl-3, 11, 15-trimethyl-2, 6, 10, 14-hexadecateraen-1-ol or 18-hydroxygeranylgeraniol).

1.2.2.2 Extraction and purification of plaunotol

Process of plaunotol isolations have been reported from independent groups of Ogiso *et al*. (1978) and Nilubol (1992). For the latter protocol was patented to the US patent (No. 5,264,638) (Nilubol, 1992). In 1978 Ogiso and co-workers isolated plaunotol from *C. stellatopilosus* acetone extract, partitioned with the non-polar solvents, which were *n*-hexane and benzene. Benzene portion was then washed with an aqueous sodium hydrogen carbonate solution. The residue of benzene fraction was dissolved in ether. The ether solution was further purified with silica gel column chromatography, using benzene and ethyl acetate as mobile phase, afforded plaunotol. Another portion of ether residue was purified using chloroform and methanol as mobile phase, afforded the furanoditerpenes. Plaunotol was registered to the World Health Organization (WHO) as compound CS-684.

However, Nilubol also patented the process for extraction and purification of plaunotol to the US patent (Patent No. 5,264,638). The protocol began with methanol extract of *C. stellatopilosus* dried leaves. Then the residue was extracted with chloroform. After treatment the chloroform fraction with activated charcoal, the filtrate was evaporated to dryness. The residue was dissolved in 80% (v/v) ethanol/water mixture. The filtrate was washed with *n*-hexane and the ethanol fraction was separated and evaporated. Adding of 10% aqueous sodium hydroxide and heated 110 $^{\circ}$C for 15 min, the solution was partitioned with *n*-hexane. The *n*-hexane layer was treated with anhydrous sodium sulphate and fullers earth. After solvent evaporation, diterpene alcohol plaunotol was obtained (Nilubol, 1992).

It can be noted that both protocols relied on several steps of organic solvent extractions and separations with silica gel column. Therefore, the percent yield of plaunotol was low, caused by multi-step isolations and high-cost isolation can be estimated. Thus, development of plaunotol extraction still challenges to the researchers nowadays.

1.2.2.3 Determination of plaunotol content

Vongchareonsathit and De-Eknamkul (1998) reported methods of plaunotol quantitative analysis by thin-layer chromatography (TLC) densitometric method and gas chromatographic method.

TLC densitometric method has been developed for rapid, simple 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 is 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 is then scanned by TLC densitometer under the wavelength of 220 nm. The developed technique showed high accuracy and precision as compared with the GC method and could detect plaunotol at a quantity of as low as 0.12 μg. This TLC densitometric method is a useful tool in the screening plants and tissue cultures containing plaunotol.

Gas chromatography method can be used for determining the plaunotol content in the microgram range. This method is 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).

1.2.2.4 Antipeptic ulcer activity and mode of action

The antiulcer effects of plaunotol were investigated on acute gastric or duodenal ulcers and on chronic gastric ulcers in several experimental animals. Studies on acute ulcers performing induced-gastric models with reserpine, stress, aspirin, indomethacin, and pyrolous ligation, plaunotol showed significant inhibitory effects at an intraduodenal dose of 300 mg/kg. Thus, plaunotol exhibited a broader antiulcer spectrum than other investigated antiulcer

drug such as cetraxate, gefarnate and sucralfate which have been regarded as potentiators of the mucosal protective factors (Ogiso *et al*., 1985). Effects of plaunotol on chronic ulcers were studied in the model of acetic-gastric ulcer in dogs and clamp-induced gastric ulcers in rats. The studies concluded that plaunotol facilitated the healing of chronic ulcers significantly in rats and dogs (Ogiso *et al*., 1985).

The mode of actions of anti-ulcer effects of plaunotol have been investigated and suggested, including increase of prostaglandin production in gastric mucosa (Ushiyama *et al*., 1987), released endogenous secretin which is a potential mediator of the anti-ulcer actions of mucosal protective agents (Shiratori *et al*., 1993). In animal experiments, plaunotol prevents indomethacin-induced gastric mucosal injury by inhibiting neutrophil activation (Murakami *et al*., 1999). Takagi and co-workers reported that gastric mucosal injury induced by *H. pylori* infection was inhibited by plaunotol in a dose-dependent manner (Takagi *et al*., 2000). Therefore, plaunotol prevents gastric mucosal injury, and has gastroprotective actions through various mucosal defensive factors. In 2002, Koga and co-workers investigated the effect of plaunotol when combined with clarithromycin or amoxicillin against *H. pylori in vitro* and *in vivo*. For *in vitro*, when combined with clarithromycin, plaunotol showed synergistic activity. When combined with amoxicillin, plaunotol showed additive activity against 10 of 14 strains of *H. pylori*. It can be concluded that plaunotol, may have a useful role in combination with anti *H. pylori* drugs in the treatment of *H. pylori*-associated diseases (Koga *et al*., 2002).

In addition, plaunotol is able to inhibit the growth of *Staphylococcus aureus.* Plaunotol was shown to be effective against all tested strains of methicillin-resistant *S. aureus* (MRSA) and of methicillin-sensitive *S. aureus* (MSSA) which had been isolated from the skin of patients with atopic dermatitis (Matsumoto *et al*., 1998). This pharmacological property allows plaunotol to apply for skin diseases protection.

1.2.2.5 Plaunotol biosynthesis

Plaunotol biosynthesis has been firstly reported from the group of De-Eknamkul and his co-workers in 1998. Plaunotol is acyclic diterpene alcohol and therefore, is derived from four isoprene units. As shown in Fig. 1.4, one molecule of DMAPP and three molecules of IPP were attached by head-to-tail condensation, catalyzed by prenyltransferases (Spurgeon and Porter, 1981), yielding geranylgeranyl diphosphate (GGPP). Recently, a membrane bound GGPP phosphatases was shown to catalyze the monophosphorylations of GGPP, afforded the geranylgeraniol (GGOH) (Nualkaew *et al*., 2005; Nualkaew *et al*., 2006). For the final step in plaunotol biosynthesis, the GGOH 18-hydroxylase catalyzed the addition of hydroxyl group at C-18 position of GGOH, yielding 18-hydroxygeranylgeraniol or plaunotol (Fig. 1.5) (Tansakul and De-Eknamkul, 1998). Study on localization of plaunotol in *C. stellatopilosus* leaves suggested that plaunotol accumulated mainly in palisade cells as oily globules (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 storaged in the chloroplast (Morimoto and Murai, 1989).

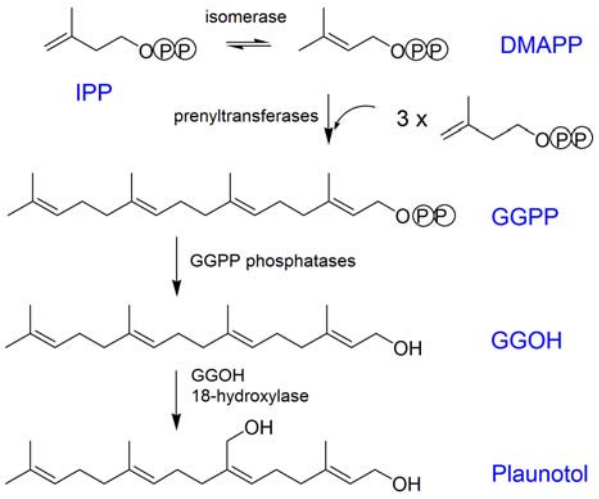


Figure 1.5 Biosynthesis pathway of plaunotol originated from IPP and DMAPP.

1.2.3 Isoprenoid biosynthesis

Isoprenoids are a large family of natural products with important representatives in all taxonomic groups. Isoprenoid serves as the original precursor of all terpenoids. Terpenoids have a wide variety of biological functions and many have potential medicinal applications. All isoprenoids share a common feature: they are derived biosynthetically from 5-carbon compound, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The biosynthesis of IPP was investigated by many research groups. They demonstrated that at least two different pathways for IPP biosynthesis exist, namely, the mevalonate pathway and the alternative deoxyxylulose phosphate pathway.

1.2.3.1 The mevalonate pathway

The classical mevalonate (MVA) pathway has been known as the route for IPP biosynthesis. This pathway starts from acetyl-CoA as precursor. Sequentially, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by thiolase. The latter compound then is condensed with another molecule of acetyl-CoA to HMG-CoA by HMG-CoA synthase. In the subsequent step, HMG-CoA is reduced to mevalonic acid in the presence of NADPH by HMG-CoA reductase. The mevalonic acid is phosphorylated twice by mevalonate kinase and mevalonate 5-phosphate kinase to form mevalonic acid 5-phosphate and mevalonic acid 5-diphosphate, respectively. Mevalonic acid 5-phosphate is then decarboxylated and dehydrated by mevalonic acid 5-diphosphate decarboxylase to form isopentenyl diphosphate (IPP). IPP is isomerized to DMAPP by IPP isomerase (Qureshi and Porter, 1981; Spurgeon and Porter, 1981). The whole biosynthesis pathway of IPP via the MVA pathway is shown in Fig. 1.6.

In this pathway, the HMG-CoA reductase is therefore attracted and of great interest since it catalyzes the rate-limiting step in the MVA pathway thus it become a key target for interruption of cholesterol biosynthesis.

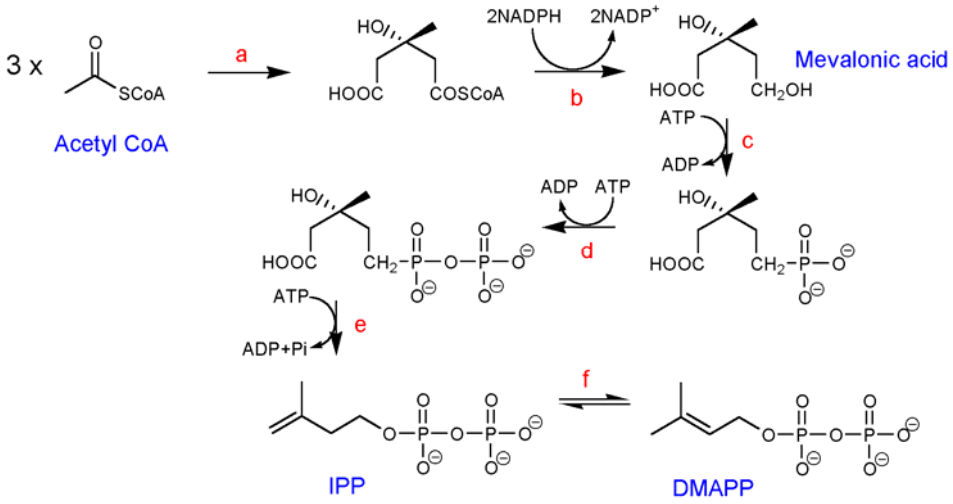


Figure 1.6 Biosynthesis of IPP via the mevalonate pathway.

a, HMG-CoA synthase; b, HMG- CoA reductase; c, mevalonate kinase; d, mevalonate 5-phosphate kinase; e, mevalonate 5-phosphate decarboxylase; f, IPP isomerase.

1.2.3.2 The alternative deoxyxylulose phosphate pathway

Evidence of the existence of an alternative isoprenoid biosynthetic pathway emerged from independent incorporation studies in the research groups of Rohmer and Arigoni who found that the isotopic labeling patterns observed in their studies could not be explained in terms of the MVA pathway (for reviews see Eisenreich *et al*., 1998; Rohmer, 1999).

During two decades, the alternative deoxyxylulose phosphate (DXP) pathway has been introduced and was discovered in 2003 (Rohdich *et al*., 2003). The DXP pathway can be named as mevalonate-independent pathway, non-mevalonate pathway, methylerythritol phosphate pathway or Rohmer's pathway. The DXP pathway composes of seven enzymatic reactions as shown in Fig. 1.7.

The DXP pathway was completely elucidated in *Escherichia coli* (for review see Rohdich *et al*., 2003). The initial step begins with the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and D-glyceraldehyde 3-phosphate, catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Sprenger *et al*., 1997). The

intermediate of DXP is served as substrate for IPP biosynthesis as well as vitamin B biosynthesis (Sprenger *et al*., 1997). In the second step, DXP is further 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, followed by reduction using NADPH (Takahashi *et al*., 1998). Further, MEP is catalyzed by 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol synthase (IspD) to form 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol in a cytidine triphosphate (CTP) dependent reaction (Rohdich *et al*., 1999). Next, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol is phosphorylated at position 2-hydroxy group of 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol, catalyzed by 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol kinase (IspE) in an ATP-dependent phosphorylation (Lüttgen *et al*., 2000). The next step in the pathway, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol-2-phosphate is cyclized to form 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, catalyzed by 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF) (Herz *et al*., 2000).

Recently, the last two enzymes have been reported. 2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate is then converted to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, catalyzed by 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (IspG) (Hecht *et al*., 2001). The last step, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate is subsequently transformed into a mixture of IPP and DMAPP, catalyzed by 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (IspH) (Rohdich *et al*., 2003). In addition, the evidence of incorporation experiments with labeled 1-deoxyxylulose into plants and microorganisms were supported by the finding of D-xylulokinase (Xyl B) in the salvage pathway. This enzyme is responsible for fluxing the 1-deoxy-D-xylulose into the IPP biosynthesis via the DXP pathway (Wungsintaweekul *et al*., 2001).

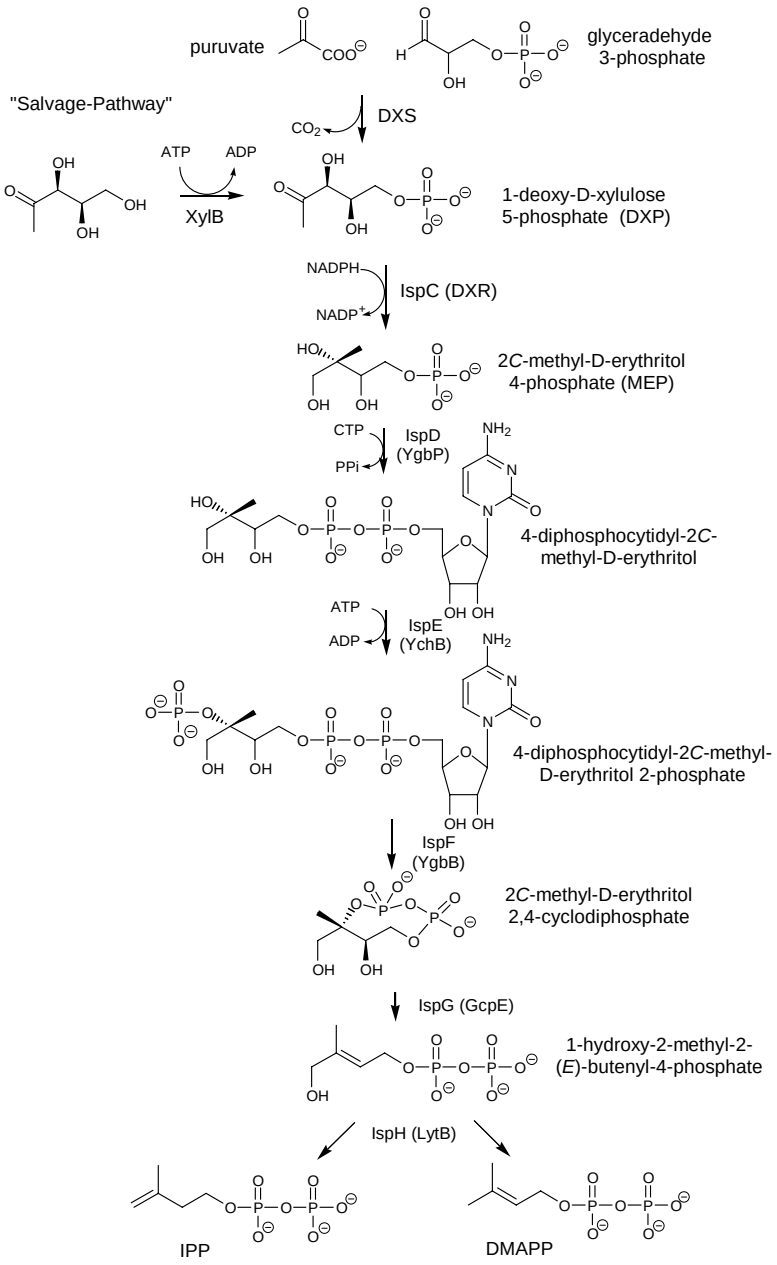


Figure 1.7 Biosynthesis of IPP and DMAPP via the alternative deoxyxylulose phosphate pathway. [DXS, deoxyxylulose 5-phosphate synthase; IspC, 2*C*-methyl-D-erythritol 4-phosphate synthase; IspD, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol synthase; IspE, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol kinase; IspF, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase; IspH, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase].

1.2.3.3 The DXP pathway is widely distributed in nature

After the DXP pathway was introduced for IPP biosynthesis, studies of isotopic incorporations were re-investigated in bacteria, certain algae, plant cell cultures and plants. In summary (Table 1.1), archea, certain bacteria, yeasts, fungi, some protozoa and animals appear to use the MVA pathway. On the other hand, many bacteria including human pathogens, green algae and malaria parasite *Plasmodium falciparum* appear to rely exclusively on the DXP pathway. *Streptomycetes*, some algae, mosses and liverworts, marine diatoms and higher plants appear to use both pathways (Eisenreich *et al*., 1998; Arigoni and Schwarz, 1999; Rohmer, 1999).

Table 1.1 Distribution in nature of the mevalonate and the deoxyxylulose phosphate pathways of IPP biosynthesis.

Organism	Mevalonate	Deoxyxylulose phosphate
Bacteria	✓	or ✓
Archea	✓	
Fungi	✓	
Algae	✓	and/or ✓
Higher plants		
Plastidic compartment		✓
Cytosolic compartment	✓	
Protozoa	✓	✓
Animals	✓	

Interestingly, in higher plants, IPP biosynthesis performs via both pathways in compartmentation dependent. The MVA route operates in the cytoplasm and mitochondria and is responsible for sterols, sesquiterpenes and ubiquinones formation. Whereas, isoprenoids synthesized in the plastids (hemiterpenes, monoterpenes, diterpenes and carotenoids) are formed predominantly via 1-deoxy-D-xylulose 5-phosphate (Arigoni and and Schwarz, 1999).

Schwarz (1994) investigated the contribution of the MVA and DXP pathway in embryo culture of *Ginkgo biloba* with different labeled glucoses. His results suggested that the compartmental separation of the two different IPP biosynthetic pathways is not absolute because at least one metabolite such as IPP, GPP or FPP can be exchanged between the compartments (Fig. 1.1). The extent of this crosstalk depends on the species as well as the concentration of exogenous precursors.

1.2.4 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) (EC 2.2.1.7)

1.2.4.1 Catalytic properties of the DXS protein

1-Deoxy-D-xylulose 5-phosphate synthase (DXS) (EC 2.2.1.7) is the first enzyme in the DXP pathway. The starting substrates of the DXS are pyruvate and glyceraldehyde 3-phosphate. A C2-unit derived from pyruvate (hydroxyethyl-thiamine) is transferred to glyceraldehyde 3-phosphate in a thiamine diphosphate (TPP)-dependent transketolase-type reaction. Then 1-deoxy-D-xylulose 5-phosphate is formed.

In 1997, Sprenger *et al*. reported the identification of a thiamine-dependent synthase in *Escherichia coli*. The gene encoding *dxs* was first identified in *E. coli* by extensive searches the database using E1 subunit of pyruvate dehydrogenase complex, pyruvate decarboxylase, and transketolase as queries. The recombinant protein was shown to catalyze the formation of 1-deoxy-D-xylulose 5-phosphate from pyruvate and glyceraldehyde 3-phosphate. The cognate *dxs* gene from *E. coli* was cloned and the corresponding gene product, 1-deoxy-D-xylulose 5-phophate synthase (DXS) was overexpressed, purified and characterized (Sprenger *et al*., 1997). The enzyme requires thiamine diphosphate (TPP) and Mg^{2+} as cofactors. *E. coli* DXS is a homodimer consisting of 2 subunits of 65 kDa. It is inhibited by fluoropyruvate with an IC_{50} of 80 μM. Fluoropyruvate is supposed to bind covalently to active site of DXS as already demonstrated for the pyruvate dehydrogenase component (E1) in the pyruvate dehydrogenase

complex (Flournoy and Frey, 1989). In addition, clomazone has been shown to inhibit the *Chlamydomonas* DXS with the IC_{50} value of 0.1 mM (Müller *et al*., 2000).

1.2.4.2 Structure of DXS protein

Multiple alignments of deduced amino acid sequences of DXS from various organisms indicated that DXS proteins have a number of conserved structural features, which are thiamine diphosphate (TPP)-binding motif, a transketolase motif, and several invariant amino acids such as histidine, glutamic acid, and arginine (Xiang *et al*., 2007). Those catalytic amino acid residues were shown to have an important role for binding to the substrate and cofactor, thiamine diphosphate (Fig. 1.9).

Consideration a primary structure of DXS, the DXS protein from *E. coli* is similar to transketolase-like enzyme and to E1 proteins from pyruvate dehydrogenase complex of various organisms (Sprenger *et al*., 1997; Lois *et al*., 1998). It shows a sequence motif that shares features with a typical binding site for the thiamine diphosphate (TPP) cofactor which is common in pyruvate decarboxylases, acetolactate synthases and transketolases (Reynen and Sahm, 1988; Hawkins *et al*., 1989). As shown in Fig. 1.9, the TPP-binding motif and a transketolase-like motif within the DXS proteins are located at the NH_2-terminus and near the COOH-terminus, respectively. The consensus sequence for the TPP-binding motif is designated as $GDG(X)_{7-8}E(X)_{3-4}A(X)_{11-13}NDN$, which is a conserved region among DXSs (Hawkins *et al*., 1989). In addition, the DXS protein contains a transketolase motif, which is designated as DRAG-X_{28}-P-X-D, where X denotes any amino acid (Schenk *et al*., 1997).

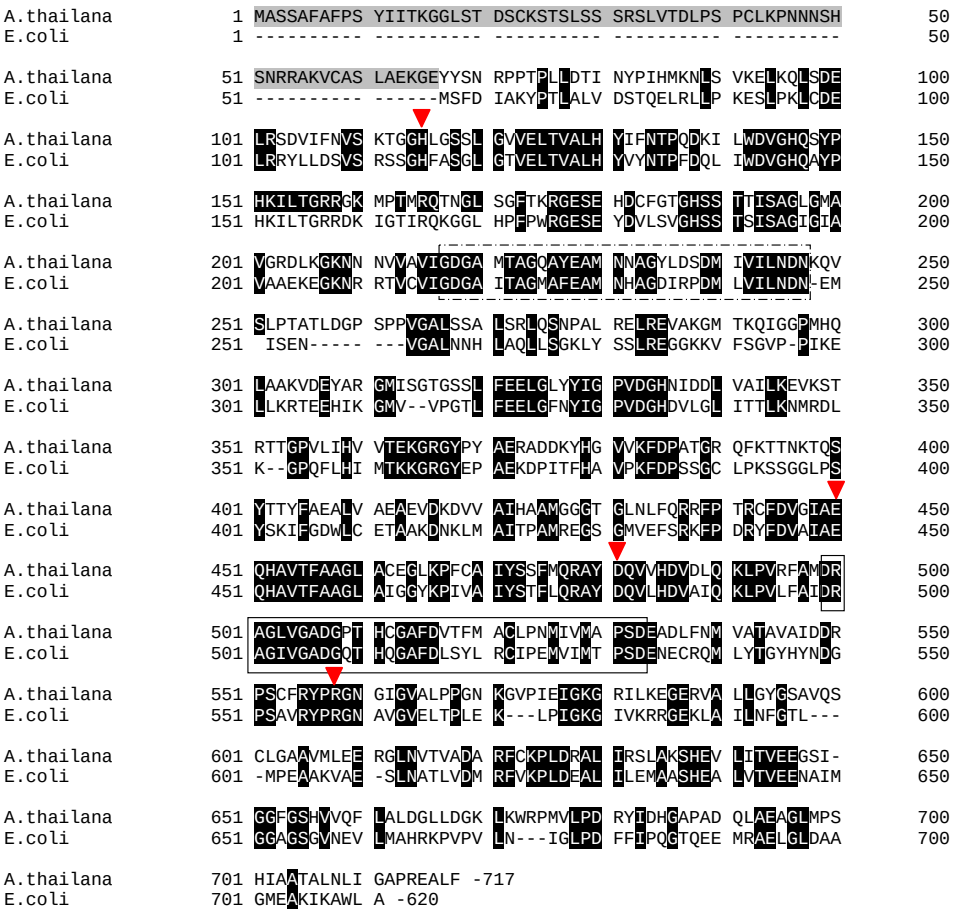


Figure 1.8 Primary structure of DXS. Amino acid sequences alignment of DXS from *Arabidopsis thaliana* and *Escherichia coli*. The shaded sequence denotes the chloroplast transit peptide (cTP). Dashed box and solid-line box indicate the regions corresponding to the putative thiamine diphosphate-binding site and transketolase motif, respectively. The arrowheads denote amino acid residues that form the substrate channel of transketolase.

In comparison to the DXS from *Arabidopsis thaliana* (formerly CLA1 gene), the primary structure is similar to *E. coli* DXS. However, it can be distinguished from *E. coli DXS* with an extra amino acid residues at NH_2-terminus (Fig. 1.9), namely the chloroplast transit peptide (cTP). The *cla1* gene was formerly defined as gene that is important for the synthesis of chlorophyll and carotenoids in *A. thaliana*. Disruption of this gene affects the expression of both nuclear- and chloroplast-encoded photosynthetic gene, resulting in an impairment of thylakoid membrane proliferation (Mandel *et al*., 1996). This study suggested that the DXS protein plays a role during chloroplast development. Later, cloning of *dxs* genes from *Mentha piperita* (Lange *et al*., 1998), *Capsicum annuum* (Bouvier *et al*., 1998), *Ginkgo biloba* (Kim *et al*., 2006) revealed that all DXSs from plants also contained the chloroplast transit peptides and distinct to the plant species. The common feature of the cTP is rich in hydroxylated amino acids such as serine and threonine, and contains some basic residues. These are common features of cTP sequence in plants (Emanuelsson *et al*., 1999). The cTP sequence is important for translocation of protein from the cytosol to the chloroplast compartment in plants (Mori and Cline, 2001).

Mutagenesis studies suggested that histidine residue (H49 of *E. coli* DXS) (Fig. 1.9) is required for growth and catalytic activity (Querol *et al*., 2001). Recently, the crystal structure of *E. coli DXS* was reported and allowed the identification of additional important two arginine residues (R398 and R478), which are essential for the DXS activity (Fig. 1.9) (Xiang *et al*., 2007). The study on active sites for substrates (glyceraldehydes 3-phosphate) and cofactor (thiamine diphosphate) suggested that glutamic acid (E370) and arginine (R398 and R478) residues in the *E. coli* DXS play a crucial role during catalysis. Mutation of those residues to alanine (E370A, R398A, and R478A) shows no DXS activities (Xiang *et al*., 2007).

1.2.4.3 Expression of the DXS protein and transcript

The expressions of *dxs* genes from different tissues of various plant species have been reported. In 1998, the putative *dxs* genes from *M. piperita* and *C. annuum* were cloned and their physiological roles were studies (Lange *et al*., 1998; Bouvier *et al*., 1998). The CapTKT2 (*dxs*) from *C. annuum* was shown to catalyze the condensation of D-glyceraldehyde 3-phosphate and pyruvate with the rate of 500 nmol/min. mg protein. The K_m values for D-glyceraldehyde 3-phosphate and pyruvate are 750 and 500 μM, respectively (Bouvier *et al*., 1998). Immunoblot analysis of organelle from chloroplast, chromoplast and mitochondria with anti-CapTKT2 suggested that the CapTKT2 presences in chloroplast and chromoplast, not in mitochondria. Analysis of CapTKT2 mRNA during chloroplast-to-chromoplast differentiation in pepper fruit indicated that the CapTKT2 is up-regulated expression when carotenoid accumulation (Bouvier *et al*., 1998). For *dxs* from *M. piperita*, putative *dxs* gene was isolated from cDNA library derived from *M. piperita* oil gland secretory cells. The DXS protein from *M. piperita* was obtained after induction and characterized by forming the 1-deoxy-D-xylulose 5-phosphate in the recombinant *E. coli*. The DXS mRNA levels were shown to relate with monoterpenoid biosynthesis in peppermint (Lange *et al*., 1998).

