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โครงการ: ชีวสังเคราะห์ของเทอร์ปีนอยด์อินโดลแอลคาลอยด์: การโคลน การศึกษาคุณลักษณะและการแสดงออกของยีน *dxs* และ *dxr* จากกระท่อม

(Biosynthesis of terpenoid indole alkaloid: molecular cloning, characterization and expressions of *dxs* and *dxr* genes from *Mitragyna speciosa* (Roxb.) Korth.)

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การศึกษาครั้งนี้ได้โคลน cDNA ของยีน 1-deoxy-D-xylulose 5-phosphate synthase (*msdxs*) และ 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*msdxr*) จากส่วนใบกระท่อม โดยเทคนิค homology-based polymerase chain reaction (PCR) และ rapid amplification of cDNA ends (RACE) ผลการศึกษาคุณลักษณะของ cDNA ของยีน *msdxs* พบว่ายีน *msdxs* ในกระท่อมปรากฏอย่างน้อย 2 รูปแบบ เรียกว่า *msdxs1* และ *msdxs2* คุณลักษณะของ *msdxs1* cDNA ประกอบด้วย open reading frame (ORF) ขนาด 2,010 คู่เบส เมื่อถอดรหัสเป็นสายกรดอะมิโน ได้ลำดับกรดอะมิโนจำนวน 669 หน่วยอะมิโน มวลโมเลกุลมีค่า 71.9 กิโลดาลตัน และมีค่า pI เท่ากับ 6.89 ส่วนคุณลักษณะของ *msdxs2* ประกอบด้วย ORF ขนาด 2,193 คู่เบส ถอดรหัสเป็นกรดอะมิโนจำนวน 730 หน่วยอะมิโน มวลโมเลกุลมีค่า 78.6 กิโลดาลตัน และมีค่า pI เท่ากับ 6.46 เมื่อเปรียบเทียบสายกรดอะมิโนระหว่าง MSDXS1 และ MSDXS2 พบว่ามีค่าความเหมือน 66% เมื่อวิเคราะห์ลำดับสายกรดอะมิโน พบว่า MSDXS1 และ MSDXS2 ประกอบด้วยส่วนของ thiamine diphosphate-binding motif และ transketolase motif ซึ่งเป็นลักษณะของโปรตีนกลุ่ม transketolase สำหรับการโคลนยีน *msdxr* คุณลักษณะของ cDNA ที่ได้ประกอบด้วย ORF ขนาด 1,317 คู่เบส ถอดรหัสเป็นกรดอะมิโนจำนวน 438 หน่วยอะมิโน คำมวลโมเลกุลมีค่า 47.5 กิโลดาลตัน และมีค่า pI เท่ากับ 6.43 เมื่อวิเคราะห์สายกรดอะมิโนของ MSDXR พบว่าประกอบด้วยส่วน chloroplast transit peptide ที่ทำหน้านำส่งโปรตีนเข้าสู่พลาสติด บริเวณของ proline rich ตำแหน่งของ NADPH binding motif และหน่วยอะมิโนที่มีส่วนสำคัญต่อการเร่งปฏิกิริยา เมื่อวิเคราะห์ความเหมือนของสายกรดอะมิโนของ MSDXR กับ DXR ในพืชอื่นๆ พบว่ามีค่าความเหมือนมากกว่า 80% เมื่อศึกษาบทบาทหน้าที่ในระดับเซลล์ของ MSDXS1 และ MSDXS2 ในต้นกระท่อม โดยใช้ต้นกระท่อมที่ผ่านกระบวนการ regenerated จากการเหนี่ยวนำรากลอยด้วย *Agrobacterium rhizogenes* เป็นพืชต้นแบบ ทั้งนี้เนื่องจากต้น regenerated มีปริมาณ mitragynine สะสมในปริมาณสูง เมื่อวัดระดับการแสดงออกของ mRNA ของยีน *msdxs1* และ *msdxs2* โดยวิธี quantitative real-time (qRT) PCR ของต้น regenerated เปรียบเทียบกับต้นในสภาวะทดลอง (เพาะได้จากเมล็ด) ผลการทดลองพบว่า mRNA ของ *msdxs1* ของต้น regenerated ส่วนของใบ ลำต้น และราก มีระดับการแสดงออกของยีนสูงกว่าต้นที่เพาะจากเมล็ด 1.5 เท่า ในขณะที่ระดับการแสดงออกของ *msdxs2* ในส่วนของใบและลำต้นของต้น regenerated มีค่าน้อยกว่าต้นเพาะจากเมล็ด ถึง 50% เนื่องจาก mitragynine มีแหล่งสะสมส่วนใหญ่อยู่ที่ใบ จากผลการทดลองนี้สรุปได้ว่า MSDXS1 มีส่วนสำคัญต่อชีวสังเคราะห์ mitragynine มากกว่า MSDXS2 การศึกษาครั้งนี้ยังได้เหนี่ยวนำการแสดงออกของยีน *msdxr* ในรูป pseudomature form (ตัดส่วน plastid-targeting sequence) ในเชื้อ *E.coli* ภายหลังจากเหนี่ยวนำด้วย IPTG พบว่าเซลล์ *E. coli* สามารถสร้าง MSDXR ในรูป soluble form โดยปรากฏมวลโมเลกุลประมาณ 42 กิโลดาลตัน บนแผ่น SDS-PAGE และ MSDXR ส่วนใหญ่อยู่ในรูป insoluble form

คำหลัก : cDNA cloning, expression, *Mitragyna speciosa*, mitragynine, terpenoid indole alkaloid biosynthesis

Abstract

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Project Title: Biosynthesis of terpenoid indole alkaloid: molecular cloning, characterization and expressions of *dxs* and *dxr* genes from *Mitragyna speciosa* (Roxb.) Korth.

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In this study, the full-length cDNA of 1-deoxy-D-xylulose 5-phosphate synthase (*msdxs*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*msdxr*) from *M. speciosa* leaves were cloned by homology-based polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) methods. For *msdxs* cDNA, *M. speciosa* leaves produced at least two isoforms, namely *msdxs1* and *msdxs2*. The full-length cDNA of *msdxs1* contained the open reading frame (ORF) of 2,010 bp encoding a deduced peptide of 669 amino acid residues with a predicted molecular mass of 71.9 kDa and isoelectric point of 6.89. The full-length cDNA of *msdxs2* contained the ORF of 2,193 bp encoding 730 amino acid residues with a predicted molecular mass of 78.6 kDa and isoelectric point of 6.46. MSDXS1 and MSDXS2 shared the identity of 66%. Analysis of deduced amino acid sequence of MSDXS1 and MSDXS2 indicated that they composed of thiamine diphosphate-binding motif and transketolase motif and, therefore, belong to transketolase family. For *msdxr* cDNA, the full-length cDNA contained the ORF of 1,317 bp encoding a deduced peptide of 438 amino acid residues with a predicted molecular mass of 47.5 kDa and isoelectric point of 6.43. MSDXR contained all important characteristics for DXR such as the chloroplast transit peptide for plastid localization, extended proline rich region, NADPH binding motif and invariant catalytic amino acid residues. Multiple alignment of MSDXR showed that MSDXR shared a high homology to other known plant DXRs (more than 80% identities). Study on physiological roles of MSDXS1 and MSDXS2 in *M. speciosa*, the regenerated plants from *Agrobacterium rhizogenes* transformed hairy roots were used as plant model as they contained high-yield of mitragynine. Transcription profiles of *msdxs1* and *msdxs2* mRNAs were measured by quantitative real-time (qRT) PCR in comparison with *in vitro* plant, in which from seed germination. The results showed that the *msdxs1* mRNA in leaves, roots and stems of the regenerated plants were expressed higher than the *in vitro* plants with a magnitude of 1.5 times. In contrast with the *msdxs2* mRNA expressions in leaves and stems of the regenerated plants were suppressed about 50%. As mitragynine is accumulated mostly in the leaves, it can be suggested that the MSDXS1 plays a regulatory role in the mitragynine biosynthesis rather than the MSDXS2. In this study, the *msdxr* gene was expressed in pseudomature form without the putative plastid targeting sequence in a recombinant *E. coli* strain. After induction with IPTG, the recombinant *E. coli* cells could produce the soluble protein and had an apparent molecular mass *ca.* 42 kDa on the SDS-PAGE and MSDXR was expressed mostly in insoluble fraction.

Keywords: cDNA cloning, expression, *Mitragyna speciosa*, mitragynine, terpenoid indole alkaloid biosynthesis

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Juraithip Wungsintaweekul

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List of Abbreviations

ATP	=	adenosine 5'-triphosphate
BA	=	benzyladenine
BSA	=	bovine serum albumin
cDNA	=	complementary deoxynucleic acid
CTP	=	cytidine 5'-triphosphate
cTP	=	chloroplast transit peptide
DMAPP	=	dimethylallyl diphosphate
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
DXP	=	1-deoxy-D-xylulose 5-phosphate
DXR	=	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	=	1-deoxy-D-xylulose 5-phosphate synthase
EDTA	=	ethylenediaminetetraacetic acid
FPLC	=	fast protein liquid chromatography
FPP	=	farnesyl diphosphate
G10H	=	geraniol 10-hydroxylase
GPP	=	geranyl diphosphate
HMG CoA	=	3-hydroxy-3-methylglutaryl-CoA
IPP	=	isopentanyl diphosphate
IPTG	=	isopropyl-β-D-thiogalactopyranoside
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
LB	=	Luria Bertani
MEP	=	2*C*-methyl-D-erythritol 4-phosphate
MIA	=	monoterpenoid indole alkaloid
mRNA	=	messenger ribonucleic acid

msdxr	=	1-deoxy-D-xylulose 5-phosphate reductoisomerase gene from *Mitragyna speciosa*
MSDXR	=	1-deoxy-D-xylulose 5-phosphate reductoisomerase from *M. speciosa*
msdxs	=	1-deoxy-D-xylulose 5-phosphate synthase gene from *M. speciosa*
MSDXS	=	1-deoxy-D-xylulose 5-phosphate synthase from *M. speciosa*
MVA	=	mevalonate
MWCO	=	molecular weight cut off
NAA	=	1-naphthaleneacetic acid
NADPH	=	nicotinamide adenine dinucleotidephosphate (reduced form)
PDA	=	photodiode array
RLM-RACE	=	RNA ligase–mediated rapid amplification of cDNA ends
RNA	=	ribonucleic acid
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	=	Tris acetate EDTA
TDZ	=	thidiazuron
WPM	=	McCown woody plant medium
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. Introduction

M. speciosa (Roxb.) Korth. (Rubiaceae) is an endemic plant found in tropical Southeast Asia. It is of particular medicinal importance known as "Kratom" in Thailand (Smitinand, 2001). For folklore medicine, Kratom has been used as an opium-substitute for pain relief and treatment of diarrhea. Many studies reported the wide varieties of indole alkaloids from Kratom's leaves including mitragynine, paynantheine, rhynchophylline, etc. (see Table 1.1). Moreover, it also contains flavone, flavonol, flavonoid, lignan, phenylpropanoid and triterpenoid (Information from NAPRALERT database). The pharmacological activities of mitragynine and its derivatives have been reported from studies in tested animals such as analgesic activity, antinociceptive activity (Watanabe et al., 1997), anti-stress activity, muscle relaxant activity (Aji et al., 2001) and inhibition of gastric acid secretion (Tsuchiya et al., 2002). The mechanism of action of mitragynine for analgesic activity is binding to the opioid receptors, similar to morphine. Interestingly, mitragynine has a characteristic of less addiction than morphine. Therefore, it highlights the relevance to be an alternative to opioid analgesic drug (Thongpraditchote et al. 1998).

Contrary to the potential of *M. speciosa*, it is recognized as an illegal plant and growing of this plant is prohibited in Thailand. As such the basic knowledge of mitragynine biosynthesis is very little. From the biosynthesis point of view, the steps of mitragynine formation are still unknown. Mitragynine is a monoterpenoid indole alkaloid (MIA). Biosynthetically, mitragynine was supplied from strictosidine as suggested by feeding experiment of strictosidine in *M. speciosa* (Rueffer et al., 1978). Therefore, mitragynine is composed of moieties of tryptamine and secologanin as same as vincristine in *Catharanthus roseus*. Although in higher plants, the origin of secologanin supplies from two existed isoprenoid pathways, namely mevalonate (MVA) pathway and deoxyxylulose phosphate (DXP) pathway. It has been suggested that loganin was originated from the isoprene unit from the DXP pathway in ajmalicine biosynthesis in *Rauwolfia serpentina* (Eichinger et al., 1999). As part of interest in mitragynine biosynthesis, we began to investigate firstly in the early step of isoprenoid biosynthesis. The branch point enzymes in the DXP pathway, which were 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) were selected. In this study, we began with cDNA cloning and functional characterization based on nucleotide and amino acid sequences. Thereafter, the expressions of *dxs* and *dxr* were investigated in terms of mRNA expression for *dxs* and protein expression

for *dxr*. Heterologous expressions of *dxs* and *dxr* in *E.coli* have been done, however, there were unsuccessful. In order to understand the regulatory role of *dxs* and *dxr*, plant tissue culture e.g. hairy root culture and *in vitro* plant culture were established. The metabolic profile in those cultures was evaluated and used it as investigated materials for following the *dxs* expression in parallel with determination of mitragynine content. The results obtained from this study will be useful for further study on mitragynine biosynthesis in *M.speciosa*.

1.1 Botanical aspects of *M. speciosa* (Roxb.) Korth.

Mitragyna speciosa (Roxb.) Korth. (Fig. 1.1) belongs to the Rubiaceae family, found in the regions of Africa and Asia. The genus, named *Mitragyna* was given by Korthals due to the shape of the stigmas in the species. However the nomenclature has frequently been confused; the genuses have been variously named and are consistently recognized as *Naucleeae*, *Sarcocephalus*, *Stephegyne* and *Uncaria*. In Thailand, there are four species of *Mitragyna*, which are *M. speciosa, M. hirtusa, M. diversifolia* and *M. rotundifolia* (Smitinand, 2001). Taxonomically, plant species can be categorized by types of indole alkaloids (Keawpradub, 1990). Recently, these species were authenticated by molecular approach, based on rDNA ITS sequence (Sukrong et al., 2007).

For botanical aspects, *M. speciosa* is characterized by the globular flowering head each containing up to 120 florets (Fig. 1.1 A). Each floret is surrounded by many overlapping bracteoles which completely cover the developing florets during the flower-bud stage. The inflorescence is a dichasial cyme. The fruit is a capsule containing numerous small flat seeds. The young woody shoots bear 10-12 leaves arranged in opposite and decussate each pair of leaves being accompanied by two interpetiolar stipules (Fig. 1.1 B) which initially are closely oppressed and protect the apical bud.

1.2 Chemical constituents of *M. speciosa*

Leaves of *M. speciosa*, found in Thailand and Malaysia, contain common indole alkaloids. Takayama (2004) reviewed the abundance of mitragynine in crude alkaloidal extract of Thai and Malay varieties. It has been reported that the *M. speciosa* of Thai contained mitragynine up to 66% of the total alkaloid content, whereas only 12% was found in the *M. speciosa* of Malay sample. Reviews of the distribution of indole alkaloids in leaves, twigs, stem barks and root barks of *M. speciosa* are summarized in Table 1.1.

Figure 1.1 Globular flowering head (A) of *Mitragyna speciosa* (Roxb.) Korth. (Rubiaceae) (B).

Table 1.1 Chemical constituents of *M. speciosa* (Roxb.) Korth.

Plant part	Category/chemical substances	Reference
leaves	Alkaloid:	Phillipson et al., 1973;
	ajmalicine, akuammigine, angustine,	Phillipson et al., 1973;
	corynantheidine, corynantheidaline,	Shellard et al., 1966;
	corynantheidalinic acid, corynoxeine,	Shellard et al., 1978a;
	corynoxine, corynoxine B, hirsutine,	Shellard et al., 1978b
	hirsuteine, isocorynoxeine, isomitraphylline,	Takayama, 2004
	isorhynchophylline,	
	isocorynantheidine, javaphylline, mitraciliatine,	
	mitragynine oxindole B,	
	mitrajavine, mitraphylline, mitrasulgynine,	
	mitragynaline, mitragynalinic acid, mitralactonal,	
	paynantheine, mitragynine, pinoresinol,	
	speciociliatine,	
	speciogynine, 3-isoajmalicine, 3,4,5,6-	
	tetradehydromitragynine, 7α-hydroxy-7*H*-	
	mitragynine	
	flavones:	Hinou and Harvala, 1988
	apigenin, apigenin-7-O-rhamnoglucoside,	
	cosmosiin	Hinou and Harvala, 1988

	flavonol: astragalin, hyperoside, kaempferol, quercetin, quercitrin, quercetin-3-galactoside-7-rhamnoside, quercitrin, rutin	
leaves	Phenylpropanoid: caffeic acid, chlorogenic acid Flavonoid: (-)-epicatechin Lignin: (+)-pinoresinol Triterpene: ursolic acid	Hinou and Harvala, 1988 Houghton and Said, 1986 Takayama et al., 1998 Said et al., 1991
young twigs, stem bark	Alkaloid: ciliaphylline, rhynchociline, ciliaphylline, isomitraphylline, isorhynchophylline, isospecionoxeine, javaphylline, mitraciliatine, mitragynine oxindole A, mitragynine oxindole B, mitraphylline, rhynchociline, rhynchophylline, speciogynine, speciociliatine, specionoxeine	Shellard et al., 1978a; Shellard et al., 1978b
root bark	Alkaloid: ciliaphylline, corynoxeine, isocorynoxeine, isomitraphylline, isorhynchophylline, isospecionoxeine, mitraciliatine, mitraphylline, rhynchociline, rhynchophylline, speciociliatine, speciogynine, specionoxeine	Shellard et al., 1978b; Houghton and Shellard, 1974

1.3 Biological activities

1.3.1 Indole alkaloids

As mentioned earlier, *M. speciosa* is a major source of indole alkaloids. Mitragynine is the most abundant component that is present in the leaves of *M. speciosa*. For biological activities of alkaloids obtained from *M. speciosa*, the crude extract and pure alkaloid-mitragynine were used as materials *in vitro* and *in vivo* experiments. In 1996, a Japanese group investigated the antinociception of mitragynine in mice and the results showed that mitragynine acted as opioid receptors agonist in the brain of mice (Matsumoto et al., 1996a). Later, they found that the mechanisms for antinociceptive effects differed from those of morphine in mice (Matsumoto et al., 1996b). Mitragynine preferred to bind μ and δ opioid receptors, unlike morphine that binds only to μ opioid receptor specifically. Therefore, mitragynine exhibits 10 fold less potent than morphine (Thongpraditchote et al., 1998). Caused by the affinity to opioid receptors, mitragynine also acts as morphine-like action on inhibition of gastric secretion (Tsuchiya et al., 2002). Studies on the synthesis of mitragynine-related indole alkaloids discovered mitragynine pseudoindoxyl and 7-hydroxymitragynine, which exhibited opioid agonistic activity with higher potency than morphine (Takayama et al., 2002). With regards to the psychological effects, *M. speciosa* extract was known to have a stimulatory effect on the dorsal raphe nucleus and an antidepressant-like activity. Stimulation of this brain area has been known to cause antinociception (Kumarnsit et al., 2007).

1.3.2 Triterpenoids : ursolic acid

Ursolic acid was found in *M. speciosa* leaves (Said et al., 1991). To date, there is no report about the biological activity of ursolic acid from *M. speciosa*. However, ursolic acid normally exists in plants, especially the Lamiaceae family. Contemporary scientific researches revealed and confirmed that ursolic acid exhibited several pharmacological effects, such as antitumour, hepatoprotective, anti-inflammatory, anti-ulcer, antimicrobial, anti-hyperlipidemic and antiviral activity (Liu, 1995). Ursolic acid was also identified as one of the active principles in *Rosmarinus officinalis* (Lamiaceae) to inhibit the growth of some food borne pathogens (Kamatou et al., 2007). Somova et al. (2003) found that African wild olives contain oleuafricein (mixture of oleanolic and ursolic acid) exhibiting the antihypertensive, diuretic/natriuretic, antihyperlipidemic, hypoglycemic and antioxidant activities (Samova et al., 2003). Ursolic acid from *Bouchea fluminensis* has anti-inflammatory activity inhibiting significantly the edema

induced by either carrageenan or serotonin (Costa et al., 2003). The anti-inflammatory effects of ursolic acid have been attributed to various mechanisms including inhibition of lipoxygenase and cyclooxygenase activities, inhibition of elastase and inhibition of C3-convertase (Costa et al., 2003).

1.4 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.

1.4.1 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. 1.2.

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.

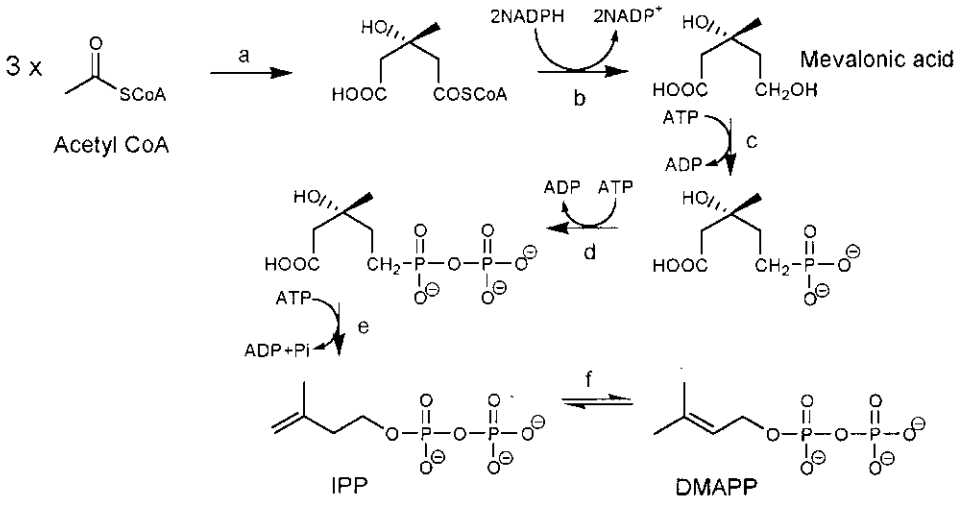

Figure 1.2 Biosynthesis of IPP via the mevalonate pathway.

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

1.4.2 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. 1.3.

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).

1.4.3 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 1.2), 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).