Many researchers are interested in the regulation role of *dxs* gene in plants. A well-established genome sequence of *A. thaliana* lead to an exploring how does the DXS act in plant. Mandel and co-workers suggested the *Arabidopsis* transketolase gene, namely *CLA1*. They reported that disruption of which results in an albino phenotype (Mandel *et al*., 1996). Later, the complementation of the *CLA1-1* mutant was shown that the *CLA*1 gene is widely expressed throughout the plants, with the higher expression levels in young tissues (Estévez *et al*., 2000). The function of the CLA1 product as DXS is then experimentally confirmed in both *in vivo* and *in vitro*. Estévez *et al*. (2000) reported that when the CLA1-1 plants were grown in the germination media supplemented with 0.02% (w/v) 1-deoxy-D-xylulose, rescued the albino-type of *Arabidopsis*. The *CLA1* gene is present as a single gene in the *A.*

thaliana genome (Mandel *et al*., 1996). Recently, a search in *A. thaliana* genome, however, results in a finding of two more *dxs*-related genes: *dxs*2 (At3g21500 or NM_113045) and *dxs*3 (At5g11380 or CAB96673). The function of these genes in relation to isoprenoid biosynthesis is still unknown. The *dxs*2 and *dxs*3 genes are scattered in different chromosomes of *A. thaliana*. The *dxs*2 and *dxs*3 genes are located on chromosome 3 and 5, respectively, while the *CLA1* or *dxs*1 gene is settled on chromosome 4. Therefore, the *A. thaliana* should contain three *dxs* genes in its genome (Rodríguez-Concepción and Boronat, 2002).

In *L. esculentum*, the expression of *dxs* mRNA also found in photo-synthetic tissue, while very low levels are detected in roots (Lois *et al*., 2000). In addition, the levels of *dxs* mRNA are correlated with the accumulation carotenoids during *L. esculentum* fruit development, suggesting that the induction of *dxs* gene is associated with the activation of carotenoid biosynthesis at the onset of ripening (Lois *et al*., 2000). The expression of *dxs* gene in correlation to carotenoid accumulation during ripening is also found in other plants such as *C. annuum* and *E. guineensis* fruits (Bouvier *et al*., 1998; Khemvong and Suvachittanont, 2005).

Since the discovery of the first two *dxs* genes in *M. truncatula* by Walter *et al*. (2002), the transcripts of these genes in different tissues of this plant were also studied. The authors found that the Mt*dxs*1 transcripts are abundant in all above-ground tissues, while the Mt*dxs*2 transcripts show very low to non-detectable levels in the above-ground tissues but they are abundant in roots interacted with mycorrhizal fungi (Walter *et al*., 2002). Upon mycorrhization, the *dxs* transcripts in *Oryza sativa*, *Zea mays*, *Triticum aestivum*, and *Hordeum vulgare* increased in relation to the accumulation of apocarotenoids i.e. mycorradicin and glycosylated cyclohexenone derivatives (Walter *et al*., 2000; Walter *et al*., 2002).

In *G. biloba*, another plant that two classes of *dxs* genes, studied by Kim *et al*. (2006), found that the expression of Gb*dxs*1 is abundant in leaves, while the transcripts of Gb*dxs*2 are abundant in roots. Also, they reported that the Gb*dxs* transcripts are correlated with the production of ginkgolides in the embryo culture of *G. biloba (*Kim *et al*., 2006).

Based on these results, it can be concluded as a general idea that the expression of *dxs* class I is required for the biosynthesis of primary isoprenoids such as chlorophylls and carotenoids (Mandel *et al*., 1996; Bouvier *et al*., 1998; Estévez *et al*., 2000; Lois *et al*., 2000; Walter *et al*., 2002; Khemvong and Suvachittanont, 2005; Kim *et al*., 2006). In contrast, the expression of *dxs* class II might be involved in secondary metabolite biosynthesis such as apocarotenoids and ginkgolides (Walter *et al*., 2002; Kim *et al*., 2006). Other secondary isoprenoids such as monoterpene from *M. piperita* and monoterpenoid derived from *Catharanthus roseus* also seemed to be correlated with the expression of their *dxs* genes, belonging to the plant *dxs* class II (Lange *et al*., 1998; Veau *et al*., 2000; Chahed *et al*., 2000; Burlat *et al*., 2004). To date, there is no information of *dxs* sequence belonging to *dxs* class I identified in these plants.

Currently, genes encoding DXS proteins have now been identified in a number of plant species. For example, the *dxs* cDNAs were cloned from *Capsicum annuum* (Bouvier *et al.*, 1998), *M. piperita* (Lange *et al*., 1998), *Catharanthus roseus* (Chahed *et al*., 2000), *Tagetes erecta* (Moehs *et al*., 2001), *Artemisia annua* (Souret *et al*., 2002*), Morinda citrifolia* (Han *et al*., 2003), *Stevia rebaudiana* (Totté *et al*., 2003), *Antirrhinum majus* (Zhang *et al*., 2005), and *Chrysanthemum morifolium* (Kishimoto and Ohmiya, 2006).

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipments

Fast Protein Liquid Chromatography (FPLC) systems

(Pharmacia LKB, Amersham Pharmacia Biotech, Uppsala, Sweden)

Columns for FPLC System: HiTrap™ Chelating HP Columns (5 ml); HiTrap™ Desalting HP Columns (5ml), GE Healthcare Life, Singapore

FRAC-100 Fraction Collector

LCC-500 Controller

MV-7 Motor valve

P-500 Pump

Recorder: EYELA Toriconder TR250, Japan

Superloop (10 ml)

UV-1 Monitor

Gas Chromatography (GC) system

HP 6850 Series, Hewlett Packard, CA, USA

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)

GC-Oven (Hewlett Packard, USA)

Injector, Agilent technology 7683B Series

Additional equipments

Autoclave	Model HA-3D (Hirayama, Japan)
Balance	Explorer (Ohaus, USA); Avery Berkel (USA); Sartorius TE 3102S (USA)
Centrifuge	Hermle Z 323 K (Germany); Kubota 5922 (Japan)
Electrophoresis	SE 250 Mighty Small II (Amersham Biosciences, USA)
Electrophoresis	Mupid α Mini Electrophoresis System (Japan)
Gel documentation	Gel Doc model 1000 (BIO-RAD, USA) equipped with Molecular Analyst® Software, Windows Software for BioRad's Image Analysis Systems Version 1.4
Gene amplification	GeneAmp, PCR system 9600 (Perkin Elmer, USA); TaKaRa PCR Thermal Cycler Dice Version III Model TP600, Japan
Hot air oven	Memmert (Germany)
Hot plate and stirrer	Fisher Scientific (USA)
Incubator	Thermomixer comfort (Eppendorf, Germany)
Laminar air flow cabinet	HT-122 ISSCO (Australia)
Microplate reader	Bio-Rad, USA
Micropipettes	Socorex: 0.1-2.0 µl, 2-20 µl, 20-200 µl, 100-1000 µl (Switzerland)
Microwave oven	LG, Thailand
pH meter	Benchtop, pH meter Model 710A (Germany)
Power supply	Model EPS301 (Amersham Biosciences, USA)
Refrigerator	Sanden Intercool (4 °C); Whirlpool (-20 °C); Deep-freezer (-80 °C) (Thailand), Forma Scientific (USA)
Shaking incubator	BIOER Technology Co., Ltd. (Tokyo, Japan)
Spectrophotometer	Labomed, Inc. (USA)

Ultrasonicator High intensity ultrasonic processor 1500 watt model with high volume flow cell (CT., USA)

2.2 Materials

2.2.1 Plant materials

Young leaves of 2 years old of *C. stellatopilosus* were collected from the horticulture of the Faculty of Pharmaceutical Sciences, Prince of Songkla University (PSU), Hat Yai Campus. Voucher specimen was collected and kept at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, PSU, Songkhla, Thailand.

2.2.2 Chemicals

Solvents used in this study were analytical grades. The reagents used for molecular biology were biotechnological grade. All solvents were purchased from Lab-Scan Asia Co., Ltd., Bangkok, Thailand. Culture media are supplied from Himedia Laboratories, India. Chemicals for buffer preparation were from Biobasic Inc., Canada. Reagents for polyacrylamide gel electrophoresis were purchased from USB Cooperation, USA and Amersham Biosciences, USA. List of chemicals is shown in Table 6.1 (Appendix).

2.2.3 Substrates and cofactors

Substrates and cofactors were used for enzyme activity test. D-Glyceraldehyde 3-phosphate and thiamine diphosphate were purchased from Sigma-Aldrich, USA. NADPH was from Biomol, Germany. Sodium pyruvate was purchased from Wako Chemicals, Japan. All compounds were pure grade and for biotechnological purposes.

2.2.4 Molecular biology kits and enzymes

Kits and restriction enzymes were used for gene cloning and expression. A-addition kit, PCR purification kit, Gel extraction kit, PCR cloning kit, RNeasy plant Mini kit and taq PCR core kit were purchased from Qiagen, Germany. Superscript™ III reverse transcriptase was from Invitrogen, USA. Marathon™ cDNA amplification kit for 5'-and 3'-rapid amplification cDNA ends (RACE) PCR was supplied from Clontech®, BD Bioscience, USA. GFX micro-palsmid prep kit was from Amersham Biosciences, USA. Wizard® PCR Midi Preps DNA purification system was purchased from Promega, USA. The restriction endonucleases were purchased from TaKaRa, Japan; Qiagen, Germany; Toyobo, Japan and NEB (New England Biolabs), New England, USA. Enzymes used in protein extraction were from Biomol, Germany. 2*C*-Methyl-D-erythritol synthase used in the couple assay of 1-deoxy-D-xylulose 5-phosphate synthase activity was purified from the recombinant strain of *E. coli* harboring pQE-ECDXR. List of kits and enzymes used in this study are shown in Table 6.2 and 6.3 (Appendix).

2.2.5 Solutions preparations

All stock solutions were prepared in distilled water. For sterilized solutions, the sterilized water was used and labwares were autoclaved and dried in hot air oven (70 °C). All solutions were prepared as listed below.

For media preparation

Ampicillin sodium salt (0.25 g) is dissolved in 10 ml of sterilized distilled water. The solution is aliquot to 1 ml and stored the aliquots at -20 °C. Final concentration of stock solution is 25 mg/ml.

5-Bromo-4-chloro-3-indolyl *β*-D-galactopyranoside (X-gal) stock solution is prepared for 20 mg/ml. X-gal (200 mg) is dissolved in 10 ml dimethylformamide. The solution is aliquot

to 1 ml, protects the solution from light by wrapping using aluminium foil and stored at -20 °C.

Isopropyl *β*-D-thiogalactopyranoside (IPTG, MW 238.3) stock solution is prepared for 1M. IPTG (1.19 g) is dissolved in 5 ml of sterilized distilled water. Aliquots are stored at -20 °C.

TB buffer contains pipes (10 mM), $MnCl_2$ (55 mM), $CaCl_2$ (15 mM), KCl (250 mM). pH of the solution is adjusted to 6.7-7.0. The solution is sterilized using autoclave. And sterilized $MnCl_2$ by filtration through filter 0.22 micron is added.

TE buffer contains Tris-HCl, pH 8.0 (10 mM), EDTA (1 mM) and volume is adjusted to 500 ml with distilled water. The solution is sterilized using autoclave.

For agarose gel electrophoresis

Ethidium bromide solution (10 µl) is added in to 100 ml distilled water. The solution is used for DNA and RNA staining.

TAE buffer (50x) is used for agarose gel preparation and 1x buffer is used as running buffer. TAE buffer contains Tris base (121 g), EDTA tetrasodium salts (19.7 g) and glacial acetic acid (35 ml). Volume is adjusted with distilled water to 500 ml. For preparation of running buffer, 20 ml of TAE (50x) is added and volume is adjusted with distilled water to 1000 ml.

Sample buffer composed of 50% (v/v) glycerin and 0.1% (w/v) bromophenol blue.

For SDS-polyacrylamide electrophoresis (SDS-PAGE)

Coomassie brilliant blue staining contains coomassie brilliant blue R-250 (2.5 g) is dissolved in methanol (454 ml) and glacial acetic acid (92 ml). The volume is adjusted with distilled water to 1000 ml.

Destaining solution contains methanol (250 ml), glacial acetic acid (100 ml) and volume is adjusted with distilled water to 1000 ml. The solution is used for protein destaining.

Stacking gel buffer composes of 0.125 M Tris-HCl, pH 6.8, 0.1% SDS

Separating gel buffer composes of 0.375 M Tris-HCl, pH 8.8, 0.1% SDS

SDS-PAGE running buffer contains 192 mM glycine, 25 mM Tris-HCl, pH 8.3 and 0.1% (w/v) SDS.

SDS-PAGE sample buffer contains 60 mM Tris-HCl, pH 6.8, 5% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, 30% (v/v) glycerin, 0.02% (w/v) bromophenol blue and 10% (w/v) sucrose.

For protein determination

Bradford reagent is prepared according to method of Bradford (1976). Coomassie brilliant blue G-250 (0.1 g) is added to absolute ethanol (10 ml) and phosphoric acid (85% v/v) (25 ml), stirred vigorously for 30 min. Volume is adjusted with Millipore water (0.22 μm membrane filter) to 250 ml and stirred for overnight. The solution is filtered through Whatman filter paper (no. 1). The filtrate is collected and stored in a dark-colored at room temperature. The solution can be kept for 1 year.

Standard protein solution is prepared for constructing the standard curve for total protein determination. Bovine serum albumin (BSA) (10 mg) is dissolved in distilled water. Volume is adjusted to 100 ml in volumetric flask and mixed.

2.2.6 Media preparations

Luria-Bertani (LB) medium is prepared and composes of casein hydrolysate (10 g), bacto yeast extract (5 g) and NaCl (5 g). Volume is adjusted to 1000 ml with distilled water. The medium is then sterilized using autoclave (121 °C, 15 pound/inch^2, 15 min). For LB-

ampicillin medium, sterilized ampicillin solution (25 mg/ml) is added to the final concentration of 50 mg/l.

Transformation agar is used for growth of bacteria, which the recombinant plasmids are transformed. The medium is firstly prepared, containing NaCl (1 g), bacto tryptone (1 g), bacto yeast extract (0.5 g) and bacto agar (1.5 g). Volume is adjusted to 100 ml with distilled water. The medium is sterilized using autoclave. Ampicillin (25 mg/ml, 200 μl), X-gal (20 mg/ml, 100 μl) and IPTG (1 M, 10 μl) are added when the temperature of the medium is about 50 $^{\circ}$C and mixed. The mixture is then poured to petri-dish (20 ml per plate) under laminar air flow cabinet.

SOB medium is used for the competent cells preparation. It contains bacto tryptone (2 g), bacto yeast extract (0.5 g) and salts are added to the final concentration (as indicated): NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), $MgSO_4$ (10 mM). Volume is adjusted to 1000 ml and mixed. pH is adjusted to 6.7-7.0 with 1 N NaOH or 1 N HCl. The mixture is sterilized using autoclave.

2.2.7 Primers

The oligonucleotides used in this study were purchased from Operon, Germany. The degenerated primers were designed based on the highly conserved regions among the plants species. The amino acid sequences of the DXSs from *Arabidopsis thaliana* (NM117647), *Capsicum annuum* (O78328), *Catharanthus roseus* (AG011840*)* and *Lycopersicon esculentum* (AF143812), which are available at http://au.expasy.org, were performed for multiple alignment using Clustal W (1.82) (http://www.ebi.ac.uk/clustalw/). The conserved regions were selected and degenerated primers were designed as shown in Table 2.1. Since the amino acid residue is encoded from triple codon of nucletic acids. Therefore, the degenerated primers contained the possible nucleic acids, can be designated to specific alphabets, which are I for A/T/C/G; D for A/G/T; H for A/C/T ; R for A/G and Y for C/T.

In this study, the full-length *csdxs* gene was determined for DNA sequence by means of PCR-direct sequencing. Therefore, the oligonucleotide sequences were designed and used for DNA sequencing. List of primers used in *csdxs* gene sequencing is shown in Table 2.2.

Oligonucleotide sequences of specific primers were designed from the nucleotide sequence that obtained from recombinant clones of core *csdxs* gene generated from the degenerated primers. Table 2.3 shows the oligonucleotide sequences of specific primers used for full-length *dxs* gene cloning and expression.

Table 2.1 Degenerated primers used in this study.

Primer[a]	T_M (°C)	Oligonucleotide sequence [b] (5' to 3')	Encoded amino acid residues
P126S	56.30	5'-CCICAYAARATHYTIGCIGG-3'	PHKILTG
P194S	59.72	5'-GCIATHGGIGAYGGIGCIATG-3'	AIGDGAM
P273S	55.96	5'-ATHGGIGTIGATGGICA -3'	IGPVDGH
P393A	48.75	5'-GTIACIGCRTTYTGYTCIGCDAT-3'	AEQHAVTF
P434A	53.17	5'-GCRAAIARIACIGGIARYTTYTGIA-3'	QKLPVRFA
P500A	54.78	5'-ATIGCRTTICCICKIGGRTAIC-3'	RYPRGNGI

[a] Name of primers were designated for *C. stellatopilosus*, number indicated the estimate position of amino acid residues and S or A indicated the direction of primers for sense and antisense, respectively. [b]I: Inosine, D: A/G/T, H: A/C/T, R: A/G, Y: C/T

Table 2.2 Primers used for *csdxs* gene sequencing.

Primer	T_M (°C)	Oligonucleotide sequence (5' to 3')
FCS6	66.1	5'-CTTTTTGGTACCATGGCTCTCTCTGCA-3'
5'P_256A	64.4	5'-GCTCCCACAGGTGGTATTGGTCCATCAA-3'
P140S	62.3	5'-GGGATGTTGGCCATCAGTC-3'
P420A	60.4	5'-CGGCGATGGAAGAGATTCAA-3'
3'P_393S	64.1	5'-ACACAGTCGTACACGACATACTTTGCAG-3'
3'P_521S	63.2	5'-GCGTGCCTTCCTAACATGGTGGTG-3'

Table 2.3 Specific primer used in this study.

Primer	T_M (°C)	Oligonucleotide sequence (5' to 3')
For reverse transcription		
RACE32	64.7	5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3'
For 5'- and 3'- ends cloning		
5'P_256A	64.4	5'-GCTCCCACAGGTGGTATTGGTCCATCAA-3'
5'P_280A	63.7	5'-ACCCTTGGCAATCTCTCTTAGTTCTCTT-3'
5'P_298A	63.9	5'-TGCATATTCATCAACCTTTGCAGCCAAC-3'
3'P_346S	63.8	5'-AATCAACTGGGCCAGTCTTGATTCATGT-3'
3'P_393S	64.1	5'-ACACAGTCGTACACGACATACTTTGCAG-3'
3'P_521S	63.2	5'-GCGTGCCTTCCTAACATGGTGGTG-3'
AP1	71	5'-CCATCCTAATACGACTCACTATAGGGC-3'
AP2	77	5'-ACTCACTATAGGGCTCGAGCGGC-3'
RACE17	58.5	5'-GACTCGAGTCGACATCG-3'
RACE32	64.7	5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3'
For full-length and truncated genes cloning		
PDXS-S1	58.1	5'-TTTTTAGCTTGCTTCCGT-3'
PDXSF-S2	64.5	5'-CTTTTT**GGTACC**GCTCTCTCTGCA-3'
PDXST-S3	70.1	5'-GGTGGG**GGTACC**GCATCACTTTCAGAAAGT-3'
PDXS-A1	56.5	5'-AGTTACAGAACTTCCATG-3'
PDXS-A2	64.6	5'-TCAC**GTCGAC**<u>TTA</u>TGCTGACATAATTTGCA-3'

Bold letters indicate the sites for restriction endonuclease (**GGTACC** for *Kpn*I, **GTCGAC** for *Sal*I). Underlined letter shows the position of 3'-stop codon for TAA.

For determination of mRNA expression using semi-quantitative RT-PCR, the primers were designed from *dxs* gene sequence at the region of NH_2-terminus, including the chloroplast transit peptide. For the house-keeping gene (*18S rRNA*), the primers were kindly provided from Mr. Yortyot Seetang-nun, Faculty of Sciences, PSU.

Table 2.4 Primers used for determination of mRNA expression.

Primer	T_M (°C)	Oligonucleotide sequence (5' to 3')	Expected size
FCS6	66.1	5'-CTTTTTGGTACCATGGCTCTCTCTGCA-3'	For *dxs*
5'P_256A	64.4	5'-GCTCCCACAGGTGGTATTGGTCCATCAA-3'	768 bp
18s-0.5F	62.45	5'-CAAAGCAAGCCTACGCTCTG-3'	For *18S rRNA*
18s-0.5R	62.45	5'-CGCTCCACCAACTAAGAACG-3'	530 bp

2.2.8 *Escherichia coli* strains

E. coli strains used in this study were used for gene cloning and gene expression. *E. coli* DH5α and *E. coli* TOP10 were used as hosts for cloning of core sequence, 5'-end, 3'-end, full-length and truncated genes. *E. coli* XL1 Blue MRF' was used as a host for protein expression. The characteristics of those *E. coli* strains are shown in Table 2.5.

Table 2.5 *E.coli* strains used in this study.

Strain	Characteristic	Source
DH5α	F^-, φ80d*lacZ*ΔM15, Δ (*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	TaKaRa, Japan
TOP10	F^-, *mcr*A, Δ(*mrr-hsd* RMS-*mcr*BC), ϕ80*lacZ*, ΔM15, Δ*lac*X74, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*G	Invitrogen, USA
XL1-Blue MRF'	F', :: Tn10, $proAB^+$, $lacI^q$, Δ(*lacZ*)M15, *rec*A1, *end*A1, gyrA96, (Nal^r), *thi*, *hsd*R17(r_k^-, m_k^+), *gln*V44, *rel*A1, *lac*	Stratagene, USA

2.2.9 Plasmid vectors

In this study, pT7Blue (Novagen, Germany) and pDrive (Qiagen, Germany) were used for gene subcloning of PCR fragments. The PCR products which ligated with pT7Blue and pDrive were transformed into either *E. coli* DH5α or *E. coli* TOP10. The pT7Blue contains the pUC19 backbone (including high-copy number origin of replication and *lac* sequences), a T7

promoter, f1 origin of replication, and modified multiple cloning region. The multiple cloning regions contains an *Eco*RV site used for T-cloning flanked by an *Nde*I site, which allows inserts to be conveniently subcloned into the *Nde*I site of many pET vectors**.** This vector allows ampicillin selection, as well as blue/white colony screening. In this study, *Pst*I and *Sal*I were used for restriction analysis for recombinant plasmids of pT7Blue. Size of pT7Blue is 2.88 kb.

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. In this study, *Eco*RI was used for restriction analysis of the recombinant plasmid of pDrive. Size of pDrive is 3.85 kb.

For gene expression, the PCR fragments were ligated to pQE30 (Qiagen, Germany) and transformed into *E. coli* XL1 Blue MRF'. This vector allows ampicillin selection, as well as 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 PT5 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 pQE30 expression vector has a Col E1 origin of replication of DNA. The pQE30 plasmid provides the histidine tag (6 residues of histidine) that makes the protein purification easy by using the Ni^{2+}-chelating affinity column. The pQE30 used for protein expression was treated with restriction endonuclease before ligation. In this study, restriction endonucleases (*Kpn*I and *Sal*I) were used. However, the pQE30 is also supplied in a linear form, namely pQE30 UA that ready-to-use for direct ligation of PCR products.

2.3 Molecular cloning methods

2.3.1 Total RNA extraction

2.3.1.1 Using Phenol-SDS/lithium chloride method

Total RNA was isolated from *C. stellatopilosus* young leaves using phenol-SDS method and lithium chloride (LiCl) precipitation (Sambrook *et al*., 1989). Batch of total RNA extraction began with five 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: $CHCl_3$: isoamyl alcohol; 25:24:1) in the ratio of 10:9:1 was added and ground vigorously until ice was melted.

The homogenate was then transferred into microcentrifuge tubes, centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected (1 ml/tube). The supernatant was deproteinized with 1/5 volume of PCI (0.2 ml/tube), well shaken and centrifuged. The organic layer was discarded and the supernatant was extracted with 1/10 of volume CI ($CHCl_3$: isoamyl alcohol; 24:1) (0.1 ml/tube). For option, the latter step was done twice. To precipitate the nucleic acids, the supernatant was aliquot into 240 µl/tube, 1/10 volume of 3 M sodium acetate (24 µl/tube) and 4 volume of absolute ethanol (*ca*. 1000 µl/tube) were added. The mixture was kept at -80 °C for 1 h and then followed by centrifugation at 13,000 rpm for 10 min at 4 °C.

The supernatant was discarded carefully without disturbing to the pellet. The pellet was then re-suspended in DEPC-treated water in minimum volume. The insoluble matter was removed by centrifugation. To precipitate RNA, the supernatant was transferred to microcentrifuge tubes (250 µl/tube). The total RNA was precipitated with 1 volume of 4 M LiCl (250 µl/tube). The mixture was kept at 4 °C for overnight.

The total RNA pellets were successively centrifuged at 13,000 rpm for 10 min at 4 °C. After remove the supernatant carefully, the pellet was washed with 0.5 volume of chilled 70% ethanol (250 µl/tube). The ethanol fraction was discarded and the pellet was dried shortly at room temperature under vacuum. Finally, the pellet containing total RNA was re-suspended in 100 µl of DEPC-treated water. The total RNA was stored at -20 °C until used.

2.3.1.2 Using RNeasy plant mini kit

For determination of mRNA expression, total RNAs were extracted using RNeasy plant mini kit (Qiagen). According to the manufacturer's instruction, the plant tissue was ground into powder in the presence of liquid N_2. Composition of buffer and reagents are shown in Table 6.2 (Appendix). 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 14,000 rpm for 2 min. 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 14,000 rpm for 15 sec. The flow-through was discarded, 700 µl of buffer RW1 was added onto the RNeasy spin column and centrifuged at 14,000 rpm for 15 sec to wash the spin column membrane. Buffer RPE (500 µl) was added and centrifuged at 14,000 rpm for 15 sec. After drying the membrane by centrifuged at 14,000 rpm for 1 min, the RNeasy spin column was removed and placed on a new microcentrifuge tube. The total RNA was eluted by adding RNase-free water (30 µl), centrifuge at 14,000 rpm for 1 min. The results of total RNA were obtained and used as material for cDNA preparation in semi-quantitative RT-PCR experiments. The total RNA was stored at -20 °C until used.

2.3.1.3 Determination of total RNA concentration and purity

Total RNAs obtained from 2.3.1.1-2.3.1.2 were determined for their concentrations and purities using a spectrophotometer. Aliquot of RNA sample was diluted with DEPC-treated water to the total volume of 200 μl in 96-well plate. The plate was directly measured for A_{260} simultaneously A_{280} using the microplate reader (Bio-Rad).

Total RNA concentration was calculated using the equation of A_{260} multiply with dilution afforded the concentration in μg/μl. The purity of total RNA was determined from the calculation of a ratio of A_{260} and A_{280} (A_{260}/A_{280}). By spectrophotometer, quality of total RNA was judged by the ratio of A_{260}/A_{280}, of which should have a ratio about 1.9-2.1. The pattern of intact RNA was evaluated by agarose gel electrophoresis and ethidium bromide staining.

2.3.2 Synthesis of the first-strand cDNA

2.3.2.1 Using Superscript™ III reverse transcriptase

The first strand cDNA used for core sequence amplification was synthesized using Superscript™ III reverse transcriptase (Invitrogen). According to the manufacturer's instruction, the solution contained total RNA, an oligo dT primer (RACE32) (Table 2.2) and dNTP mix in the volume as shown below. The highest amount of total RNA was used in the mixture. The reaction was incubated at 65 °C for 5 min and quickly chilled on ice for 1 min. The content was centrifuged briefly. Then, 5x first strand buffer, 0.1 M dithiothreitol and Superscript™ III RT were added. The reaction was incubated at 50 °C for 1 h. The enzyme was inactivated by heating at 70 °C for 15 min. The complementary RNA was removed from the resulting cDNA by treatment with 2 units of RNase H and incubated at 37 °C for 20 min. The resulting cDNA was used as template for core sequence amplification.

First-strand cDNA synthesis reaction	
Total RNA	4 μl
Oligo dT (RACE32) (50 μM)	1 μl
dNTP mix (2.5mM)	4 μl
Distilled water	4 μl
5x first strand buffer	4 μl
dithiothreitol (0.1 M)	1 μl
Superscript™ III RT (200 U/μl)	1 μl
RNase out	1 μl

2.3.2.2 Using Marathon™ cDNA amplification kit

The cDNA that used for 5'-and 3'-end amplification was synthesized using Marathon™ cDNA amplification kit (Clontech®, BD Biosciences). Steps of synthesis composed of three steps of first-strand cDNA synthesis, second-strand cDNA synthesis and adaptor ligation. The resulting double strand cDNA is illustrated in Fig. 2.1.