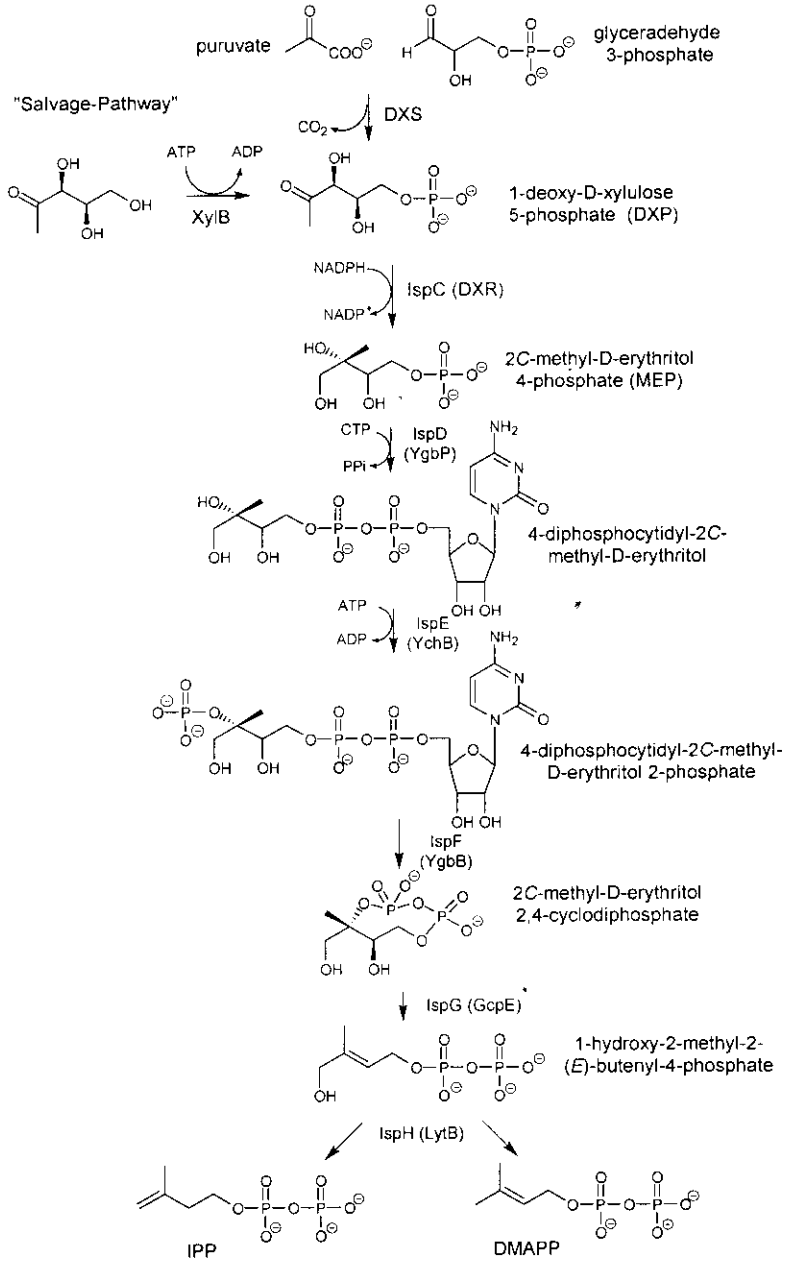

Figure 1.3 Biosynthesis of IPP and DMAPP via the alternative deoxyxylulose phosphate pathway. [DXS, deoxyxylulose 5-phosphate synthase; IspC, 2*C*-methyl-D-erythritol 4-phosphate synthase; IspD, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol synthase; IspE, 4-(diphosphocytidyl)-2*C*-methyl-D-erythritol 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].

Table 1.2 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, which are 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 (Schwarz, 1994). 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. The extent of this crosstalk depends on the species as well as the concentration of exogenous precursors.

1.4.4 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) (EC 2.2.1.7)

1.4.4.1 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).

1.4.4.2 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.

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 TPP cofactor which is common in pyruvate decarboxylases, acetolactate synthases and transketolases (Reynen and Sahm, 1988; Hawkins et al., 1989). As shown in Fig. 1.5, 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 $DRAG\text{-}X_{28}\text{-}P\text{-}X\text{-}D$, where X denotes any amino acid (Schenk et al., 1997).

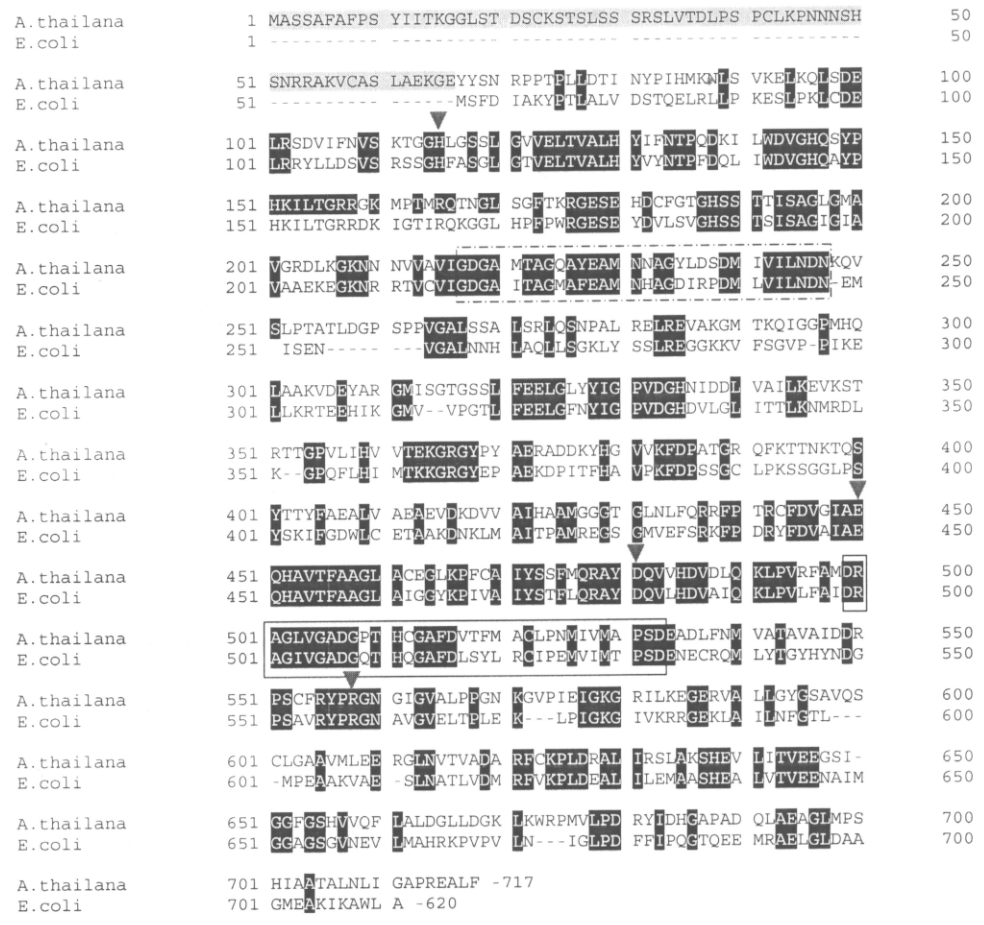

Figure 1.4 Primary structure of DXS. Amino acid sequences alignment of DXS from *Arabidopsis thaliana* and *Escherichia coli*. The shaded sequence denotes the chloroplast transit peptide (cTP). Dashed box and solid-line box indicate the regions corresponding to the putative thiamine diphosphate-binding 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. 1.4), 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. 1.4) 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. 1.4) (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).

1.4.4.3 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).

1.4.5 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (EC 1.1.1.267)

Gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in the DXP pathway was firstly reported by Seto and his group in 1998. By the mutagenesis approach, genes responsible for the DXP pathway were cloned by using a strategy to prepare *E. coli* mutants with a metabolic block(s) between DXP and MEP (Kuzuyama et al., 1998; Takahashi et al., 1998). The mutant that required 2*C*-methyl-D-erythritol for growth was screened. By complementation of these mutants, only the *yaeM* gene from *E. coli* was cloned and the gene product was purified and characterized (Takahashi et al., 1998). The *yaeM* gene was later renamed to *dxr* or *ispC* (Takahashi et al., 1998).

1.4.5.1 Catalytic properties of the DXR protein

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

Naturally, the DXR protein does not utilize 1-deoxy-D-xylulose. It should be noted that the successful incorporation of 1-deoxy-D-xylulose into terpenoids of various organisms required a phosphorylation step catalyzed by the unspecific D-xylulose kinase (Wungsintaweekul et al., 2001). The DXR prefers to use NADPH rather than NADH as co-factor. It transfers the pro-s proton to 1-deoxyxy-D-xylulose 5-phosphate from NADPH and later rearrange to form the branch polyol, 2*C*-methyl-D-erythritol 4-phosphate. Therefore, the DXR protein is belonging to the class B dehydrogenases (Arigoni et al., 1999; Radykewicz et al., 2000; Proteau et al., 1999). The K_M of DXP and V_{max} values are 171 μM and 18 μmol $min^{-1}mg^{-1}$ (turnover number 13 s^{-1} per subunit). It utilizes NADPH as cofactor with a K_M of 25 μM (Wungsintaweekul, 2001).

Fosmidomycin [3-(N-formyl-N-hydroxyamino) propyl phosphate] inhibits the DXR protein (Kuzuyama et al., 1998). The inhibitory effect of fosmidomycin on DXR can be explained by a structural

similarity of fosmidomycin to 2*C*-methyl-D-erythrose 4-phosphate, a putative intermediate of the enzyme reaction. Fosmidomycin had been reported as a mixed-type inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* with a *Ki* value of 38 nM (Kuzuyama et al., 1998). For the DXR of *Zymomonas mobilis*, fosmidomycin served as a competitive inhibitor with a *Ki* value of 600 nM (Grolle et al., 2000). Currently, the DXR of *A. thaliana* was inhibited by fosmidomycin with *Ki* value of 85 nM (Rohdich et al., 2006).

Table 1.3 Characterization of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase protein.

Organisms	Characteristics				References
	Molecular weight (kDa)	Kinetics			
		K_M (μM)		Activity	
		DXP	NADPH	μmol/min.mg	
Bacteria					
Escherichia coli	42	171	25	18	Wungsintaweekul, 2001 Altincicek et al., 2000
Pseudomonas aeruginosa	43.9			0.0258	Yin and Proteau, 2003;
Synechocystis sp.	39	134	5.0	19.5	Woo et al., 2005
Zymomonas mobilis	39	300	5.0	5.6	Groll et al., 2000
Plants					
Arabidopsis thaliana	52	132	30		Rohdich et al., 2006
Lycopersicon esculentum	51.5				Rodriguez- Concepcion et al., 2001
Mentha piperita	43.5				Lange and Croteau, 1999

1.4.5.2 Structure of DXR protein

Primary structure of DXR composes of the characteristics of chloroplast transit peptide (cTP), the proline-rich region and NADPH-binding site. As shown in Fig. 1.5, NH_2-terminus of DXR of *A. thaliana* showed the feature of cTP with the cleavage site at 86 amino acid residues. Glu-231 of the *E. coli* enzyme is important in the conversion of DXP into MEP, and His-153, His-209 and His-257 are part of the binding site of DXP to the enzyme (Kuzuyama et al., 2000).

The structure of the *E. coli* DXR protein has been published (Reuter et al., 2002; Yajima et al., 2002; Steinbacher et al., 2003; Mac Sweeney et al., 2005). Each subunit of the homodimer consists of an N-terminal dinucleotide binding domain, a connecting domain with the catalytic site and C-terminal helical domain. The structure of a complex with NADPH (Yajima et al., 2002) confirmed the essential role of Gly-14, Glu-231, His-153, His 209 and His-257 in the catalytic process.

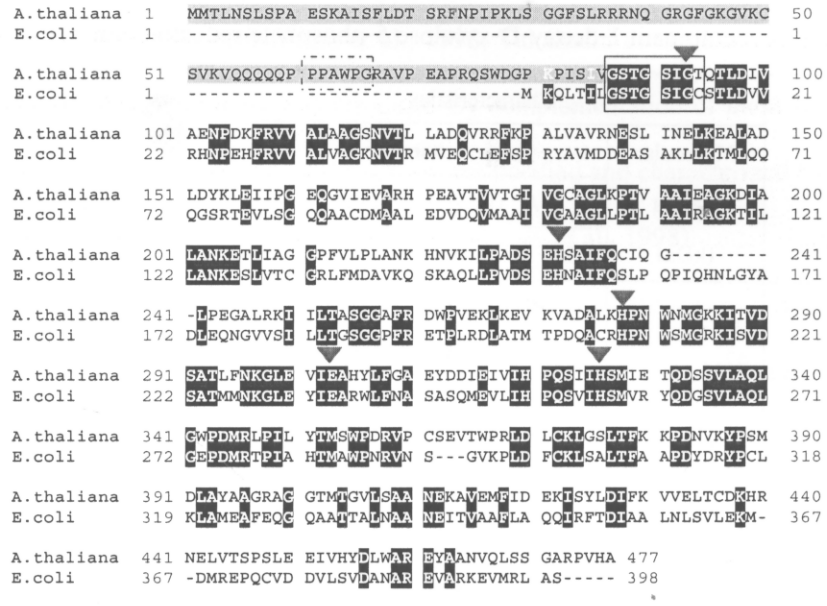

Figure 1.5 Primary structure of DXR. Amino acid sequences alignment of DXR from *A. thaliana* and *E.coli*. The shaded sequence denotes the chloroplast transit peptide (cTP). Dashed box and solid-line box indicate the regions corresponding to the proline-rich region and NADPH-binding site, respectively. The arrowheads denote amino acid residues that essential for catalytic actions.

1.4.5.3 Expression of the DXR protein and transcripts

In transgenic peppermint (*M. piperita*), overexpressing DXR led to an increase of essential oil monoterpenes in its leaf tissues compared to the wild type. Gene silencing of partial *dxr* gene of the engineered peppermint plants led to a reduction of essential oil accumulation (Mahmoud and Croteau, 2001). A positive correlation was found between the accumulation of apocarotenoids in mycorrhizal roots from monocots with increase in DXR transcript (Walter et al., 2000), and similar observation was also reported with indole alkaloids accumulation in *Catharanthus roseus* cell suspension culture (Veau et al., 2000). However, in tomato (*Lycopersicon esculentum*) fruit during ripening, neither DXR transcripts nor protein level increased, despite the massive carotenoids accumulation, suggesting a non-limiting role for DXR in this system (Rodriguez-Concepcion et al., 2001). Later, the expression level of *dxr* gene in relation to terpenoid production was reported from *Gingko biloba* (Gong et al., 2005) and *Camptotheca accuminata* (Yao et al., 2007). It has been demonstrated that *dxr* gene associated with terpenoid biosynthesis and the gene product does not exhibited as rate-limiting step in the DXP pathway.

1.5 Mitragynine biosynthesis

Mitragynine is corynanthe-type indole alkaloid. Generally, the indole alkaloid skeleton is obtained from the condensation of one molecule of tryptamine, a decarboxylation product of trytophan and one molecule of secologanin, an iridoid moiety. Tryptamine is supplied from the shikimate pathway, whereas secologanin originated from terpenoid pathway. Therefore, the alkaloid group is named as "monoterpenoid-indole alkaloids" (MIAs). Condensation of tryptamine and secologanin is catalyzed by strictosidine synthase, forming the first intermediate strictosidine (Mizukami et al., 1979) (Fig. 1.6). Distance between strictosidine and mitragynine is still unknown.

Monoterpenoid indole alkaloids (MIAs) are a large class of pharmaceutically valuable and structurally complex natural products (Kutchan, 2000). Strictosidine serves as a precursor for MIAs e.g. vinblastine, camptothecin, reserpine, which are medically used as anticancers and antihypertension, respectively. MIA biosynthesis has been studied extensively in *Catharanthus roseus*. For the route of terpenoid pathway, it has been reported that geraniol 10-hydroxylase (G10H) is the first committed step in secologanin biosynthesis and responsible for the hydroxylation of gernaiol at the C-10 position (Meijer et al., 1993). For the route of shikimate pathway, tryptophan decarboxylase (TDC) plays an important role in the

formation of indole alkaloid in *C. roseus* (Meijer et al., 1993). Those reports demonstrated that the early step enzymes regulated the indole alkaloid biosynthesis as well as the later step enzymes.

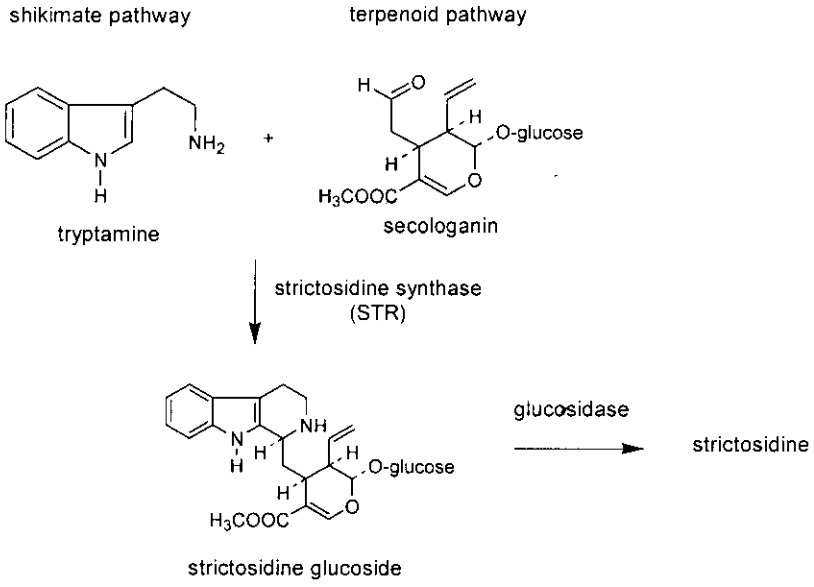

Figure 1.6 Formation of strictosidine, a common precursor of MIAs.

1.6 Plant tissue culture of the Rubiaceous plants

For the studies on plant tissue culture of the Rubiaceous plants, they reported the establishment of callus, cell suspension and organ cultures under specific conditions. Table 1.4 summarized the plant tissue cultures of the Rubiaceous plants. It can be noted that they were able to produce the alkaloids and triterpenoids. An example of the hairy roots of *Ophiorrhiza pumila* convinced the utility of hairy root culture for camptothecin production in the industrial scale (Saito et al., 2007). Suspension culture of *Uncaria tomantosa* could produce ursolic acid and oleanolic acid but no alkaloid was present in the culture (Feria Romero et al. 2005).

Table 1.4 Plant tissue culture of the Rubiaceous plants.

Plant source	Chemicals	Type of culture	Reference
Cinchona ledgerina	cinchonine, quinidine, quinine	suspension culture	Hamill et al., 1989
Ophiorrhiza pumila	Camptothecin	hairy root culture	Saito et al., 2007
Psychotria umbellata	umbellatine	embryogenic regeneration	Paranhos et al., 2005
Rudgea jasminoides	-	callus cell culture	Stella and Graba, 2002
Uncaria rhynchopylla	hirsuteine, hirsutine,3α-dihydrocadambine, ursolic acid	callus cultûre :	Kohda et al., 1996
U. tomentosa	ursolic acid, oleanolic acid	suspension culture	Feria Romero et al., 2005

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^{TM} Chelating HP Columns (5 ml); HiTrap^{TM} 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

High Pressure Liquid Chromatography: Agilent

Agilent 1100 series equipped with photodiode-arrays detector (PDA); autosampler

Additional equipments

Autoclave	Model HA-3D (Hirayama, Japan)
Balance	Explorer (Ohaus, USA); Avery Berkel (USA); Sartorius TE 3102S (USA)
Centrifuge	Kubota 5922 (Japan)
Electrophoresis	SE 250 Mighty Small II (Amersham Biosciences, USA); 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	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)
Micropipettes	Socorex: 0.1-2.0 µl, 2-20 µl, 20-200 µl, 100-1000 µl (Switzerland)
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

For RNA isolation, young leaves of 1 year old of *M.speciosa* were collected from the botanical garden 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. All compounds were pure grade and for biotechnological purposes.

2.2.3 Substrates

The authentic mitragynine was kindly provided by Assist. Prof. Dr. Niwat Keawpradub, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

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, Plasmid isolation kit and taq PCR core kit were purchased from Qiagen, Germany. Superscript™ III reverse transcriptase was from

Invitrogen, USA. GFX micro-palsmid prep kit was from Amersham Biosciences, USA. Wizard® PCR Midi Preps DNA purification system was purchased from Promega, USA. Taq DNA polymerase were from Qiagen, germany and New England Biolab (NEB, 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,

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 ^{o}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 ^{o}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) and *Catharanthus roseus* (AG011840), 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 degenerate primers were designed as shown in Table 2.1. Primers design for DXR cloning, the amino acid sequences of *A. thaliana* (NP201085), *Mentha piperita* (AAD24768) and *Artemisia annua* (AAD66391) were aligned and degenerate primers were designed.

Table 2.1 Degenerate primers for amplification of partial cDNA fragments of *dxs* and *dxr* sequences.

Primer names	Nucleotide sequence (5′⟶3′)	For amino acid sequence
PIH-DXS1	CC(A/T)(G/A)TTCACATGAA(G/A)AA(C/T)CT	PIHMKN
IAE-DXS1	ACTGC(G/A)TGTTGTTC(C/T)GCTAT	IAEQHA
MIS- DXS2	ATGAT(C/T)AGTGGTTCTGGATC	MISGSG
ITV-DXS2	GA(C/T)CCTTCTTC(G/A/T)AC(G/C/A)GTGAT	ITVEEG
WDG-DXR1	GGATGG(C/T)CCAAAGCCTAT	WDGPKP
EVK-DXR1	GCATC(G/A)GCTACTTTAAC(A/T)TCTT	EVKVAD
GIV-DXR2	AGGAATAGTTGG(C/T)TGTGC	GIVGCA
EKA-DXR2	ACAT(C/T)TCAAC(G/A)GCTTTCTTCA	EKAVEM

Oligonucleotide sequences of specific primers were designed from the nucleotide sequence that obtained from recombinant clones of partial fragments of *dxs* and *dxr* genes. Table 2.2 shows the oligonucleotide sequences of specific primers used for 5'-end, 3'-end and full-length *dxs* and *dxr* genes clonings.

Table 2.2 Specific primers for amplification of the 5'-, 3'-ends and full-length genes of *dxs1*, *dxs2* and *dxr* from *M. speciosa*.

Primer name	Nucleotide sequence (5′⟶3′)	T_M (°C)
dxs amplification for 3'- and 5'-ends		
3DXS1	CTGTTTGGCTGCTGCTGCTTTGGT	66.3
3DXS1 Nested	CAGATGCACGATTCTGCAAACCATTAG	64.6
5DXS1	AGGACTGATGACCAACATCCCAGAGT	66.2
5DXS1 Nested	CAACACCGAGGCTCGAACCAAGAG	67.9
3DXS2	ATGCCTGCGCCAGGGCCAGTCCT	71.7
3DXS2 Nested	GCTTCTTCAAGGGGTTGGAATATCAGC	66.1
5DXS2	GTTGAACTATGACCAGCTCCAAATGC	64.6
5DXS2 Nested	GCATTCTTGACCTCCTTCCTGTCAAG	66.2

Primer name	Nucleotide sequence (5′⟶3′)	T_M (°C)
dxr amplification for 3'- and 5'-ends		
3DXR	CTGAGATAACTTGGCCTCGCCTTG	66.3
3DXR Nested	AGCAGGAGGGACGATGACTGGA	66.4
5DXR	CAACCCACTATTCCCGTGACAACAG	66.2
5DXR Nested	ATCAGGGTGGCGAGCTACCTCAACT	67.9
RACE Kit		
3RACE Kit	GCTGTCAACGATACGCTACGTAACG	76
3RACE Nested	CGCTACGTAACGGCATGACAGTG	72
5RACE Kit	CGACTGGAGCACGAGGACACTGA	74
5RACE Nested	GGACACTGACATGGACTGAAGGAGTA	78
Full-length amplification		
DXS1For	TCAGTTAATCGGGTTTTCAAGCCTA	61.3
DXS1Rev	GGTTGTATTGGAGACTGGACTTA	61.0
DXS2For	CATCAACAGAAAGCTGGTÇGCAG	64.5
DXS2Rev	ACGAGTTGCAGATCAGCTAGGCAT	64.6
DXRFor	GCTGTTTAGATCAAAGTGTAGTT	57.4
DXRRev	GAATACGATTATTACACTGCAGT	57.4

Information of the partial DNA sequences of 5'-end, internal sequence and 3'-end allowed us to design the primers for the full-length of *msdxs1, msdxs2* and *msdxr* genes amplifications. Analysis of the open reading frame (ORF) of those proteins revealed position of ATG-start codon and stop codon. Since MSDXS1, MSDXS2 and MSDXR proteins carry the chloroplast transit peptides (from NH_2-terminus), thus, truncated gene encoding the mature proteins were also amplified. Primers used for amplification of the full-length and truncated genes are shown in Table 2.3.

Table 2.3 Primers used for the full-length genes amplifications.

Primer name	Nucleotide sequence (5′⟶3′)	T_M (°C)
For msdxs1		
MSDXS1For1	GATATA**GGTACC**ATGGCTTTCAGTGCAC	66.1
MSDXS1For2	AATGGA**GGTACC**GCATCATTGTCAGAAAG	66.0
MSDXS1Rev	GAAAGC**GTCGAC**TCAAACCATCAAATTGC	66.0
Bold letters indicate the sites for restriction endonuclease (GGTACC for *KpN* I and GTCGAC for *Sal*I).		
For msdxs2		
MSDXSIIFor0	GCTGGTCGCAGCCACAGAGAC	68.4
MSDXSIIFor1	ATGGCATCAGCATCTTATGGTGTTCTC	64.6
MSDXSIIFor2	GCAACTGAGGAAATTGATGTCCTGTTG	64.6
MSDXSIIRev0	AGTTGCAGATCAGCTAGGCA	61.7
MSDXSIIRev1	TTATAGATTGAGAAGGTGTAGACTATCC	61.7
For msdxr		
MSDXRFor1	**GAATTC**CAGCCACCTCCTCCGGCATG	64.6
MSDXRFor2	**GAATTC**ATTTCAATAGTTGGATCAACA	68.3
MSDXRRev	**CTCGAG**TCACAGAAAAAGAACTTTGTG	63.3
Bold letters indicate the sites for restriction endonuclease (GATTC for *EcoR* I and CTCGAG for *Xho*I).		

For determination of mRNA expression using semi-quantitative RT-PCR, the primers are shown in Table 2.4. For the house-keeping gene (*18S rRNA*), the primers were kindly provided from Mr. Yortyot Sectang-nun, Faculty of Sciences, PSU.

Table 2.4 Primers used for determination of *dxs1* and *dxs2* mRNA expressions.

Primer	T_M (°C)	Oligonucleotide sequence (5' to 3')	Expected size
DXS1-936S	67.75	5'-GGTGGTTCAGTGCATGAACTTGCAGC-3'	For *dxs*
DXS1-1560A	67.74	5'-CGGAAGGCATGCCATAAATGCCACATC-3'	624 bp
DXS2-1068S	64.61	5'-TGTGACAAAGCAACTAGGAAACCAAGCT-3'	For *dxs*
DXS2-1747A	67.64	5'-AGGCAGACAAGCCATGTAAGTGGTGTC-3'	679 bp
18s-0.5F	59.4	5'-CAAAGCAAGCCTACGCTCTG-3'	For *18S rRNA*
18s-0.5R	59.4	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* TOP10 was 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.

2.2.9 Plasmid vectors

In this study, pGEM-T Easy vector (Promega, USA) and pDrive (Qiagen, Germany) were used for gene subcloning of PCR fragments. The PCR products which ligated with pGEM-T Easy vector and pDrive were transformed into *E. coli* TOP10.

The pGEM-T Easy and pDrive vectors are supplied in a linear form, ready-to-use for direct ligation of PCR products. These vectors allow 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, *EcoR*I was used for restriction analysis of the recombinant plasmid of pDrive and pGEM-T easy vectors. Sizes of pDrive and pGEM-T easy are 3.85 kb and 3.105 kb, respectively.

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. 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

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. 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 total RNA was stored at -20 ^{o}C until used.

Total RNAs 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 First strand cDNA synthesis

The extracted RNA was then used as a template to synthesize first-strand cDNA using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instruction. Reverse transcription reaction containing 1.5 μg total RNA, 0.2 mM dNTP, 1 μl of ImProm-II reverse transcriptase, 1 μM oligo-d(T) primer were incubated for 1 h at 42 °C in a 20 μl reaction.

2.3.3 Preparation of the RACE-ready cDNA

Invitrogen GeneRacer kit was used to prepare RACE-ready cDNA for RACE amplifications. According to the GeneRacer protocol, one micrograms of total *M. speciosa* leaf RNA was treated with calf intestinal alkaline phosphate to remove the 5'-terminal phosphatase from any RNA that is not full-length mRNA. These RNA were then treated with tobacco acid pyrophosphatase to remove the 5'-cap structure of the full-length mRNA thus leaving a 5'-PO_4 only on full-length mRNA. RNA ligase was then used to ligate the GeneRacer RNA-oligo primer to only the full length mRNA. First-strand cDNA synthesis was accomplished with SuperScript III Reverse Transcriptase, GeneRacer oligo (dT) primer, and RNA treated as described above as template in 20 ml reaction according to the manufacturer's protocol. Two microliters of this reverse transcriptase reaction mixture was used as templates for second-stranded cDNA synthesis and amplification.

2.3.4 Polymerase chain reaction (PCR) for DNA amplifications

2.3.4.1 Amplification of internal sequences of *dxs* and *dxr*

Scheme of amplification of internal sequence of *dxs* and *dxr* is demonstrated in Fig. 2.1. PCR reaction composed of 1x ThermoPol buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 0.1% Triton X-100, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$), 2.5 U Taq DNA polymerase, 0.2 mM dNTPs, 50 μM of each primer and 5% template from 2.3.2. PCR reaction was performed with MasterCycler (Model 5333, Eppendorf, Germany). Temperature profiles were [1 cycle (94 °C, 3 min); 5 cycles (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min); 10 cycles (94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min); 20 cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min); followed by a 5 min extension at 72 °C and cooling to 4 °C] for internal sequence of

dxs and [1 cycle (94 °C, 5 min); 30 cycles (94 °C, 1 min; 42 °C, 2 min; 72 °C, 3 min); followed by a 10 min extension at 72 °C and cooling to 4 °C] for the internal sequence of *dxr*.

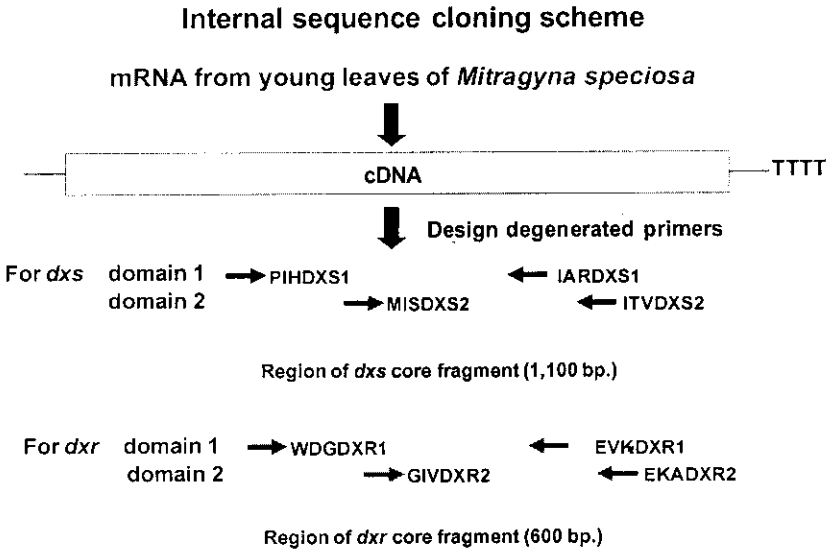

Figure 2.1 Amplifications of internal sequences of *dxs* and *dxr* genes.

2.3.4.2 Amplification of 5'-end and 3'-end of *dxs* and *dxr*

DNA fragments of 5'-end and 3'-end were amplified using RNA ligase-mediated rapid amplification of 5' and 3'- cDNA ends (RLM) technique (GeneRacer™ kit, Invitrogen, CA, USA) as described in 2.3.3. Fig. 2.2 demonstrates the amplifications of the 5'-end and 3'-end of *dxs* and *dxr* genes. Amplification protocols for 3'-ends of *dxs* and *dxr* composed 3 steps: step 1 [3RACE Kit – 3DXS(3DXR)], step 2 [3RACE Kit – 3DXS Nested(3DXR Nested) and step 3 [3RACE Nested – 3DXS Nested(3DXR Nested)]. PCR reactions were performed with Taq PCR core kit (Qiagen), contained 5 µl 10x QIAGEN PCR Buffer, 200 µM of each dNTP, 0.5 µM of each primers, 2.5 units/reaction *Taq* DNA Polymerase, ≤ 1 µg/reaction RACE-ready cDNA. Temperature profiles were 1 cycle (94 °C, 3 min); 5 cycles (94 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s); 10 cycles (94 °C, 30 s; 60 °C, 30 s; 72 °C, 45 s); 20 cycles (94 °C, 30 s; 62 °C, 30 s; 72 °C, 45 s); followed by a 5 min extension at 72 °C and cooling to 4 °C. Amplification protocols for 5'-ends of *dxs* and *dxr* composed 3 steps: step 1 [5RACE Kit – 5DXS(5DXR)], step 2 [5RACE Kit – 5DXS Nested(5DXR Nested) and step 3 [5RACE Nested – 5DXS Nested(5DXR Nested)]. Temperature profiles were similar to 3'-end amplification.

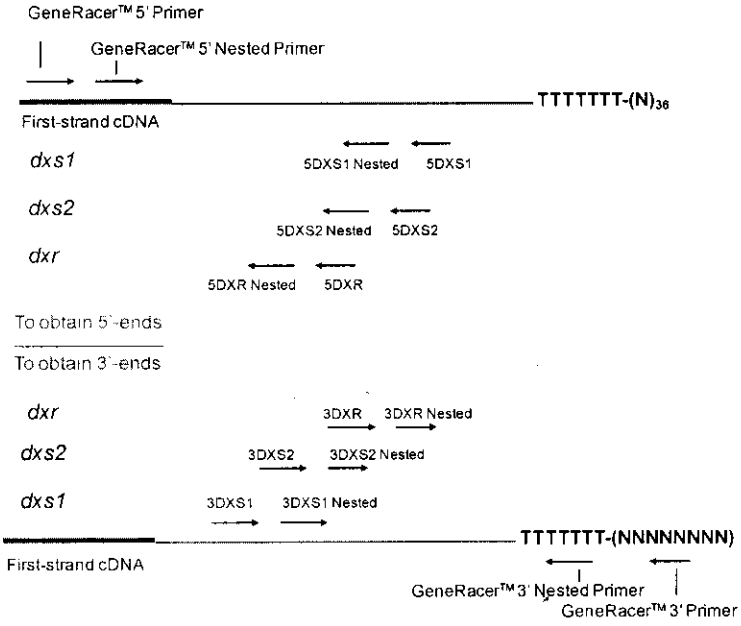

Figure 2.2 Amplifications of 5'-end and 3'-end of *dxs* and *dxr*.

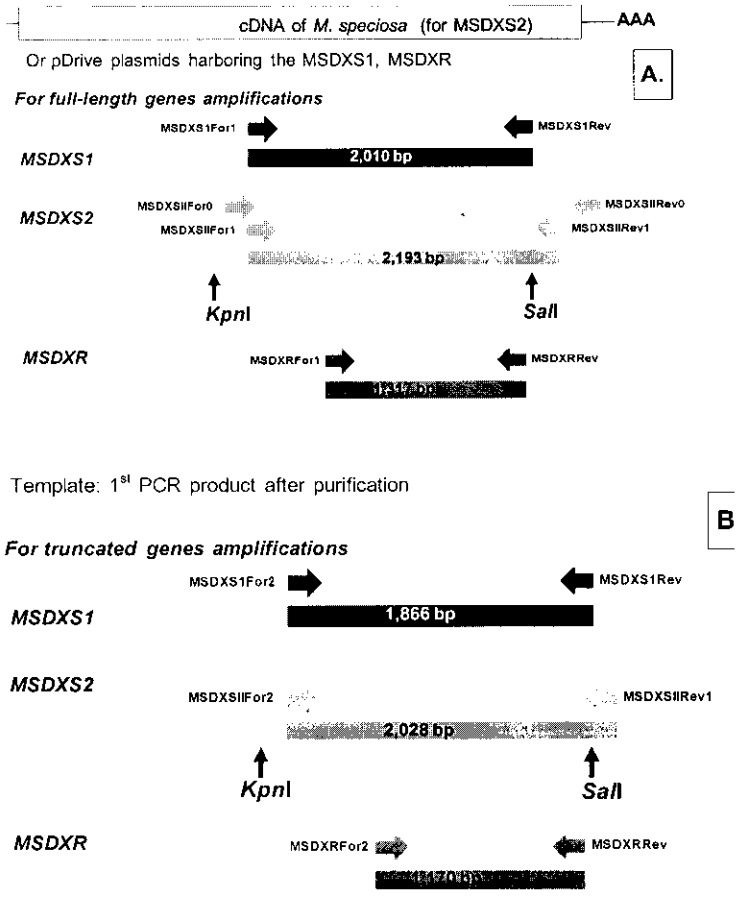

Figure 2.3 Amplifications the full-length (A) and truncated genes (B) of the *msdxs1*, *msdxs2* and *msdxr*, respectively.

2.3.4.3 Amplification of full-length and truncated clonings of the *msdxs1*, *msdxs2* and *msdxr*

The PCR fragments of full-length and truncated *MS* genes were amplified using the hot start ExTaq DNA polymerase (TaKaRa). The general composition of the PCR reaction was 5 µl of 10x Ex Taq buffer, 4 µl of dNTP mix (2.5 mM), 0.5 µl of each primer (100 µM), 1 µl of of cDNA template (1:10 dilution), and 1 µl of *Ex Taq*^{TM} polymerase (5 unit/ µl). For the full-length genes of *msdxs1* and *msdxr*, single step PCR reactions were performed using appropriate templates. In case of *msdxs2*, two steps PCR reactions were performed using similar thermal profile. Expected sizes of the full-length and truncated genes are shown in Fig. 2.3. The thermal profiles were [1 cycle (95 °C, 5 min); 35 cycles (95 °C, 1 min; 61 °C, 2 min; 72 °C, 3 min); followed by a 10 min extension at 72 °C and cooling to 4 °C] for *msdxs1*, [1 cycle (95 °C, 5 min); 35 cycles (95 °C, 1 min; 42 °C(1^{st}): 54 °C (2^{nd}) , 2 min; 72 °C, 3 min); followed by a 10 min extension at 72 °C and cooling to 4 °C] for *msdxs2*, and [1 cycle (95 °C, 5 min); 35 cycles (95 °C, 1 min; 58 °C, 2 min; 72 °C, 3 min); followed by a 10 min extension at 72 °C and cooling to 4 °C] for *msdxr*.

2.3.5 DNA cloning

2.3.5.1 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.

2.3.5.2 Ligation the DNA fragment to vector

2.3.5.2.1 Ligation with pDrive and pGEM-T Easy vectors

Since the pDrive and pGEM-T Easy 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

2.3.5.2.2 Ligation with pQE30 vector

The PCR products of the full-length and truncated genes were purified on agarose gel electrophoresis. The purified PCR products were then ligated to pQE30 UA vector (as shown below). The ligation mixtures were incubated at 16 °C for 2 h and chemically transformed into *E. coli* XL1 blue MRF' by heat-shock at 42 °C for 30 s. After pre-incubation in SOC medium for 1 h, 200 µl of mixture was plated on the LB-medium supplemented with ampicillin for selection the recombinant plasmid.

Ligation mixture: with pQE30 UA	
DNA fragment	4 μl
pQE30 UA	0.5 μl
2x ligation master mix	5 μl
Sterile dH_2O	0.5 μl

The vector pQE30 UA is supplied in linear form that ready-to-use for direct ligation of the PCR product. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of the recombinant plasmids. The pQE30 plasmid provides the histidine tag (6 residues of histidine) at the N-terminal part that makes the protein purification easy by using the Ni^{2+}-chelating affinity column. The construction of pQE30 UA and insert region is shown in Fig. 2.4.

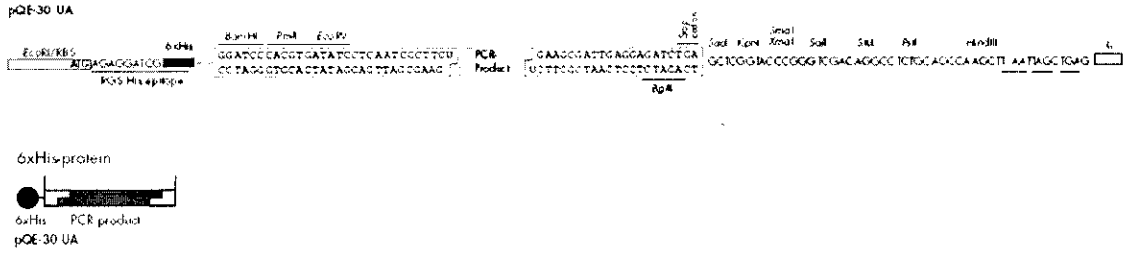

Figure 2.4 The construction of pQE30 UA supplied in a linear form.

To check the construction of the recombinant plasmids, the restriction endonucleases of *BamHI*/*Kpn*I and *EcoRI*/*Xho*I were selected for cutting the plasmids carrying *msdxs2* and *msdxr*, respectively. For checking the plasmid harboring *msdxs1*, the designed recognition sites of *Kpn*I/*Sal*I were used. The cutting reactions were incubated at 35 °C and 37 °C for 2 h. The fragments were then separated on 1.2% agarose gel electrophoresis.

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

2.3.5.3.1 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. 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.

2.3.5.3.2 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 *E. coli* TOP10, whereas the recombinant DNA was transformed into *E. coli* XL1 blue MRF' for protein expression. 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.

2.3.6 Isolation and identification of the recombinant DNA

2.3.6.1 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 pGEM-T Easy, 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 $^{\circ}$C until used.

2.3.6.2 Identification of the recombinant DNA

For pDrive and pGEM-T Easy containing inserts, the restriction sites analyses were performed in the presence of *EcoR*I. 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/pGEM-TEasy
Plasmid DNA	5 µl
10x Buffer	1 µl (H buffer)*
*EcoR*I (20 units/µl)	0.5 µl
*Pst*I (20 units/µl)	-
Distilled water	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.

2.3.7 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.

2.3.8 DNA sequencing and sequencing analysis

The nucleotide sequences were analyzed at 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. 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).

2.4 Quantitative real time-polymerase chain reaction (qRT-PCR)

Transcription levels of *msdxs1* and *msdxs2* were measured by quantitative real-time (qRT) PCR technique using Sybr® GreenER™ pPCR Supermix (Invitrogen®, USA). The PCR product was measured directly using Applied Biosystems (ABI) 7300 real-time PCR (SEC, PSU). The PCR reaction was composed of 1x Sybr® GreenER™ pPCR Supermix for ABI PRISM®, 0.2 μM forward primer, 0.2 μM reverse primer, 10% (v/v) of undiluted cDNA and DEPC-treated water in total volume of 25 μl. The reactions were performed in 96-well plate. The reaction plate was placed in preheated real-time instrument. The annealing temperature for *msdxs1*, *msdxs2* and *18S rRNA* was 60 °C. Data were collected and analyzed. The data were automatically normalized with data from *18S rRNA*, an endogenous gene. The relative quantitation (RQ) was measured.

2.5 Proteinchemical methods

2.5.1 Heterologous expression and purification of the truncated MSDXR protein

For expression of truncated MSDXR protein, the recombinants *E. coli* XL1-blue MRF' harboring pQE30-T MSDXR (designated for truncated MSDXR 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 1.0 mM. The cells were further grown at 30 °C for 5 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 protein purification, the recombinant *E. coli* cells (0.4 g) were suspended in 2 ml of 1X buffer A (20 mM phosphate buffer, pH 7.0 containing 10 mM imidazole and 300 mM NaCl). Lysozyme (0.4 mg) was added to the suspension and subsequently incubated at 37 °C for 30 min. The suspension was cooled in an ice bath for 20 min. Ultrasonication was then performed (60% duty cycle, pulse on/off 9.9, 4 × 15 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.

Protein purification was performed using the ProPur™ IMAC purification kit (Nunc™, Roskilde, Denmark). The Nunc™ ProPur™ mini spin column was firstly equilibrated with 2 x 0.65 ml of binding buffer (50 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl, 10 mM imidazole), centrifuge at 1,800g for 1 min. The cell-free extract (0.65 ml) was loaded on the top of the pre-equilibrated column and centrifuged at 640g for 6 min. The spin column was washed with 5 x 0.65 ml of washing buffer 50 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl, 30 mM imidazole) and centrifuged at 1,800g for 1 min. The bound protein was eluted with 0.65 ml of elution buffer (50 mM sodium phosphate buffer, pH 7.4, 300 mM NaCl, 300 mM imidazole) and centrifuged at 1,800g for 1min. The elution fraction was desalted using ultrafiltration concentrator (10kDa MWCO PES, Nunc™). The protein solution was loaded and the concentrator was centrifuged at 12,000g for 5 min. The retentate was collected and used for SDS-PAGE analysis.