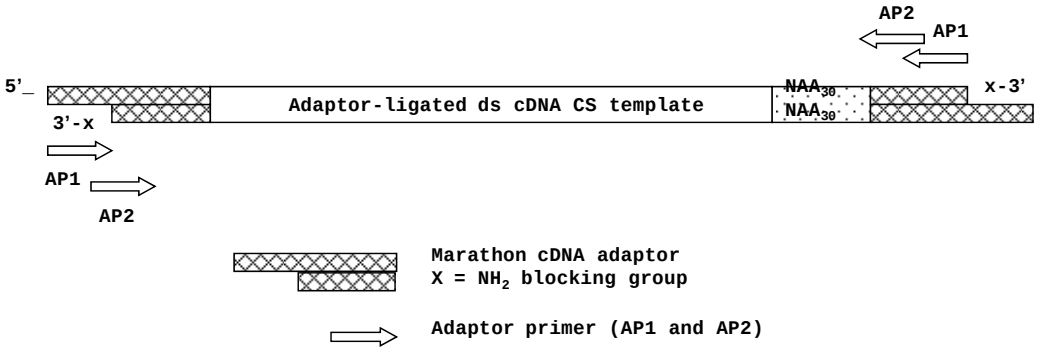


Figure 2.1 Construction of double-strand cDNA obtained from the method of Marathon™ cDNA amplification kit (Clontech®, BD Bioscience).

Firstly, total RNA and cDNA synthesis primer were mixed, incubated at 70 °C for 2 min, cooled on ice for 2 min. The 5x First-strand buffer, dNTP mix and AMV-reverse transcriptase were added and volume was adjusted with distilled water to 10 µl. The contents were mixed gently by pipetting, spinned briefly. The mixture was incubated at 42 °C for 60 min. The first-strand synthesis reaction was terminated by placing the tube on ice. The first-strand synthesis was performed according to protocol and the reaction contains the components as shown below.

First-strand cDNA synthesis reaction	
Total RNA (0.26 µg/µl)	4 µl
cDNA synthesis primer	1 µl
5x first strand buffer	2 µl
dNTP mix (10 mM)	1 µl
AMV-reverse transcriptase (20 units/µl)	1 µl
Distilled water	1 µl

Second-strand synthesis began with addition of 5x second-strand buffer, dNTP mix (10mM) and 20x second-strand enzyme cocktail to first-strand reaction as shown below. Volume was adjusted with distilled water to 80 µl. The reaction was at 16 °C for 90 min. Then, T4 DNA ligase (2 µl, 10 units) was added, mixed, and incubated at 16°C for 45 min. The reaction was terminated by adding 4 µl of EDTA/glycogen. To purify the double-strand cDNA, 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortex vigorously. The mixture was centrifuged at 14,000 rpm for 10 min to separate the phases. The top layer (70 µl) was carefully transferred and placed to the clean-tube. Mixture of chloroform:isoamyl alcohol (24:1) (100 µl) was added to the aqueous layer, vortex thoroughly and centrifuged. The top layer (70 µl) was removed and placed to the clean-tube. Sodium acetate (4 M) (35 µl) was added and mixed. The 99.5% Ethanol (chilled, 263 µl) was added, mixed and centrifuged at 14,000 rpm for

20 min. The supernatant was removed carefully. The pellet was washed with 80% (v/v) ethanol, 300 µl and centrifuged at 14,000 rpm for 10 min. After removing the supernatant, the pellet was dried at room temperature. The pellet was dissolved in 10 µl of distilled water and ready to perform an adaptor ligation.

Second-strand cDNA synthesis reaction	
First-strand reaction	10 µl
5x second-strand buffer	16 µl
dNTP mix (10 mM)	1.6 µl
20x second-strand enzyme cocktail	4 µl
Distilled water	48.4 µl

The second-strand cDNA (5 µl) was mixed with Marathon cDNA adaptor, 5x DNA ligation buffer and T4 DNA ligase as shown below. The reaction was incubated at 16 °C for overnight (16 h). After activated the ligase by heating at 70 °C for 5 min, the adaptor-ligated DNA was kept at -20 °C until use. The aliquot (1 µl) of the adaptor-ligated was diluted with Tris-EDTA buffer (50 µl). The diluted solution was heated at 94 °C for 2 min, cooled tube on ice for 2 min. The content was centrifuged briefly and stored at -20 °C.

Adaptor ligation reaction	
Double-strand cDNA (ds cDNA)	5 µl
Marathon cDNA adaptor (10 µM)	2 µl
5x DNA ligation buffer	2 µl
T4 DNA ligase (1 unit/µl)	1 µl

2.3.3 Polymerase chain reaction for DNA amplification of *csdxs* gene

The DNA fragments of core sequence, 5'-end, 3'-end, full-length corresponding to the *dxs* gene from *C. stellatopilosus* (is now designated as *csdxs*) were amplified using TaKaRa *Ex Taq*^{TM} (Japan). In case of the full-length and truncated *csdxs* gene for expression, the KOD *taq* polymerase (Toyobo) was used. For semi-quantitative RT-PCR, the partial DNA fragments were amplified using TaKaRa *Ex Taq*^{TM} (Japan). The compositions of PCR reactions are shown in Table 2.6.

Table 2.6 The general compositions of PCR reactions.

Component	
Using TaKaRa *Ex Taq*^{TM}	
10x Ex Taq buffer	5 µl
dNTP mix (2.5mM)	4 µl
Sense primer (100 µM)	0.5 µl
Antisense primer (100 µM)	0.5 µl
cDNA template (1:10 dilution)	1 µl
Ex Taq^{TM} polymerase (5 unit/µl)	1 µl
Distilled water	Adjust to 50 µl
Using KOD *taq* polymerase	
10x KOD plus buffer	5 µl
dNTP mix (2 mM)	5 µl
$MgCl_2$ (25mM)	2 µl
Sense primer (100 µM)	1 µl
Antisense primer (100 µM)	1 µl
cDNA template	1 µl
KOD Plus *taq* polymerase (5 unit/µl)	1 µl
Distilled water	Adjust to 50 µl

2.3.3.1 The core sequence of *dxs* gene

The degenerated primers (Table 2.1) were used to amplify the core sequence of *csdxs* gene. The amplifications were performed by two steps PCR. The 1st PCR were investigated with 4-combinations (P126S-P500A, P126S-P434A, P273S-P500A, P273S-P434A). After purified, the PCR products were used for the 2nd PCR, which were performed with 9 combinations based on 1st PCR template and nested region of corresponded template. The scheme of core sequence amplification is shown in Fig. 2.2. The thermal profile is shown in Table 2.7.

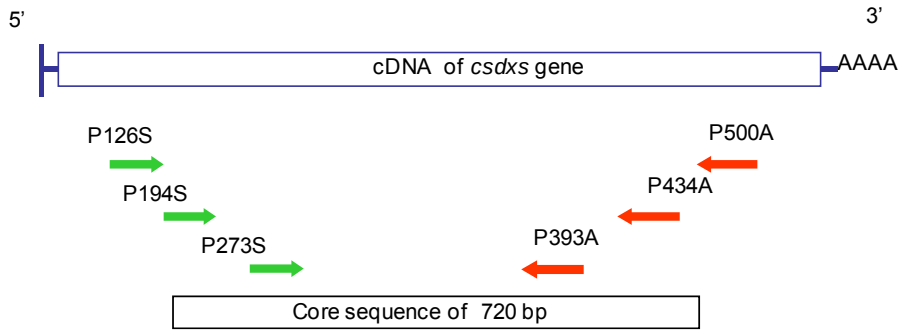


Figure 2.2 Scheme of amplification of *csdxs* core sequence using the degenerated primers and cDNA obtained from Superscript™ III RT (2.3.2.1).

Table 2.7 Thermal profile for amplification of core sequence of *csdxs*.

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	95	5	1
2	Denaturing Annealing Extension	95 42 72	1 2 3	30
3	Extension Holding	72 4	10 ∞	1

2.3.3.2 Amplification of 5'- and 3'- ends of *csdxs* gene

The 5'- and 3'-ends of *csdxs* gene was obtained by RACE method. The specific primers (Table 2.3) were designed according to core sequence (720 bp). The double strand with adaptors was prepared as described in 2.3.2.2 and used as templates for 5'- and 3'-ends. In this case, in 50 µl PCR reaction contained 5 µl 10x cDNA PCR reaction buffer, 1 µl dNTP mix (10mM), 1 µl Advantage 2 polymerase mix (50x), 5 µl adaptor-ligated cDNA (1:50), 1 µl AP1 primer and 1 µl sense or antisense primer. For 5'-end amplification, the PCR reactions were performed with 3 combinations (5'_298A-AP1, 5'_280A-AP1, 5'_256A-AP1). For 3'-end amplifications, the PCR reactions were performed using the combination 3 combinations (3'_346S-AP1, 3'_393S-AP1, 3'_521S-AP1). Schemes of 5'- and 3'- ends amplification are shown in Fig. 2.3. The thermal profile is shown in Table 2.8.

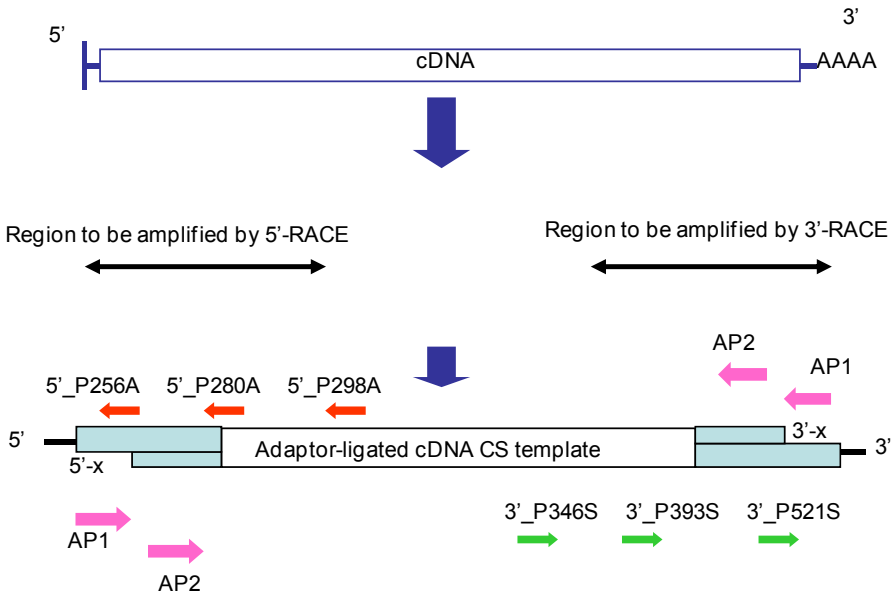


Figure 2.3 Scheme of amplification of 5'-and 3'-ends of *csdxs* using the cDNA obtained from Marathon™ cDNA amplification kit (Clontech®, BD Biosciences).

Table 2.8 Thermal profile for amplification of 5'-and 3-ends of *csdxs*.

Segment	Step	Temperature (°C)	Time	Number of cycles
1	Denaturing	94	30 sec	1
2	Denaturing Annealing	94 68	5 sec 3 min	30
3	Holding	4	∞	1

2.3.3.3 Amplification of full-length and truncated *csdxs* gene

Information of the partial DNA sequences of 5'-end, core sequence and 3'-end allowed us to design the primers of full-length of *csdxs* gene. Analysis of the open reading frame (ORF) revealed position of ATG-start codon and TAA-stop codon. Since *csdxs* gene carries the chloroplast transit peptide (57 amino acid residues from NH_2-terminus), thus, truncated gene encoding the mature protein was also investigated. Primers used for amplification of full-length and truncated *csdxs* genes are shown in Table 2.3. The PCR fragments of full-length and truncated *csdxs* gene were amplified using proofreading *Taq* polymerase (Toyobo). Two steps PCR reactions were performed. For 1st PCR, primers of PDXS-S1 and PDXS-A1 were amplified and the PCR product was used as templates for nested PCR. After purify PCR fragments, 2nd PCRs were performed with PDXSF-S2 and PDXS-A2 for full-length *csdxs* gene and PDXST-S3 and PDXS-A2 for truncated *csdxs* gene. Expected sizes of full-length and truncated *csdxs* genes were 2160 bp and 1989 bp, respectively. Scheme of amplification for full-length and truncated *csdxs* genes is shown in Fig. 2.4. The thermal profile is shown in Table 2.9.

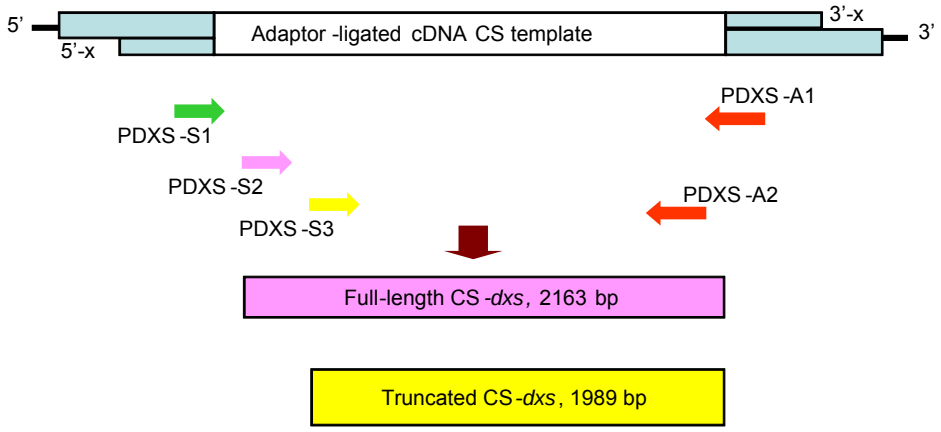


Figure 2.4 Scheme of amplification of full-length and truncated *csdxs* genes.

Table 2.9 Thermal profile for amplification of full-length and truncated *csdxs* genes.

Segment	Step	Temperature (°C)	Time (min)	Number of cycles
1	Denaturing	94	2	1
2	Denaturing	94	15 sec	30
	Annealing	52 (1st)	30 sec	
	Annealing	58 (2nd)	30 sec	
	Extension	68	2	
3	Extension	68	10	1
	Holding	4	∞	

2.3.3.4 Semi-quantitative RT-PCR of *dxs* gene

The level of *csdxs* mRNA expression was determined from different tissues including leaf, twig and root using semi-quantitative RT-PCR technique. The partial DNA sequence located at the NH_2-terminus was selected to monitor the transcription level of *csdxs* gene due to its distinct region for *dxs* gene in *C. stellatopilosus*. The partial *csdxs* gene was

amplified with primers of FCS6 and 5'_256A. For control gene, the house-keeping gene (*18S rRNA*) was also amplified in parallel reactions on each RNA sample. The scheme of amplifications and the thermal profile are performed as shown below.

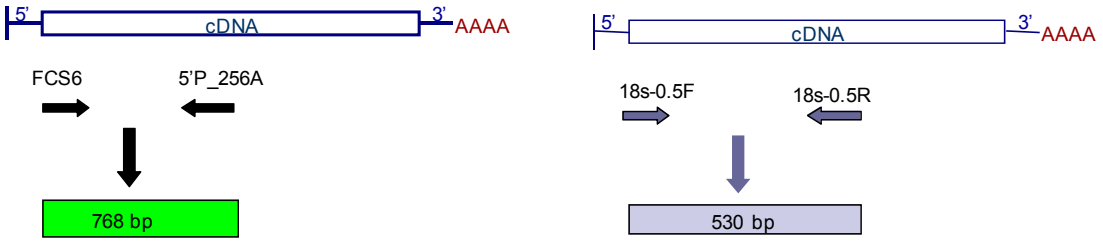


Figure 2.5 Scheme of amplification of partial *csdxs* gene and house-keeping gene (*18S rRNA*).

Table 2.10 Thermal profile for amplification of partial *csdxs* gene and house-keeping gene (*18S rRNA*).

Segment	Step	Temperature (°C)	For *csdxs* gene		For *18S rRNA*	
			Time (min)	Number of cycles	Time (min)	Number of cycles
1	Denaturing	94	3	1	3	1
2	Denaturing Annealing Extension	94 58 72	1 2 3	30	1 2 3	35
3	Extension Holding	72 4	5 ∞	1	10 ∞	1

2.3.4 DNA cloning

2.3.4.1 Purification of DNA fragments

DNA fragments obtained from the PCR reactions were purified using PCR purification kit (Qiagen) and using gel extraction kit (Qiagen). PCR purification kit is used

to purify the PCR product that is used as template for nested PCR. Whereas, gel extraction kit is used to purify the PCR product that is separated on agarose gel and is used for further gene cloning.

DNA fragments purification using PCR purification began with dilution of the PCR reaction with 3 volumes of buffer PB. The mixture was loaded on the mini column, allowed to stand at room temperature for 1 min and centrifuged at 14,000 rpm for 1 min. The column was washed with buffer PE (0.75 ml) and centrifuged. The flow through was discarded. After drying the column by centrifugation at 14,000 rpm for 1 min, DNA fragment was harvested by elution with 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5), left at room temperature for 1 min and centrifuged.

For DNA fragment purification on agarose gel, the expected DNA fragment was excised with a clean razor from the agarose gel and transferred into a microcentrifuge tube. Three volumes of buffer QG (TBE: Tris-borate/EDTA) were added to 1 volume of the gel (100 mg of gel ~ 100 µl), then the mixture was incubated at 50 °C until the gel slice had completely dissolved. Then, the mixture was applied to the mini column, allowed to stand at room temperature for 1 min and centrifuged at 13,000 rpm for 1 min. The flow through was discarded. The column was washed by adding 0.75 ml of buffer PE to the column, left at room temperature for 1 min, and then centrifuged at 13,000 rpm for 1 min. The flow through solution was discarded and the column was centrifuged for 1 additional min at 13,000 rpm to dry the matrix. The column was then placed in a new microcentrifuge tube, 50 µl of buffer EB (10 mM Tris-HCl, pH 8.5) was added into this column and left to stand at room temperature for 1 min and centrifuged for 1 min at maximum speed to elute DNA.

2.3.4.2 Ligation the DNA fragment to vector

2.3.4.2.1 Ligation with pDrive and pT7Blue vectors

Since the pDrive and pT7 blue vectors are supplied in a linear form and the PCR product carries the A-overhang at 3'-end. In addition, using *Ex Taq*™ polymerase allows the sticky end of DNA fragement. Therefore, DNA fragment and vector can be directly performed the ligation. The ligation mixture contained the molar ratio of 5-10 times of the DNA fragment than the vector as followed. The ligation mixture was incubated at 16°C for 2 h and ready for transformation.

Ligation mixture: with pDrive vector or pT7 Blue	
DNA fragment	4 μl
pDrive or pT7 Blue vector	1 μl
2x ligation master mix	5 μl

2.3.4.2.2 Ligation with pQE30 vector

The DNA fragment and pQE30 vector need to treat with the restriction endonucleases (before performing ligation. In this study, the restriction sites were designed for *Kpn*I and *Sal*I for NH_2-terminus and COOH-terminus, respectively. After purification of DNA fragment (2.3.4.1) by gel extraction kit, the reaction contained 10 μl of 10x H-buffer, 47 μl of DNA, 5 μl of *Kpn*I (10 units/μl) and 5 μl of *Sal*I (15 units/μl). The mixture was incubated at 37 °C for 2 h. The treated DNA fragment was purified using agarose gel and gel extraction kit.

For restriction endonucleases treatment of pQE30 vector, the pQE30 vector was prepared from *E. coli* XL1blue MRF' harbouring pQE30. The *E. coli* cells were cultured for overnight and plasmids were purified using Wizard® PCR midi preps DNA purification kit. The resulting plasmid was concentrated using ethanol precipitation, and used in

the reaction as mentioned above. The mixture was incubated at 37 °C for 2 h. The resulting treated pQE30 fragment was purified using agarose gel and gel extraction kit. The ligation mixture contained the solutions as shown below. The ligation mixture was incubated at 16 °C for 2 h and ready for transformation.

Ligation mixture: with pQE30 vector	
DNA fragment (E/N)	4 µl
pQE30 (E/N)	1 µl
T4 DNA ligase (5 units/µl)	0.5 µl
10x ligase buffer	1 µl
Distilled water	Adjust to 10 µl

2.3.4.3 Transformation the plasmid DNA to *E. coli* host

2.3.4.3.1 Preparation of the *E. coli* competent cells

The *E. coli* strains (Table 2.5) were prepared for using as hosts (competent cells) (Sambrook *et al*. 1989). *E. coli* strain kept at -80 °C was activated by streak on LB agar containing ampicillin (2.2.6). The plate was incubated at 37 °C for overnight. The *E. coli* cells were culture in liquid medium (5 ml) at 37 °C for overnight from a single colony. The culture was then cultured in 50 ml SOB medium, incubated at 25 °C until the OD_{600} reached 0.4-0.6. The suspension was kept on ice for 10 min, cells were harvested by centrifugation at 5,000 xg for 10 min at 4 °C. The cell pellet was 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 re-suspended in 2 ml of ice-cold TB and DMSO (final concentration of 7%). The portions (50 µl) of resulting competent cells were kept frozen at -80 °C until used.

2.3.4.3.2 Transformation the plasmid DNA to *E. coli* cells

Plasmid DNA was transformed into an appropriate *E. coli* host cells. Plasmid DNA for subcloning of *csdxs* gene was transformed into either *E. coli* DH5α or *E. coli* TOP10, whereas the recombinant DNA was transformed into *E. coli* XL1 blue MRF' for protein expression (2.2.8). The transformation protocol was chemically transformed according to method of Sambrook *et al*. (1989). The plasmid DNA (1 μl) or ligation mixture (5 μl) was mixed gently, placed on ice for 30 min. The cells were heat-shocked at 42 °C for 30 sec and placed immediately on ice. SOC medium (250 μl) was added in the mixture and the solution was constantly shaken at 300 rpm at 37 °C for 1 h. Cells suspension was spread on transformation agar (LB medium containing ampicillin, X-gal and IPTG). The transformed agar plate was incubated at 37 °C for 16 h. The transformant was selected by ampicillin and screened by blue/white colony.

2.3.5 Isolation and identification of the recombinant DNA

2.3.5.1 Isolation of the recombinant DNA

A single white colony of *E. coli* cells grown on transformation agar was picked. In case of recombinant DNA derived from pDrive and pT7 Blue, the transformant is detected from blue/white screening. Cells were cultured at 37°C for 16 h in LB-medium (3 ml) containing ampicillin with vigorous shaking 200 rpm. A plasmid DNA was isolated from 1.5 ml of overnight *E.coli* cells culture using GFX Micro Plasmid Prep Kit. According to the manufacture's protocol, cell culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 30 sec to pellet the cells. The pellet was re-suspended in 150 μl of solution I (100 mM Tris-HCl; pH 7.5, 10 mM EDTA, 400 μg/ml RNase I) with vigorous vortex and then 150 μl of solution II (1 M NaOH, 5.3% (w/v) SDS, 65 ml of distilled water) was added and mixed by inverting the tube 10-15 times. The protein was precipitated by adding 300 μl of

solution III (acetate and chaotropethen), mixed by inverting the tube until a flocculent precipitate appeared. The mixture was centrifuged at 14,000 rpm 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 14,000 rpm for 1 min. The column was washed by adding 400 µl of washing buffer and centrifuged at 14,000 rpm for 1 min. The matrix was dried prior to elution. Finally, the mini column was transferred to a fresh microcentrifuge tube and 100 µl of TE (Tris-EDTA buffer, 100 ml absolute ethanol) 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 14,000 rpm for 1 min. The resulting DNA was obtained and stored at -20 °C until used.

2.3.5.2 Identification of the recombinant DNA

For pDrive and pT7Blue containing inserts, the restriction sites analyses were performed in the presence of *Eco*RI and *Eco*RI / *Pst*I, respectively. For pQE30 containing insert, in this study, restriction enzymes of *Kpn*I and *Sal*I were used to identify the recombinant DNA. The reaction mixture contained components as shown below, and incubated at 37°C for 2 h. To analyze the DNA fragments, the solution then mixed with sample buffer, loaded on 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide solution.

Restriction reaction	For pDrive	For pT7Blue
Plasmid DNA	5 µl	5 µl
10x Buffer	1 µl (H buffer)*	1 µl (T buffer)*
*Eco*RI (20 units/µl)	0.5 µl	0.5 µl
*Pst*I (20 units/µl)	-	0.5 µl
Distilled water	Adjust to 10 µl	Adjust to 10 µl

Restriction reaction: pQE30 vector	
Plasmid DNA	5 µl
*Kpn*I (10 units/µl)	0.5 µl
*Sal*I (15 units/µl)	0.5 µl
10x H buffer *	1 µl
Distilled water	Adjust to 10 µl

*obtained from supplier (TaKaRa, Japan)

10x H buffer 500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM dithithreitol, 1000 mM NaCl; 10x T buffer (BSA free) 330 mM Tris-HCl (pH 7.9), 100 mM Mg-acetate, 5 mM dithithreitol, 660 mM K-acetate.

2.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis technique was used to analyze the DNA fragments from PCR and restriction reactions. Agarose gel was prepared in the concentration of 1.2% (w/v). To prepare 20 ml agarose gel/mini plate, the mixture contained 0.24 g agarose, 0.4 ml TAE (50x) and volume was adjusted with distilled water to 20 ml. The mixture was boiled using microwave oven until obtained the cleared solution. The solution was poured into the tray and comb was placed. The agarose gel was set at room temperature for 1 h. The tray was carefully removed and placed on the platform in the electrophoresis tank containing 1x TAE buffer. 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 DNA pattern was observed under UV transluminator (312 nm) and the picture was developed using Gel documentation.

2.3.7 DNA sequencing and sequencing analysis

The nucleotide sequences were analyzed either at Bioservice Unit (BSU, Ratchathewi, Bangkok) or Scientific Equipment Center (SEC, PSU, Songkhla). The DNA fragments were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied

Biosystems, USA). Amplifications were performed using the M13-forward and M13-reverse primers for subcloning gene and specific primers for *csdxs* gene direct sequencing. After amplification, the samples were separated in an ABI PRISM®, Applied Biosystems 3700 DNA analyzer, equipped with computer workstation Model 3100, Version 3.7 (ABI PRISM, Applied Biosystems 3730 DNA analyzer).

Comparative analyses of nucleotide sequences and deduced amino acid sequences were performed using Blast programs (http://www.ncbi.nlm.nih.gov/BLAST/) and DNASIS v3.5 software (Hitachi software engineering). Multiple alignments were conducted through Clustal W (2.0), ClustalW XXL (http://www.ebi.ac.uk/clustalw/) using default parameters and BioEdit v7.0.5. A phylogenetic tree was constructed using TreeView version 1.6.6. TargetP V1.0 program and ChloroP 1.1 (http://www.cbs.dtu.dk/services/TargetP/; http://www.cbs.dtu.dk/services/Chloro/) was used to predict for the possible chloroplast transit leader sequence (Nielsen *et al*., 1997; Nielsen *et al*., 1999).

2.3.8 Gel documentation

Gel documentation is used to photograph an image of DNA pattern on agarose gel. The image is acquired from gel documentation systems and saved in a digitized form. The image can be adjusted or optimized for performing volume analysis. Data obtained by volume analysis used for molecular weight or concentration determination. The intensity of the various signals is directly proportional to the intensity of the gray levels displayed on the computer monitor.

To determine the mRNA expression of *csdxs* gene from tissues of *C. stellatopilosus*, the PCR products (2.3.3.4) were separated by 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. Band intensities was measured by a Gel Doc Model 1000 (Bio-Rad, USA) equipped with Molecular Analyst® software (Window Software for

Bio-Rad's Image Analysis Systems, Version 1.4). Expressions of transcripts were calculated as the ratio of bands of intensities of genes and *18S rRNA* house-keeping gene.

2.4 Proteinchemical methods

2.4.1 Expression of CSDXS proteins from recombinant *E. coli* strains

The *csdxs* genes were cloned for full-length and truncated genes and overexpressed in the high copy vectors pQE30 (Qiagen) in *E.coli* XL1 blue MRF' host strains. For expression of *csdxs* proteins from recombinant *E. coli* strains, the recombinants *E. coli* XL1-blue MRF' harbouring either pQE30-FCSDXS (designated for full-length DXS protein) or TCSDXS (designated for truncated DXS protein) were cultured on LB-medium supplemented ampicillin. Cells were grown at 37 °C for 16 h with shaking at 200 rpm. The overnight culture was inoculated to the same medium at a ratio of 1:50, culture at the same condition. At on OD_{600} of 0.6-0.7, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM. The cells were further grown at 25 °C for 8 h with shaking at 200 rpm. The cells were harvested by centrifugation at 3,500 rpm for 10 min. The pellets were washed with buffer (100 mM Tris-HCl, pH 8.0) and stored at -20 °C until used.

For optimal condition for protein expression, factors including IPTG concentrations (0.5-4 mM), induction temperatures (22 °C, 30 °C, 37 °C) and induction periods (4 h, 8 h) were investigated. Each factor was varied when other standard conditions were constant.