2.5.2 SDS-polyacrylamide gel electrophoresis

Proteins were separated on 10% SDS- polyacrylamide gel, which was prepared according to Laemmli method (1970). The electrophoresis system was powered from a power supply with 20 mA per gel. Gel was stained with Coomassie Blue R-250 and destained. Broad-range molecular weight (6-175 kDa, NEB, USA) were used as proteins markers.

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 ^{o}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)

2.6 Induction of hairy culture of *M. speciosa*

2.6.1 Bacterial strain and chemicals

Agrobacterium rhizogenes ATCC 15834 was obtained from the Microbiology Resource Center, Pathumtani, Thailand. Mitragynine was isolated from *M. speciosa* leaves (Keawpradub, 1990). Ursolic acid was purchased from Sigma-Aldrich Pte., Ltd. (Singapore). McCown woody plant (WPM) and plant agar

were from Duchefa Biochemie (Haarlem, The Netherlands). *N*-Benzyladenine (BA), 1-naphthylacetic acid (NAA) (95% GC), and 6-furfurylamino-purine (kinetin) were purchased from Fluka Chemie (Buchs, Switzerland). Thidiazuron (TDZ) was purchased from Supelco (Bellefonte, PA, USA). Bacterial media were purchased from Himedia Laboratories (Mumbai, India). All other chemicals were standard commercial products of analytical grade.

2.6.2 Plant materials

M. speciosa seeds were collected from Hat Yai District, Songkhla, Thailand. They were surface-sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox® (NaClO, Selangor, Malaysia) for 5 min and finally rinsing with sterile distilled water thoroughly. Sterile seeds were germinated on WPM medium supplemented with 1.0 mg/l BA, incubated at 25 °C under 16 h daily light. Two-month-old plantlets were used for bacterial infection.

2.6.3 Induction of M. speciosa hairy root cultures

A. rhizogenes ATCC 15834 were prepared freshly on yeast extract (YE) solid medium [containing 5.0 g/l beef extract, 1.0 g/l peptone, 5.0 g/l sucrose, 50 ml/l of 10% (w/v) $MgSO_4$ solution and 15 g/l agar] at 28 °C for 16 h. A single bacterial colony was inoculated into 5 ml YE broth medium, placed on a rotary shaker (218 rpm) and incubated at 28 °C for 16 h. The bacterial suspension was harvested by centrifugation at 4,000 rpm for 5 min and the pellet was re-suspended in sterile WPM medium. The OD_{600} value was adjusted to 0.5 - 0.6. The explants (stems and leaves) were wounded by needle and submerged in bacterial suspension for 30 min. The infected explants were washed with sterile water thoroughly and transferred to WPM containing 0.7% (w/v) plant agar. After 3 d of infection, explants were transferred to the WPM solid medium containing 500 mg/l cefotaxime disodium (M&H, Bangkok, Thailand) and, for further culture, the cefotaxime disodium concentrations were decreased to 250, 100 and 50 mg/l each week. Cultures free of *A. rhizogenes* were transferred to hormone-free WPM solid medium. Hairy roots were initiated within 10 d after infection, at (25 ± 2) °C under darkness. Hairy roots were excised from explants and maintained in WPM liquid medium. For untransformed roots, the seedling roots were cut and cultured in WPM liquid medium. Both types of culture were kept at (25 ± 2) °C in the darkness and rotary-shaken at 80 rpm.

2.6.4 Identification of transformed hairy roots by PCR analysis

Genomic DNA was isolated from untransformed roots and hairy roots using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The transformed genes in hairy roots were identified by PCR analysis for the rooting locus genes *rol*A and *rol*B.

For *rol*A (300-bp) forward primer : 5'-CAGAATGGAATTAGCCGGACTA-3' and

reverse primer : 5'-CGTATTAATCCCGTAGGTTTGTTT-3'

For *rol*B (780-bp) forward primer : 5'- ATGGATCCCAAATTGCTATTCCTTCCACGA -3'

reverse primer :5'- TTAGGCTTCTTTCTTCAGGTTTACTGCAGC -3'

The PCR reactions were performed in a total volume of 50 µl, containing 50 pg of genomic DNA, 0.5 µM of each primer, 200 µM dNTP, 2.5 U of Taq DNA polymerase (New England Biolab, MA, USA), and 1x ThermolPol buffer (New England Biolab). PCR conditions were 94 ^{o}C for 2 min, 24 cycles of 94 ^{o}C for 1 min, 58 ^{o}C for 1 min, 72 ^{o}C for 1 min for *rol*A and 30 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, 72 ^{o}C for 3 min for *rol*B and a final extension at 72 ^{o}C for 10 min. PCR fragments were analyzed by 1.2% (w/v) agarose gel electrophoresis and visualized by UV transluminator (312 nm) after ethidium bromide staining.

2.6.5 Isolation of ursolic acid and phytosterols

Dried hairy roots of *M. speciosa* (37.4 g) were macerated with 300 ml methanol for 3 d and filtered. The marc was re-macerated and the methanol fractions were pooled and evaporated to dryness. The residue (8.52 g) was dissolved in 100 ml methanol, partitioned with 100 ml *n*-hexane for three times, and evaporated. The crude *n*-hexane extract (403 mg) was further purified by loading on the top of a silica gel column (3 x 18 cm; Scharlau, La Jota, Spain) and eluted with *n*-hexane/ethyl acetate (9:1; 8:2; 7:3; 5:5, v/v), ethyl acetate, ethyl acetate/methanol (8:2; 5:5, v/v) and methanol. From TLC analysis, 11 fractions were obtained. Fractions F6 and F10 were further purified. F6 was re-crystallized with the ratio of solvents of chloroform/methanol (7:3, v/v). White needle crystals were obtained, affording MSF1 (6.1 mg). F10 was washed with *n*-hexane. A white amorphous solid was obtained, affording MSF2 (10.11 mg).

Concerning the structure of MSF2, the ^{1}H NMR spectra it exhibited signals as typical as for the structure of triterpenoid compounds. The NMR data were obtained as follows.

^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$): 3.20 (t, 1H, J = 7.5 Hz, H-3), 5.24 (m, J = 3.5 Hz 1H, H-12), 2.20 (d, J = 11.0 Hz, 1H, H-18), 2.02-1.15 (m, H-22), 0.98 (s, 3H, Me-23), 0.78 (s, 3H, Me-24), 0.93 (s, 3H, Me-25), 0.82 (s, 3H, Me-26), 1.10 (s, 3H, Me-27), 0.86 (d, J = 6.5 Hz, 3H, Me-29), 0.95 (d, J = 6.5 Hz, 3H, Me-30)

^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$): 38.4 (C-1), 26.5 (C-2), 78.5 (C-3), 39.2 (C-4), 55.0 (C-5), 18.0 (C-6), 32.8 (C-7), 39.2 (C-8), 47.3 (C-9), 36.7 (C-10), 23.9 (C -11), 125.2 (C-12), 137.9 (C-13), 41.8 (C-14), 23.9 (C-15), 23.0 (C-16), 47.6 (C-17), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20), 30.4 (C-21), 36.6 (C-22), 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 180.5 (C-28), 16.7 (C-29), 20.9 (C-30)

DEPT 90 experiment CH ($CDCl_3/CD_3OD$): 78.5 (C-3), 55.0 (C-5), 47.3 (C-9), 125.2 (C-12), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20)

DEPT 135 experiment CH_2 ($CDCl_3/CD_3OD$): 38.4 (C-1), 26.5 (C-2), 18.0 (C-6), 32.8 (C-7), 23.9 (C-11), 23.9 (C-15), 23.0 (C-16), 30.4 (C-21), 36.6 (C-22); CH_3: 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 16.7 (C-29), 20.9 (C-30)

2.6.6 Quantification of ursolic acid content

The ursolic acid content was determined using the HPLC method as described in Chen et al. (2003). Dried hairy root powder (100 mg) was refluxed thrice with 50 ml of *n*-hexane for 1 h at 50 °C and filtered. Pooled *n*-hexane fractions were evaporated to dryness. For HPLC analysis, the residue was dissolved in 5 ml of acetonitrile, filtered through a 0.45 μm membrane prior to HPLC injection. An HPLC system (Agilent 1100 Series LC System, Agilent Technologies, Wilmington, USA) was equipped with a C18 reverse phase column (Bondapak, Waters, USA, 3.9 x 300 mm, 10 μm) and UV detector (photodiode array) set at 206 nm. Ursolic acid was eluted with isocratic elution of acetonitrile/0.1% (v/v) H_3PO_4 in water (70:30, v/v) with a flow rate of 1 ml/min at 15.5 min. A calibration curve of authentic ursolic acid (Sigma-Aldrich) was established. Linearity of the calibration curve was observed in the range 15 - 120 μg/ml with r^2 of 0.9999 (% RSD of 0.09 - 0.45%). Each calibration point was carried out in triplicate.

2.6.7 Quantification of mitragynine content

The TLC-densitometric method was established for determination of the mitragynine content developed by Keawpradub (1990). The regenerated plants were harvested, dried at 50 °C for 12 h, ground and used as material for preparation of the crude alkaloid extract. The dried powder (200 mg) was refluxed with 50 ml methanol three times at 60 °C for 1 h and filtered. The filtrates were pooled and evaporated to dryness. The dried residue was re-dissolved in 30 ml of 7% (v/v) acetic acid/H_2O and filtered. The acidic filtrate was washed with petroleum ether, and then the solution was basified with 25% (v/v) ammonia solution to pH 9. The filtrate was partitioned with 50 ml chloroform three times. The chloroform fractions were pooled and evaporated to dryness. The crude alkaloid extract was dissolved in 5 ml chloroform and the solution (5 µl) was subjected to a TLC plate (Silica gel F_{254}, Merck). The mobile phase was chloroform/ methanol (9:1). The R_F-value of mitragynine was 0.7. After removing from the tank, the TLC plate was dried and placed in a CAMAG TLC scanner (Muttenz, Switzerland) equipped with Cats version 4.01 software. The UV detector was set at 254 nm, and peak areas were integrated and converted to concentrations using a calibration curve. The linearity of the calibration curve of authentic mitragynine was in the range of 0.3 - 5.0 mg/ml with R^2 of 0.9984. The peak identity was performed by scanning the UV absorption at 200 - 600 nm.

2.6.8 Spectroscopy

^1H (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Unity Inova NMR spectrophotometer (Varian, Darmstadt, Germany). A mixture of $CDCl_3$ and CD_3OH (1:1) was used as solvent and tetramethylsilane (TMS) was used as the internal standard.

3. Results

In this study, we aimed to study on mitragynine biosynthesis. To succeed the goal, we firstly investigated the genes, which involved in the early step of isoprenoid biosynthesis. An evidence of loganin, a precursor for secologanin, is supplied from the isoprene units via the DXP pathway in *Rauwolfia serpentina* (Eichinger et al., 1999). Therefore, we hypothesized that secologanin in the structure of mitragynine should also be supplied from isoprene unit originated from the DXP pathway. Study on the genes, which involved in the DXP pathway, may lead us to get more knowledge on the mitragynine biosynthesis.

Genes encoding 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) were studied designated as *msdxs* and *msdxr*, respectively. Since the *msdxs* and *msdxr* genes from *M. speciosa* have not been reported in the GenBank, however, there are reported from others plants *dxs* and *dxr*. By homology-based alignment, we could design the degenerate primers from the conserved regions of known *dxs* and *dxr* genes and used for cDNA clonings of *msdxs* and *msdxr* genes. The nucleotide sequences of the internal sequences of *msdxs* and *msdxr* genes allowed us to design the specific primers for 5'- and 3'-end clonings. Together with technology of RLM-RACE amplifications, the 5'- and 3'-ends DNA fragments were eased to amplify by PCR reactions. The nucleotide sequences of the DNA fragments, including internal sequences, 5'- and 3'- ends were assembled. The putative nucleotide sequences of the full-length *msdxs* and *msdxr* genes were analyzed for their functional characteristics. The *msdxs* and *msdxr* genes were then cloned and expressed. According to previously report, the DXS and DXR proteins in higher plants function maturely in plastid. In this study, the truncated (pseudo-mature) proteins were investigated by preparing the PCR products to the expression vectors. The recombinant plasmids were transformed and heterologous expressed in *E. coli* XL1blue MRF'. By using the pQE30 plasmid, the fusion proteins were obtained and made the purification possible in a single step of metal-chelating column. For heterologous expression of *msdxs*, construction of recombinant *msdxs* gene in the expression vector was unsuccessful. However, the recombinant *msdxr* in pQE vector was transformed and expressed. The *msdxr* gene product was low amount, which is not enough for further activity test. In parallel, the hairy root culture of *M. speciosa* was established in this study. To determine the expression profiles of *msdxs1* and *msdxs2* genes in the *in vitro* plants, mRNA

expressions of *msdxs1* and *msdxs2* genes were determined using qRT-PCR technique. Transcription profiles of *msdxs1* and *msdxs2* genes figured the regulatory roles in mitragynine biosynthesis.

3.1 cDNA preparation from total RNA

From 300 mg of young leaves, the total RNA was isolated using RNeasy plant mini kit. 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. To determine the total RNA concentration, dilution of total RNA was measured for A_{260} and A_{280} using a spectrophotometer. The concentration of the resulting RNA was 97 μg/ml in total volume of 92 μl, afforded 8.92 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 1.97, in which pure RNA should have an A_{260}/ A_{280} ratio of 1.9-2.1. This total RNA was used for preparation of cDNA by means of ImProm II reverse transcriptase and 5'- and 3'-end GeneRacer amplification kit.

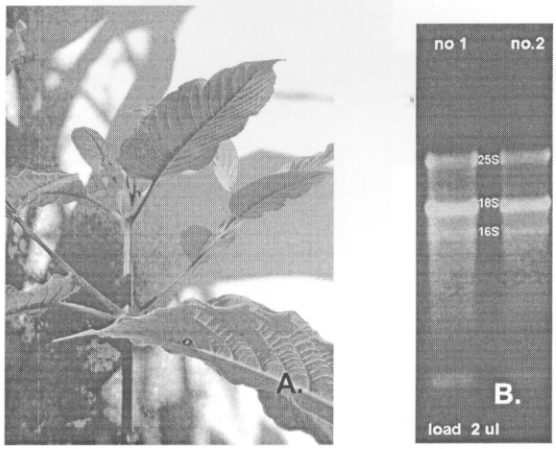

Figure 3.1 Isolation of total RNA from *M. speciosa*. A. young leaves of 2-year old plant; B. Analysis of intact RNAs on 1.2% (w/v) agarose gel electrophoresis stained with ethidium bromide solution and visualized under UV transluminator (312 nm).

3.2 cDNA cloning of 1-deoxy-D-xylulose 5-phosphate synthase (*msdxs*)

3.2.1 The internal sequences of *msdxs*

Amplifications of the internal sequences were performed as described in 2.3.4.1. With 2 combinations of [PIH-IAE] and [MIS-ITV] afforded the resulting PCR products at size of *ca.* 1,100 bp and 1,000 bp, respectively as shown in Fig. 3.2. The PCR products were purified and ligated into pDrive vectors. The recombinant plasmids were sequences. The results showed that the [PIH-IAE] domain contained 1,169 bp and the [MIS-ITV] domain contained 1,010bp.

The [PIH-IAE] domain clonings were shown to have at least 2 isoforms of nucleotide sequences. Five recombinant plasmids were sequenced. As shown in Fig. 3.3, the [PIH-IAE] domain could be classified into 2 groups, which are class I (contig 11 & 16) and class II (contig 12, 13, 14). The *msdxs* class I and class II was renamed as *msdxs1* and *msdxs2*, respectively.

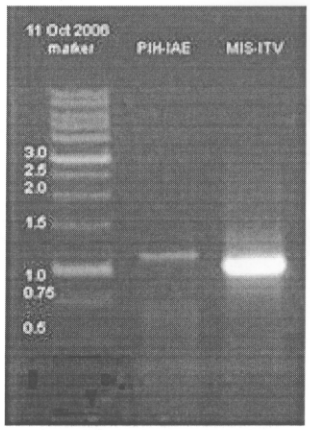

Figure 3.2 1.2% Agarose gel electrophoresis of the internal sequences of *msdxs*. Lane 1: DNA ladder; Lane 2: PCR product from PIHDXS1-IAEDXS2; Lane 3: PCR product from MISDXS2-ITVDXS2.

The [MIS-ITV] domain nucleotide sequences were obtained from three recombinant plasmids. Alignment of [MIS-ITV] domain revealed that it has 100% similarity to *msdxs1*. Assembling of *msdxs1* from [PIH-IAE] domain and [MIS-ITV] domain with CAP3 (Huang and Madan, 1999) provided the *msdxs1* internal sequence of 1,697 bp. Deduced amino acids of the *msdxs1* showed similarity to *dxs* from *Andrographis paniculata* (accession no. AAP14353), *Lycopersicon esculentum* (accession no. AAT97962.1) and *Capsicum annuum* (accession no. 07828). The informations of internal sequences were further used to design for 5'-end and 3'-end of *msdxs1* and *msdxs2*.

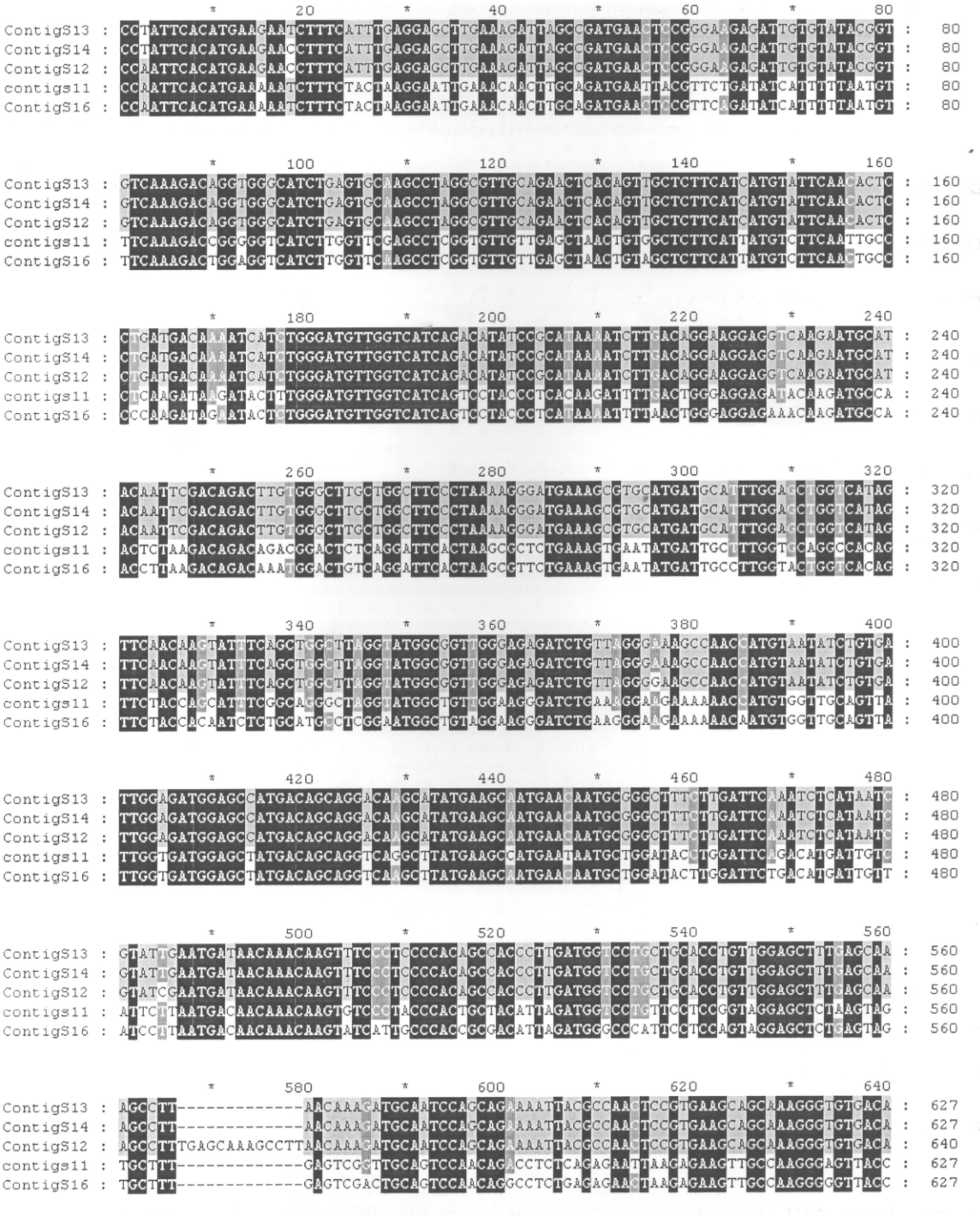

Figure 3.3 Alignments of five clones of internal sequences obtained form [PIH-IAE] domain.

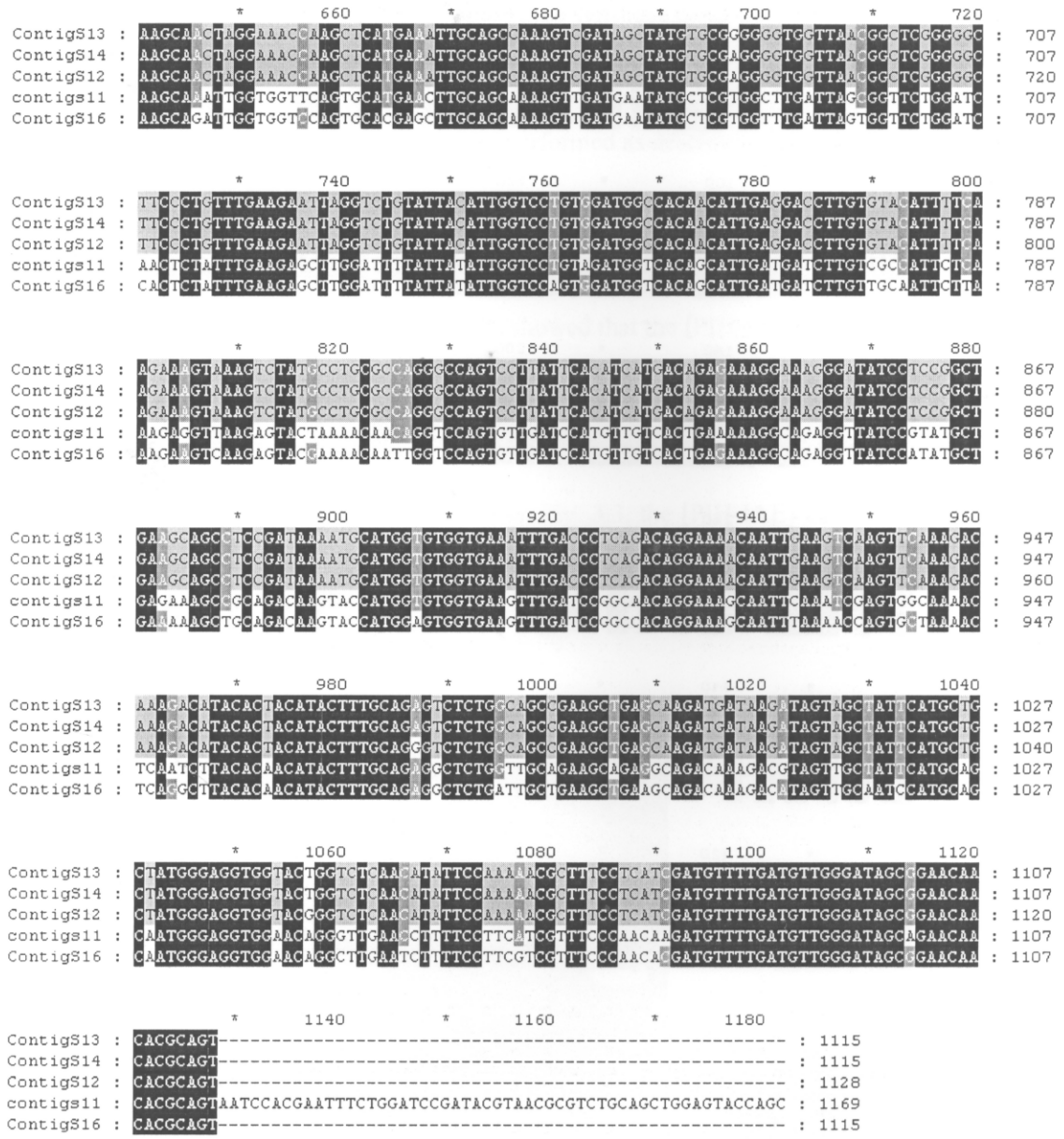

Figure 3.3 (continued).

3.2.2 The 5'-and 3'-ends of *msdxs*

For *msdxs1*, amplifications of the 5'-end and 3'-end were performed as described in 2.3.4.2. The PCR products of 5'-end was 450 bp and 3'-end was 300 bp. After clonings, the resulting nucleotide sequences were obtained. The 5'-end, internal sequence and 3'-end were assembled, affording cDNA *msdxs1* as shown in Fig. 3.4. For *msdxs2*, primers for 5'-end and 3'-end amplifications were designed based on the class II internal sequence, which obtained from [PIH-IAE] domain. The PCR products were obtained, cloning and sequenced. The 5'-end, internal sequence and 3'-end were assembled, affording cDNA *msdxs2* as shown in Fig. 3.5.

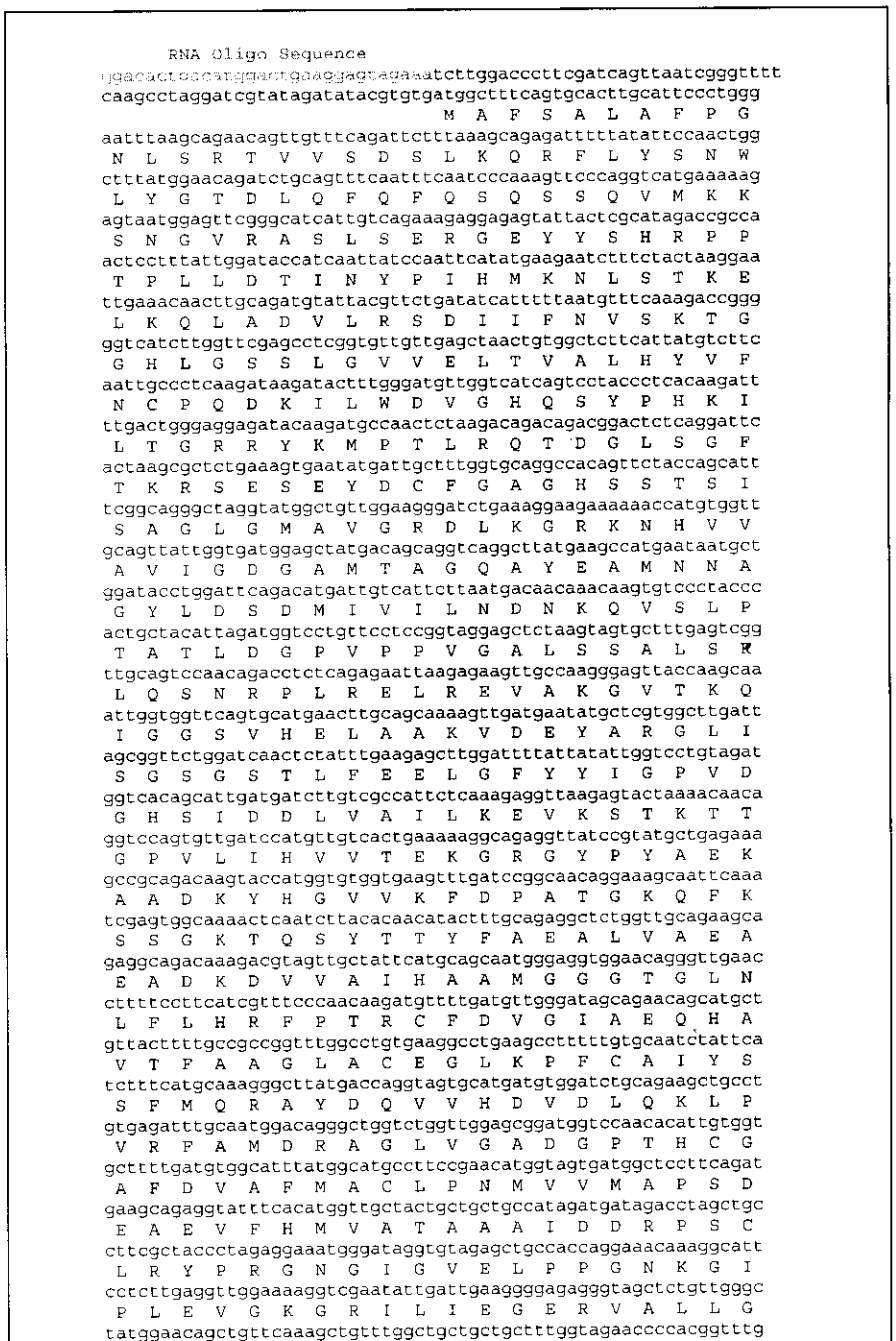

Figure 3.4 cDNA of *msdxs1* from *M.speciosa*.

3.2.3 The full-length cDNA of *msdxs*

Information of the partial DNA sequences of 5'-end, internal sequences and 3'-end allowed us to design the specific primers for the full-length of *msdxs1* and *msdxs2* genes amplifications. The PCR products were achieved as shown in Fig. 3.6. The full-length genes were cloned and sequenced, confirmed the nucleotide sequences of the previously information. For truncated *msdxs1* and *msdxs2* genes, were further purified, digested and ligated into the pQE30 expression vectors for protein expression.

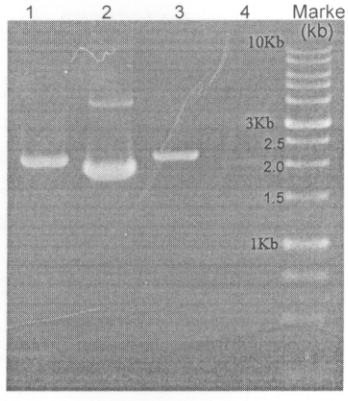

Figure 3.6 Analysis of the PCR products of the full-length and truncated genes of msdxs1 and msdxs2 on 1.2% agarose gel electrophoresis after staining with ethidium bromide solution and visualized under transluminator (312nm). Marker: DNA ladder (SibEnzyme); 1: full-length *msdxs1* (*ca.* 2.0 kb); 2: truncated *msdxs1* (*ca.* 1.9 kb); 3: full-length *msdxs2* (*ca.* 2.2 kb); 4: truncated *msdxs2* (*ca.* 2.0 kb).

In this study, at least two isoforms of *dxs* existed in *M. speciosa* leaves, which are *dxs* class I (*msdxs1*) and *dxs* class II (*msdxs2*). The full-length cDNA of *msdxs1* contained 2,010 bp, which encoded the open reading frame (ORF) of MSDXS1 for 669 amino acid residues. Molecular weight and isoelectric point (pI) have been predicted to be 71,963.26 dalton and 6.89, respectively (http://au.expasy.org/cgi-bin/pi_tool). The full-length of cDNA of *msdxs2* contained 2,193 bp, encoded the open reading frame (ORF) of MSDXS2 for 730 amino acid residues. Molecular weight and isoelectric point (pI) have been predicted to be 78,562.74 dalton and 6.46, respectively.

3.2.4 Primary structure of MSDXS1 and MSDXS2

The full-length genes of the *msdxs1* and *msdxs2* were translated into amino acid sequences using DNASIS software. Primary structures of MSDXS1and MSDXS2 were analyzed using PSI-BLAST. Localizations of proteins were predicted using ChloroP and Target P programs.

The amino acid sequences of MSDXS1 (669 residues) and MSDXS2 (730 residues) were separately submitted to http://blast.ncbi.nlm.nih.gov/Blast.cgi in order to perform the identity of MSDXS to other known DXS in plants. Percent identity of MSDXS1 and MSDXS2 are summarized in Table 3.1.

BLAST P suggested that the MSDXS1 and MSDXS2 proteins belong to the transketolase and dehydrogenase E1 component family. Searching for the TPP-binding and the transketolase motifs in the MSDXS revealed that the MSDXS 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) (Hawkins et al., 1989) and the consensus sequence for the transketolase motif (designated as DRAG-X_{28}-P-X-D) (Schenk et al., 1997), respectively. In comparison with the *E. coli* DXS found that the MSDXS1 and MSDXS2 carry the catalytic amino acid residues as shown in Fig. 3.7 as same as the previously report in *E. coli* (Sprenger et al., 1997; Lois et al., 1998), and in pepper (*Capsicum annuum*) (Bouvier et al., 1998). The bioinformatic results from primary structures suggested that MSDXS1 and MSDXS2, obtained from this study, are member of DXS enzyme family. However, the functional characterization of MSDXS1 and MSDXS2 in the intact plant is necessary in order to understand the regulatory roles of MSDXS1 and MSDXS2 in mitragynine biosynthesis

The topology search for subcellular prediction, the MSDXS protein were predicted with ChloroP 1.1 to have chloroplast transit peptide (cTP = 0.533 for MSDXS1 and cTP = 0.551 for MSDXS2). Prediction of length of cTP with ChloroP suggested those 55 residues for MSDXS1 and 48 residues for MSDXS2 from the N-terminal region (Fig 3.7). From this predictions indicated that both proteins located in the chloroplast organelle.

Table 3.1 Comparison the sequence of MSDXS1 and MSDXS2 with other plants DXSs

Plant species	% identity to	
	MSDXS1	MSDXS2
Mitragyna speciosa DXS1	**100**	**66**
M. speciosa DXS2	**66**	**100**
Adonis aestivalis var. palaestina	75	74
Andrographis paniculata	88	72
Antirrhinum majus	73	79
Arabidopsis thaliana	84	71
Arabidopsis thaliana, DXS1	74	69
Artemisia annua	85	74
Brassica rapa	83	74
Capsicum annuum	87	74
Catharanthus roseus	75	77
Chrysanthemum x morifolium	75	80
Croton stellatopilosus	84	69
Elaeis guineensis	84	73
Ginkgo biloba	84	75
Hevea brasiliensis	87	79
Lycopersicon hirsutum, DXS2	71	78
Lycopersicon esculentum	87	78
Medicago truncatula	69	79
Medicago truncatula1	88	72
Mentha x piperita	70	78
Morinda citrifolia	76	77
Narcissus pseudonarcissus	71	79
Oryza sativa Japonica Group	88	76
Physcomitrella patens subsp. patens	84	74
Picea abies	84	73
Picea abies2	77	73
Picrorhiza kurrooa	67	75
Populus trichocarpa	90	75
Pueraria montana var. lobata	88	74
Salvia miltiorrhiza	86	74
Stevia rebaudiana	75	76
Tagetes erecta	73	77
Taxus x media	70	74
Vitis vinifera	83	81
Zea mays	87	78

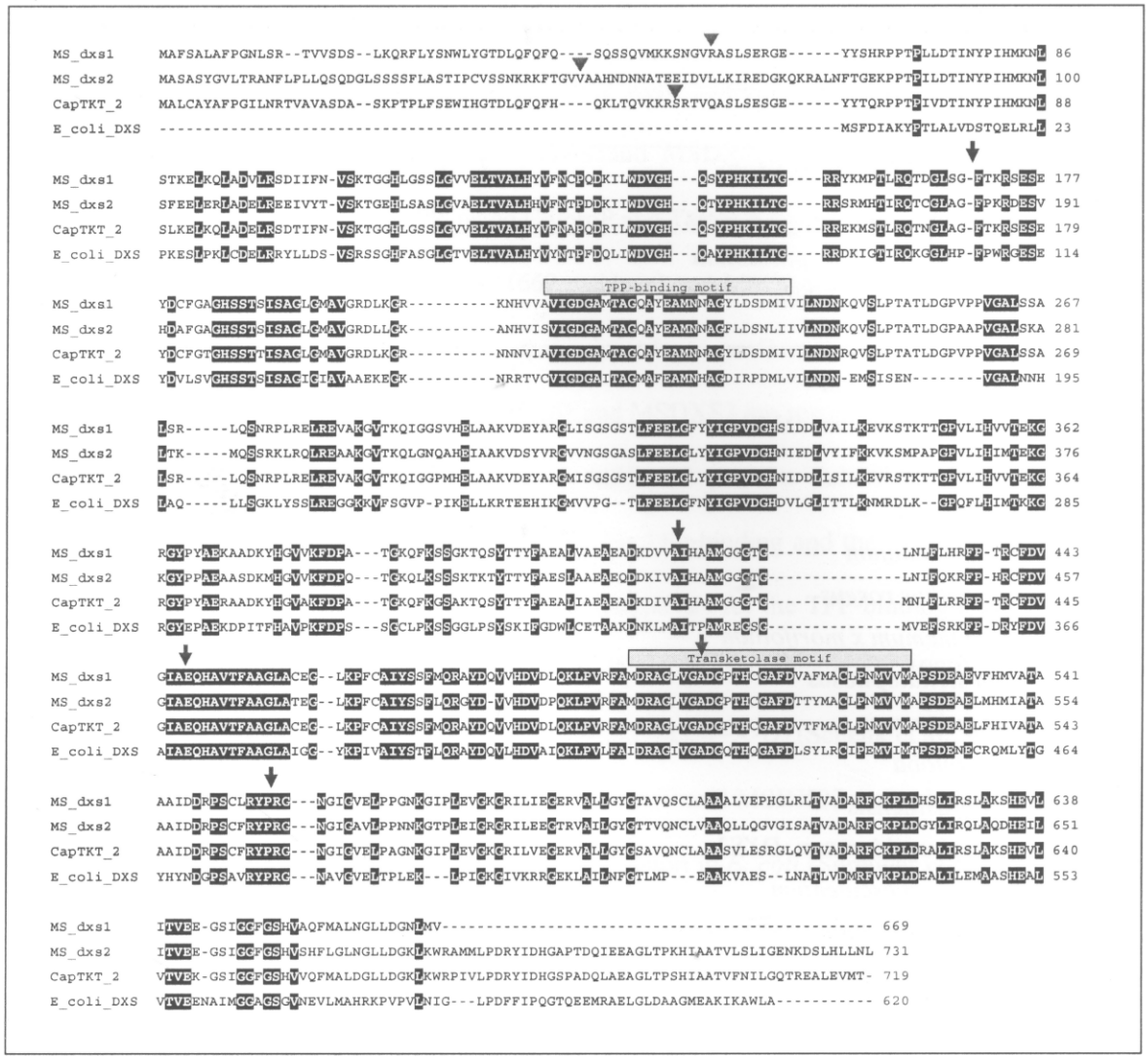

Figure 3.7 Alignment of DXS sequence from *M. speciosa*, *Capsicum annuum* and *E. coli*. Invert triangles are the N-terminal putative chloroplast transit peptide (ChloroP). Shaded boxes indicate TPP-binding motif and transketolase motif (Bouvier et al., 1998). Arrows denote amino acid residues that form the substrate channel of transketolase (Sprenger et al., 1997).

MSDXS1 and MSDXS2 share 66% identities [DNASIS software] to each other. Although, the DXS family is divided into two classes which are DXS class I and DXS class II, based on the phylogenetic tree. According to classification of Walter et al. (2002), DXS sequences from *Arabidopsis*, *Artemisia*, *Lycopersicon* and *Capsicum* fall into class I (DXS1) category, while sequences from *Catharanthus*, *Mentha*, and *Tagetes* fall into class II (DXS2) category. However, some evidences were observed as misclustering of the genes (Krushkal et al., 2003). It may cause from sequences represent paralogous

rather than orthologous genes, wrong position of the tree root, a violation of the molecular clock, and difference in the rates of DXS evolution and different functional roles of DXS among plant species (Krushkal et al., 2003). In the case of *M. speciosa* DXS, we named them according to the distance of MSDXS1and MSDXS2 to known classified DXS (Fig. 3.8).

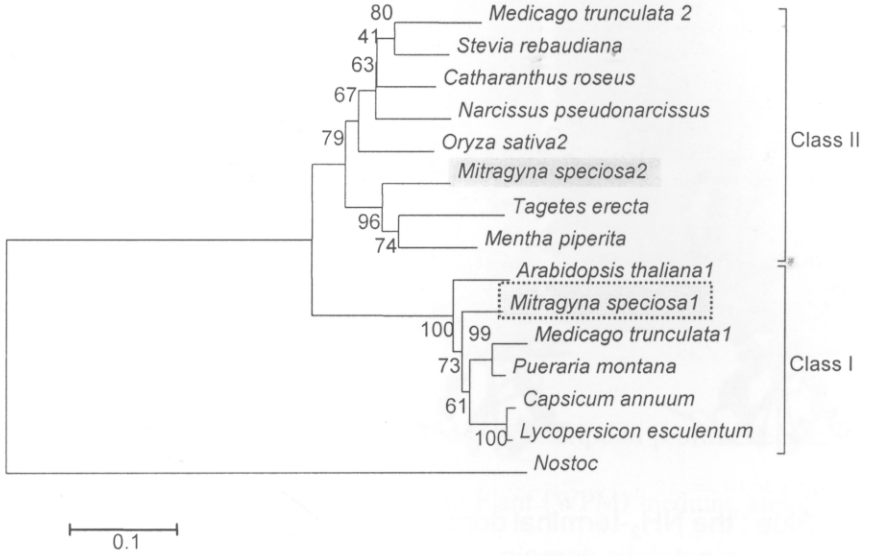

Figure 3.8 Phylogenetic analysis of deduced amino acid of DXS class I & II from *M. speciosa* to other plants DXS class I & II.

```
MS_dxs1   1   ------------------MAFSAIAFPGNFSRTVVSDSLKQRFLYSNWLYGTFLQFQFQSQSSQVMKKSN  52
MS_dxs2   1   MASASYGVLTRANFLPLLQSQDGFSSSSFLASTIPCVSSNKRKFTGVVAAHNENNATEEIDVLLKIREDG  70

MS_dxs1  53   GVRASLSERGEYYSHRPPTPLLDTINYPIHMKNLSTKELKQLADVLRSDIIFNVSKTGGHLGSSLGVVEL  122
MS_dxs2  71   KQKRALN----FTGEKPPTPILDTINYPIHMKNLSFEELERLADELREEIVYTVSKTGEHLSASLGVAEL  136

MS_dxs1 123   TVALHYVFNCFQDKILWDVGHQSYPHKILTGRRYKMPTLRQTDGLSGFTKRSESEYDCFGAGHSSTSISA  192
MS_dxs2 137   TVALHHVFNTFDDKIIWDVGHQTYPHKILTGRRSRMHTIRQTCGLAGFPKRDESVHDAFGAGHSSTSISA  206
                            ##########################
MS_dxs1 193   GLGMAVGRDLKGRKNHVVAVIGDGAMTAGQAYEAMNNAGYLDSDMIVILNDNKQVSLPTATLDGPVPPVG  262
MS_dxs2 207   GLGMAVGRDLLGKANHVISVIGDGAMTAGQAYEAMNNAGFLDSNLIIVLNDNKQVSLPTATLDGPAAPVG  276

MS_dxs1 263   ALSSALSRLQSNRPLRELREVAKGVTKQICGSVPELAAKVDEYARGLISGSGSTLFEELGFYYIGPVDGH  332
MS_dxs2 277   ALSKALTKMQSSRKLRQLREAAKGVTKQLGNQAHEIAAKVDSYVRGVVNGSGASLFEELGLYYIGPVDGH  346

MS_dxs1 333   SIDDLVAILKEVKSTKTTGPVLIHVVTEKGRGYPYAEKAADKYHGVVKFDPATGKQFKSSGKTQSYTTYF  402
MS_dxs2 347   NIEDLVYIFKKVKSMPAPGPVLIHIMTEKGKGYPPAEAASDKMHGVVKFDPQTGKQLKSSSKTKTYTTYF  416

MS_dxs1 403   AEALVAEAEADKDVVAIHAAMGGGTGLNLFLHRFPTRCFDVGIAEQHAVTFAAGLACEGLKPFCAIYSSF  472
MS_dxs2 417   AESLAAEAEQDDKIVAIHAAMGGGTGLNIFQKRFPHRCFDVGIAEQHAVTFAAGLATEGLKPFCAIYSSF  486
                            ..........................................
MS_dxs1 473   MQRAYDQVVHDVDLQKLPVRFAMDRAGLVGADGPTHCGAFDVAFMACLPNMVVMAPSDEAEVFHMVATAA  542
MS_dxs2 487   LQRGYDEVVHDVDFQKLPVRFAMDRAGLVGADGPTHCGAFDITYMACLPNMVVMAPSDEAELMHMIATAA  555

MS_dxs1 543   AIDDRPSCLRYPRGNGIGVELPEGNKGIPLEVGKGRILEGERVALLGYGTAVQSCLAAAALVEPHGLRL  612
MS_dxs2 556   AIDDRPSCFRYPRGNGIGAVLPENNKGTPLEIGRGRILEEGTRVALLGYGTTVQNCLVAAQLLQGVGISA  625

MS_dxs1 613   TVADARFCKPLDHSLIRSLAKSHEVLITVEEGSIGGFGSHVAQFMALNGLLDGNLMV-------------- 669
MS_dxs2 626   TVADARFCKPLDGYLIRQLAQDHEILITVEEGSIGGFGSHVSHFLGLNGLLDGKLKWRAMMLPDRYIDHG 695

MS_dxs1 669   ------------------------------------ 669
MS_dxs2 696   APTDQIEEAGLTPKHIAATVLSLIGENKDSLHLLNL 731
```

Figure 3.9 Alignment of deduced amino acids of DXS class I & II showing the 66% identity [DNAsis] ### shows region of TPP-binding motif; ---- shows the region of transketolase motif.