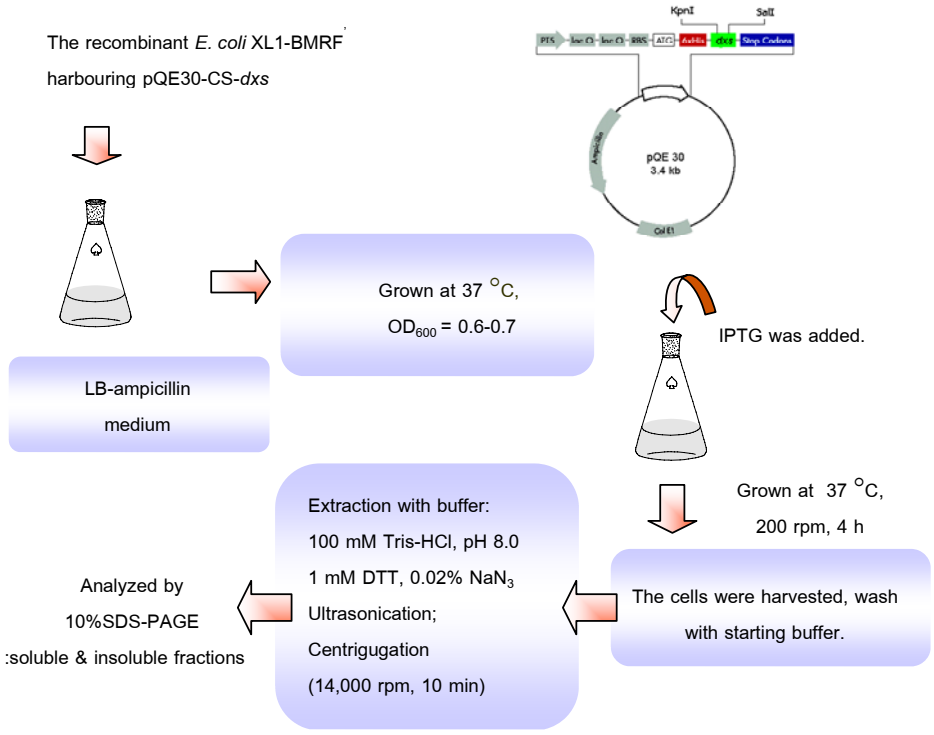


Figure 2.6 General protocol of the FCSDXS and TCSDXS proteins expressions.

2.4.2 Purification of CSDXS proteins

2.4.2.1 Extraction of recombinant *E. coli* XL1 blue MRF' pQE30-CSDXS cells

The recombinant *E. coli* cells obtained from this study are *E. coli* XL1 blue MRF' harboring plasmid pQE30-FCSDXS for full-length protein plasmid pQE30-TCSDXS for truncated protein. For protein purification, the recombinant *E. coli* cells (8 g) were suspended in 40 ml of buffer A (20 mM imidazole, 0.5 M NaCl, 100 mM Tris-HCl, pH 8.0). Lysozyme and DNaseI were added to the suspension to a final concentration of 1 mg/ml and 0.1 mg/ml, respectively. The mixture was subsequently incubated at 37 °C for 30 min. The suspension was cooled in an ice bath for 20 min. Ultrasonication was then performed (40% duty cycle, pulse on/off 9.9, 4 × 10 sec, 10 sec pause) during cooling on ice. Cell debris was centrifuged at 9,000 rpm for 30 min. The supernatant was used as cell extract to purify by column chromatography.

2.4.2.2 HiTrap™ chelating affinity column chromatography

Protein purification was performed using the FPLC system (Pharmacia). HiTrap™ chelating HP column (size 5 ml, GE Bioscience) was used to purify the FCSDXS and TCSDXS proteins. It contains His-bind resin, charged with Ni^{2+} ion. The column was equilibrated with 50 ml of buffer A (20 mM imidazole, 0.5 M NaCl, 100 mM Tris-HCl, pH 8.0). The protein in buffer A (2.4.2.1) was loaded on to the column at flow rate of 3 ml/min. The column was washed with about 50 ml buffer A (monitor at OD_{280} to baseline). Linear gradient was performed from buffer A to buffer B (250 mM imidazole, 0.5 M NaCl, 100 mM Tris-HCl, pH 8.0) in 50 ml at flow rate of 3 ml/min. The protein was eluted from the column at *ca*. 100-150 mM imidazole. The protein fraction was pooled and concentrated to volume of 1 ml using membrane (Amicon® Ultra-15, MWCO 10 kDa, size 15 ml), centrifuged at 3,500 rpm for 30 min. The retentate was collected and further purified by the desalting column.

2.4.2.3 HiTrap™ desalting column chromatography

The desalting column was equilibrated with 5 column volumes of buffer C (100 mM Tris-HCl, pH 8.0, 0.02% NaN_3) at the flow rate of 2 ml/min. The protein obtained from the concentrator (2.4.2.2, 1 ml) was loaded on the HiTrap™ desalting column (5 ml). The column was eluted with buffer C at the flow rate of 1 ml/min. The protein fraction was collected after void volume (3 ml). The protein fraction was collected and concentrated using the concentrator (as described above). The purified FCSDXS and TCSDXS were then analyzed for purity on SDS-PAGE and DXS activity.

2.4.3 Determination of DXS activity

Based on previously report of DXS activity from periwinkle (Chahed *et al*., 2000), it was mentioned that the DXS activity can be measured from mature or truncated DXS protein, which the chloroplast transit peptide has been removed. Therefore, in this study, only the TCSDXS protein was measured for the enzyme activity by mean of thin layer chromatographic method. The TCSDXS protein was measured for DXS activity using the couple assay with 2*C*-methyl-D-erythritol synthase (MEPS) according to the methods of Sprenger *et al*. (1997) and Wungsintaweekul (2001) with modification.

Assay mixture contained 100 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM sodium pyruvate, 10 mM DL-glyceraldehyde 3-phosphate, 2 mM dithiothreitol, 2 mM thiamine diphosphate and 50 μg TCSDXS protein in a total volume of 50 μl. For confirmation of the reaction product, 1-deoxy-D-xylulose 5-phosphate, additional assay mixture was performed as above and 50 μg of MEPS (from *E. coli*), 1 mM NADPH and 1 mM $MnCl_2$ were added. The assay mixtures were incubated at 37 °C for 1 h. To remove the phosphate moiety, 10 units of alkaline phosphatase was added. The reactions were further incubated at 37 °C for 30 min. Aliquots (50 μl) were spotted on Silica gel 60 F_{254} plates. After drying the TLC plate, it was

developed in solvent system of isopropanol: ethyl acetate: H_2O (6:2:1). To detect the sugar moiety, the TLC plate was detected by spraying with anisaldehyde/H_2SO_4 reagent. The *Rf* values of the dephosphorylated products of 1-deoxy-D-xylulose and 2*C*-methyl-D-erythritol are 0.56 and 0.44, respectively (Wungsintaweekul, 2001).

2.4.4 Determination of total protein

The amount of protein was determined by the dye-binding method (Bradford assay) (Bradford, 1976). The solution of Bradford reagent was prepared (2.2.5). The protein sample or standard protein solution (50 µl) and Bradford reagent (950 µl) were mixed in 1 ml cuvette. The mixture was measured at 595 nm. For a reference, 50 µl of which buffer was mixed with 950 µl of Bradford reagent. In this study, bovine serum albumin (BSA) was used as standard protein in a range of 0.01-0.1 mg/ml. The amount of protein was estimated from a standard curve.

2.4.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The determination of the purity of protein fractions was measured using discontinuous SDS-PAGE according to the system of Laemmli (1970). SDS is an anionic detergent which solubilizes and denatures proteins. SDS confers net negative charges to proteins. The proteins have then a mobility which inversely proportional to their size or molecular mass.

The gel was prepared using the SDS-PAGE discontinuous buffer system with vertical slab gels as shown below. The components of the separating gel solution were mixed together and then loaded into the slab deposited between two glass plates on the gel caster. The top of the gel was overlayed with isopropanol. The polymerization of acrylamide was completed after 1 h. After removing of the isopropanol, the stacking gel solution was prepared and loaded on the top of separating gel in the presence of 10-well comb. The stacking gel completed its polymerization after 20 min. The slab gel was placed on the SE 250 Mighty Small II electrophoresis system (Hoefer Scientific) which was connected to a cooling system.

Protein samples were mixed with SDS-sample buffer at a ratio of 1:1. The mixture was heated at 95 °C for 5 min. Then the protein samples were loaded into wells. Broad-range molecular weight proteins markers (6-175 kDa, New England Biolab) were used. The electrophoresis system was powered from a power supply with 20 mA per gel. After running for 1 h, the gel was removed carefully and stained in staining solution for 30 min. The gel was destained with destaining solution for 1 h.

For 2 gels preparation

Stock solution	3.6% acrylamide in stacking gel	10% acrylamide in separating gel
Acrylamide (40%)	0.45 ml	2.5 ml
Bis-acrylamide (2%)	0.225 ml	1.34 ml
2x Stacking gel buffer	2.5 ml	-
4x Separating gel buffer	-	2.5 ml
Water	1.825 ml	3.66 ml
10% w/w APS	30 µl	50 µl
TEMED	5 µl	5 µl
Total volume	10 ml	10 ml

Separating gel buffer: 0.375 M Tris-HCl, pH 8.8, 0.1% SDS

Stacking gel buffer: 0.125 M Tris-HCl, pH 6.8, 0.1% SDS

Running buffer: 0.192 M Glycine, 0.025 M Tris-HCl, pH 8.3, 0.1% SDS

10% (w/w) Ammonuim persulfate (APS): 1 mg in 10 ml of water (freshly prepared)

2.4.6 MALDI-TOF Mass spectrometry

The protein was subjected to Bruker Reflex IV matrix assisted laser desorption ionization (MALDI) mass spectrometer. MALDI-TOF Mass spectrometry was used to determine the molecular weight and estimate the degradation of purified protein. The FCSDXS and TCSDXS protein were sent to Proteomic Service Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Pathumthani.

2.5 Quantitative analysis of plaunotol

2.5.1 Preparation of *n*-hexane extracts

Six-month old of *C. stellatopilosus* was used for plaunotol content determination in this study. Mature leaves, twigs and roots were separately collected, dried at 50 °C for overnight in a hot air oven and ground into powder. Samples including leaves, twigs and roots were separately extracted as described previously (Vongchareonsathit, 1994). Dried samples (2 g) were extracted with 10 ml of absolute ethanol under reflux for 1 h, and filtered. The filtrate was evaporated (Speedvac Plus SC210A, Savant, Holbrook, NY, USA) and the residue was re-dissolved in 50% (v/v) methanol. A solution of 10% (w/v) NaOH was added to give a final content of 1% (v/v) and the mixture was heated at 70 °C for 30 min. The solution was then partitioned with *n*-hexane three times. Pooled *n*-hexane fractions were dried under reduced pressure.

2.5.2 Quantitative analysis of plaunotol using gas chromatography

2.5.2.1 Construction of the plaunotol calibration curve

The calibration curve was constructed using the authentic plaunotol (kindly provided from Thai Sankyo Co. Ltd). 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 mg/ml. The solutions were analyzed using gas chromatography. The areas under curves of each concentration were plotted against to the concentration of standard plaunotol.

2.5.2.2 Gas chromatographic method

For gas chromatography analysis, the extract was dissolved in 200 µl of *n*-hexane. The clear solution was subjected to a GC Agilent 6850 instrument equipped with an FID detector. The GC conditions were HP1 methylsiloxane column (GC-Hewlett Packard, CA, USA), 30 m x 0.32 mm i.d., 0.25 µm film thickness, flow rate 1.0 ml/min helium; splitless injection; temperature program from 235 °C (hold 1 min) to 280 °C (rate 15 °C/min) and hold of 2.5 min; injector temperature 220 °C; detector temperature 280 °C and sample size 1.0 µl. Plaunotol was eluted at 5.2 min. The peak areas were converted to concentration by using a plaunotol standard calibration curve (linearity range 0.01-0.5 µg/µl with R^2 of 0.999). Samples were carried out in four replicates.

CHAPTER 3

RESULTS

The 1-deoxy-D-xylulose 5-phosphate synthase gene from *C. stellatopilosus* (designated as *csdxs*) was cloned from young leaves using the rapid amplification cDNA ends (RACE) technique. As described in materials and methods (2.2.7), the degenerated primers were designed from the conserved regions of well known *dxs* genes from others plants and used for cloning of the core sequence. The nucleotide sequence of the core sequence allowed us to design the specific primers for 5'- and 3'-end cloning. Together with technology of cDNA amplification kit, the *C. stellatopilosus* cDNA was prepared and ligated with adaptors, which specific to the AP1 primers (2.3.2.2). Thus the 5'- and 3'-ends DNA fragments were ease to amplify by PCR. The nucleotide sequences of the DNA fragments, including core sequence, 5'- and 3'- ends were assembled. The putative nucleotide sequence of the full-length *csdxs* was analyzed for their functional characteristics. To determine the physiological role of *csdxs* gene in plaunotol biosynthesis, the mRNA expressions levels from different tissues of *C. stellatopilosus* were determined using the semi-quantitative RT-PCR technique (2.2.3.4). The *csdxs* genes were then cloned and expressed. According to previously report, the DXS proteins in higher plants function maturely in plastid. They need the chloroplast transit peptide to enter the chloroplast envelope (Scheiff and Klösgen, 2001). In this study, the full-length (premature) CSDXS and the truncated (mature) proteins were investigated by preparing the recombinant plasmids of those genes to the expression vectors (2.2.9). The recombinant plasmids were heterologous expressed in *E. coli* XL1blue MRF'. By using of the pQE30 plasmid, the fusion proteins were obtained and made the purification possible in a single step of metal-chelating column. The purified proteins were purified and characterized.

3.1 cDNA cloning of *csdxs* gene of *Croton stellatopilosus*

3.1.1 cDNA preparation from total RNA

Total RNA was isolated from *C. stellatopilosus* young leaves (2.3.1.1). Using phenol-SDS method and LiCl precipitation technique (Sambrook *et al*., 1989), mRNA and rRNA but not tRNA and DNA were precipitated from the DNA/RNA solution. Quantification and purity of resulting RNA were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), respectively.

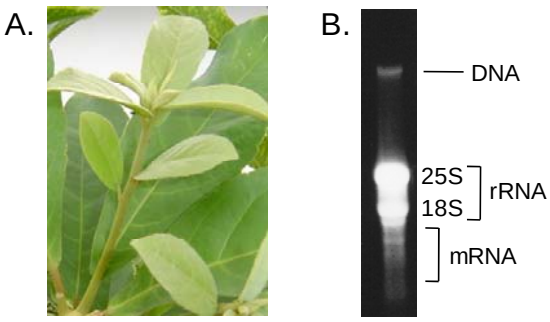


Figure 3.1 Preparation of total RNA by phenol/SDS method and LiCl precipitation from 2-year old of *Croton stellatopilosus*. A. young leaves of 2-year old plant; B. Analysis of intact RNAs on 1.2% (w/v) agarose gel electrophoresis stained with ethidium bromide solution and visualized under UV transluminator (312 nm).

Table 3.1 Determination of amount of total RNA and its purity.

Sample	A_{260}	A_{280}	Concentration of total RNA sample (μg/ml)*	Total volume (ml)	Total amount (μg)	Purity [A_{260}/ A_{280}]
C. stellatopilosus leaves (5 g)	0.0671	0.0351	268.4	0.088	23.6	1.91

*Concentration of RNA sample = 40 μg/ml x A_{260} x dilution factor

From 5 g of young leaves, the total RNA was diluted with DEPC-treated water to 1:100 dilution. The solution was measured for A_{260} and A_{280} using a spectrophotometer. As shown in Table 3.1, the concentration of the resulting RNA was 268.4 μg/ml in total volume of 88 μl, afforded 23.6 μg of total RNA. The ratio of A_{260} and A_{280} was determined for estimating the RNA purity. The isolated RNA had a ratio of 1.91 (Table 3.1), in which pure RNA should have an A_{260}/ A_{280} ratio of 1.9-2.1. The integrity and size distribution of total RNA can be checked by agarose gel electrophoresis and ethidium bromide staining. As shown in Fig.3.1, the intact RNAs contain 2-subunits of rRNA and small fragment of mRNA with minor contamination of DNA. This total RNA was used for preparation of cDNA by means of Superscript™ III reverse transcriptase and 5'- and 3'-end amplification kit.

For the first strand cDNA synthesis using Superscript™ III reverse transcriptase (2.3.2.1), the total RNA (1 μg) was used as starting material. According to method 2.3.2.1, the cDNA was treated with 1 unit of RNase H (*E. coli*) to remove RNA complementary. The resulting cDNA was used as template for core sequence amplification.

For cDNA preparation using Marathon™ cDNA amplification kit (2.3.2.2), the total RNA (1 μg) was used to prepare the first-strand cDNA synthesis. After performing the second-strand cDNA synthesis and DNA purification, the double strand cDNA was ligated to adaptor (Marathon™ cDNA adaptor) with T4 ligase. The resulting double strand cDNA with adaptor was used as template for 5'- and 3'-ends amplification.

3.1.2 The core sequence of *csdxs* gene

Amplification of the core sequence was performed as described in 2.3.3 using the *Ex Taq*™ polymerase. The annealing temperature was 42 °C. With 4 combinations of 1st PCR and 9 combinations of 2nd PCR afforded the resulting PCR products. Five of them were successful. The PCR products of 597 bp, 360 bp, 360 bp, 483 bp and 360 bp were obtained.

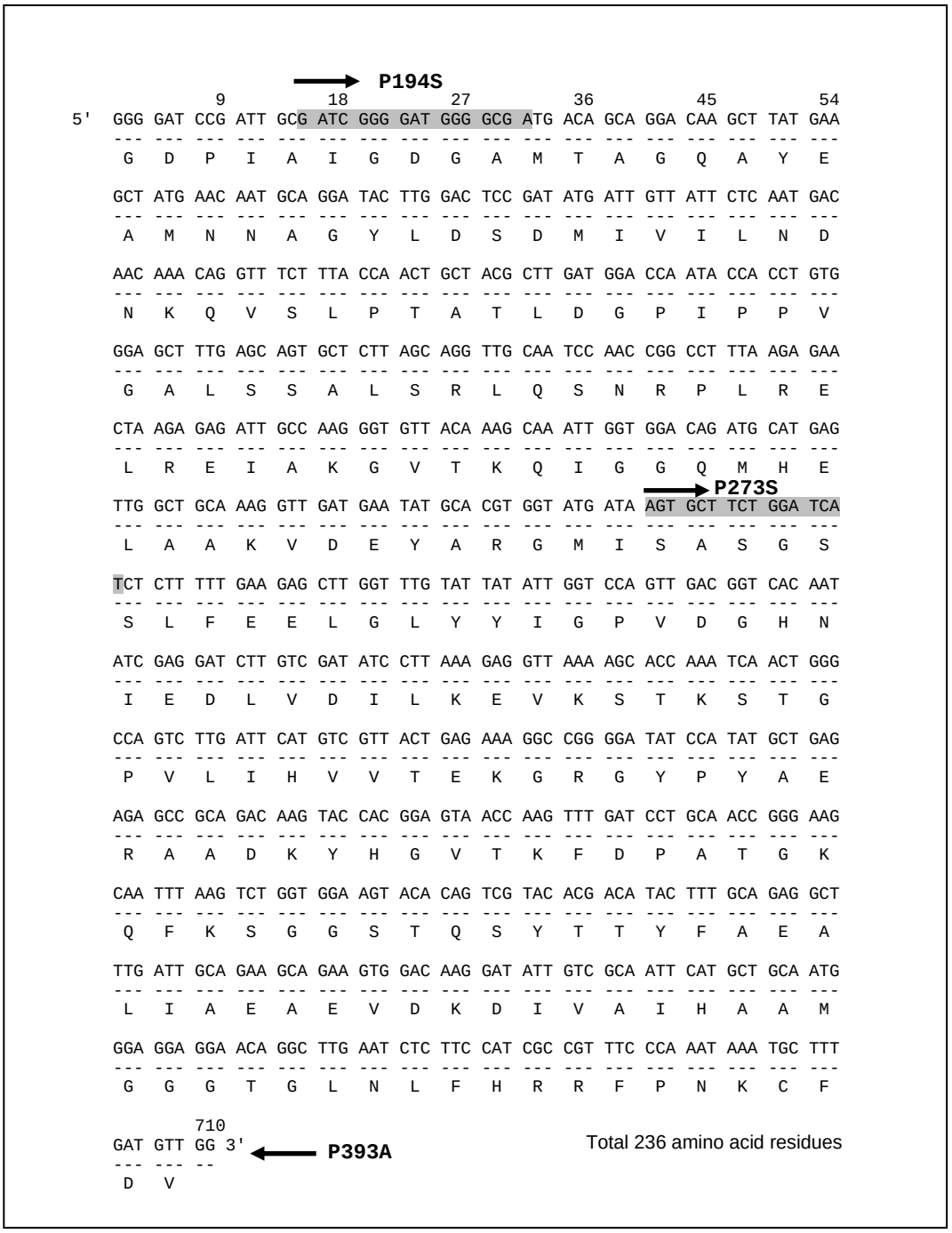


Figure 3.2 The core sequence of *csdxs* gene using the degenerated primers. Nucleotide sequence was translated to amino acid residues using DNASIS software. Arrows indicate names of the degenerated primers and their directions.

The PCR product (597 bp) was ligated with pT7Blue vector and transformed into *E. coli* DH5α. The recombinant plasmids were isolated and determined for the nucleotide sequence. By using pDrive vector, M13 forward and M13 reverse primers were used for amplification of the fragment. After separation by ABI PRISM®, Applied Biosystems 3700 DNA analyzer, the nucleotide sequence was obtained. Information of core sequence was analyzed using the DNASIS v3.5 as shown in Fig.3.2. The DNA sequence analysis revealed the 708 bp DNA fragment. The location of primers, which used for amplification, was found in the inner part of the core sequence (Fig. 3.2). The translate amino acids sequence contains 236 amino acid residues. Alignment of amino acids sequence of the resulting core sequence with partial DXS of *Arabidopsis thaliana* was performed using Clustal W 2.0. Using the DNASIS v3.5 for % identity calculation, the core sequence has an identity about 75.6%. From DNA and amino acid sequences analyses suggested that the core sequence was a part of *csdxs* gene. Thus the DNA sequence of the core sequence was then used to design specific primers for further cloning of the 5'-and 3'-ends.

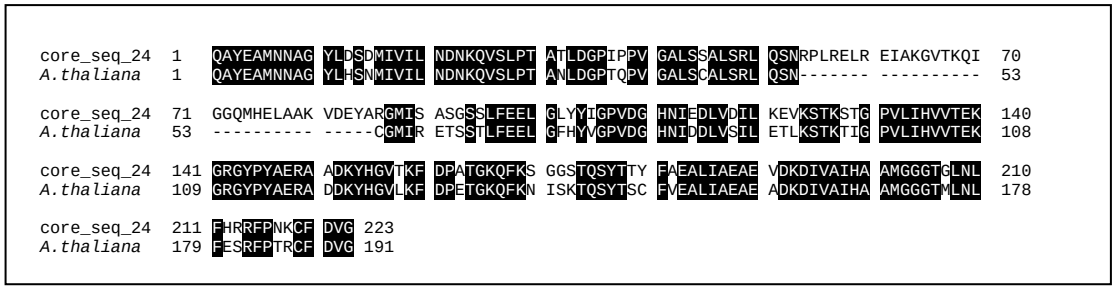


Figure 3.3 Alignment of the core sequence and partial sequence of DXS from *Arabidopsis thaliana* using Clustal W 2.0 and viewed with BioEdit v7.0.5. Solid shade shows the identical amino acid residues.

3.1.3 The 5'- and 3'-ends sequence of *csdxs* gene

For 5'-end amplification, the PCR reactions were performed with 3 combinations of 5'P_298A-AP1, 5'P_280A-AP1, 5'P_256A-AP1. According to the protocol for PCR reactions (2.3.3.2), the resulting PCR product was obtained from the pairs of 5'P_280-AP1, its size about 1.2 kbp, which was purified on agarose gel and eluted with gel extraction kit. The purified PCR product was ligated with pT7Blue vector and transformed into *E. coli* DH5α competent cells. The double strands DNA were sequences. The DNA sequence analysis revealed the 1,112 bp DNA fragment, which overlapped to the 5'-region of the core sequence about 186 bp. The translated amino acid sequence was shown to 78.0% identity to partial *A. thaliana dxs* gene.

For 3'-end amplification, the PCR reactions were performed using the combinations of 3'P_346S-AP1, 3'P_393S-AP1 and 3'P_521S-AP1. According to the PCR program, the PCR products were obtained of the sizes of 1.4 kbp, 1.2 kbp and 850 bp, respectively. The PCR product of 1.4 kbp was further purified, ligated with pT7Blue vector and transformed into *E. coli* DH5α competent cells. The DNA were sequenced and analyzed. The sequence of 393-AP1 was shown to overlap with the core sequence. However, the DNA sequence of the 3'-end could not afford to the end of *csdxs* gene as the poly A-tail had not been found. To complete the 3'-end, the PCR product of 850 bp was investigated with the same procedure, afforded the complete 3'-end fragment.

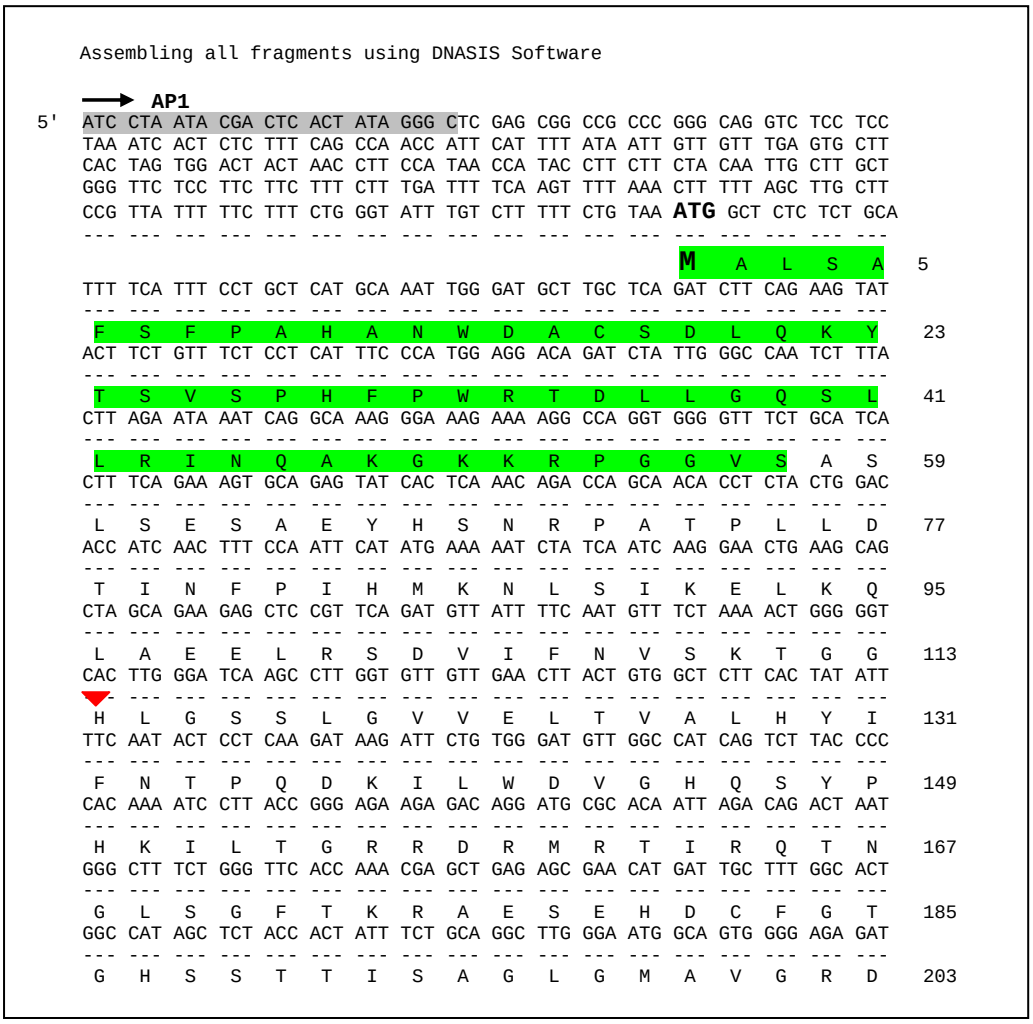


Figure 3.4 Assembling of the nucleic acids of the core sequence, 5'-and 3'-ends of the *csdxs* gene and the deduced amino acids of the cDNA encoding the CSDXS protein (DNASIS v3.5). Grey regions indicate the sites of primers which are used for 5'- and 3'-ends cloning. Bold letters mark the open reading frame (ORF) of the CSDXS protein. Green region denotes the chloroplast transit peptide (ChloroP 1.1). Yellow and pink colors indicate the regions corresponding to the putative thiamine diphosphate-binding site and the transketolase motif, respectively. The arrowheads denote the catalytic amino acid residues at H114, E431, D462 and R539 of the CSDXS protein. X (s) indicate the un-translated amino acid residues.