3.2.5 Three-dimensional structure of MSDXS of *M. speciosa*

Based on the primary sequence of MSDXS1, 3-D structure has been predicted for protein architecture. In comparison with *E. coli* DXS, MSDXS1 shows similar architecture (Fig. 3.10). The locations of NH_2-terminal domain, a catalytic domain and the COOH-terminal domain of MSDXS1 appeared in parallel with *E. coli* DXS. Since each DXS has a unique catalysis in each organism, therefore, the catalytic velocity as well as Michaelis-Menten constants should be measured.

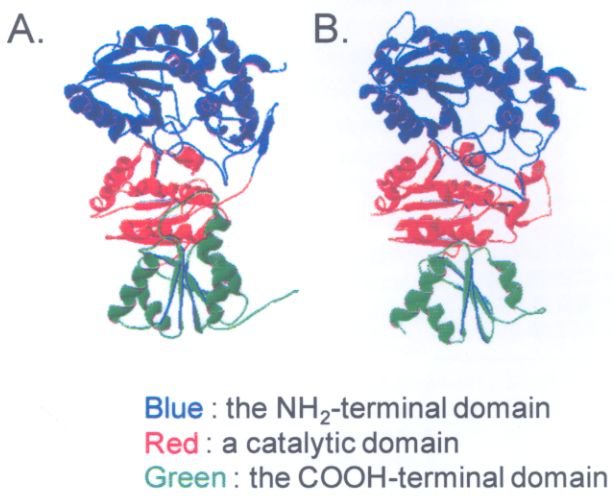

Figure 3.10 The predicted 3-D structures; A. *E. coli* DXS; B. MSDXS1

3.2.6 Transcription profiles of *msdxs1* and *msdxs2* in *M. speciosa*

1-Deoxy-D-xylulose 5-phosphate synthase (DXS; EC 4.1.3.37) catalyzes the first step of the early step in mitragynine biosynthesis. As mentioned above, there are two isoforms of DXS in *M. speciosa*, namely MSDXS1 and MSDXS2. However, the physiological roles of the *msdxs1* and the *msdxs2* in mitragynine biosynthesis are still unknown.

To determine the regulatory roles of *msdxs1* and *msdxs2* in mitragynine biosynthesis, the mRNAs expressions of both genes were measured, when *18S-RNA* (house-keeping gene) was used as endogenous gene. Plants generated from hairy root culture were used as our plant material, so called transgenic plant. As described in section 3.3, the transgenic plants of *M. speciosa* were obtained from shoot regeneration of the hairy root cultures by infection of *Agrobacterium rhizogenes* ATCC15834. Interestingly, transgenic plants produced and accumulated mitragynine about 3 times higher than the *in vitro* plants, which

obtained from seed germination. Hypothetically, the biosynthesis of mitragynine in the transgenic plant might be different from the *in vitro* plants. Fig. 3.11 shows the characteristics of transgenic plant and *in vitro* plant, which used for expression profiles determination.

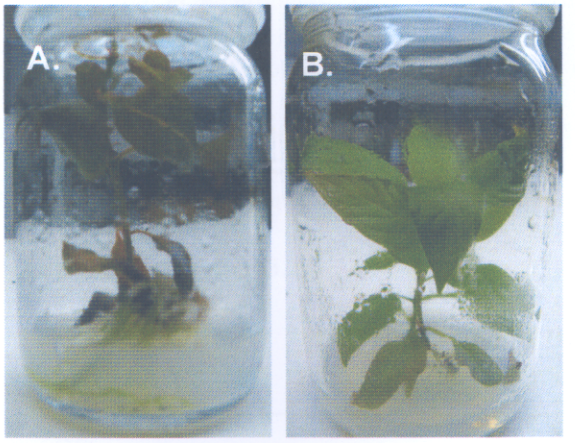

Figure 3.11 *M. speciosa* (6-week old) used in transcription profiles determination. A.; transgenic plant; B. *in vitro* plant, grown in McCown Woody Plant (WPM) medium, supplemented with 1 mg/ml indole butyric acid (IBA).

Thus, in order to follow the mitragynine biosynthesis in the transgenic and the *in vitro* plants, the transcription profiles of the *msdxs1* and the *msdxs2* were determined by quantitative real-time (qRT)-polymerase chain reaction (PCR) technique using Sybr® GreenER™ qPCR Supermix and Applied Biosystems 7300 Real-time PCR. The relative quantitation (RQ) was measured when the *18S rRNA*, a house-keeping gene, was used as an endogenous gene. As shown in Table 3.2 and Fig. 3.12, the results showed that the *msdxs1* in leaves, roots and stems of the transgenic plants were expressed higher than the *in vitro* plants with a magnitude of 1.5 times. In addition, the *msdxs2* in roots of the transgenic plants were expressed tremendously higher than the *in vitro* plants with a magnitude of 34 times. In contrast with the *msdxs2* expressions in leaves and stems of the transgenic plants were suppressed about 50%. As mitragynine is accumulated mostly in the leaves, it can be suggested that the *msdxs1* plays a regulatory role in the mitragynine biosynthesis rather than the *MSDXS2*. Therefore, the *msdxs2* is a potential target for further metabolic engineering for the production of mitragynine in *M. speciosa*.

Table 3.2 Relative quantitation of the *msdxs1* and *msdxs2* in transgenic *M. speciosa.*

mRNA/part of plant	*M. speciosa*		Ratio [T/I]
	in vitro plant [I]	transgenic plant [T]	
msdxs1			
leaf	1	1.316	1.316
root	0.355	0.789	2.223
stem	0.738	0.841	1.140
msdxs2			
leaf	1	0.460	0.460
root	0.024	0.819	34.125
stem	1.011	0.617	0.619

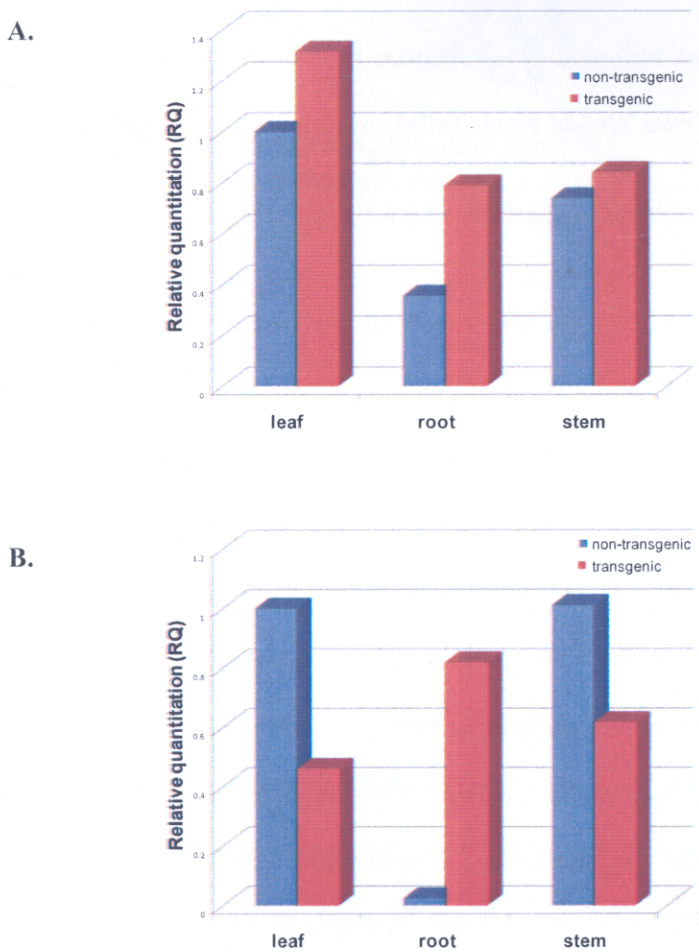

Figure 3.12 Relative quantitation of *msdxs1* (A) and *msdxs2* (B) of *in vitro* and transgenic plants of *M. speciosa*. Data were normalized with endogenous gene (*18S rRNA*) and RQ of leaves.

3.3 cDNA cloning of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*msdxs*)

3.3.1 The internal sequence of *msdxr*

Amplifications of the internal sequences were performed as described in 2.3.4.1. With 2 combinations of [WDG-EVK] and [GIV-EKA], the PCR products were expected sizes for 594 bp and 719 bp, respectively. Fig. 3.13 shows the PCR product, obtained from [GIV-EKA] domain. The PCR products were purified and ligated into pDrive vectors. Three recombinant plasmids were sequenced. Nucleotide sequences of the two domains were overlapped, therefore, the internal sequence of *msdxr* was obtained for 1,022 bp after assembling by CAP3 program (Fig. 3.14).

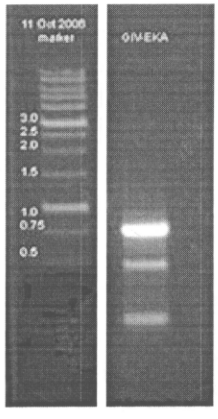

Figure 3.13 1.2% Agarose gel electrophoresis of the internal sequences of *msdxr*. Lane 1: DNA ladder; Lane 2: PCR product from [GIV-EKA].

```
Assembling DXR domain 1 and 2 using CAP3
>CloneR11R22, 1022 bp
GGATGGTCCAAAGCCTATTTCAATAGTTGGATCAACAGGGTCCATTGGAACTCAGACGCT
GGACATAGTTGCGGAAAATCCAGACAAATTCAGAGTCGTTGCACTTGCAGCTGGTTCAAA
TGTGACTCTTCTTGCTGATCAGGTTAAGACATTTAAACCTCAATTAGTTGCAGTCAGAAA
TGAGTCGTTAGTTGATGAACTCGAAGAAGCTTTGGCTGATTTTGAATACAAACCTGAAAT
TATTCCGGGAGAAGAGGGAGTAGTTGAGGTAGCTCGCCACCCTGATGCTGTTTCTGTTGT
CACGGGAATAGTGGGTTGTGCTGGTCTAAAGCCAACAGTGGCTGCCATAGAAGCAGGGAA
AGATATTGCCTTGGCCAATAAAGAGACACTAATTGCTGGTGGTCCATTTGTCCTTCCTCT
TGCGCACAAGCACAAAGTGAAGATTCTTCCTGCTGATTCAGAACATTCTGCCATATTCCA
GTGTATACAAGGTTTGCCTGAAGGTGCTCTTAGGCGTATCATTTTAACAGCATCTGGAGG
TGCTTTCAGGGATTTGCCAGTTGAGAAATTGAAAGAAGTTAAAGTAGCAGATGCTTTGAA
ACATCCCAACTGGAACATGGGAAAAAAGATTACAGTAGATTCTGCGACTCTATTTAATAA
GGGCCTTGAAGTAATTGAAGCCCATTACCTTTTTGGCGCTGAGTACGACAACATTGAAAT
TGTTATTCATCCCCAATCTATCATCCACTCAATGGTTGAAACACAGGATTCATCTGTATT
GGCACAACTGGGATGGCCTGATATGCGTTTACCCATTCTTTACACTATGTCTTGGCCAGA
CAGAATTTACTGTTCTGAGATAACTTGGCCTCGCCTTGATCTTTGCAAGCTTGGGTCTCT
GACATTTAAAACACCAGATAATGTGAAGTACCCGTCCATGGACCTGGCATATGCTGCCGG
ACGAGCAGGAGGGACGATGACTGGAGTTCTAAGTGCTGCAAATGAGAAAGCCGTTGAGAT
GT
```

Figure 3.14 Assembling the nucleotide sequences of [WGD-EVK] and [GIV-EKA].

3.3.2 The 5'- and 3'ends of *msdxr*

Data from internal sequences allowed us to design specific primers for 5'-end and 3'-end amplifications. Using template from RLM-RACE cDNA preparation, amplifications of 5'-end and 3'-end were performed. The PCR products were ligated in pDrive vector and transformed into *E. coli* TOP10. After DNA sequencing, 5'-end and 3'-end contained 500 bp and 240 bp, respectively.

3.3.3 The full-length cDNA of *msdxr*

The partial sequences of 5'-end, internal sequence and 3'-end were assembled and revealed that the partial sequences are overlapped. Thus, the primers were designed for full-length cDNA of *msdxr*. After amplification of the full-length *msdxr* cDNA, the PCR product was cloned and sequenced. The result showed that *msdxr* cDNA contained 1,317 bp, which encoded the deduced amino acid of 438 residues (Fig. 3.14). Molecular weight and isoelectric point (pI) were predicted to 47.5 kDa and 6.43, respectively.

3.3.4 Primary structure of MSDXR

The nucleotide sequence of the resulting MSDXR was translated to the amino acid sequences. It composed of 438 amino acid residues. Its length is rather shorter than other plant DXR. Identities search was performed using the deduced amino acid sequence of MSDXR as query to perform the PSI-BLAST in http://blast.ncbi.nlm.nih.gov/Blast.cgi. The MSDXR exhibits the high percent identities to other plant DXRs between 71- 89 % identities. Percent identity of MSDXR is summarized in Table 3.3.

As shown in Fig. 3.15, the MSDXR contained the major characteristic of the chloroplast transit peptide at the NH_2-terminus. This leader sequence is essential for translocation of the enzyme into a plastid to the terpenoid biosynthetic site. The transit peptide cleavage site of the MSDXR appeared in front of a conserved CS motif (residues 49 of MSDXR) as suggested by the ChloroP 1.1 (Fig. 3.15). Downstream of the N-terminal, an extended proline-rich region was found, that contained seven amino acid residues (PPPAWPG). The most important characteristic of the DXRs is the NADPH binding motif (GSTGSIGT), which is essential for the catalytic activity. In addition, catalytic amino acid residues of DXR, suggested from the *E. coli* DXR (Kuzuyama et al., 2000), such as histidine (residues 223, 269 and 318 of MSDXR) and glutamate (residue 291 of MSDXR) were also found (Fig. 3.14).

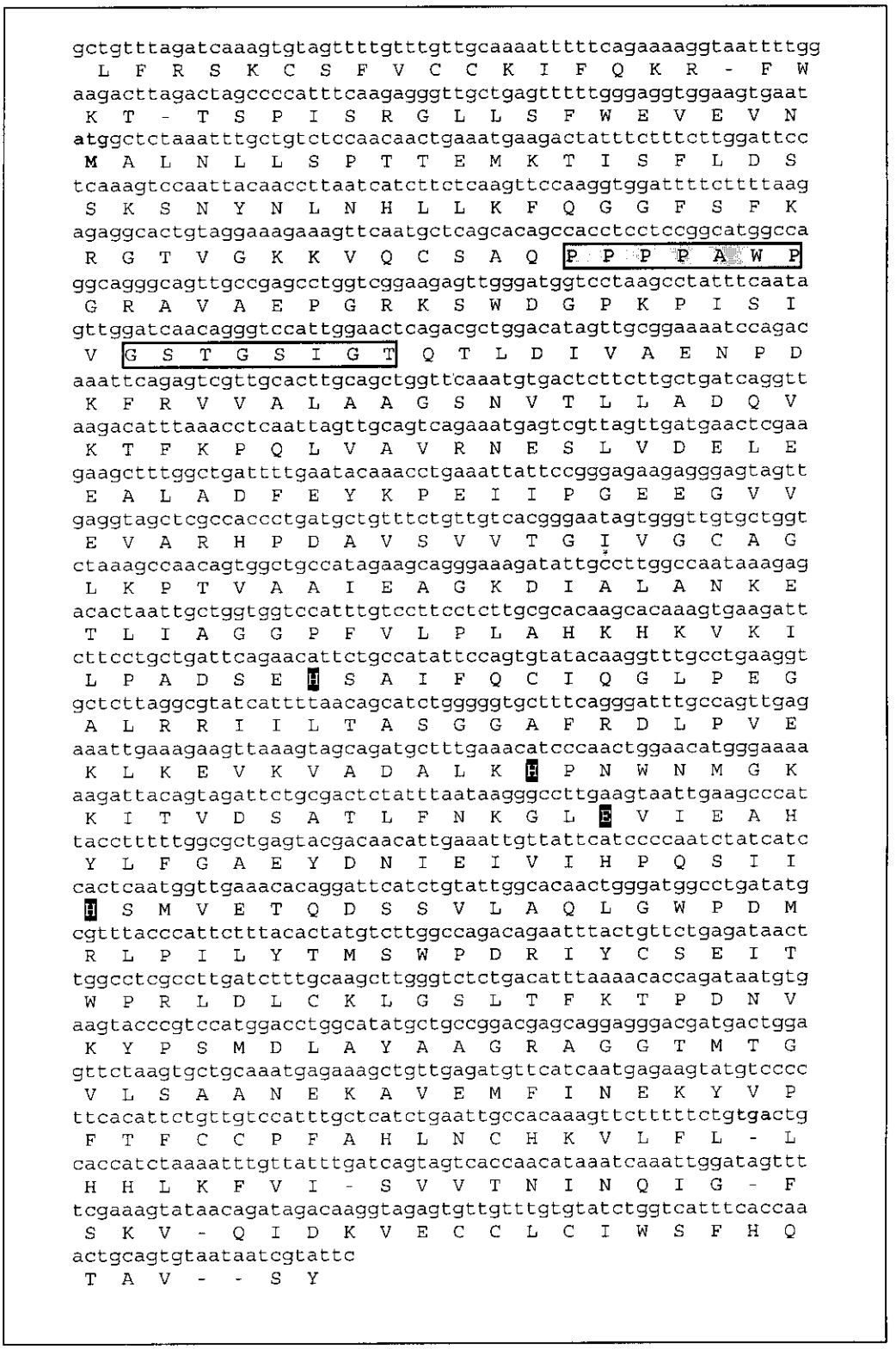

Figure 3.15 Full-length cDNA of *M. speciosa dxr*, shading indicates the chloroplast transit peptide, shade/box and box indicate the proline-rich region and NADPH-binding site, respectively; inverse-contrast letter shows the position of catalytic residues.

Table 3.3 Comparison the sequence of MSDXR with other plants DXRs.

Plant species	% identity to MSDXR
Antirrhinum majus	84
Arabidopsis thaliana	82
Artemisia annua	80
Camptotheca acuminata	85
Catharanthus roseus	87
Chrysanthemum x morifolium	80
Cistus incanus subsp. creticus	80
Croton stellatopilosus	87
Ginkgo biloba	78
Hevea brasiliensis	87
Hevea brasiliensis	86
Hordeum vulgare subsp. vulgare	87
Linum usitatissimum	80
Lycopersicon esculentum	87
Mentha x piperita	85
Nicotiana tabacum	87
Oryza sativa (indica cultivar-group	85
Physcomitrella patens subsp. patens	81
Picrorhiza kurrooa	84
Plectranthus barbatus	86
Populus alba x Populus tremula]	78
Populus trichocarpa	89
Pueraria montana var. lobata	71
Rauvolfia verticillata	89
Salvia miltiorrhiza	85
Stevia rebaudiana	82
Taxus cuspidata	81
Taxus x media	79
Vanda hybrid cultivar	83
Vitis vinifera	85
Zea mays	87

The cDNA of the *msdxr* contained 1,317 bp nucleotides and the MSMEPS protein had 438 amino acid residues with a predicted molecular mass of 47.5 kDa and a calculated isoelectric point of 6.43 (http://au.expasy.org/tools/pi_tool.html). The TargetP 1.1 predicted that the MSDXR was located in the chloroplast (cTP 0.519) and the transit peptide contained 49 amino acid residues downstream from the N-

terminal. Thus, the mature MSDXR after cleavage from the chloroplast transit peptide consisted of 389 amino acids. Based on this finding, the MSDXR, thus, belongs to the oxidoreductase family (Kuzuyama et al., 2000).

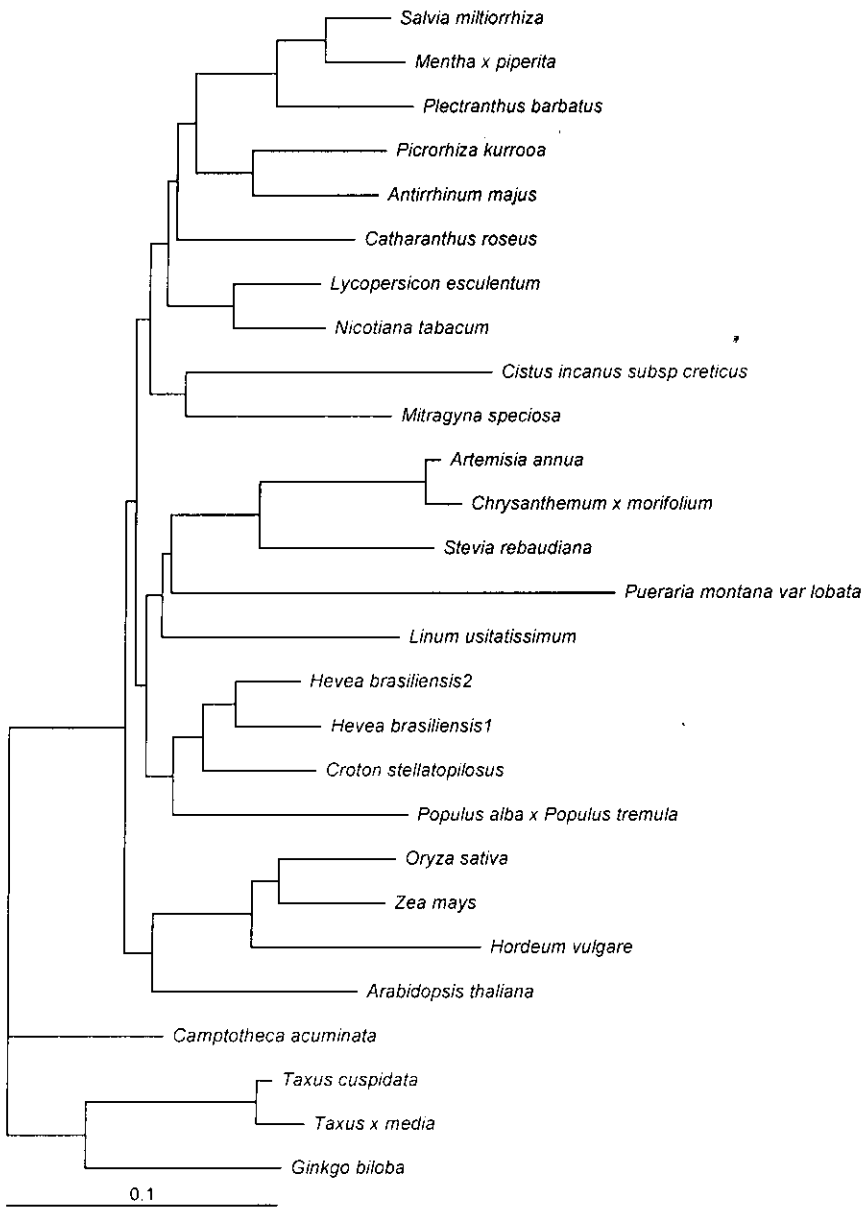

Figure 3.16 Phylogenetic analysis of *M. speciosa* DXR to other plant DXR.