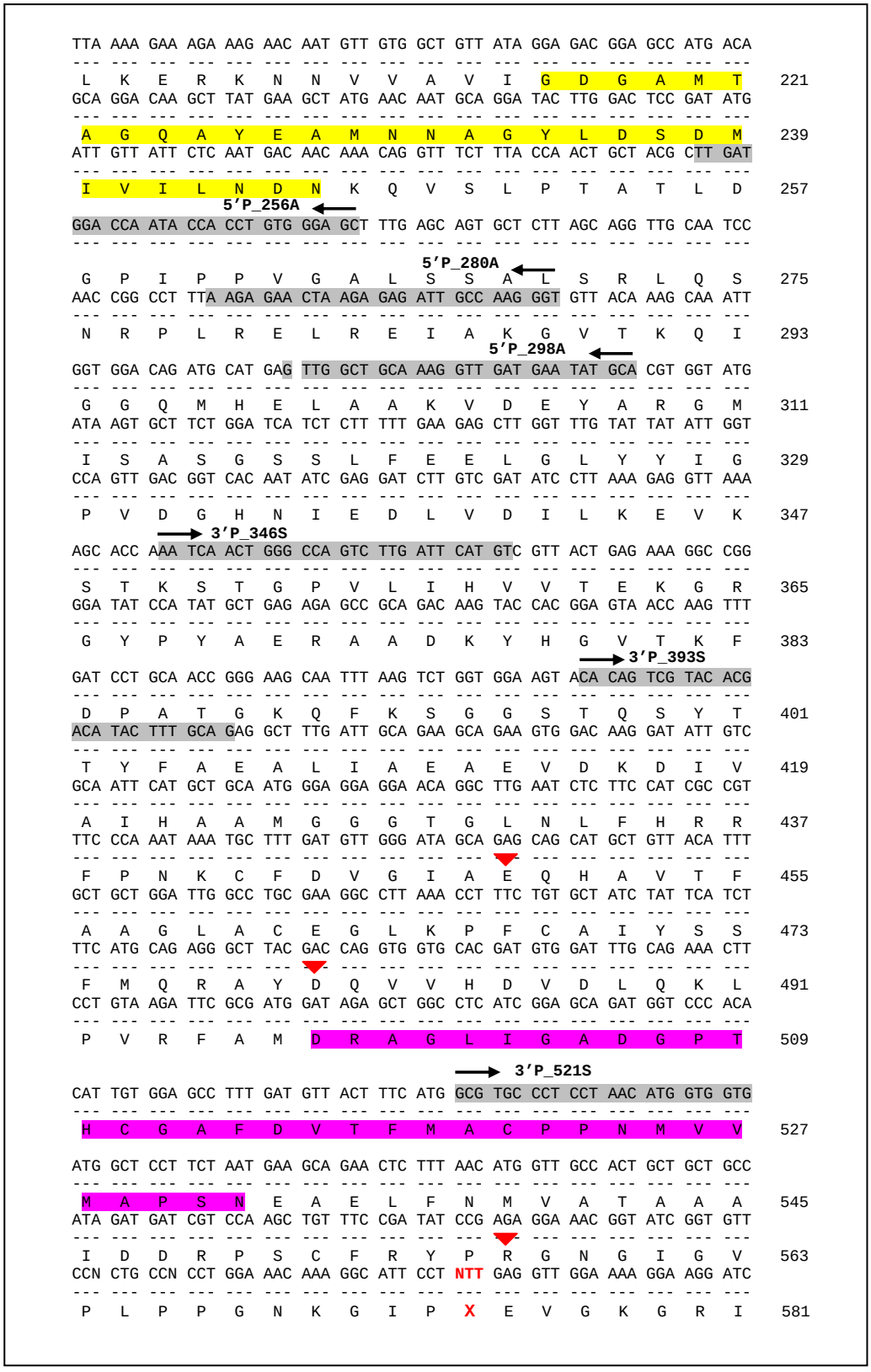


Figure 3.4 (continued).

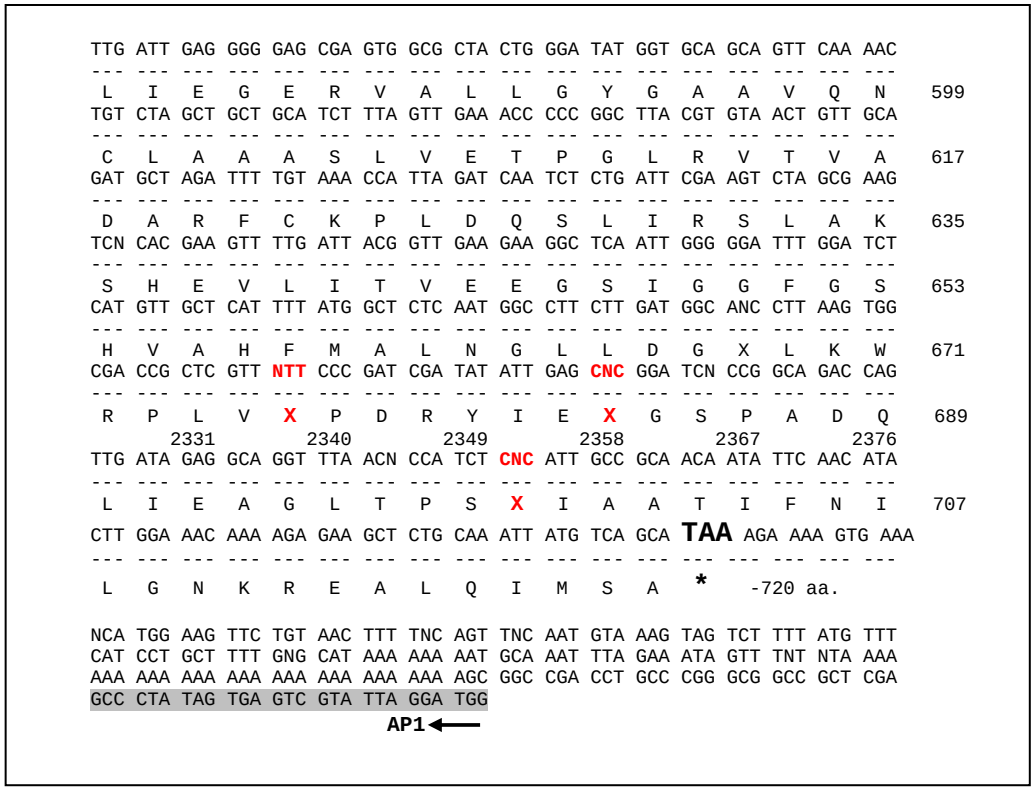


Figure 3.4 (continued).

3.1.4 The cDNA of *csdxs* gene

Assembling of 5'-end, core sequence and 3'-end of the cloning *csdxs* cDNA revealed that the putative nucleotide sequence composes of 2,607 bp, which can be found the primers used during cloning (Fig. 3.4).The *csdxs* cDNA composes of 2,308 bp (from ATG to poly A-tail). The deduced amino acid analysis revealed that the *csdxs* cDNA contains the open reading frame (ORF) at the ATG starting codon at NH_2-terminal and TAA stop codon at COOH-terminal (Fig. 3.4). The *csdxs* cDNA contains 2,163 bp, encoding 720 amino acid residues. However, the missing of nucleotide in the assembling caused the un-translated amino acid residues (designated as X) at COOH-terminal region. To complete the DNA sequence, the cloning of the full-length of the *csdxs* gene was performed.

The CSDXS obtained from partial clonings was submitted for BLAST P search (http://www.ncbi.nlm.nih.gov/BLAST/), revealed that this protein is similar to the DXSs proteins

from higher plants such as *A. thaliana*, *Artemisia annuua*, *Lycopersicon esculentum*, etc. BLAST P suggested that this protein belongs to the transketolase and dehydrogenase E1 component family. Searching for the TPP-binding and the transketolase motifs in the CSDXS revealed that the CSDXS has the consensus sequence for the TPP-binding motif (designated as $GDG(X)_8E(X)_4A(X)_{11}NDN$, where X denotes any amino acid) at position 216-246 (Hawkins *et al*., 1989) and the consensus sequence for the transketolase motif (designated as DRAG-X_{28}-P-X-D) at position 498-532 of CSDXS protein (Schenk *et al*., 1997), respectively. In comparison with the *E. coli* DXS, the CSDXS carried the catalytic amino acid residues at H114, E431, D462 and R539 as same as the previously reports in *E. coli* (Sprenger *et al*., 1997; Lois *et al*., 1998), *Mentha piperita* (Lange *et al*., 1998) and pepper (*Capsicum annuum*) (Bouvier *et al*., 1998).

The CSDXS protein was analyzed for their characteristic. It has p*I* of 6.80 and calculated molecular weight of 77,623 predicted with http://au.expasy.org/cgi-bin/pi_tool. The topology search for subcellular prediction, the CSDXS protein was predicted with TargetP v1.0 to have a chloroplast transit peptide (cTP) of 0.325. The cleavage site of the transit peptide was predicted with ChloroP 1.1 to have the chloroplast transit peptide of 57 amino acid residues from NH_2-terminus. From this results indicated that the CSDXS protein locates in the chloroplast organelle.

3.1.5 The full-length *csdxs* gene

Information of *csdxs* cDNA obtained from 3.1.4 allowed us to design the specific primers for full-length gene cloning. Due to the missing amino acid sequence (X residues) found in the translate amino acid sequence of the CSDXS protein, the DNA fragment of the full-length gene were sequenced and analyzed. However, attempts to perform the full-length *csdxs* gene were unsuccessful by means of cloning to pDrive vector. Therefore, the DNA sequencing was done by PCR-direct sequencing. The full-length *csdxs* gene was amplified and

purified on agarose gel (Fig. 3.5). The purified PCR product was sent for DNA sequencing. Primers, which used for sequencing, are shown in Fig 3.6.

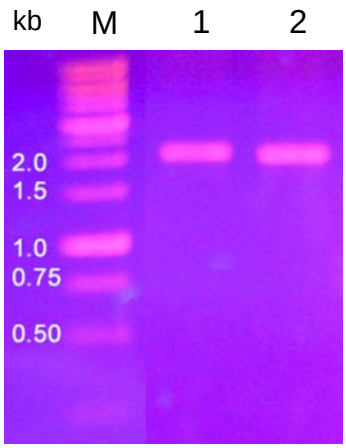


Figure 3.5 Analysis of the PCR products of the full-length and truncated genes of *csdxs* on 1.2% agarose gel electrophoresis stained with ethidium bromide and visualized under transluminator (312 nm). M: DNA ladder (SibEnzyme); 1: full-length *csdxs* (*ca.*2.1 kb); 2: truncated *csdxs* (*ca.* 1.9 kb).

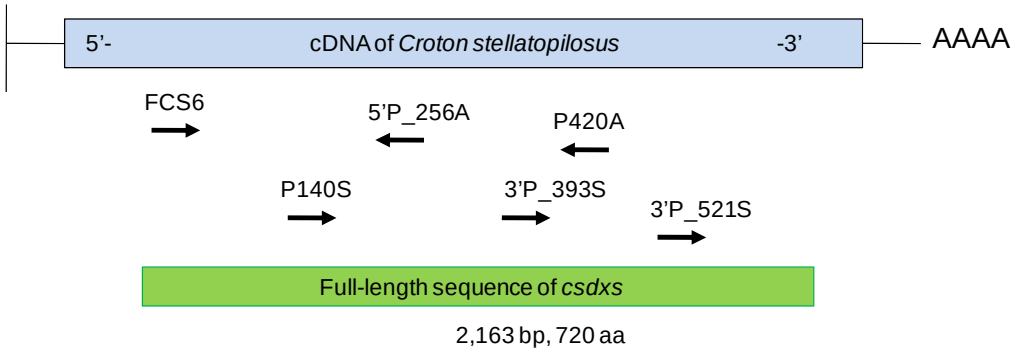


Figure 3.6 Schematic illustration of the DNA sequencing procedure using PCR-direct technique of the full-length *csdxs* gene. Arrows show the direction of primers of sense (S) and antisense (A).

By using different primers, the partial DNA fragments were obtained. Assembling all DNA fragments were performed using the CAP Contig assembly program in the

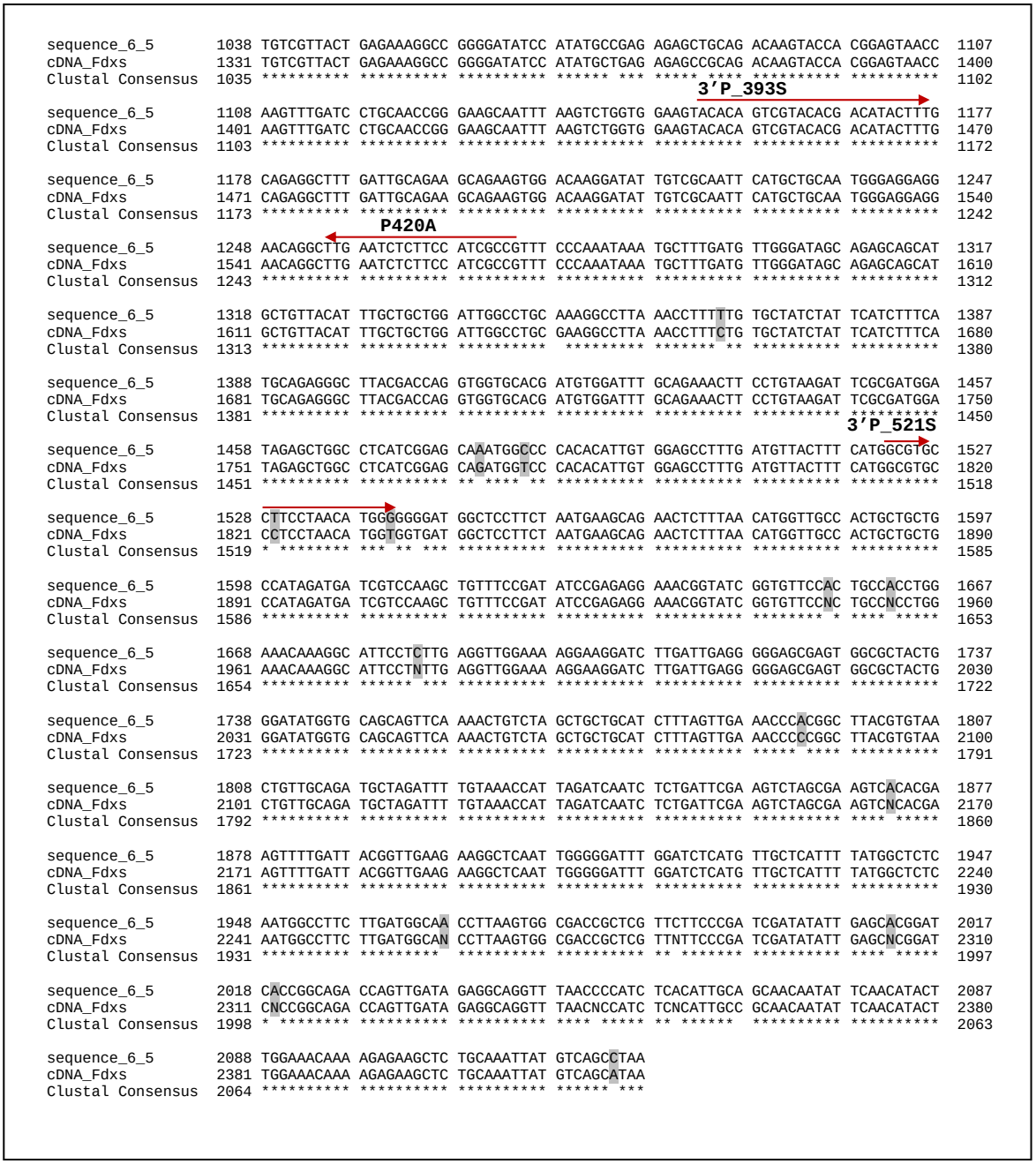
BioEdit v 7.0.5. The resulting DNA assemble is aligned with the ORF of *csdxs* gene as shown in Fig. 3.7. The results reveal the positions of nucleic acid residues which are different from each other. The DNA sequence obtained from PCR-direct sequencing is then translated to amino acids as shown in Fig. 3.8. Alignment of the deduced amino acid sequence with the amino acid sequence from *A. thaliana* DXS by ClustalW 2.0 revealed that 17 amino acid residues were different. Consideration of the conserved regions of the DXSs and the frequency of the occurrence of amino acid residue in higher plants, the CSDXS protein was corrected at the doubted amino acid residue, affording the FCSDXS consensus (Fig. 3.8). The nucleotide sequence of *csdxs* cDNA was submitted to the DDBJ database (http://www.ddbj.nig.ac.jp/) as an accession number of AB354578.

3.1.6 Comparative and bioinformatic analyses of CSDXS

The deduced amino acid sequence of the CSDXS was submitted to NCBI for PSI-BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/). As shown in Fig. 3.9, the CSDXS has high homology with DXSs sequences from other plant species with 89% identity with DXS from *Hevea brasiliensis*. The CSDXS was also highly similar more than 80% identity to DXRs from *Pueraria Montana*, *Medicago truncatula*, *Capsicum annuum*, *Lycopersicon esculentum*, *Artemisia anuua*, *Arabidopsis thaliana*, *Brassica rapa*, *Elaeis guineensis*, *Oryza sativa*, *Zea mays*, *Physcomitrella patens*, *Picea abies* and *Ginkgo biloba*. Some of them have been shown to belong to the DXS class II such as *C. annuum* (Bouvier *et al*. 1998), *H. brasiliensis* (Seetang-Nun *et al*., 2007). Besides, the CSDXS also had about 68-75% identity with other plants DXS including *Morinda citrifolia*, *P. abies*, *Taxus x media*, *Adonis aestivalis var. palestina*, *Chrysanthemum x moriifolium*, *Stevia rebaudiana*, *Catharanthus roseus*, *Lycopersicon hirsutum*, *Narcissus pseudonarcissus*, *M. truncatula*, *Tagetes erecta*, *Antirhinum majus* and *Mentha x piperita*.

```
                                                                    FCS6
sequence_6_5         1    ---------- ---------- ---------- ---------- ---------- ---------- ----------  1
cDNA_Fdxs            211  GCTTGCTTCC GTTATTTTTC TTTCTGGGTA TTTGTCTTTT TCTGTAAATG GCTCTCTCTG CATTTTCATT  280
Clustal Consensus    1                                                                                 1

sequence_6_5         1    ---------- ---AATTGGG ATGCTTGCTC AGATCTTCAG AAGTATACTT CTGTTTCTCC TCATTTCCCA  57
cDNA_Fdxs            281  TCCTGCTCAT GCAAATTGGG ATGCTTGCTC AGATCTTCAG AAGTATACTT CTGTTTCTCC TCATTTCCCA  350
Clustal Consensus    1               ******* ********** ********** ********** ********** **********  57

sequence_6_5         58   TGGAGGACAG ATCTATTGGG CCAATCTTTA CTTAGAATAA ATCAGGCAAA GGGAAAGAAA AGGCCAGGTG  127
cDNA_Fdxs            351  TGGAGGACAG ATCTATTGGG CCAATCTTTA CTTAGAATAA ATCAGGCAAA GGGAAAGAAA AGGCCAGGTG  420
Clustal Consensus    58   ********** ********** ********** ********** ********** ********** **********  127

sequence_6_5         128  GGGTTTCTGC ATCACTTTCA GAAAGTGCAG AGTATCACTC AAACAGACCA GCAACACCTC TACTGGACAC  197
cDNA_Fdxs            421  GGGTTTCTGC ATCACTTTCA GAAAGTGCAG AGTATCACTC AAACAGACCA GCAACACCTC TACTGGACAC  490
Clustal Consensus    128  ********** ********** ********** ********** ********** ********** **********  197

sequence_6_5         198  CATCAACTTT CCAATTCATA TGAAAAATCT ATCAATCAAG GAACTGAAGC AGCTAGCAGA AGAGCTCCGT  267
cDNA_Fdxs            491  CATCAACTTT CCAATTCATA TGAAAAATCT ATCAATCAAG GAACTGAAGC AGCTAGCAGA AGAGCTCCGT  560
Clustal Consensus    198  ********** ********** ********** ********** ********** ********** **********  267

sequence_6_5         268  TCAGATGTTA TTTTCAATGT TTCTAAAACT GGGGGTCACT TGGGATCAAG CCTTGGTGTT GTTGAACTTA  337
cDNA_Fdxs            561  TCAGATGTTA TTTTCAATGT TTCTAAAACT GGGGGTCACT TGGGATCAAG CCTTGGTGTT GTTGAACTTA  630
Clustal Consensus    268  ********** ********** ********** ********** ********** ********** **********  337
                                                                         P140S
sequence_6_5         338  CTGTGGCTCT TCACTATATT TTCAATACTC CTCAAGATAA GATCCTGGGG GATGTTGGCC ATCAGTCTTA  407
cDNA_Fdxs            631  CTGTGGCTCT TCACTATATT TTCAATACTC CTCAAGATAA GATTCTGTGG GATGTTGGCC ATCAGTCTTA  700
Clustal Consensus    338  ********** ********** ********** ********** *** *** ** ********** **********  405

sequence_6_5         408  CCCCCACCAA ATCCTTACCG GGAGAAGAGA CAGGATGCGC ACAATTAGAC AGACTAATGG GCTTTCTGGG  477
cDNA_Fdxs            701  CCCCCACAAA ATCCTTACCG GGAGAAGAGA CAGGATGCGC ACAATTAGAC AGACTAATGG GCTTTCTGGG  770
Clustal Consensus    406  ******* ** ********** ********** ********** ********** ********** **********  474

sequence_6_5         478  TTCACCAAAC GAGCTGAGAG CGAACATGAT TGCTTTGGCA CTGGCCATAG CTCTACCACT ATTTCTGCAG  547
cDNA_Fdxs            771  TTCACCAAAC GAGCTGAGAG CGAACATGAT TGCTTTGGCA CTGGCCATAG CTCTACCACT ATTTCTGCAG  840
Clustal Consensus    475  ********** ********** ********** ********** ********** ********** **********  544

sequence_6_5         548  GCTTGGGAAT GGCAGTGGGG AGAGATTTAA AAGAAAGAAA GAACAATGTT GTGGCTGTTA TAGGAGACGG  617
cDNA_Fdxs            841  GCTTGGGAAT GGCAGTGGGG AGAGATTTAA AAGAAAGAAA GAACAATGTT GTGGCTGTTA TAGGAGACGG  910
Clustal Consensus    545  ********** ********** ********** ********** ********** ********** **********  614

sequence_6_5         618  AGCCATGACA GCAGGACAAG CTTATGAAGC TATGAACAAT GCAGGATACT TGGACTCCGA TATGATTGTT  687
cDNA_Fdxs            911  AGCCATGACA GCAGGACAAG CTTATGAAGC TATGAACAAT GCAGGATACT TGGACTCCGA TATGATTGTT  980
Clustal Consensus    615  ********** ********** ********** ********** ********** ********** **********  684
                                                                                         5′_256A
sequence_6_5         688  ATTCTCAATG ACAACAAACA GGTTTCTTTA CCAACTGCTA CGCTTGATGG ACCAATACCA CCTGTGGGAG  757
cDNA_Fdxs            981  ATTCTCAATG ACAACAAACA GGTTTCTTTA CCAACTGCTA CGCTTGATGG ACCAATACCA CCTGTGGGAG  1050
Clustal Consensus    685  ********** ********** ********** ********** ********** ********** **********  754

sequence_6_5         758  CTTTGAGCAG TGCTCTTAGC AGGTTGCAAT CCAACCGGCC TTTAAGAGAA CTAAGAGAGA TTGCCAAGGG  827
cDNA_Fdxs            1051 CTTTGAGCAG TGCTCTTAGC AGGTTGCAAT CCAACCGGCC TTTAAGAGAA CTAAGAGAGA TTGCCAAGGG  1120
Clustal Consensus    755  ********** ********** ********** ********** ********** ********** **********  824

sequence_6_5         828  TGTTACAAAG CAAATTGGTG GACAGATGCA TGAGTTGGCT GCAAAGGTTG ATGAATATGC ACGTGGTATG  897
cDNA_Fdxs            1121 TGTTACAAAG CAAATTGGTG GACAGATGCA TGAGTTGGCT GCAAAGGTTG ATGAATATGC ACGTGGTATG  1190
Clustal Consensus    825  ********** ********** ********** ********** ********** ********** **********  894

sequence_6_5         898  ATAAGTGCTT CTGGATCATC TCTTTTTGAA GAGCTTGGTT TGTATTATAT TGGTCCAGTT GACGGTCACA  967
cDNA_Fdxs            1191 ATAAGTGCTT CTGGATCATC TCTTTTTGAA GAGCTTGGTT TGTATTATAT TGGTCCAGTT GACGGTCACA  1260
Clustal Consensus    895  ********** ********** ********** ********** ********** ********** **********  964

sequence_6_5         968  ATATCGAGGA TCTTGTCGAT ATCCTTAAAG AGGTTAAAAG CACCAAATCA ACTGGGCCAG TCTTGATTCA  1037
cDNA_Fdxs            1261 ATATCGAGGA TCTTGTCGAT ATCCTTAAAG AGGTTAAAAG CACCAAATCA ACTGGGCCAG TCTTGATTCA  1330
Clustal Consensus    965  ********** ********** ********** ********** ********** ********** **********  1034
```

Figure 3.7 Alignment of nucleotide sequences obtained from assembling of DNA sequencing data (sequence_6_5) and the putative cDNA encoding the *csdxs* gene (cDNA_Fdxs). Clustal consensus indicates the identical residue and is denoted by asterisks (*). Shade letters denote un-identical and missing nucleic acids (N).




```
sequence_6_5       1038 TGTCGTTACT GAGAAAGGCC GGGGATATCC ATATGCCGAG AGAGCTGCAG ACAAGTACCA CGGAGTAACC  1107
cDNA_Fdxs          1331 TGTCGTTACT GAGAAAGGCC GGGGATATCC ATATGCTGAG AGAGCCGCAG ACAAGTACCA CGGAGTAACC  1400
Clustal Consensus  1035 ********** ********** ********** ****** *** ***** **** ********** **********  1102

sequence_6_5       1108 AAGTTTGATC CTGCAACCGG GAAGCAATTT AAGTCTGGTG GAAGTACACA GTCGTACACG ACATACTTTG  1177
cDNA_Fdxs          1401 AAGTTTGATC CTGCAACCGG GAAGCAATTT AAGTCTGGTG GAAGTACACA GTCGTACACG ACATACTTTG  1470
Clustal Consensus  1103 ********** ********** ********** ********** ********** ********** **********  1172

sequence_6_5       1178 CAGAGGCTTT GATTGCAGAA GCAGAAGTGG ACAAGGATAT TGTCGCAATT CATGCTGCAA TGGGAGGAGG  1247
cDNA_Fdxs          1471 CAGAGGCTTT GATTGCAGAA GCAGAAGTGG ACAAGGATAT TGTCGCAATT CATGCTGCAA TGGGAGGAGG  1540
Clustal Consensus  1173 ********** ********** ********** ********** ********** ********** **********  1242

sequence_6_5       1248 AACAGGCTTG AATCTCTTCC ATCGCCGTTT CCCAAATAAA TGCTTTGATG TTGGGATAGC AGAGCAGCAT  1317
cDNA_Fdxs          1541 AACAGGCTTG AATCTCTTCC ATCGCCGTTT CCCAAATAAA TGCTTTGATG TTGGGATAGC AGAGCAGCAT  1610
Clustal Consensus  1243 ********** ********** ********** ********** ********** ********** **********  1312

sequence_6_5       1318 GCTGTTACAT TTGCTGCTGG ATTGGCCTGC AAAGGCCTTA AACCTTTTTG TGCTATCTAT TCATCTTTCA  1387
cDNA_Fdxs          1611 GCTGTTACAT TTGCTGCTGG ATTGGCCTGC GAAGGCCTTA AACCTTTCTG TGCTATCTAT TCATCTTTCA  1680
Clustal Consensus  1313 ********** ********** **********  ********** ******* ** ********** **********  1380

sequence_6_5       1388 TGCAGAGGGC TTACGACCAG GTGGTGCACG ATGTGGATTT GCAGAAACTT CCTGTAAGAT TCGCGATGGA  1457
cDNA_Fdxs          1681 TGCAGAGGGC TTACGACCAG GTGGTGCACG ATGTGGATTT GCAGAAACTT CCTGTAAGAT TCGCGATGGA  1750
Clustal Consensus  1381 ********** ********** ********** ********** ********** ********** **********  1450

sequence_6_5       1458 TAGAGCTGGC CTCATCGGAG CAAATGGCCC CACACATTGT GGAGCCTTTG ATGTTACTTT CATGGCGTGC  1527
cDNA_Fdxs          1751 TAGAGCTGGC CTCATCGGAG CAGATGGTCC CACACATTGT GGAGCCTTTG ATGTTACTTT CATGGCGTGC  1820
Clustal Consensus  1451 ********** ********** ** **** ** ********** ********** ********** **********  1518

sequence_6_5       1528 CTTCCTAACA TGGGGGGGAT GGCTCCTTCT AATGAAGCAG AACTCTTTAA CATGGTTGCC ACTGCTGCTG  1597
cDNA_Fdxs          1821 CCTCCTAACA TGGTGGTGAT GGCTCCTTCT AATGAAGCAG AACTCTTTAA CATGGTTGCC ACTGCTGCTG  1890
Clustal Consensus  1519 * ******** *** ** *** ********** ********** ********** ********** **********  1585

sequence_6_5       1598 CCATAGATGA TCGTCCAAGC TGTTTCCGAT ATCCGAGAGG AAACGGTATC GGTGTTCCAC TGCCACCTGG  1667
cDNA_Fdxs          1891 CCATAGATGA TCGTCCAAGC TGTTTCCGAT ATCCGAGAGG AAACGGTATC GGTGTTCCNC TGCCNCCTGG  1960
Clustal Consensus  1586 ********** ********** ********** ********** ********** ******** * **** *****  1653

sequence_6_5       1668 AAACAAAGGC ATTCCTCTTG AGGTTGGAAA AGGAAGGATC TTGATTGAGG GGGAGCGAGT GGCGCTACTG  1737
cDNA_Fdxs          1961 AAACAAAGGC ATTCCTNTTG AGGTTGGAAA AGGAAGGATC TTGATTGAGG GGGAGCGAGT GGCGCTACTG  2030
Clustal Consensus  1654 ********** ****** *** ********** ********** ********** ********** **********  1722

sequence_6_5       1738 GGATATGGTG CAGCAGTTCA AAACTGTCTA GCTGCTGCAT CTTTAGTTGA AACCCACGGC TTACGTGTAA  1807
cDNA_Fdxs          2031 GGATATGGTG CAGCAGTTCA AAACTGTCTA GCTGCTGCAT CTTTAGTTGA AACCCCCGGC TTACGTGTAA  2100
Clustal Consensus  1723 ********** ********** ********** ********** ********** ***** **** **********  1791

sequence_6_5       1808 CTGTTGCAGA TGCTAGATTT TGTAAACCAT TAGATCAATC TCTGATTCGA AGTCTAGCGA AGTCACACGA  1877
cDNA_Fdxs          2101 CTGTTGCAGA TGCTAGATTT TGTAAACCAT TAGATCAATC TCTGATTCGA AGTCTAGCGA AGTCNCACGA  2170
Clustal Consensus  1792 ********** ********** ********** ********** ********** ********** **** *****  1860

sequence_6_5       1878 AGTTTTGATT ACGGTTGAAG AAGGCTCAAT TGGGGGATTT GGATCTCATG TTGCTCATTT TATGGCTCTC  1947
cDNA_Fdxs          2171 AGTTTTGATT ACGGTTGAAG AAGGCTCAAT TGGGGGATTT GGATCTCATG TTGCTCATTT TATGGCTCTC  2240
Clustal Consensus  1861 ********** ********** ********** ********** ********** ********** **********  1930

sequence_6_5       1948 AATGGCCTTC TTGATGGCAA CCTTAAGTGG CGACCGCTCG TTCTTCCCGA TCGATATATT GAGCACGGAT  2017
cDNA_Fdxs          2241 AATGGCCTTC TTGATGGCAN CCTTAAGTGG CGACCGCTCG TTNTTCCCGA TCGATATATT GAGCNCGGAT  2310
Clustal Consensus  1931 ********** *********  ********** ********** ** ******* ********** **** *****  1997

sequence_6_5       2018 CACCGGCAGA CCAGTTGATA GAGGCAGGTT TAACCCCATC TCACATTGCA GCAACAATAT TCAACATACT  2087
cDNA_Fdxs          2311 CNCCGGCAGA CCAGTTGATA GAGGCAGGTT TAACNCCATC TCNCATTGCC GCAACAATAT TCAACATACT  2380
Clustal Consensus  1998 * ******** ********** ********** **** ***** ** ******  ********** **********  2063

sequence_6_5       2088 TGGAAACAAA AGAGAAGCTC TGCAAATTAT GTCAGCCTAA
cDNA_Fdxs          2381 TGGAAACAAA AGAGAAGCTC TGCAAATTAT GTCAGCATAA
Clustal Consensus  2064 ********** ********** ********** ****** ***
```

Figure 3.7 (continued).

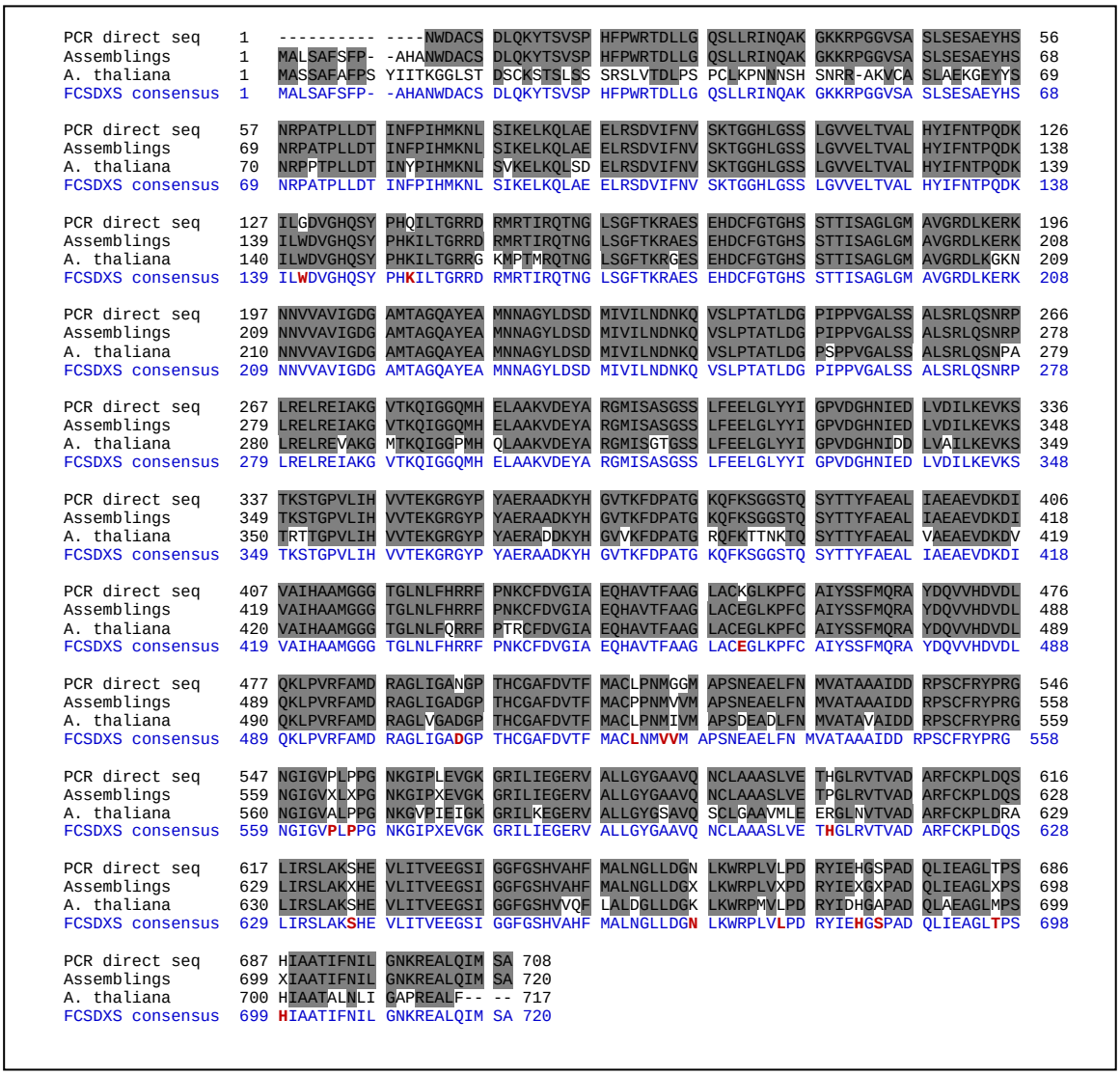


Figure 3.8 Alignment of the amino acid sequences of the CSDXS protein in comparison to *Arabidopsis thaliana*. PCR direct seq and assembling provide amino acid sequences obtained from results of the PCR product from direct sequencing and from cDNA (3.1.4). Blue sequence exhibits the consensus of the FCSDXS protein, which is corrected for the amino acid residues as shown in red letter.

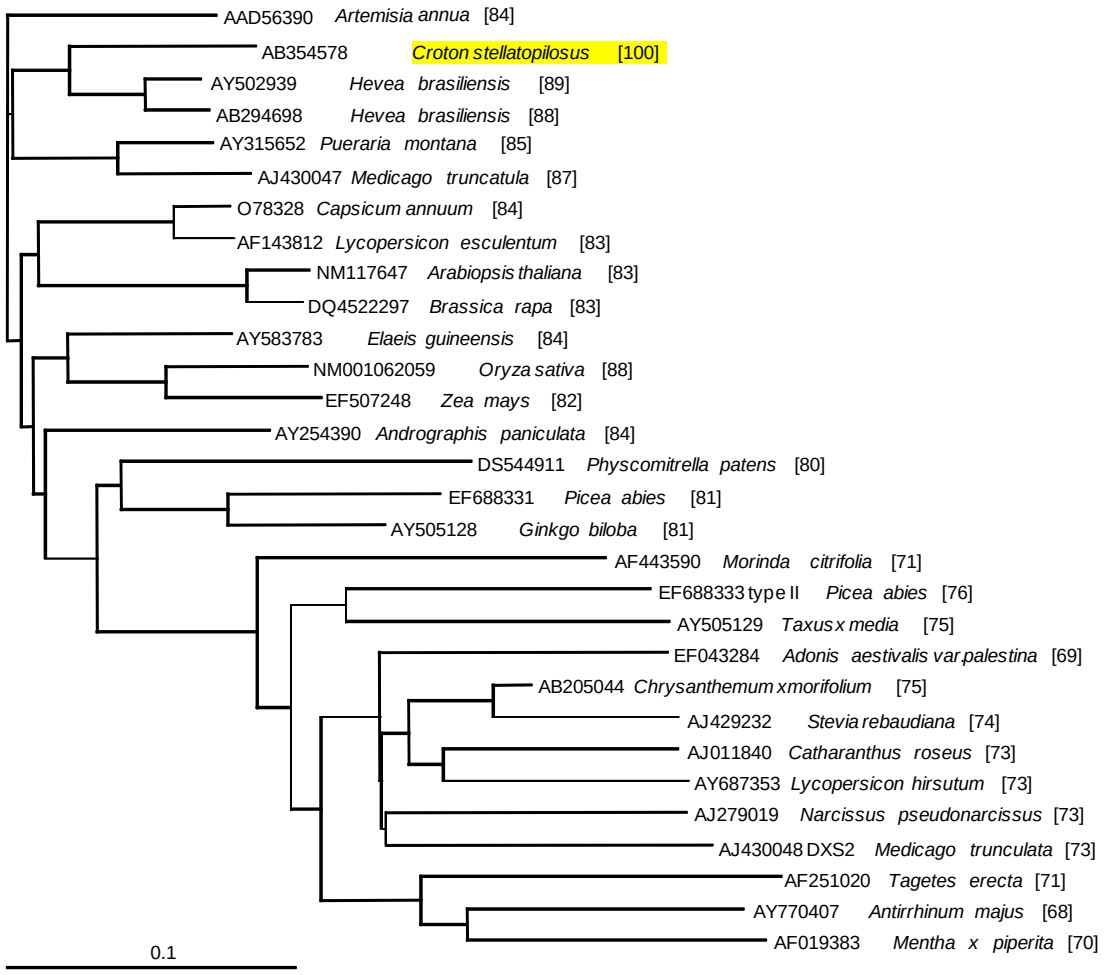


Figure 3.9 Phylogenetic tree analysis of the DXSs from plants from ClustalW XXL alignments and viewed with TreeView v 1.6.6. Accession numbers are indicated in front of the plant names. Numbers in blanket indicate the %identity from the PSI-BLAST.

3.1.7 Physiological role of the *csdxs* in plaunotol biosynthesis

3.1.7.1 mRNA expression profiles in different tissues of *C. stellatopilosus*

The mRNA expression of *csdxs* in different tissues including leaf, twig and root of *C. stellatopilosus* were investigated. Total RNAs were isolated and prepared for

cDNA. The transcription level of each mRNA was determined using the semi-quantitative RT-PCR using primers FCS6 and 5'P_256A. Due to band intensity, it should be related to mRNA expression, the optimal cycle for amplification was investigated. The results indicated that the amplification cycles of partial *csdxs* and *18S rRNA* were 30 cycles and 35 cycles, respectively. After analysis on agarose gel, band intensities were measured by gel documentation (Fig. 10A). Table 3.2 and Fig. 3.10B show the relative intensities of *csdxs* and *18S rRNA* from different tissues. The result shows that *csdxs* expression could be detected in leaf and twig in a similar level but very rare in root.

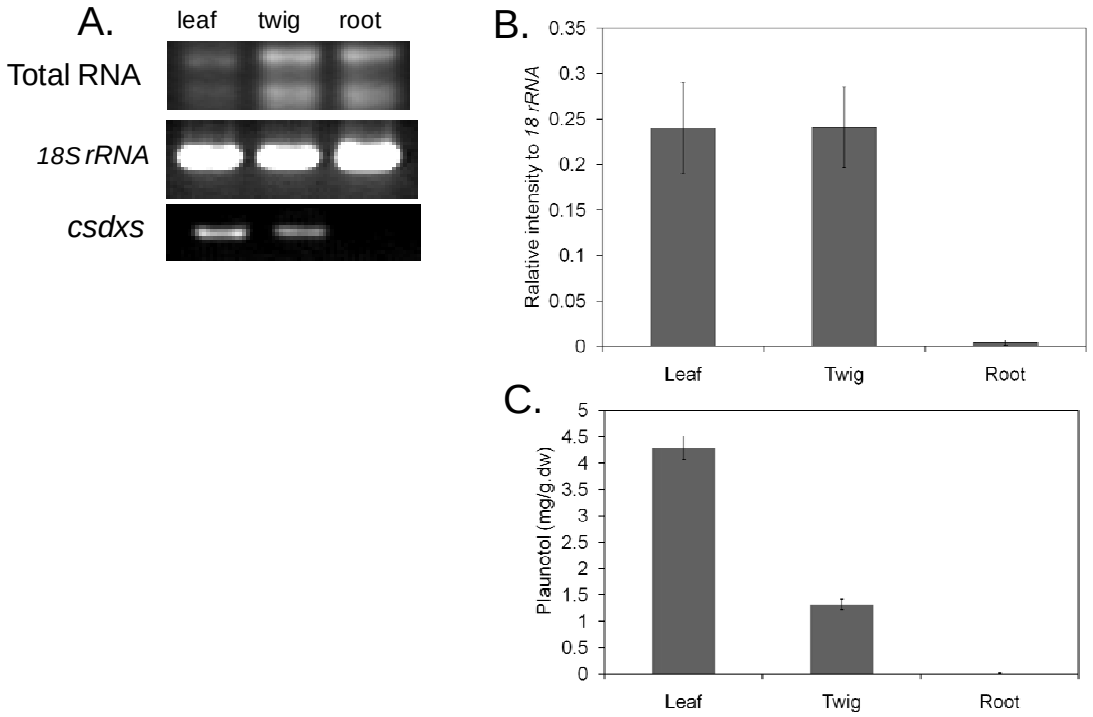


Figure 3.10 The mRNA expression of the *csdxs* gene from leaf, twig and root of *C. stellatopilosus* using semi-quantitative RT-PCR. A. 1.2% (w/w) agarose gel analysis of the *csdxs* gene and *18S rRNA*, analyzed by gel documentation; B. and C. illustrate the relationships of the relative intensities and plaunotol contents, respectively.

Table 3.2 The relative intensities of the *csdxs* gene from leaf, twig and root of *C. stellatopilosus* in comparison with the house-keeping gene (*18S rRNA*) measured by gel documentation. Samples were analyzed from four replicates.

sample	Intensity (volume)		Relative intensity (*csdxs*/*18S rRNA*)	Mean of relative intensity (Average ± S.D.)
	csdxs	*18S rRNA*		
Leaf No. 1	4.10	20.20	0.20	0.24± 0.05
Leaf No. 2	4.20	20.20	0.21	
Leaf No. 3	6.30	20.20	0.31	
Leaf No. 4	4.80	20.20	0.24	
Twig No. 1	3.80	19.60	0.19	0.24 ± 0.04
Twig No. 2	4.50	19.60	0.23	
Twig No. 3	4.70	19.60	0.24	
Twig No. 4	5.90	19.60	0.30	
Root No. 1	0.10	21.50	0.01	0.01± 0.00
Root No. 2	0.10	21.50	0.01	
Root No. 3	0.20	21.50	0.01	
Root No. 4	0.00	21.50	0.00	

3.1.7.2 Plaunotol content in different tissues of *C. stellatopilosus*

Leaves, twigs and roots were collected from 6-month old of *C. stellatoplilosus* and prepared for *n*-hexane extract (2.5.2.1). The extracts were subjected to the GC system (2.5.2.2). The GC chromatogram is shown in Fig. 3.12. Quantification of plaunotol content was performed using the authentic plaunotol calibration curve (R^2 = 0.999). The area under peak was converted to concentration and calculated the plaunotol content in mg/g dry wt. Table 3.3 shows that plaunotol is accumulated highly in leaves and lower in twig. Nevertheless,

the plaunotol was not detected in roots. It can be concluded that plaunotol is accumulated in specific tissue, especially in leaf of *C. stellatpilosus*.

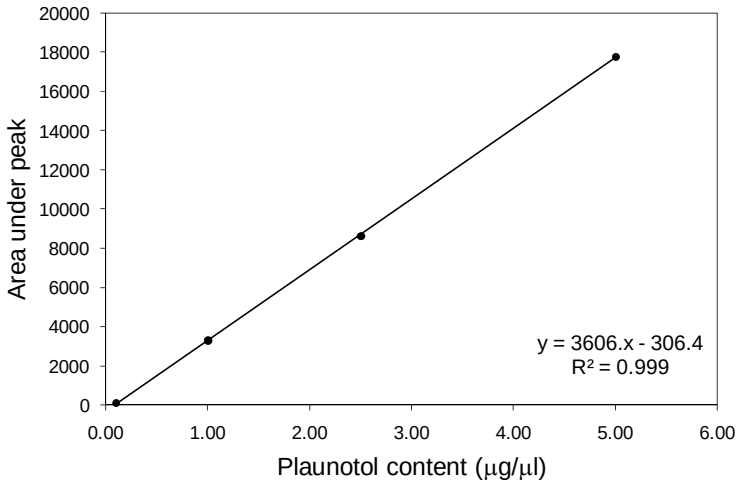


Figure 3.11 Calibration curve of authentic plaunotol by gas chromatography.

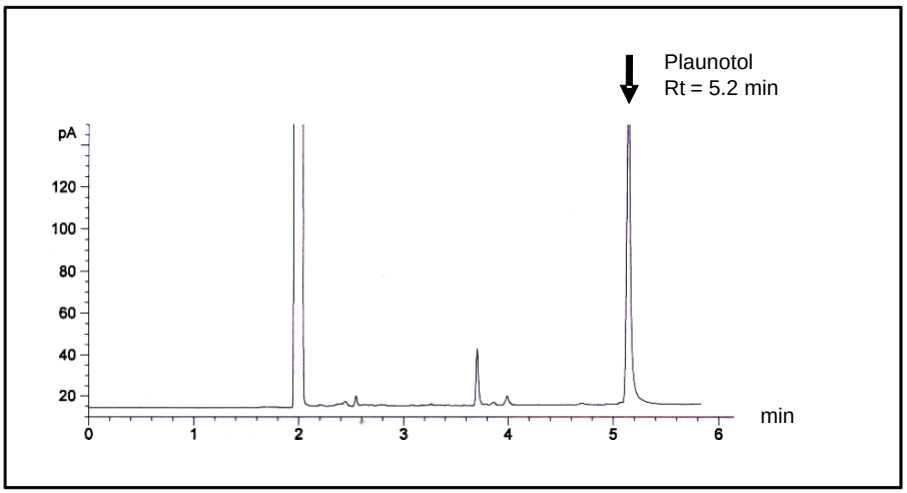


Figure 3.12 GC chromatogram of *n*-hexane extract from leaves.

Table 3.3 Quantitative analysis of plaunotol content from leaves, twigs and roots of *C. stellatopilosus* by gas chromatography. Samples were investigated in four replicates.

Sample	Plaunotol content (mg/g dry wt.)				
	No.1	No.2	No.3	No.4	Average ± S.D.
Leaf	4.59	4.38	4.17	4.04	4.29 ± 0.22
Twig	1.17	1.33	1.40	1.40	1.32 ± 0.10
Root	0.02	0.01	0.02	0.02	0.02 ± 0.00

3.2 1-Deoxyxylulose 5-phosphate synthase from *C. stellatopilosus* (CSDXS)

1-Deoxy-D-xylulose 5-phosphate synthase catalyzed the condensation of pyruvate and D-glyceraldehyde 3-phosphate (G3P) to 1-deoxy-D-xylulose 5-phosphate (DXP) in the presence of a cofactor thiamine diphosphate (TPP) (Fig. 3.13). This enzyme was firstly identified in *E. coli* (Sprenger *et al*., 1997). For the DXS in higher plants, there were reported from *M. piperita* (Lange *et al*., 1998), *C. annuum* (Bouvier *et al*., 1998), *A. thaliana* (Estévez *et al*., 2000), and *C. roseus* (Chahed *et al*., 2000). To determine the CSDXS activity from *C. stellatopilosus*, the *csdxs* cDNA gene was constructed into expression vector and transformed to expression host. The protein was purified and characterized.

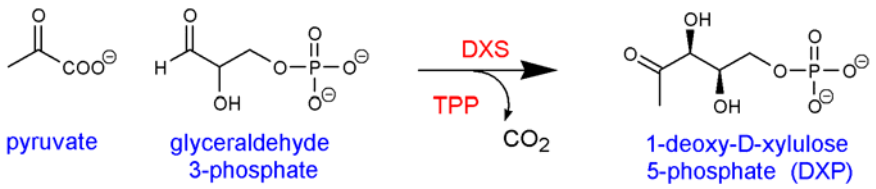


Figure 3.13 Catalytic reaction of 1-deoxy-D-xylulose 5-phosphate synthase, endoced by *dxs* .

3.2.1 Constructions of the full-length and truncated CSDXS

A *csdxs* cDNA gene has been isolated and characterized (2.1). Analysis of the CSDXS enzyme revealed that it carries the chloroplast transit peptide for 57 amino acid residues from NH_2-terminus. Therefore, the heterologous expression of the *csdxs* cDNA was prepared for the full-length and truncated protein. On the basis of data from ChloroP v1.1, suggested that at position 57 (before AS motif) is the cleavage site of the CSDXS protein (Fig. 3.14A). In order to express the full-length CSDXS (FCSDXS), PDXS-S2 primer was used for sense primer in the PCR amplification. For an expression of the truncated CSDXS (TCSDXS), PDXS-S3 primer was used.

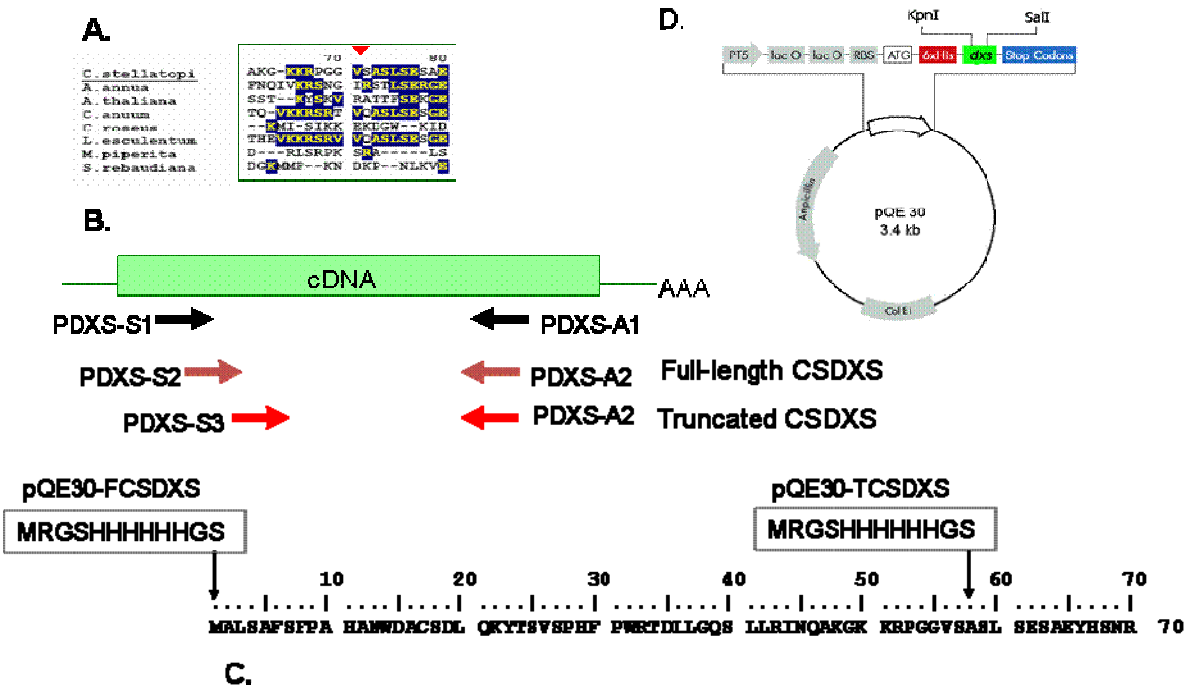


Figure 3.14 Construction of the recombinant plasmids carrying genes encoding of the full-length and truncated CSDXS proteins. A. NH_2-terminus region of the DXS protein from *C. stellatopilosus* aligned with other plants; B. Schematic illustration of primers used; C. Starting regions and N-terminal extensions of expressed constructs; D. pQE30 constructed with *csdxs* gene carrying *Kpn*I and *Sal*I restriction sites.

The scheme of the plasmids constructions is illustrated in Fig. 3.14B. Both sense primers contained *Kpn*I restriction site at the 5'-end, whereas the antisense of PDXS-A2 carries the *Sal*I for restriction site at the 5'-end. In order to enable rapid affinity chromatography purification of protein, the specify N-terminal polyhistidine tags was added to the recombinant constructs. The pQE30-FCSDXS construct contained an ORF of 2,163 codons including the vector-derived coding region MRGSHHHHHHGT followed by the complete CSDXS protein (1-720). On the other hand, the pQE30-TCSDXS construct contained 1,989 codons (cleavage of the region of chloroplast transit peptide) of the vector-derived coding region MRGSHHHHHHGT followed by the truncated CSDXS protein sequence (57-720) (Fig 3.14C-D). The constructions were transformed into *E. coli* XL1blue MRF' competent cells for proteins expressions.

3.2.2 Heterologous expression of full-length and truncated CSDXS

Heterologous expression was performed using a high copy expression vector pQE30 under the control of a T5 promoter and a *lac* operator in *E. coli* XL1 blue MRF' host strain. The T5 promoter is a strong promoter which is recognized by the RNA polymerase of *E.coli.* Expression of recombinant proteins encoded by these vectors is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. The *E.coli* host cells strain XL1-BMRF' contain a mutated *lacI* gene (*lac*l) (encoding the lac repressor). Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter.

For protein expressions, the recombinants *E.coli* were induced using the IPTG as described in 2.4.1. Cells were harvested and the protein was extracted. The soluble protein and insoluble protein were collected and analyzed on 10% SDS-PAGE. As shown in Fig. 3.15, the expression patterns of the soluble fractions from the recombinant strains differed from that *E. coli* XL1 blue MRF' and *E. coli* XL1 blue MRF'-pQE30, but showed no visible expression of the

recombinant protein as analyzed by SDS-PAGE (Fig. 3.15 AA). The calculated mass of the FCSDXS and the TCSDXS proteins are 77,623 Da and 71,353 Da, respectively.