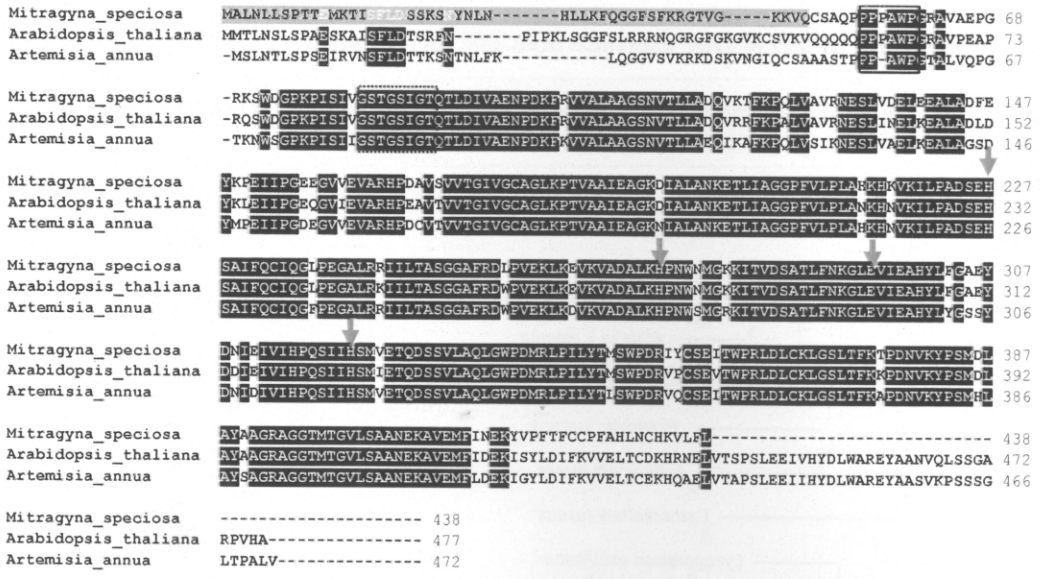

Figure 3.17 Multiple alignments of the deduced amino acids of *M. speciosa* DXS to DXRs from *Arabidopsis thaliana* and *Artemisia annua*. Shade denotes the putative chloroplast transit peptide suggested by ChloroP. Solid box indicates the extended proline rich region. Dotted box indicates the NADPH binding site. Arrows indicate the putative catalytic residues (Kuzuyama et al., 2000).

3.3.5 Three-dimensional of MSDXR

Based on the primary sequence of MSDXR, 3-D structure has been predicted for protein architecture. In comparison with *E. coli* DXR, MSDXS1 shows different at the catalytic domain (Fig 3.18).

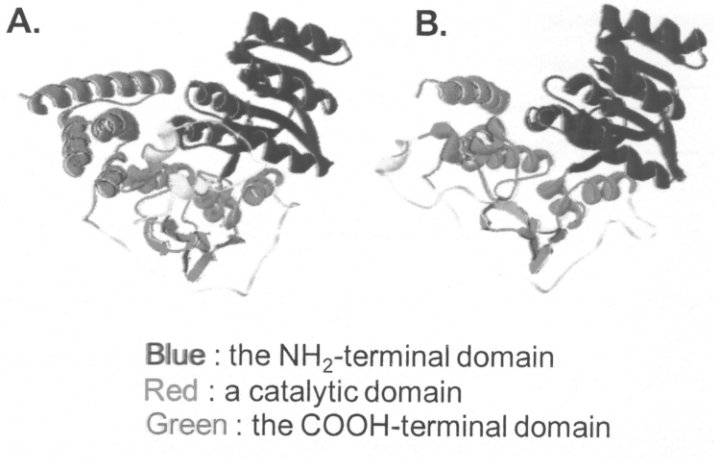

Figure 3.18 The predicted 3D structures of MSDXR: A. *E. coli* DXR, B. MSDXR.

3.3.6 Heterologous expression of truncated MSDXR

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267) is the second enzyme in the DXP pathway. It catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate to branch polyol 2*C*-methyl-D-erythritol 4-phosphate in the presence of NADPH as cosubstrate and divalent cations of Mn^{2+} or Mg^{2+} (Fig.3.19).

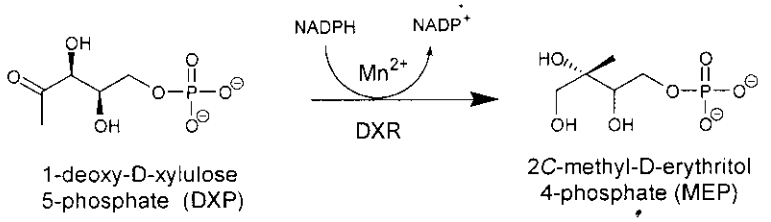

Figure 3.19 Catalytic action of 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR).

In this study, in order to follow the catalytic action of MSDXR, heterologous expression of the truncated DXR protein was performed using the pQE30-UA expression vector and transformed into *E.coli* XL1 blue MRF'. Based on the construction of the pQE30 vector (Fig. 3.20), the recombinant protein carries the polyhistidine tags at the NH_2-terminus.

Analysis of the MSDXR protein revealed that it carries the chloroplast transit peptide for 49 amino acid residues from NH_2-terminus. Therefore, the heterologous expression of the cDNA MSDXR was prepared for the truncated protein. On the basis of data from ChloroP v1.1, suggested that at position 49 (before CS motif) is the cleavage site of the MSDXR protein (Fig. 3.15). The scheme of the construction of plasmid is illustrated in Fig. 3.20. In order to enable rapid affinity chromatography purification of protein, the recombinant gene constructs were designed to specify N-terminal polyhistidine tags. The construct pQE30-MSDXR contains an ORF of 1,170 codons specifying the vector-derived coding region MRGSHHHHHHGT followed by the truncated (cleavage of the region of chloroplast transit peptide) MSDXR protein sequence (50-438). For protein expression, the recombinant plasmids were transformed to *E. coli* XL1blue MRF' competent cells.

After selection the recombinant plasmid harboring MSDXR with LB-medium plus ampicillin, 16 colonies were selected for plasmid isolation and cutting with *EcoRI*/*Xho*I, at 35 °C for 2 h. The fragments were separated on 1.2% agarose gel elcterophoresis. Only one sample was positive (band at 3.4 kb for pQE30 and band at 1.2 kb for insert MSDXR), designated as clone1. Two samples contained insert band at ca. 2.5 kb (approximately double size of MSDXR) were observed, designated as clone2 and clone3, respectively. These clones were prepared for cells and protein extraction.

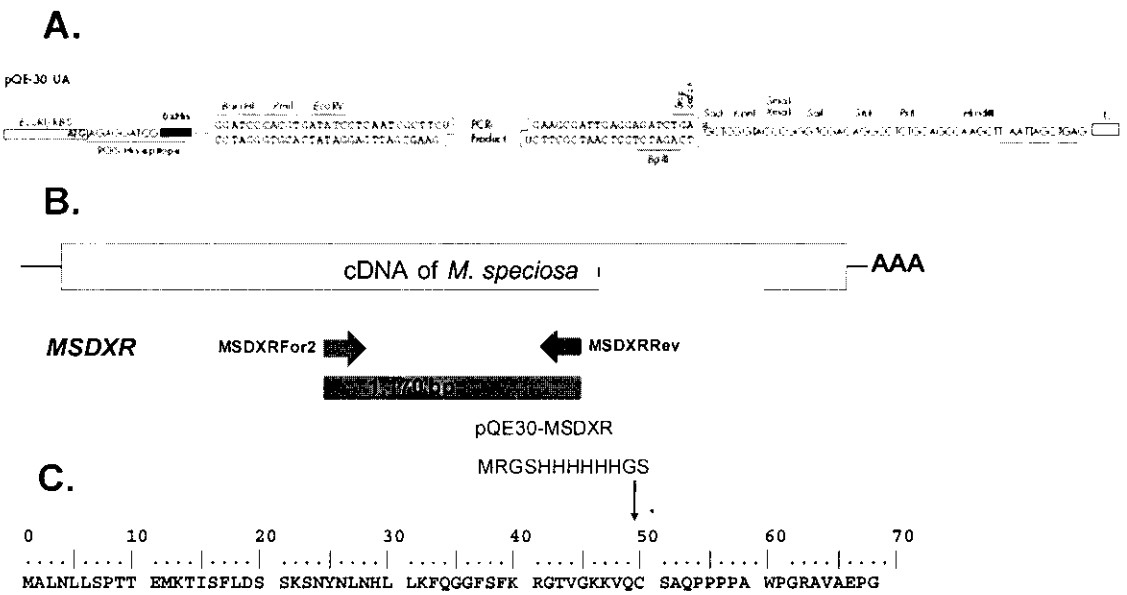

Figure 3.20 Construction of the recombinant plasmids carrying genes encoding of the truncated MSDXR protein. A. mapping of pQE30 UA (Qiagen) carrying six-residues of histidine at NH_2-terminus region of the DXR protein; B. schematic illustration of primers used; C. starting regions and N-terminal extensions of expressed constructs.

Heterologous expression was performed using a high copy 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-blue MRF' contain a mutated

lacI gene (*lacI*) (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 recombinant strains of *E.coli* harboring pQE30-MSDXR were induced using the IPTG (1 mM final concentration). Cells were harvested and extracted for protein. The soluble protein and insoluble protein were collected and analyzed on 12% SDS-PAGE. From the preliminary result (data not shown), cell-free extract from *E. coli* cells from clone1 shows proteins band distinguish from clone2 and clones3. Consideration the pellet (insoluble) fraction, proteins appeared on 12% SDS-PAGE seem higher amount than soluble fraction. We proposed that the MSDXR is mostly produced in pellet fraction.

To check the fusion protein produced from clone1 *E. coli*, we prepared the *E. coli* cells (0.4 g fresh weight). After treatment with lysozyme, protein was extracted using ultrasonication. Cell debris was removed by centrifugation at 13,000 rpm for 30 min. The fusion protein was purified using ProPur™ IMAC affinity chromatography. After concentration and changing the buffer using ultrafiltration concentrator, the bound protein was checked its purity on SDS-PAGE. Since the construction of the truncated MSDXR gene (Fig. 3.20) was performed. Calculation of pI and molecular weight of the truncated protein MSDXR suggested that the truncated MSDXR has a pI of 5.74 and the apparent molecular mass of 42,125.72 Dalton. Fig. 3.21 shows the SDS-PAGE of the soluble fraction of the *E. coli* harboring control vector and the truncated MSDXR. The protein patterns of lane 1 and lane 2 are not different, caused by the low expression level of MSDXR. However, an apparent protein band at *ca.* 42 kDa is observed on SDS-PAGE, which closed to the predicted size of the truncated MSDXR. In addition, band at *ca.* 28 kDa is also observed.

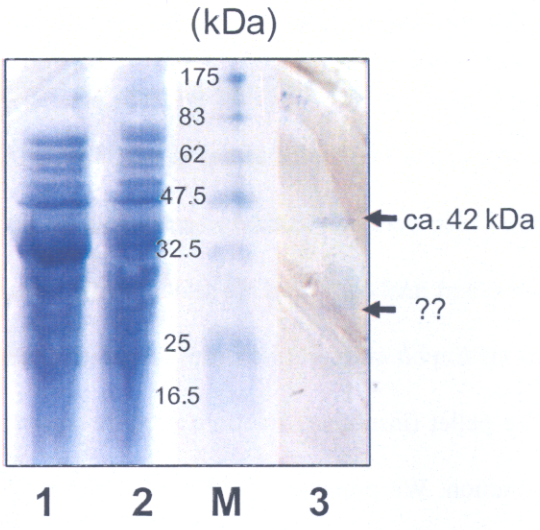

Figure 3.21 12% SDS-PAGE analysis after Coomassie blue staining. Lane 1: cell-free extract of *E. coli* harboring pQE30 (control vector); lane 2: cell-free extract of *E. coli* harboring pQE30-MSDXR; M: prestained protein marker (NEB); lane 3: eluted fraction and concentrated with ultrafiltration concentrator.

3.4 Hairy root cultures of *M. speciosa* and high yield of mitragynine by regenerated plants

Study on genes, involved in mitragynine biosynthesis, establishment of model plant is necessary. It is another system to follow the expression profiles of investigated genes. Therefore, the transformed hairy root culture was established by infection with *Agrobacterium rhizogenes* ATCC 15834. Secondary metabolites productions in the culture were evaluated. Surprisingly, the hairy roots were differentiated to shoots and later complete plants were obtained. Regenerated plants were then evaluated for mitragynine accumulation and used as plant materials to follow the expression profiles.

3.4.1 Establishment of hairy root culture

The *M. speciosa* hairy root culture was established by infection of 2-month-old plantlets with *A. rhizogenes* ATCC 15834. Steps of the hairy root induction are summarized in Fig 3.22. Optimization of the site of infection, leaf veins and stems was performed for the explants. The result as shown in Fig. 3.23 suggested that an appropriate explant was from the leaf vein with a percentage of hairy root induction of 85%, while a percentage of hairy root induction of 67% was found when using the stems as the explants.

The hairy roots obtained from leaf veins appeared as thin roots and contained small pubescent. In contrast, the hairy roots obtained from the stems contained nodules (undeveloped hairy roots) and thick roots.

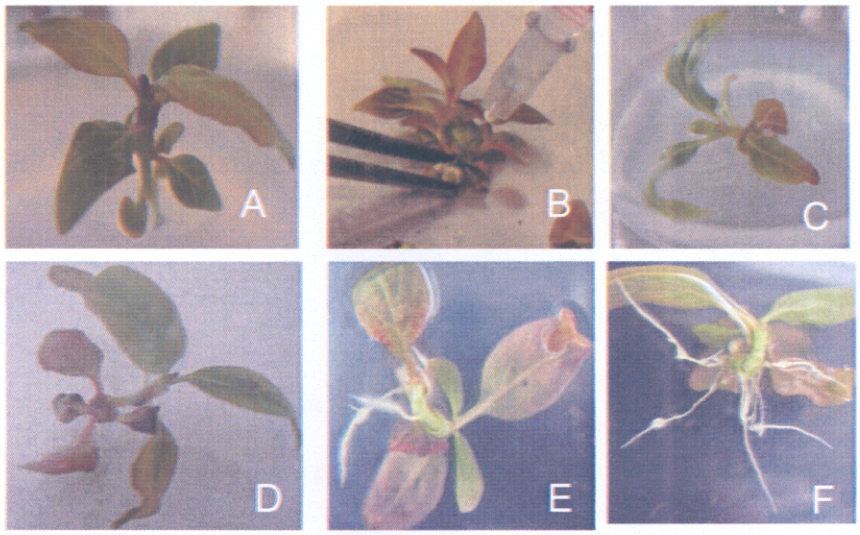

Figure 3.22 Steps of the hairy roots induction from *M. speciosa* plantlet

A. 2 months plantlets
B. infection with *A. rhizogenes* ATCC 15834
C. incubated the infected plantlet for 3 days
D. culture in WPM plus cefotaxime 500 mg/l for 7 days
E. culture in WPM plus cefotaxime 250 mg/l for 7 days
F. culture in WPM plus cefotaxime 100 mg/l for 7 days

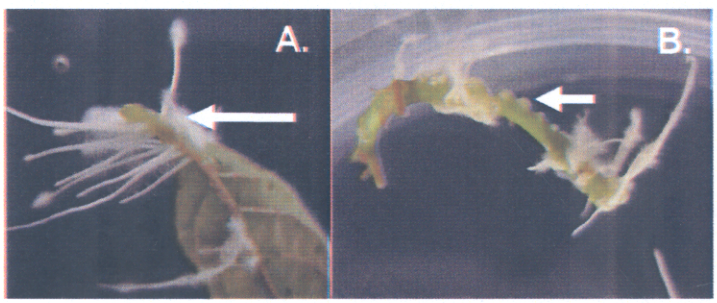

Figure 3.23 Characteristics of hairy roots, induced from leaf veins (A) and stems (B).

Genotypes of the hairy roots were identified for the rooting locus genes *rol*A and *rol*B by polymerase chain reaction. The presence of *rol*A and *rol*B genes of *A. rhizogenes* ATCC 15834 in the transformed hairy roots was accounted by 4/6 clones (Fig. 3.24).

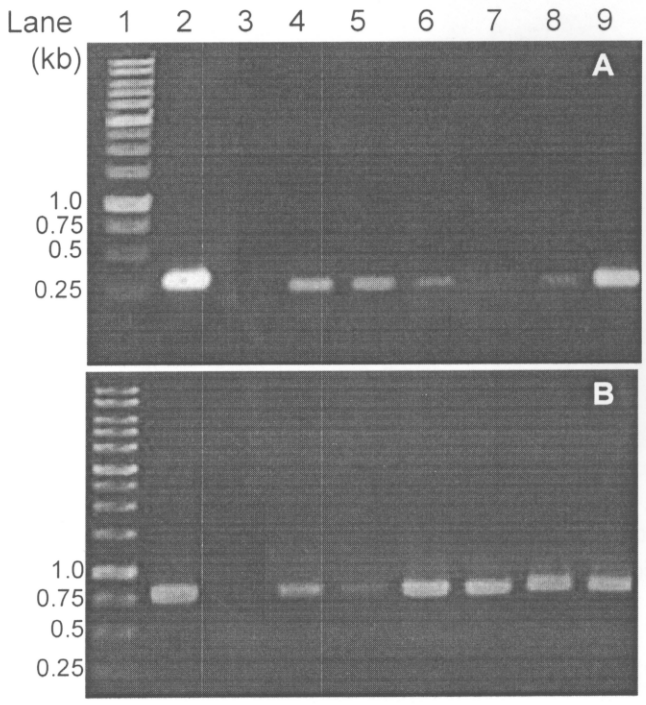

Figure 3.24 PCR analyses of the hairy roots. PCR was performed with primers for the rooting locus genes (A) *rol*A (300 bp) and (B) *rol*B (780 bp). Lane 1, marker (10 kb DNA ladder); lane 2, *A. rhizogenes* ATCC 15834; lane 3, untransformed roots; lane 4 - 9, transformed hairy roots (lines 1 - 6).

Due to the slow growth rate of the hairy roots in WPM medium, other types of media were manipulated. Hairy roots in half-strength of WPM, B5 and MS medium grew badly (data not shown). Comparison of the growth curves of the hairy roots in WPM and WPM plus NAA revealed that the addition of NAA (0.5 mg/l) to WPM medium stimulated the growth of hairy roots. As shown in Fig. 3.25, doubling time of the hairy roots was decreased from about 2 months of culture to about 30 d. The hairy roots were, therefore, maintained in WPM medium supplemented with NAA (0.5 mg/l) and used as materials for evaluation of secondary metabolites production.

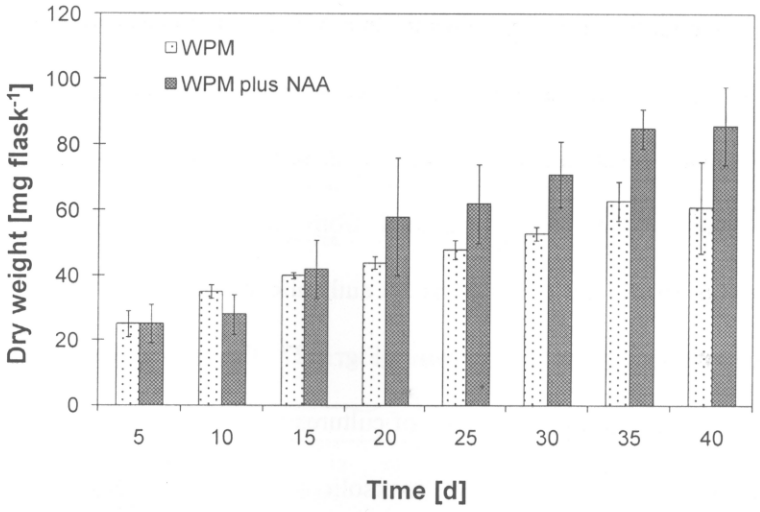

Figure 3.25 Effect of NAA (0.5 mg/l) in WPM medium on growth of the *M. speciosa* hairy root culture. Values are means of triplicate. Error bars present standard deviation.

3.4.2 Accumulation of triterpenoids and phytosterols in the hairy roots

Preliminary screening on TLC after detection with Dragendroff's reagent showed that *M. speciosa* hairy roots were unable to produce alkaloid. To identify the secondary metabolites present in the hairy roots, an *n*-hexane extract was prepared and isolated by silica gel column chromatography. NMR data of MSF1 and MSF2 were accomplished by analysis of ^{1}H, 1D and 2D ^{13}C NMR. For the structure of MSF1, analysis of ^{1}H NMR data was in agreement with NMR data of the published report as plant sterols (Subhadhirasakul and Pechpongs, 2005). From ^{13}C NMR spectra, δ at 138.32 (C-22) and at 129.21 (C-23) were observed. Both signals corresponded to the double bond in the structure of stigmasterol. Integration of allylic proton at H 22 and H 23 indicated that the MSF1 was a mixture of β-sitosterol and stigmasterol, present in the ratio of 1:1 (Fig. 3.26).

The presence of δ 78.5 (C-3; COH), 125.2 (C-12), 137.9 (C-13), 180.5 (C-28; C=C) suggested that MSF2 contained ursolic acid (**2**). 2D-NMR: COSY, HMQC and HMBC indicated the correlation of ^{13}C-^{13}C, ^{1}H-^{13}C and long-length coupling ^{1}H-^{13}C, respectively. Analysis of NMR data of MSF2 was in agreement with a previous report as triterpenoids (Güvenalp *et al.*, 2006). The presence of the signal at δ 5.28 in the ^{1}H NMR spectra indicated that MSF2 contained the isomer of ursolic acid, oleanolic acid.

Integration of the H-12 signal in ^{1}H NMR indicated that MSF2 contained a mixture of ursolic acid and oleanolic acid in the ratio of 5:1 (Fig 3.26).

To construct the growth curve and production curve, samples were taken every 5 d over 40 d of culture. The ursolic acid content was determined from the hairy roots in comparison with the untransformed roots. The HPLC chromatograms of authentic ursolic acid, and the extracts from the untransformed roots and the hairy roots are shown in Fig. 3.27. The ursolic acid contents are summarized in Table 3.4. The results indicated that both types of cultures were able to produce ursolic acid. It can be noted that the hairy roots, at 30 d, accumulated the ursolic acid with the yield of (3.47 ± 0.03) mg/g dry weight (DW), whereas the unstransformed roots could produce (2.41 ± 0.01) mg/g DW.