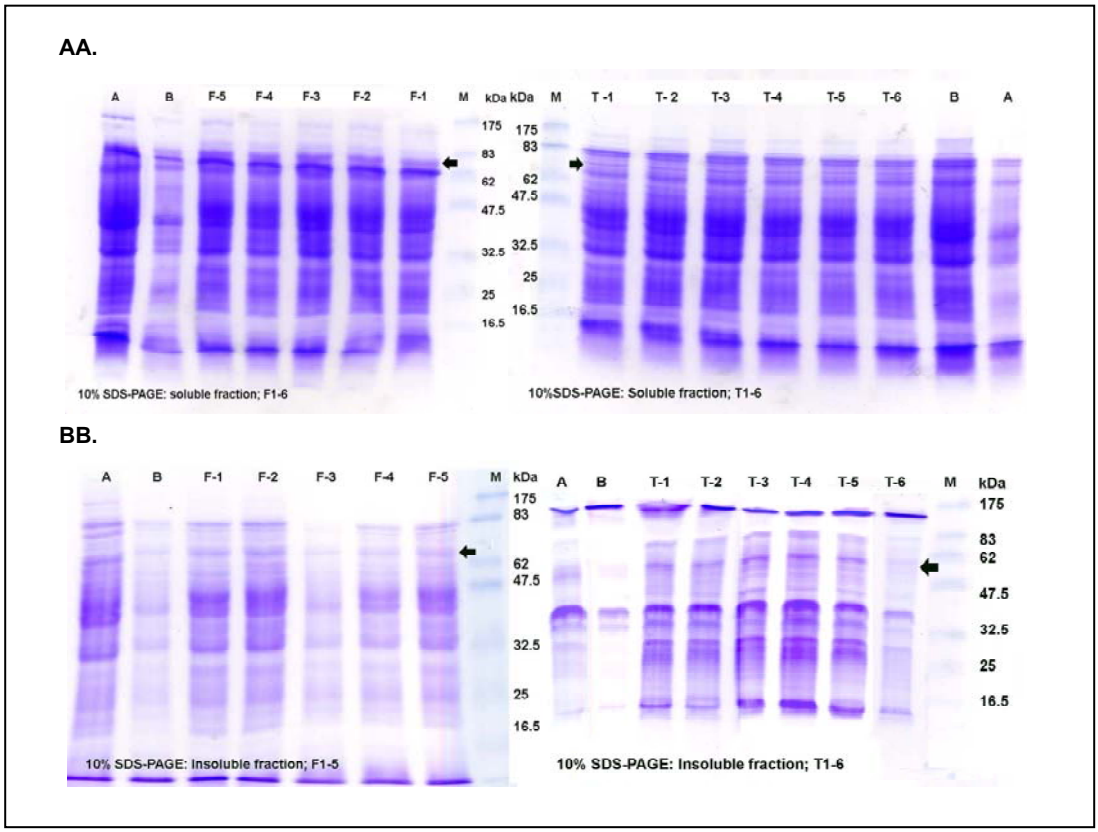


Figure 3.15 The Coomassie blue staining 10% SDS-PAGE of the FCSDXS and TCSDXS protein. AA. soluble fractions and BB. insoluble fractions; F, T designated for full-length and truncated proteins. Lane A and lane B are soluble fractions of *E. coli* XL1 blue MRF' and *E. coli* XL1 blue MRF'-pQE30. M represents rainbow protein markers and the arrow indicates the expected protein band.

3.2.3 Factors affecting on the DXS expression

In order to increase the protein expression, various parameters were investigated including induction temperatures (22 °C, 30 °C and 37°C), induction periods (4 h and 8 h) and inducer concentration (IPTG at 0.5, 1.0, 2.0 and 3.0 mM). After growing the recombinant under

tested conditions, the soluble fractions were prepared and analyzed on 10% SDS-PAGE. Firstly, cells grown at 22 °C, 30 °C and 37°C were manipulated after induction with IPTG (2.0 mM). As shown in Fig. 3.16A, the apparent proteins at 70 kDa show no difference between both CSDXS. Further experiment, the effect of induction period (4 h, 8 h) on the CSDXS expression was investigated. The soluble fractions of FCSDXS and TCSDXS proteins were harvested from 8 h growing cell (Fig. 3.16B). The concentration of IPTG at 0.5, 1, 2, 3, 4 mM were also studied. The 10% SDS-PAGE results from Fig. 3.16-D revealed that IPTG at tested concentrations did not effect on the expression levels of FCSDXS and TCSDXS. From those results, the optimal condition for induction of the FCSDXS and TCSDXS expression is growing recombinant *E. coli* cells at 30 °C for 8 h after induction with 0.5 mM IPTG.

3.2.4 Purifications of CSDXS proteins on HiTrap™ chelating HP column

Based on the construction of the pQE30 vector, the recombinant protein carries the polyhistidine tags at the NH_2-terminus. Affinity chromatography on nickel-chelating Sepharose (HiTrap™ chelating HP column) was performed. The protein bound to Ni^{2+} specifically and eluted with the concentration of imidazole.

The crude proteins were extracted from 8 g recombinant *E.coli*. Total protein was estimated using the Bradford assay. The cell extracts have a total protein of 508 mg for the crude FCSDXS and 624 mg for TCSDXS proteins. The purifications of both proteins were performed as described in 2.4.2.2. The CSDXS proteins were eluted with imidazole at *ca*.100-150 mM. Purification of the FCSDXS and TCSDXS proteins on HiTrap™ chelating HP column is shown in Fig. 3.17. High concentration of salts in the elution buffer was removed by HiTrap™ desalting column (Sephadex G-25, Superfine). Purified FCSDXS and TCSDXS proteins were further characterized.

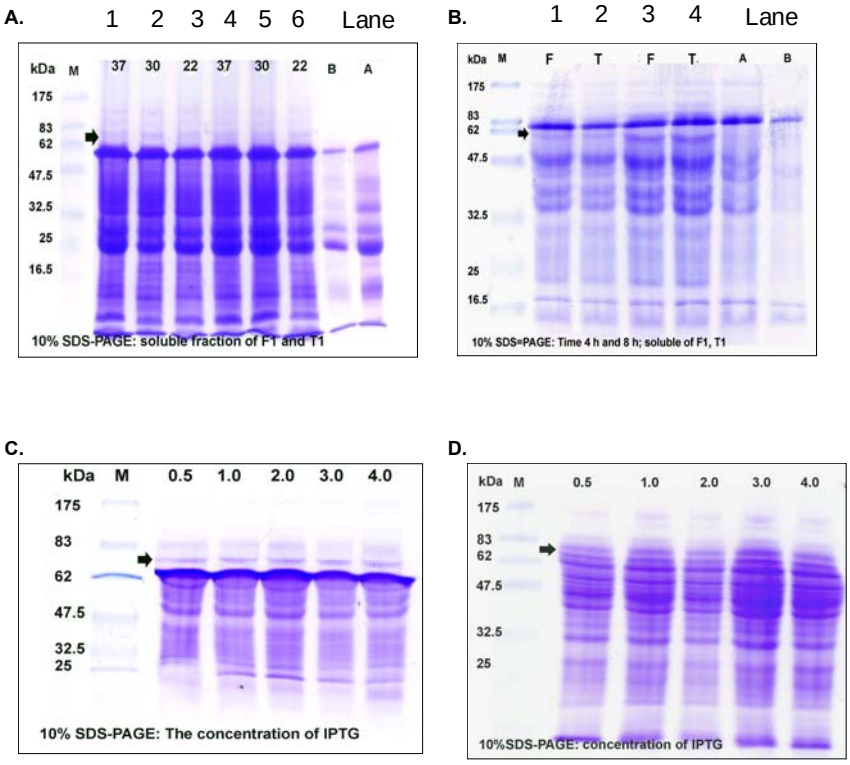


Figure 3.16 Optimization of inductied conditions on the FCSDXS and TCSDXS proteins expressions analyzed by coomassie blue staining 10% SDS-PAGE. Lane A and B are soluble fractions of *E. coli* XL1 blue MRF' and *E. coli* XL1 blue MRF'-pQE30.

A. Effects of induction temperatures at 37°C , 30°C, 22°C for truncated (lane 1-3) and full-length (lane 4-6),

B. Effects of induction periods for 4 h and 8 h for truncated (lane 1, 3) and full-length (lane 2, 4),

C. Effects of IPTG concentrations at 0.5, 1.0, 2.0, 3.0 and 4.0 mM for full-length protein,

D. Effects of IPTG concentrations at 0.5, 1.0, 2.0, 3.0 and 4.0 mM for truncated protein.

Table 3.4 Table of purification of the FCSDXS and TCSDXS proteins expressed from *E. coli* XL1 blue MRF' harboring pQE30-FCSDXS and *E. coli* XL1 blue MRF' harboring pQE30-TCSDXS, respectively.

Procedure	Full-length CSDXS		Truncated CSDXS	
	Total protein (mg)	Yield (%)	Total protein (mg)	Yield (%)
Cell extract	508	100	624	100
HiTrap™ chelating/HiTrap™ desalting	1.45	0.28	1.45	0.23

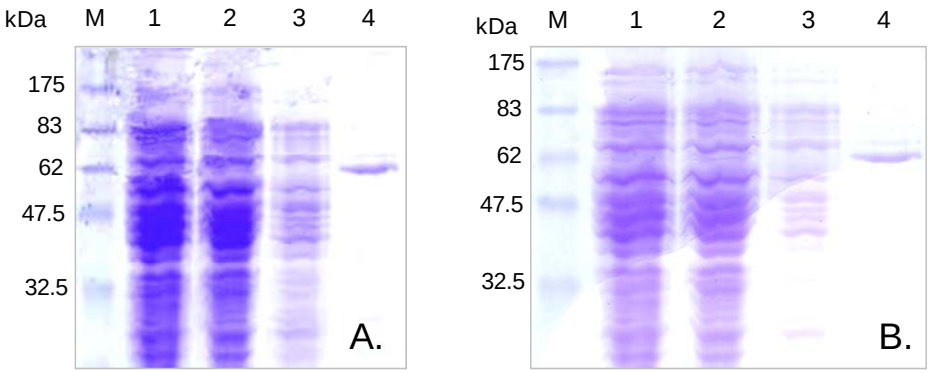


Figure 3.17 The coomassie blue staining 10% SDS-PAGE of the FCSDXS (A) and TCSDXS (B) proteins after purification by HiTrap™ chelating column. M is protein marker; lane 1: cell extract; lane 2: flow through fraction; lane 3: washed fraction; lane 4: eluted fraction.

As judged by SDS-PAGE, the purified proteins contain major band at 62 kDa and minor band at 70 kDa. This may suggested that the FCSDXS and TCSDXS proteins probably degraded during protein purification. The purified FCSDXS and TCSDXS have % yield of 0.28 and 0.23, respectively (Table 3.4).

3.2.5 Determination of the CSDXS enzyme activity

In this study, the CSDXS enzyme activity of the purified TCSDXS was determined as described in 2.4.3. Theoretically, the DXS catalyzed the reaction in the presence of substrates, which are sodium pyruvate and D-glyceraldehyde 3-phosphate and cofactor, thiamine diphosphate. The product of catalytic reaction is 1-deoxy-D-xylulose 5-phosphate (DXP). However, the DXP must be verified. Therefore, the couple assay was designed to couple DXP with 2*C*-methyl-D-erythritol 4-phosphate synthase (MEPS), the second enzyme in the DXP pathway that obtained from the recombinant strain of *E. coli* XL1 blue MRF' harboring ECDXR (cf. Seetang-Nun).

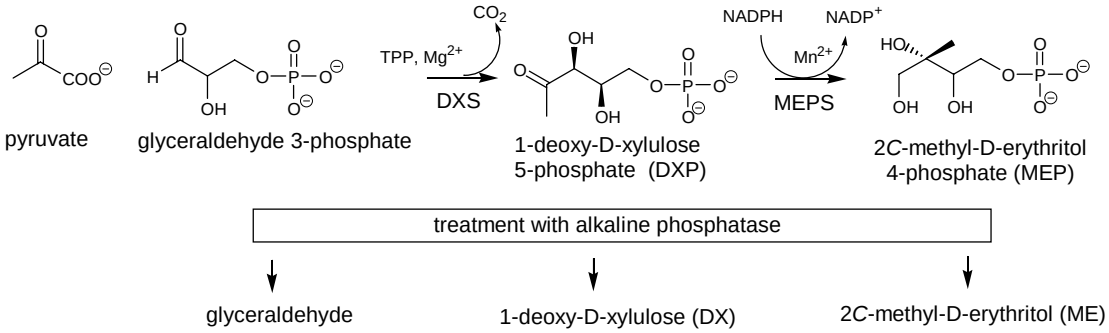


Figure 3.18 The catalytic reaction of the DXS protein coupled with 2*C*-methyl-D-erythritol 4-phosphate synthase (MEPS) and reaction products after alkaline phosphatase treatment.

The utilization of DXP in the couple reaction confirmed that the DXP was formed in the assay mixture (Fig. 3.18). Since the phosphate compounds could not separate on Silica gel 60 TLC plate, the reaction mixture was therefore treated with alkaline phosphatase. As shown in Fig.3.19, lanes 1-3 are control assay mixtures in which omitted of the enzyme, co-substrate and co-factor, respectively. Lane 4 and 5 are assay mixtures of the TCSDXSs after storage at -20 °C and freshly prepared. The assay reaction of couple with the MEPS is shown in lane 6. The result shows that the reaction product was formed from reaction of freshly prepared TCSDXS at *Rf* value of 0.47 (blue), which is transformed to the product at *Rf* value of 0.62 in reaction of lane 6. This result suggested that the freshly prepared TCSDXS could catalyze the reaction of condensation of pyruvate and D-glyceraldehyde 3-phosphate, afforded the reaction product of 1-deoxy-D-xylulose 5-phosphate. To elucidate the reaction product by mean of NMR spectrometry, the preparation of isotopic labeled of [2-^{13}C]pyruvate in the assay mixture may be used according to method of Hecht *et al*. (2001).

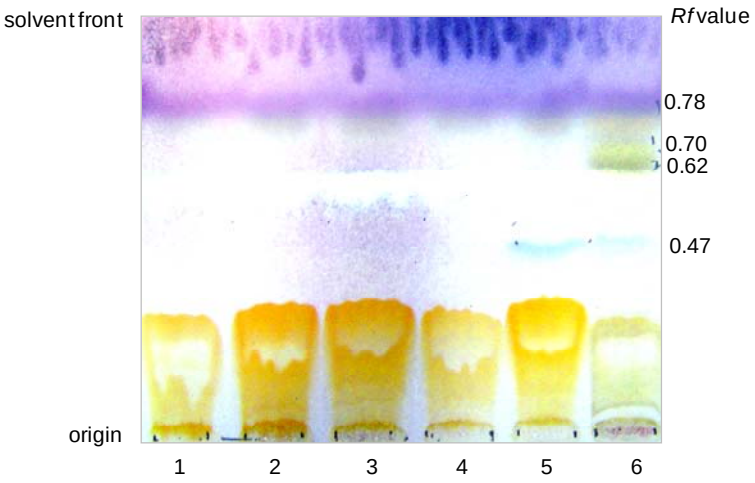


Figure 3.19 TLC chromatogram of the DXS activity assay mixtures after spraying with anisaldehyde/H_2SO_4 reagent . TLC plate: Silica gel GF_{254}; solvent system: isopropanol/ethyl acetate/H_2O (6:2:1). Loading volume is 50 µl.

Lane 1, 2, 3 are control assay mixtures without protein fraction, DL-glyceraldehyde 3-phosphate and thiamine diphosphate, respectively. Lane 4, 5 are assay mixtures containing the TCSDXS proteins (4: after -20 °C storage; 5: freshly prepared). Lane 6 is assay mixture containing 2*C*-methyl-D-erythritol 4-phosphate synthase and NADPH in the presence of Mn^{2+}. All assay mixtures were treated with alkaline phosphatase before loading.

3.2.6 Molecular weight determination of the CSDXS proteins

The lost of CSDXS activity during storage and degraded proteins found during protein purification led us consideration of the CSDXS protein carefully. In order to check the proteins expressed from recombinant *E.coli* are correct, the isolated FCSDXS and the TCSDXS proteins were determined for the molecular weight (2.4.6). Two forms of samples were prepared, in solution and in SDS-polyacrylamide gel. The partial amino acid sequences of tryptic peptides was determined. Nevertheless, as shown in Fig. 3.20A, the proteins were separated on 10% SDS-PAGE before excising gel. The apparent bands are about 62 kDa (lower than expected). The bands at 25 kDa appeared in the FCSDXS protein (Fig. 3.20A). However, this 62 kDa protein samples was eluted and reanalyzed by SDS-PAGE at BIOTEC. Unfortunately, SDS-PAGE (Fig.

3.20B) shows many bands of protein and more degraded proteins were found in the FCSDXS than the TCSDXS. This result indicates that our proteins are unstable and ease to be degraded. With this experiment, the amino acid sequences were not obtained.

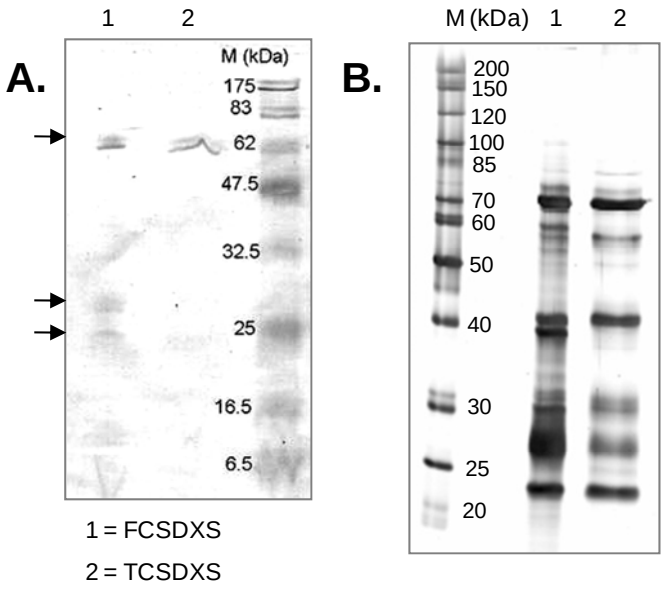


Figure 3.20 SDS-PAGE of the purified FCSDXS and TCSDXS proteins. A. protein pattern visualized by coomassie blue stain; B. protein pattern of 62 kDa eluted from the SDS-PAGE visualized by silver stain.

On the other hand, the 62 kDa sample eluted from SDS-PAGE were performed a MALDI-TOF MS for their molecular weights determination. The range of molecular weight up to 100 kDa has been plotted and recorded. The MALDI-TOF MS spectra of the FCSDXS and TCSDXS are shown in Fig. 3.21. Partial spectrum is displayed since the protein with higher molecular weight than 25 kDa, was not detected. The base peaks at 11 kDa were found in both spectra. The data concluded that the purified proteins were degraded easily.

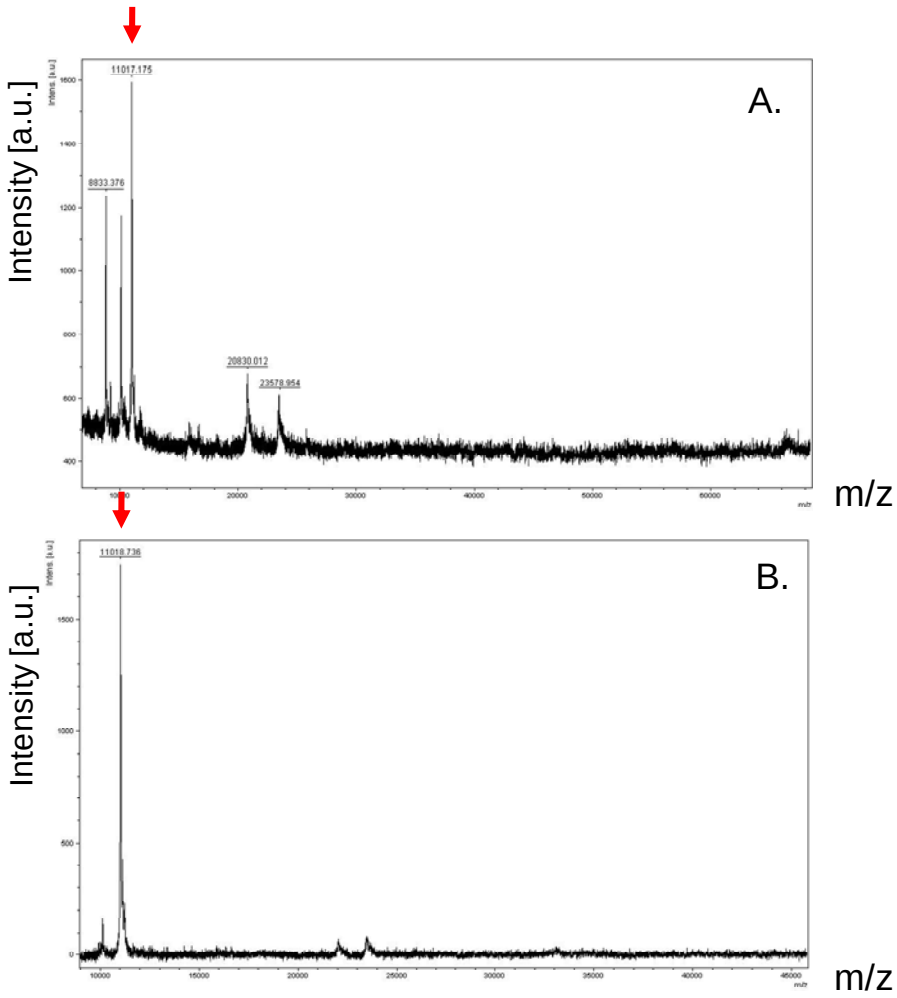


Figure 3.21 The MALDI-TOF MS spectra of the FCSDXS (A) and TCSDXS (B) proteins. The spectra were recorded in the linear positive ion mode.

CHAPTER 4

DISCUSSION

In this study, a *csdxs* cDNA has been isolated from *C. stellatopilosus* young leaves. By homology-based PCR and RACE techniques, the *csdxs* cDNA of 2,308 bp was obtained and submitted to the DDBJ/EMBL/GenBank databases (http://www.ddbj.nig.ac.jp/) as an accession number of AB354578. The ORF of CSDXS contains 2,163 bp, encoding 720 amino acid residues. Multiple alignments of CSDXS revealed high identity of CSDXS to other plant DXSs of about 68% - 89% (Fig. 3.9). As shown in Fig. 4.1, the CSDXS showed identity to the DXS from *C. annuum* (84%), *A. thaliana* (83%) and bacteria *E. coli* (47%).

In the NCBI data bank suggested that at least two isoforms of DXSs, namely DXS class I and DXS class II, existed. However, the evolution of structure and function of DXS could not be related to the phylogenetic tree as suggested by Krushkal *et al*. (2003). For instance (Fig. 3.9), the DXSs class 2 from *M. truncatula* (AJ430048), *P. abies* (EF688333), *C. anuuum* (O78328) and *H. brasiliensis* (AY502939) distributes different distances in the phylogenetic tree. It can be noted that the phylogenetic tree is constructed based on the relationship between the level of mRNA expression and the location of secondary metabolites biosynthesis. For example, the *dxs* genes isolated from leaves of *C. annuum* and *L. esculentum,* play an important role during chromoplast development (Bouvier *et al*., 1998; Lois *et al*., 2000) belonged to the same class. Whereas, the *dxs* genes isolated from secretory glands of *M. piperita* and from cell suspension culture of *C. roseus* belong to another group of DXS (Lange *et al*., 1998; Chahed *et al*., 2000). It seems the structure and function of DXS an organ-dependent. It is likely that the *csdxs* cDNA was isolated from leaves, therefore, it may be closed to the DXS of *C. annuum* and *L. esculentum* than *M. piperita*

and *C. roseus*. According to this finding, it is clearly shown that the *csdxs* gene belonged to the same group as *C. annuum* and proposed to exhibit the role in the chloroplast development.

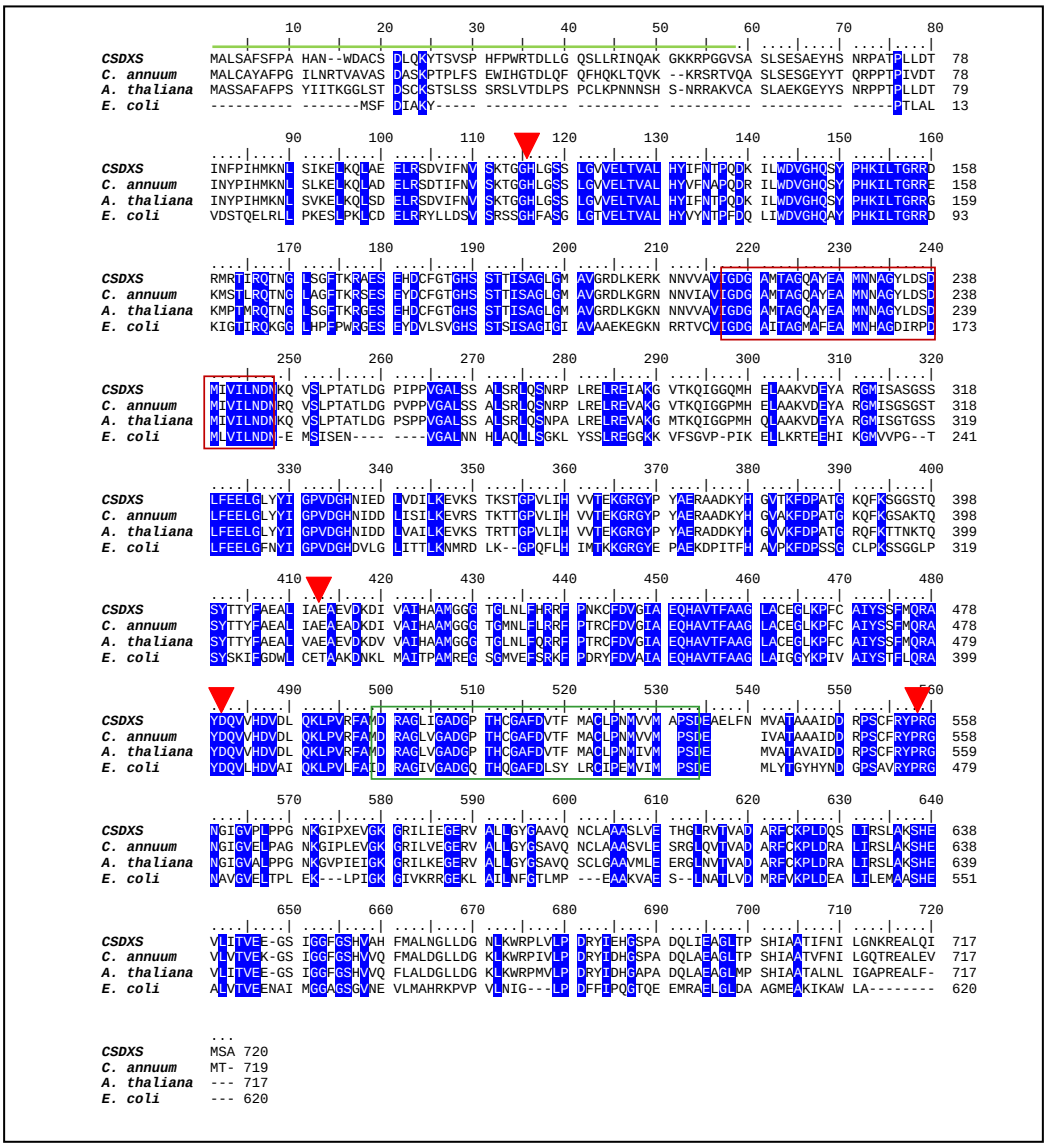


Figure 4.1 Multiple alignments of the deduced amino acid sequence of DXSs obtained from *C. stellatopilosus* (this study), *C. annuum, A. thaliana*, and *E.coli*. Green line indicates length of chloroplast transit peptide. Blue color denotes the identities between sequences. Red and green boxes indicate the motifs corresponding to the putative thiamine diphosphate-binding site and the transketolase motif, respectively.

Consideration of the CSDXS with DXS from plant and bacteria, it is clear that the DXSs from plants are distinct from bacterial DXS as they contain the chloroplast transit peptide, but bacteria do not. This feature suggested that the translocation of the DXS into the plastid appear during chloroplast development. Study on the CLA1 of *A. thaliana*, it was shown to be a transketolase-like enzyme and essential for chloroplast development. It is a nuclear gene but the expression of CLA1 occurs in the chloroplast (Estévez *et al*. 2000). The chloroplast transit peptide is a short chain peptide that located at the NH_2-terminus region. It needs to enter the double-membrane of chloroplast. The common features of the NH_2-terminus suggest that it penetrates the membrane, possibly aiding in forming a channel through which the function protein is transported. After entering the chloroplast, the transit sequence is removed (Schleiff and Klösgen, 2001).

Usually, the chloroplast transit peptide can be predicted by TargetP and ChloroP. However, the results need to be confirmed experimentally. Although, the enrichment of serine and threonine in the chloroplast transit peptide dedicates its characteristic for hydrophobicity (Krushkal *et al*., 2003). However, characteristics of chloroplast transit peptide among three plants are distinct (Fig. 4.1). The multiple alignments of CSDXS and DXS from *C. annuum* and *A. thaliana*, indicates that they do not share any identity and a common characteristic for the DXS in higher plants.

Finding the motifs of TPP-binding site and transketolase motif in the CSDXS suggests that the CSDXS belongs to the transketolase family. The conserved residues are found in the alignment. As shown in the Fig 4.1., the histidine residue (H114 of CSDXS) is suggested to be involved in proton transferring during catalysis (Bouvier *et al*., 1998). The glutamic residue (E431 of CSDXS) is thought to be specific for transketolase activity (Sprenger *et al*., 1997). Others residues of D462 and R539 are putative catalytic residue in the substrate (glyceraldehydes 3-phosphate) binding site (Xiang *et al*., 2007). From this finding, the data is suggested that the

CSDXS obtained from this study is the genuine DXS, catalyzing the transketolase-dependent reaction. However, its function needs to be confirmed.

The physiological role of *csdxs* gene in plaunotol biosynthesis was determined in different tissues including the chloroplastic tissue of leaf and twig and non-chloroplastic tissue of root at the transcriptional levels. The results clearly suggested that the *csdxs* transcripts appear in leaf and twig, where plaunotol are accumulated. In contrast, the *csdxs* transcript and plaunotol content were not detected in root. These results suggested that the absence of the *csdxs* gene in the root may be caused by an absence of essential organelle such as chloroplast in root. Altogether with the results of the CSDXS protein located in chloroplast and the plaunotol accumulated in the leaf, the CSDXS may, therefore, regulate the plaunotol biosynthesis. Interestingly, the *csdxs* gene is expressed in leaf and twig but the different amount of plaunotol were detected. Plaunotol content in twig is ¼-fold of which found in leaf. Based on the finding that plaunotol accumulates in the palisade layer of leaf as oil globules (Sitthithaworn *et al*., 2006), it could suggest that plaunotol probably is translocated to the storage site in leaf.

Our results concluded that the expression of *csdxs* mRNA appeared in the chloroplastic tissues and played a role in plaunotol biosynthesis. It has been shown that the DXS is the rate limiting enzyme in isoprenoid biosynthesis in plastid (Estévez *et al*., 2001). The transgenic *dxs* in *A. thaliana* could accumulate different levels of various isoprenoids such as chlorophylls, tocopherols, carotenoids, abscisic acids and gibberellins in related to the change of the DXS level. Therefore, the expression of *csdxs* gene in *C. stellatopilosus* may increase the plaunotol production.

To characterize the CSDXS protein, heterologous expressions of genes encoding the full-length and truncated CSDXS were investigated in *E. coli*. The FCSDXS and the TCSDXS were successfully expressed and purified with a single affinity column chromatography. The apparent molecular masses of both proteins were 62 kDa, which did not agree with the calculated masses of 77 kDa for the FCSDXS and 71 kDa for the TCSDXS. For determination of CSDXS activity,

the TLC analysis was developed to follow the reaction, however, low sensitivity still remained. Our results showed that the TCSDXS could catalyze the condensation of pyruvate and D-glyceradehyde 3-phosphate, which the reaction product was detected by couple assay with 2*C*-methyl-D-erythritol 4-phosphate synthase. The disadvantage of TLC analysis is that it is unable to determine the kinetic parameters such as K_M and V_{max}. Unlike the previous studies, the [2-^{14}C]pyruvate was used to assay the DXS activity reaction of DXS from *E. coli* (Sprenger *et al*., 1997), *M. piperita* (Lange *et al*., 1998) and *C. annuum* (Bouvier *et al*., 1998). Limitation of using the radiolabeled compound in our lab is difficult to analyze and characterize the CSDXS enzyme. In addition, the reaction product needs to compare with the authentic deoxyxylulose. One may suggest to use [2-^{13}C]pyruvate for following the reaction product by NMR spectroscopy.

Attempts on the reaction assay have also suffered from the stability of enzyme. The TCSDXS activity lost when the protein was storage in the solution, even though at -20 $^{\circ}$C. This evidence is not surprise since there have been reports the DXS activities from *E. coli* and *C. roseus*. They suggested that the DXS is a very unstable enzyme and easily destroyed by the proteases (Sprenger *et al*., 1997; Chahed *et al*., 2003). This supports our results that the CSDXS tends to degrade and lose its activity.

Plaunotol biosynthesis in *C. stellatopilosus* has been studied for a decade. Up to now, 5 enzymes from *C. stellatopilosus* have been studied and reported. In this study, the first enzyme, 1-deoxy-D-xylulose 5-phosphate synthase was cloned and characterized. It catalyzes the condensation of pyruvate and glyceraldehydes 3-phosphate in the TPP-dependent reaction. Earlier, the 2*C*-methyl-D-erythritol 4-phosphate synthase (MEPS) has been cloned and characterized. It catalyzes the skeleton rearrangement of 1-deoxy-D-xylulose 5-phosphate to 2*C*-methyl-D-erythritol 4-phosphate in the NADPH-dependent reaction. The expression of 2*C*-methyl-D-erythritol 4-phosphate synthase mRNA appeared in leaf but not in root (Sirisuntipong, 2006, Wungsintaweekul *et al.*, 2008).

For the late step after formation of isoprene units, Sitthithaworn *et al*. (2000) reported that geranylgeranyl diphosphate synthase (GGPPS) from *C. stellatopilosus* catalyzes the condensation of farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP) (Sitthithaworn *et al*., 2000). Next step, the GGPP is then cleaved by GGPP phosphatases, yielding geranylgeraniol (GGOH) and phosphate moieties (Nualkaew *et al*., 2005; Nualkaew *et al*., 2006). In the final step, GGOH is hydroxylated at position 18, forming plaunotol catalyzed by GGOH 18-hydroxylase (Tansakul and De-Eknamkul, 1998). In *C. stellatopillosus*, an electron microscope study by Sitthithaworn *et al*. (2006) showed that plaunotol was accumulated in the palisade layer of the leaf as oil globules. This is suggesting that the plaunotol biosynthesis is catalyzed by the enzymes present in chloroplasts. This evidence is in agreement with the presences of a putative chloroplast targeting sequences that found in the DXS (this study), MEPS (Sirisuntipong 2007; Wungsintaweekul *et al*., 2008) and GGPPS (Sitthithaworn *et al*. 2001).

Information of the *csdxs* gene and the CSDXS protein expression obtained from this study fulfills the knowledge of plaunotol biosynthesis, which may be useful in the application for plaunotol production via the molecular approach and metabolic engineering.

CHAPTER 5

CONCLUSIONS

5.1. The *csdxs* cDNA has been isolated from *C. stellatopilosus* young leaves. By homology-based PCR and RACE techniques, the *csdxs* cDNA contains 2,308 bp and the ORF composes of 2,163 bp, encoding 720 amino acid residues. The *csdxs* cDNA was submitted to the DDBJ database (http://www.ddbj.nig.ac.jp/) as an accession number of AB354578.

5.2. PSI-BLAST search revealed the CSDXS showed high identity (68-89%) to other plants DXSs. BLAST P suggested that this protein belongs to the transketolase and dehydrogenase E1 component family. Searching for the TPP-binding and the transketolase motifs in the CSDXS revealed that the CSDXS has the consensus sequence for the TPP-binding motif (designated as $GDG(X)_8E(X)_4A(X)_{11}NDN$, where X denotes any amino acid) at position 216-246 and the consensus sequence for the transketolase motif (designated as DRAG-X_{28}-P-X-D) at position 498-532 of the CSDXS protein. The CSDXS carries the catalytic amino acid residues at H114, E431, D462 and R539, which are essential for enzyme catalysis and glyceraldehydes 3-phosphate binding site.

5.3. The topology search for subcellular prediction, the CSDXS protein was predicted with TargetP v1.0 to have a chloroplast transit peptide (cTP) of 0.325, suggesting that the CSDXS protein locates in the chloroplast. The cleavage site of the transit peptide is at 57 amino acid residues from NH_2-terminus as predicted with ChloroP 1.1. The CSDXS protein has calculated masses of 77,623 Da for full-length protein and 71,353 Da for truncated protein.

5.4. The *csdxs* gene existing in leaf and twig is shown to play an important role in plaunotol biosynthesis. The *csdxs* gene is a nuclear gene, which is expressed and located in the chloroplast. Localization of plaunotol and *csdxs* gene suggests that the plaunotol biosynthesis is likely to appear in chloroplast.

5.5. Heterologous expressions of the FCSDXS and the TCSDXS proteins in *E. coli* afforded the soluble proteins as apparent band of 62 kDa as judged by SDS-PAGE. The FCSDXS and the TCSDXS are purified with the affinity column, simultaneously with the desalting column with a yield of 0.28% and 0.23%, respectively. Only “the pseudomature” TCSDXS catalyzes a condensation of pyruvate and D-glyceraldehyde 3-phosphate in the TPP-dependent reaction. SDS-PAGE and MALDI-TOF MS analyses suggest that the FCSDXS and the TCSDXS are very unstable and tend to degrade.

REFERENCES

นันทวัน บุณยะประภัศร, บรรณาธิการ. 2532. เปล้าน้อย. *จุลสารโครงการศูนย์ข้อมูลสมุนไพร มหาวิทยาลัยมหิดล*. 6(3), 1-6.

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

Altincicek, B., Kollas, A-K., Sanderbrand, S., Wiesner, J., Hintz, M., Beck, E., Jomaa, H. 2001. GcpE is involved in the 2-*C*-methyl-D-erythrito 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli. J. Bacteriol*. 183, 2411–2416.

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

Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A. and Camara, B. 1998. Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiol*. 117, 1423-1431.

Bouvier, F., Isner, J.C., Dogbo, O., Camara, B. 2005. Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. *Trends Plant Sci*. 10, 187-194.

Bradford, MM. 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72, 248-254.

Burlat, V., Oudin, A., Courtois, M., Rideau, M., St-Pierre, B. 2004. Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. *Plant J*. 38, 131-141.

Chahed, K., Oudin, A., Guivarch'h, N., Hamdi, S., Chnieux, J.C., Rideau, M., Clastre, M. 2000. 1-Deoxy-D-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. *Plant Physiol. Biochem.* 38, 559-566.

De-Eknamkul, W. and Potduang, B. 2003. Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. *Phytochemistry.* 62, 389–398.

Eisenreich, W., Rohdich, F., Bacher, A. 2001. Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant. Sci*. 6, 78–84.

Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M., and Bacher, A. 1998. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chem. Biol.* 5, 221-233.

Emanuelsson, O., Nielsen, H., von Heijne, G. 1999. CholroP, a neural networks-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* 8, 978-984.

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

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S. and León, P. 2001. 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* 276, 22901-22909.

Estévez, J.M., Cantero, A., Romero, C., Kawaide, H., Jimmenez. L. F., Kuzuyama, T., Seto, H., Kamiya, Y. and León, P. 2000. Analysis of the expression of *CLA1*, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-*C*-methyl-D-erythritol-4-phosphate pathway in Arabidopsis. *Plant Physiol*. 124, 95-103.

Flournoy, D.S., and Frey,P.A. 1989. In activation of the pyruvate dehydrogenase complex of *Escherichia coli* by fluoropyruvate. *Biochemistry.* 28, 9594-9602.

Gong, Y.F., Liao, Z.H., Guo, B.H., Sun, X.F., Tang, K.X. 2006. Molecular cloning and expression profile analysis of *Ginkgo biloba DXS* gene encoding 1-deoxy-D-xylulose 5-phosphate synthase, the first committed enzyme of the 2-*C*-methyl-D-erythritol 4-phosphate pathway. *Planta Med.* 72, 329-335.

Han, Y.S., Roytrakul, S., Verberne, M.C., Heijden der van, R., Linthorst, H.J.M. and Verpoorte, R. 2003. Cloning of a cDNA encoding 1-deoxy-D-xylulose 5-phosphate synthase from

Morinda citrifolia and analysis of its expression in relation to anthraquinone accumulation. *Plant Sci*. 164, 911-917.

Hawkins CF, Borges A, Perham RN. 1989. A common structural motif in thiamin pyrophosphate-binding enzymes. *FEBS Lett.* 255, 77-82.

Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kis, K., Bacher, A., Arigoni, D., Rohdich, F. 2001. Studies on the nonmevalonate pathway to terpenes: the role of the GcpE (ispG) protein. *Proc. Natl. Acad. Sci*. *USA*. 26, 14837–14842.

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-Derythritol 2, 4-cyclodiphos-phate. *Proc. Natl. Acad. Sci. USA*. 97, 2486-2490.

Hirai, N., Yoshida, R., Todoroki, Y., Ohigashi, H., 2000. Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. *Biosci. Biotechnol. Biochem*. 64, 1448–1458.

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 Sci*. 169, 571-578.

Kim, 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.

Kishimoto, S. and Ohmiya, A. 2006. Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium* Ramat.). *Physiol. Plant*. 128, 436-447.

Kitazawa, E. and Ogiso, A. 1981. Two diterpene alcohols from *Croton sublyratus*. *Phytochemistry.* 20, 287-289.

Koga, T. Kawada, H., Utsui, Y., Domon, H., Tshii, C. and Yasuda, H. 1996. *In vitro* and *in vivo* antibacterial activity of plaunotol, a cytoprotective antiulcer agent, against *Helicobacter pylori*. *J. Antimicrob. Chemother*. 37, 919-929.

Koga, T., Inoue, H., Ishii, C., Okazaki, Y., Domon, H. and Utsui, Y. 2002. Effect of plaunotol in combination with clarithromycin or amoxicillin on *Helicobacter pylori in vitro* and *in vivo*. *J. Antimicrob. Chemother*. 50, 133-136.

Krushkal, J., Pistilli, M., Ferrell, K.M., Souret, F.F. and Weathers, P.J. 2003. Computational analysis of the evolution of the structure and function of 1-deoxy-D-xylulose-5-phosphate synthase, a key regulator of the mevalonate-independent pathway in plants. *Gene*. 313, 127-138.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lange, B.M., Wildung, M.K., McCaskill, D., and Croteau, R. 1998. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proc. Natl. Acad. Sci. USA*. 95, 2100-2104.

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

Lois, L.M., Rodríguez-Concepción, M., Gallego, F., Campos, N. and Boronat, A. 2000. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *Plant J*. 22, 503-513.

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 terpeniods: YchB protein of *Escherichia coli* phosphorylates the 2-hydroxy group of 4-diphosphocytidyl-2*C*-methyl-D-erythitol. *Proc. Natl. Acad. Sci. USA*. 97, 1062-1067.

Mandel, M. A., Feldmann, K. A., Herrera-Estrella, L., Rocha-Sosa, M., and Leon, P. 1996. CLA1, a novel gene required for chloroplast development, is highly conserved in evolution. *Plant J.* 9, 649-658.

Matsumoto, Y., Hamashima, H., Masuda, K., Shiojima, K., Sasatsu, M. and Arai, T. 1998. The antibacterial activity of plaunotol against *Staphylococcus aureus* isolated from the skin of patients with atopic dermatitis. *Microbios*. 96, 149-155.

Moehs, C.P., Tian, L., Osteryoung, K.W. and Della Penna, D. 2001. Analysis of carotenoid biosynthetic gene expression during marigold petal development. *Plant Mol. Biol.* 45, 281-293.

Mori, H. and Cline, K. 2001. Post-translational thylakoid transport by the ΔpH-dependent (TAT) and cpSecA-dependent (Sec) translocation pathways. *Biochim. Biophys. Acta*. 1541, 80-90.

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.

Müller, C., Schwender, J., Zeidler, J. and Lichtenthaler, H.K. 2000. Properties and inhibition of the first two enzymes of the non-mevalonate pathway of isoprenoid biosynthesis. *Biochem. Soc. Trans.* 28, 792-793.

Murakami, K., Okajima, K., Harada, N., Isobe, H., Liu, W., Johno, M. and Okabe, H. 1999. Plaunotol prevents indomethacin-induced gastric mucosal injury in rats by inhibiting neutrophill activation. *Aliment. Pharmacol. Ther*. 13, 521-530.

Nilubol, N. 1992. Process for extraction and purification of plaunotol. *United States patent* Patent No. 5,264, 638.

Nualkaew, N., De-Eknamkul, W., Kutchan, T.M. and Zenk, M.H. 2005. Geranylgeraniol formation in *Croton stellatopilosus* proceeds via successive monodephosphorylations of geranylgeranyl diphosphate. *Tetrahedron Lett*. 46, 8727-8731.

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, 1613-1620.

Ogiso, A., Kitazawa, E., Kurabayashi, S., Komai, N., Natsunuma, N., Kataumi, S. 1985. (CS-684), A new antipeptic ulcer agent, *Sankyo Kenkyusho Nempo*. 37, 1–39.

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

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, 3117-3123.

Ponglux, D., Wongseripipatana, S., Phadungcharoen, T., Ruangrungsri, N., Likhitwitayawuid, K. 1987. *Medicinal Plants*. The First Princess Chulabhorn Science Congress Bangkok, Thailand.

Querol, J., Rodríguez-Concepción, M., Boronat, A., Imperial, S. 2001. Essential role of residue H49 for activity of *Escherichia coli* 1-deoxy-D-xylulose 5-phosphate synthase, the enzyme catalyzing the first step of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. *Biochem. Biophys. Res. Commun*. 289, 155-160**.**

Qureshi, N. and Porter, J. W. 1981. Conversion of acetyl-coenzyme A to isopentenyl pyrophosphate in J. W. Porter and S. L. Spurgeon (eds.) *Biosynthesis of isoprenoid compounds*. John Wiley and Sons, New York: 47-94.

Reynen, M., Sahm, H., 1988. Comparison of the structural genes for pyruvate decarboxylase in different *Zymomonas mobilis* strains. *J. Bacteriol.*170, 3310-3313.

Rodríguez-Concepcion, M., and Boronat, A. 2002. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol.* 130, 1079-1089.

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 isopreniods: YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2-*C*-methylerythritol. *Proc. Natl. Acad. Sci. USA.* 96, 11758-11763.

Rohdich, F., Hecht, S., Bacher, A. and Eisenreich, W. 2003a. Deoxyxylulose phosphate pathway of isoprenoid biosynthesis. Discovery and fuction of *ispDEFGH* genes and their cognate enzymes. *Pure Appl. Chem*. 75, 393-405.

Rohdich, F., Zepeck, F., Adam, P., Hecht, S., Kaiser, J., Laupitz, R., Grawert, T., Amslinger, S., Eisenreich, W., Bacher, A. and Arigoni, D. 2003b. The deoxyxylulose phosphate pathway of isoprenoid biosynthesis: studies on the mechanisms of the reactions catalyzed by *Isp*G and *Isp*H protein. *Proc. Natl. Acad. Sci. USA*. 100, 1586-1591.

Rohmer, M. 1999. The discovery of a mevalonate independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep*. 16**,** 565-574.

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

Schenk, G., Layfield, R., Candy, J.M., Duggleby, R.G., Nixon, P.F. 1997. Molecular evolutionary analysis of the thiamine-diphosphate-dependent enzyme, transketolase. *J. Mol. Evol*. 44, 552-572.

Schleiff, E. and Klösgen, R.B. 2001. Without a little help from 'my' friend: direct insertion of proteins into chloroplast membranes? *Biochim. Biophys. Acta*. 1541, 22-33.

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.

Seetang-Nun, Y., Sharkey, T.D. and Suvachittanont,W. 2007. Isolation and characterization of two distinct classes of *DXS* genes in *Hevea brasiliensis*. *DNA seq.* DOI: 10.1080/10425170701576768.

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.

Sirisuntipong, T. 2007. cDNA Cloning of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Croton stellatopilosus* Ohba leaves. Master Thesis, Prince of Songkla University.

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, 197-202.

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

Souret, F.F.,Weathers, P.J., Wobbe, K.K. 2002. The mevalonate-independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. *In Vitro Cell. Dev. Biol.* 38, 581-588.

Sprenger, G.A., Schörken, N., Wiegert, T., Grolle, S., Graaf, A.A., Taylor, S., Begley, T.P., Bringer-Meyer, S. and Sahm, H. 1997. Identification of thiamine-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isopreniods, thiamine and pyridoxal. *Proc. Natl. Acad. Sci. USA*. 94, 12857-12862.

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

Takagi, A., Koga, Y., Aiba, Y., Kabir, A.M., Watanabe, S., Ohta-Tada, U., Osaki, T. Kamiya, S. and Miwa, T. 2000. Plaunotol suppresses interleukin-8 secretion induced by *Helicobacter pylori*: therapeutic effect of plaunotol on *H. pylori* infection. *J. Gastroenterol. Hepatol*. 15, 374-380.

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

Takahashi, S., Kuzuyama, T., Watanabe, H., and Seto, H. 1998. A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2*C*-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpeniod biosynthesis. *Proc. Natl. Acad. Sci. USA.* 95, 9879-9884.

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.

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

Umlauf, D., Zapp, J., Becker, H., Adam, K.P. 2004. Biosynthesis of the irregular monoterpene artemisia ketone, the sesquiterpene germacrene D and other isoprenoids in *Tanacetum vulgare L*. (Asteraceae). *Phytochemistry*. 65, 2463-2470.

Ushiyama, S., Matsuda, K., Asai, F. and Yamazaki, M. 1987. Stimulation of prostaglandin production by (2*E*, 6*Z*, 10*E*)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadeca-tetraen-1-ol (plaunotol), a new anti ulcer drug, *in vitro* and *in vivo*. *Biochem*. *Pharmacol*. 36, 369-375.

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. 1994. Shoot regeneration from tissue cultures of *Croton sublyratus* Kurz. Master Thesis, Chulalongkorn University.

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., 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.

Walter, H.M., Hans, J., Strack, D., 2002. Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorhizal roots. *Plant J.* 31, 243-254.

Wungsintaweekul, J. 2001. Enzymes of the alternative terpenoid pathway in *Escherichia coli*. Ph.D Thesis, Technical University of Munich, 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.

Wungsintaweekul, J., Sirisuntipong, T., Kongduang, D., Losuphanporn, T., Ounaroon, A., Tansakul, P. and De-Eknamkul, W. 2008. Transcription profiles analysis of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and 2*C*-methyl-D-erythritol 4-phosphate synthase in plaunotol biosynthesis from *Croton stellatopilosus. Biol. Pharm. Bull*. 31, 852-856.

Xiang, S., Usunow, G., Lange, G., Busch, M and Tong, L**.** 2007. Crystal structure of 1-deoxy-D-xylulose 5-phosphate synthase (Dxs), a crucial enzyme for isoprenoids biosynthesis. *J. Biol. Chem*. 282, 2676-2682.

Zhang, D., Yang, Q., Bao, W., Zhang, Y., Bin Han, B., Xue, Y and Cheng, Y. 2005. Molecular cytogenetic characterization of the *Antirrhinum majus* genome. *Genetics***,** 169, 325-335.

Appendix

Table 6.1 Chemicals and solvents used in this study

Chemical	Source
1,4-Dithiothreitol (DTT)	Biomol, Germany
3,5-Diaminobenzoic acid	Sigma, USA
Acetic acid, glacial	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Acrylamide (40%)	Plus One^{TM} Acrylamide, Amersham Biosciences, USA
Agar, Bacto	Himedia laboratories, Ltd., India
Agarose D-1 Low EEO	Research Organics, USA
Ammonium persulfate	USB cooperation, USA
Ampicillin disodium	Bio Basic INC, Canada
Bis-acrylamide (2%)	PlusOne^{TM} Acrylamide, Amersham Biosciences, USA
Brilliant Blue G-250	USB cooperation, USA
Brilliant Blue R-250	USB cooperation, USA
Bovine serum albumin	Sigma-Aldrich, USA
Calcium chloride	Merck, USA
Casein hydrolysate	Himedia laboratories, Ltd., India
Chloroform	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Dimethylformamide	Bio Basic INC, Canada
Ethanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Ethidium bromide	Biolabs, UK
Ethyl acetate	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Glycerol	Vidhyasom Co., Ltd., Bangkok, Thailand
Glycine	USB cooperation, USA
Hydrochloric acid	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Imidazole	USB cooperation, USA
Isopropanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand

Appendix

Table 6.1 Chemicals and solvents used in this study (continued).

Chemical	Source
Magnesium chloride	Bio Basic INC, Canada
Mercaptoethanol	Sigma, USA
Methanol	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Phosphoric acid (85%)	Merck, USA
Pipes	Bio Basic INC, Canada
Potassium chloride	Bio Basic INC, Canada
Sodium azide	USB cooperation, USA
Sodium chloride	Lab-scan Asia Co., Ltd., Bangkok, Thailand
Sodium dodecyl sulfate	USB cooperation, USA
Sulfuric acid	Lab-scan Asia Co., Ltd., Bangkok, Thailand
TEMED	USB cooperation, USA
Trichloroacetic acid	Merck, USA
Tris-(hydroxymethyl) amino	Research Organic, Inc., USA
Tryptone, Bacto	Himedia laboratories Pvt, Ltd., India
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	USB cooperation, USA
Yeast extract, Bacto	Lab-scan Asia Co., Ltd., Bangkok, Thailand

Appendix

Table 6.2 Kits used in this study.

Kit	Composition	Source
A-addition kit	QIAGEN A-Addition Master Mix (5x) Distilled water	Qiagen, Germany
Gel extraction kit	Buffer QG: TAE: Tris-acetate/EDTA or TBE: Tris-borate/EDTA Buffer PB: guanidine hydrochloride and isopropanol Buffer PE add ethanol (2x concentrated) Buffer EB: 10 mM Tris·Cl, pH 8.5	Qiagen, Germany
GFX Micro Plasmid Prep Kit	Solution I: 100 mM Tris-HCl; pH 7.5, 10 mM EDTA, 400 μg/ml RNase I Solution II: 1 M NaOH, 5.3% (w/v) SDS, 65 ml of distilled water Solution III: acetate and chaotropethen Wash buffer: Tris-EDTA buffer, 100 ml absolute ethanol	Amersham Biosciences, UK
PCR cloning kit	Ligation Master Mix (2x concentrated) pDrive Cloning Vector, distilled water	Qiagen, Germany
PCR purification kit	Buffer PB: guanidine hydrochloride and isopropanol Buffer PE add ethanol (2x concentrated) Buffer EB: 10 mM Tris·Cl, pH 8.5	Qiagen, Germany

Appendix

Table 6.2 Kits used in this study (continued).

Kit	Composition	Source
Marathon™ cDNA amplification Kit	BD Marathon™ cDNA Adaptor, AMV reverse transcriptase, first-strand reaction buffer, Second-strand enzyme cocktail, Second-strand buffer, T4 DNA polymerase, T4 DNA ligase, DNA ligation Buffer, Adaptor primer 1 (AP1), Nested adaptor primer 2 (AP2), dNTP mix (2.5 mM), EDTA/glycogen Mix, ammonium acetate, Sterile deionized H_2O	Clontech®, BD Biosciences, USA
RNeasy Plant Mini Kit	Buffer RLT: lysis buffer containing guanidine isothiocyanate Buffer RW1: buffer containing ethanol Buffer RPE: washing buffer containing ethanol Buffer RLC: guanidine hydrochloride RNase-free water	Qiagen, Germany
SuperScript™III reverse transcriptase (RT)	SuperScript™ III reverse transcriptase (RT) 2X reaction Mix: 0.4 mM of each dNTP, 3.2 mM $MgSO_4$, 5 mM $MgSO_4$	Invitrogen, USA
Wizard® PCR Preps DNA purification Kit	Cell Resuspension Solution (CRA) 75 ml Cell Lysis Solution (CLA) 75 ml Neutralization Solution (NSA) 150 ml Wizard® Midipreps DNA Purification Resin 250 ml Column Wash Solution (CWB) 355 ml Midicolumns with Reservoirs 25 ml	Promega, USA

Appendix

Table 6.3 Enzymes used in this study.

Enzyme	EC number	Source
2*C*-Methyl-D-erythritol synthase	EC: 4.6.1.12	Y. Seetang-nun
DNaseI	EC: 3.1.21.1	Biomol, Germany
*EcoR*I	EC: 3.1.23.13	TaKaRa, Japan
KOD *taq* polymerase	EC: 2.7.7.7	Toyobo, Japan
*Kpn*I	EC: 3.1.23.26	NEB, New England Biolabs, USA
Lysozyme from chicken egg white	EC: 3.2.1.17	Biomol, Germany
*Sal*I	EC: 3.1.23.37	TaKaRa, Japan
T4 DNA ligase	EC: 6.5.1.1	NEB, New England Biolabs, USA
TaKaRa *Ex Taq*™	EC: 2.7.7.7	TaKaRa, Japan

VITAE

Name Mr. Thanaisawan Losuphanporn

Student ID 4752016

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Chemistry)	Prince of Songkla University	2003

Scholarship Awards during Enrolment

The grants from the National Center for Genetic Engineering and Biotechnology (BIOTEC), the International for Science (IFS, grant no. 4093/1), the Graduate School and Faculty of Pharmaceutical Sciences, Prince of Songkla University.

List of Publication and Proceeding

Proceeding:

Wungsintaweekul, J., Losuphanporn, T., Sirisuntipong, T., Tansakul, P., Sitthithaworn, W., Shibuya, M., Ebizuka, Y. and De-Eknamkul, W. cDNA cloning and molecular analysis of *dxs* gene from *Croton stellatopilosus*. Proceeding of the 4th Indochina Conference on Pharmaceutical Sciences: Pharma Indichina IV Pharmacy incooperation for development and integration, Ho Chi Minh City, Vietnam, November 10-13, 2005.

Publication:

Wungsintaweekul, J., Sirisuntipong, T., Kongduang, D., Losuphanporn, T., Ounaroon, A., Tansakul, P. and De-Eknamkul, W. 2008. Transcription profiles analysis of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and 2*C*-methyl-D-erythritol 4-phosphate synthase in plaunotol biosynthesis from *Croton stellatopilosus*. *Biol. Pharm. Bull.* 31(5), 852-856.