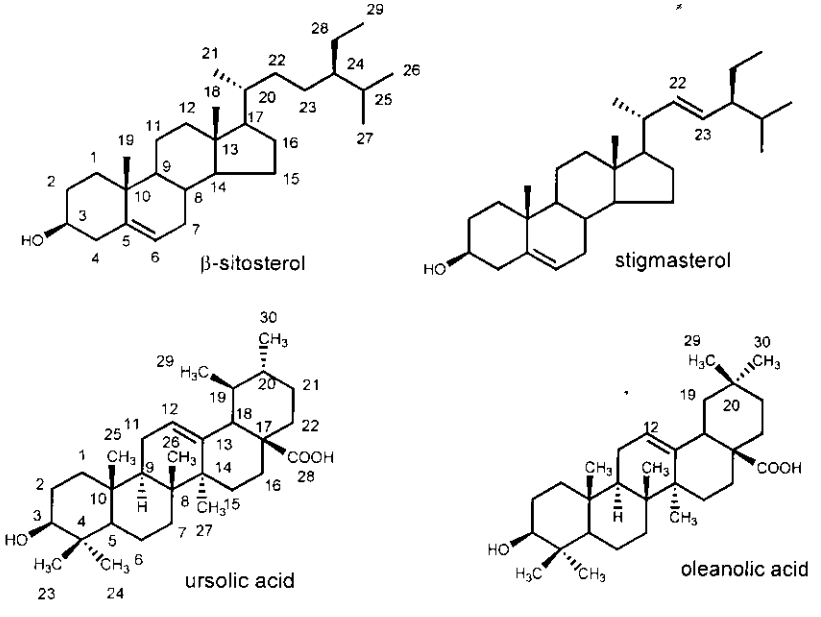

Figure 3.26 Structures of isolated compounds from the hairy roots.

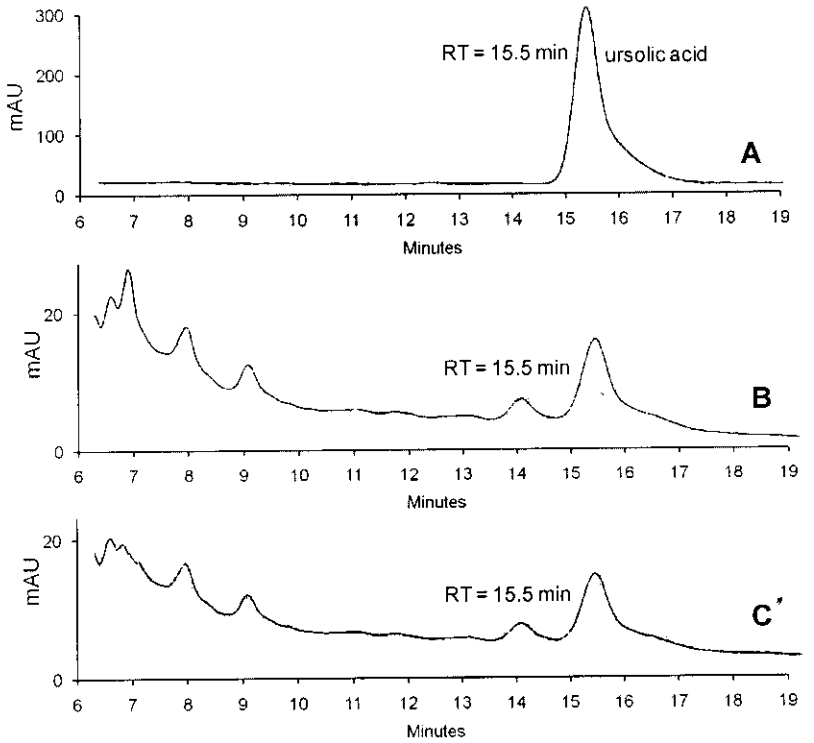

Figure 3.27 HPLC chromatograms of (A) the authentic ursolic acid, (B) the *n*-hexane extracts of the transformed hairy roots and (C) the untransformed hairy roots.

Table 3.4 Ursolic acid contents in transformed hairy roots and untransformed roots ($n = 4$).

Time	Ursolic acid content [mg/g DW ± SD]	
[d]	Transformed hairy roots	Untransformed roots
5	1.52 ± 0.00	1.58 ± 0.01
10	1.90 ± 0.02	1.74 ± 0.01
15	2.49 ± 0.02	1.89 ± 0.01
20	1.65 ± 0.01	1.69 ± 0.01
25	2.00 ± 0.04	2.09 ± 0.01
30	3.47 ± 0.03	2.41 ± 0.01
35	1.98 ± 0.03	1.74 ± 0.02
40	2.40 ± 0.01	2.01 ± 0.00

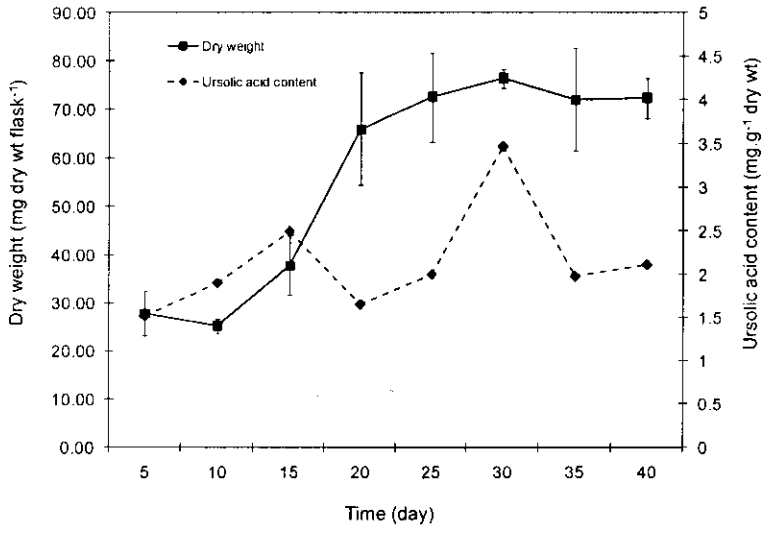

Figure 3.28 Growth curve and production curve of *M. speciosa* hairy root culture. Error bars represent standard deviations.

Fig. 3.28 indicates that the cell cycle of the *M. speciosa* hairy roots was within 30 days of culture. It can be seen that the cycle composed on 3 phases: days 1-10 for lag phase, days 10-20 for exponential phase, days 20-30 for linear phase and days 30-40 for stationary phase. The production curve of the hairy roots shows that ursolic acid was usually present in the cells and maintained at a particular level of *ca.* 2 mg/g dry wt. However, the hairy roots were able to accumulate maximum yield at the late linear phase. Thus, the ursolic acid was utilized by the cells and acted as a secondary metabolite in this *in vitro* culture.

3.4.3 Plant regeneration containing high-yields of mitragynine

During the establishment of *M. speciosa* hairy root culture, shoot regeneration was observed from the cultures. This evidence was observed when the hairy roots contained part of the stem (cut from the original explant). After 2 months, the regenerated shoots were removed and placed in the hormone-free WPM solid medium. The roots were then initiated after culture for 10 d to complete the plantlets. The regenerated plantlets were then used as materials for micropropagation. Axillary buds were excised from the stems and used as explants for shoot multiplication. Since the hairy roots have been obtained from infection with *A. rhizogenes* ATCC 15834, therefore, the regenerated plantlets were determined for the presence of the *rol*A and the *rol*B genes. However, the data indicated that the regenerated plantlets did not carry the genes from *A. rhizogenes*.

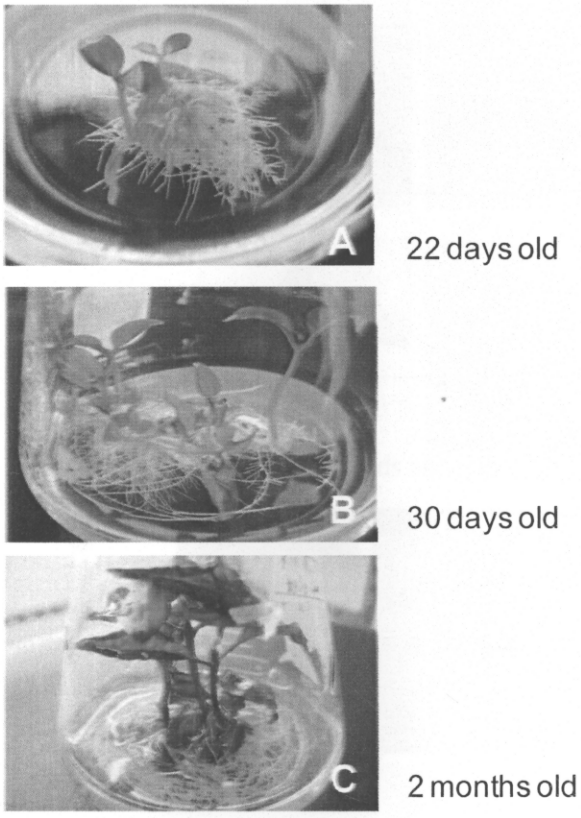

Figure 3.29 Shoot regeneration from *M. speciosa* hairy root culture

To increase the number of shoots in the *M. speciosa* plantlet, the axillary buds were excised and manipulated in WPM solid medium supplemented with three kinds of cytokinin. In this study, BA (1 mg/l) and TDZ (0.1 and 0.5 mg/l) were used for the shoot inductions. After 4 weeks, the shoot numbers were counted and calculated as the shoots number per explant. The results indicated that the shoot numbers of (2.8 ± 1.5), (6.3 ± 1.6) and (6.3 ± 1.3) per explants were obtained from the WPM supplemented with 1 mg/l of BA, 0.1 mg/l of TDZ and 0.5 mg/l of TDZ, respectively. These results showed that TDZ was an appropriate plant growth regulator for shoot multiplication. Root initiation of the regenerated plantlet of *M. speciosa* was simply performed. The shoots (containing 2 - 4 leaves) were cut from the explants and placed on hormone-free WPM medium. Roots were initiated after 1 week of culture.

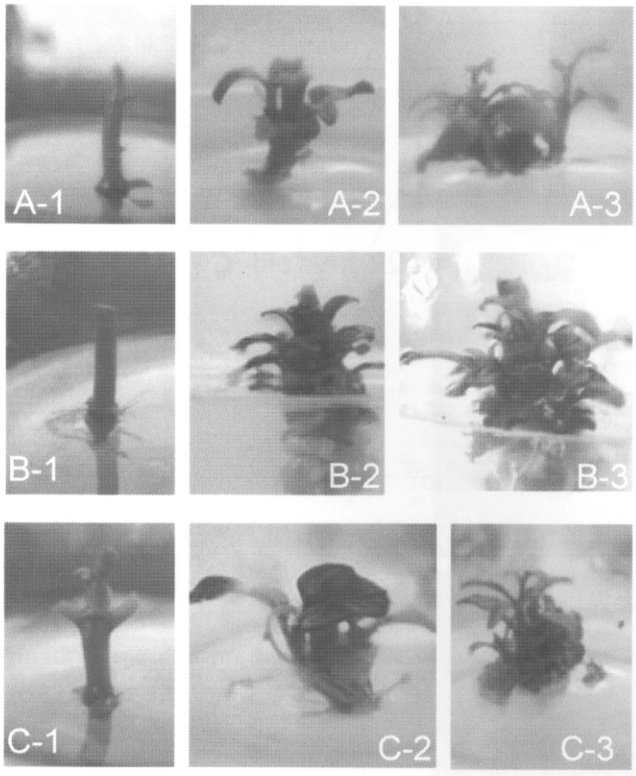

Figure 3.30 Shoot multiplication of the axillary buds, cultured in different cytokinins.

A: in WPM supplemented with 1 mg/l BA; A-1, 1st day of culture; A-2, 12 days old, A-3: 26 days old

B: in WPM supplemented with 0.1 mg/l TDZ; B-1, 1st day of culture; B-2, 12 days old, B-3: 26 days old

C: in WPM supplemented with 0.5 mg/l TDZ; C-1, 1st day of culture; C-2, 12 days old, C-3: 26 days old

To evaluate the mitragynine production in the regenerated plantlets, the *in vitro* plants, obtained from seeds germination in WPM medium supplemented with 1 mg/l BA and grew under controlled conditions, were used as control. Mitragynine contents of *in vitro* plantlets and the regenerated plantlets were determined. A simple and rapid method of mitragynine quantification was established using the TLC-densitometric method. The extract from the whole plant of the *in vitro* and the regenerated plantlets (5 months old) were prepared for alkaloid extracts and determined for mitragynine contents. As shown in Table 3.5, mitragynine content in the regenerated plantlet was about 3.2 times higher than that *in vitro* plantlet. From this experiment it was concluded that the mitragynine was produced and accumulated mostly in leaves.

Table 3.5 Mitragynine content determination from 5-month-old plants.

Sample	Mitragynine content[a] [mg/g DW ± SD]
In vitro plantlet[b]	4.45 ± 0.09
Regenerated plant[c]	14.25 ± 0.25
Leaves from regenerated plant	12.5 ± 0.25

[a]Samples were determined in triplicate.

[b]From plantlets that germinated from seeds and grown in WPM medium supplemented with BA (1 mg/l).

[c]From regenerated plants that were maintained in the hormone-free WPM medium.

4. Discussion

Monoterpenoid indole alkaloids (MIAs) are plant alkaloids with a wide variety of pharmaceutical values. Plant-derived alkaloids currently in clinical use include the anticancer agents camptothecin, vincristine and vinblastine (Lorence and Nessler, 2004). The MIA is biosynthesized from the common precursor, strictosidine. Evidence of S-3α-strictosidine incorporated into α-yohimbine and reserpiline in *Rauvolfia canescens* (Rueffler et al., 1978) suggested that strictosidine served as the precursor for MIA. This key enzyme was first identified and characterized from *Catharanthus roseus* suspension culture (Treimer and Zenk, 1979). The enzyme catalyzed the Pictet-Spengler condensation of tryptamine and secologanin. As shown in Fig. 4.1, tryptamine is a decarboxylated product from tryptophan, by tryptophan decarboxylase. Secologanin is supplied from monoterpenoid origin from loganin, catalyzed by secologanin synthase. MIA biosynthesis in *M. speciosa*, however, there are lack of data in terms of mitragynine biosynthesis. It has been reported that cDNA of strictosidine synthase was cloned and characterized by Ounaroon and De-Eknamkul (2007). Nevertheless, early step and late step of mitragynine biosynthesis is still unknown. As mentioned earlier, strictosdine is supplied from tryptamine (from shikimate pathway) and secologanin (from terpenoid pathway). For the latter, prenyl transferase catalyzed the formation of geranyl diphosphate. After cleavage of phosphate moiety, geraniol 10-hydroxylase catalyzed the intermediate 10-hydroxygeraniol, which is served for loganin. Loganin is transformed to secologanin by secologanin synthase.

Previous studies have shown that molecule of loganin proceeds from terpenoid biosynthesis via the deoxyxylulose phosphate (DXP) pathway (non-mevalonate pathway) (Eichinger et al., 1999). Unlike to classical mevalonate pathway, the DXP pathway begins with condensation of pyruvate and glyceraldehydes 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXP), catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*) (Sprengler et al., 1997). DXP is further rearranged to branch polyol to 2*C*-methyl-D-erythritol 4-phosphate (MEP), catalyzed by 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (*dxr*). MEP is sequentially transformed to IPP and DMAPP by enzymes IspD, IspE, IspF, IspG and IspH, respectively (see 1.4.2). Studies on the involvement of DXP pathway in MIA biosynthesis were shown in *Rauvolfia serpentina* suspension culture and *C. roseus* hairy root culture. Analysis of ^{13}C-NMR

of loganin after feeding of [1-^{13}C]glucose indicated that the contribution of MVA and DXP pathway in *R. serpentina* suspension culture (Eichinger et al., 1999).

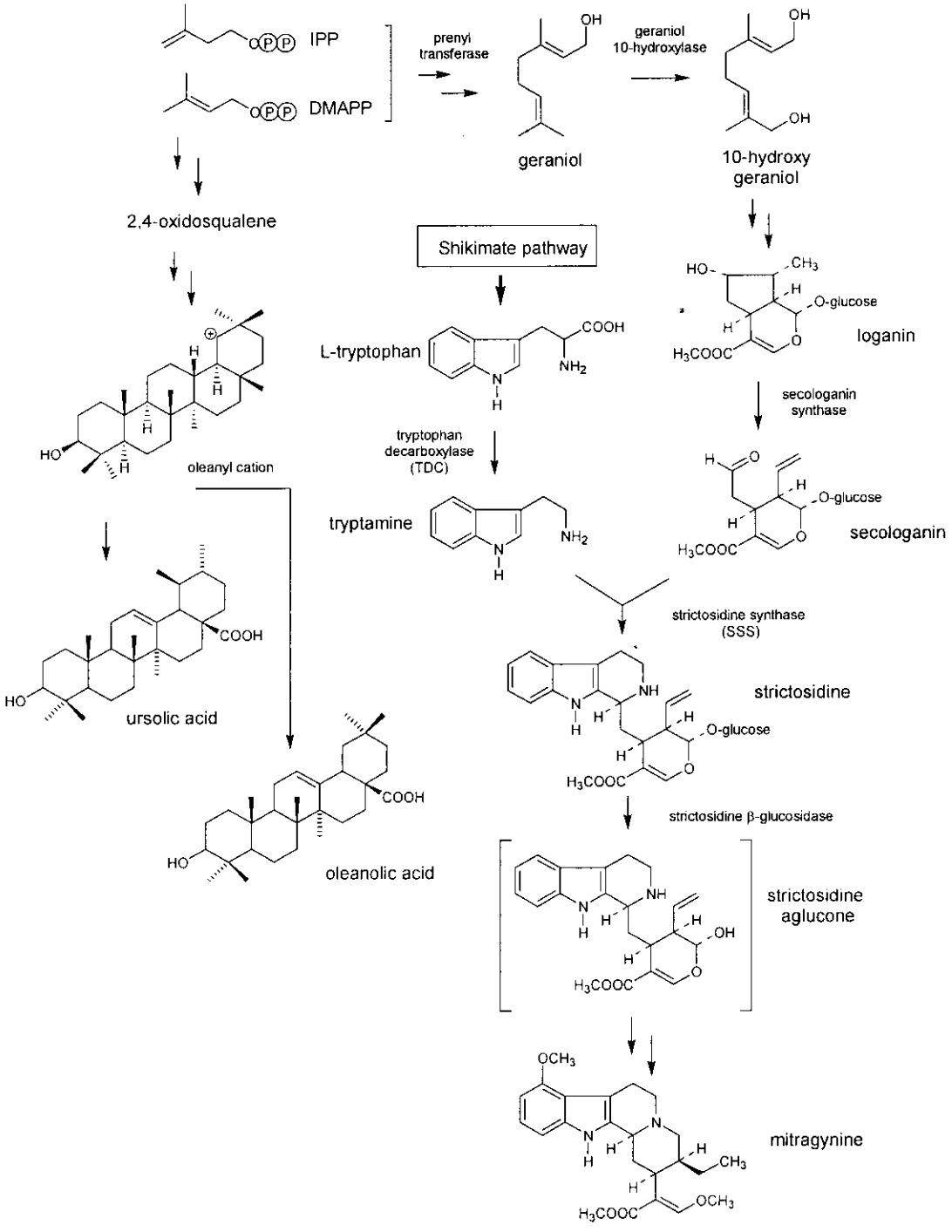

Figure 4.1 Biosynthesis of mitragynine and triterpenoid, proposed in *M. speciosa* plant and hairy root culture

For *C. roseus* hairy root culture, the MIAs accumulations such as ajmalicine, tabersonine and lochnericine were inhibited by addition of fosmidomycin, an inhibitor of DXR. In addition, feeding of 1-deoxy-D-xylulose, 10-hydroxygeraniol, or loganin resulted in significant increases in alkaloid production in the hairy roots (Hong et al., 2003). These evidences strongly suggested that the DXP pathway is a major provider of carbon for the MIA pathway. Based on these results, we proposed that mitragynine, a MIA in *M.speciosa*, should be originated dominantly from the DXP pathway. However, studies on the carbon block of mitragynine in this plant has been not yet investigated.

In this report, we focus on the cDNA cloning and characterization of *msdxs* and *msdxr* from *M. speciosa*. The full-length cDNA of *msdxs* and *msdxr* from *M. speciosa* leaves were cloned by homology-based polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE) methods.

For *msdxs* gene, in this study, at least two isoforms of *dxs* existed in *M. speciosa* leaves, which are *dxs* class I (*msdxs1*) and *dxs* class II (*msdxs2*). The full-length cDNA of *msdxs1* contained 2,010 bp, which encoded the open reading frame (ORF) of MSDXS1 for 669 amino acid residues. Molecular weight and isoelectric point (pI) have been predicted to be 71,963.26 dalton and 6.89, respectively (http://au.expasy.org/cgi-bin/pi_tool). The full-length of cDNA of *msdxs2* contained 2,193 bp, encoded the open reading frame (ORF) of MSDXS2 for 730 amino acid residues. Molecular weight and isoelectric point (pI) have been predicted to be 78,562.74 dalton and 6.46, respectively. The DXS of *M.speciosa* was classified based phylogenetic tree. Alignment of deduced amino acid sequences of the MSDXS1 and MSDXS2 revealed that they shared the identity of 66%. The MSDXS1 and MSDXS2 carry characteristics of transketolase enzyme e.g. thiamine diphosphate binding site and transketolase motif. The presence of cTP suggested that MSDXS1 and MSDXS2 located in the chloroplast.

Occurrence of DXS isoforms was found commonly in higher plant e.g. *Arabidopsis thaliana*, *Capsicum annuum*, *Medicago truntacula*, *Andrographis paniculata, Ginkgo biloba* for instance. It can be suggested that the expression of *dxs* class I is required for the biosynthesis of primary isoprenoids such as chlorophylls and carotenoids and the expression of *dxs* class II might be involved in secondary metabolite biosynthesis such as apocarotenoids and ginkgolides. In case of *M.speciosa*, we have demonstrated that the MSDXS1 plays a regulatory role in the mitragynine biosynthesis rather than the MSDXS2. The results were in contrast with the classification of DXS, that DXS class II generally plays a role in secondary

metabolism. From the results, it can be noted that the MSDXS1 and MSDXS2 located mostly in leaves and stems, but lower abundance in roots.

For *msdxr* gene, cDNA of the *msdxr* contained 1,317 bp nucleotides and the MSMEPS protein had 438 amino acid residues with a predicted molecular mass of 47.5 kDa and a calculated isoelectric point of 6.43 (http://au.expasy.org/tools/pi_tool.html). The TargetP 1.1 predicted that the MSDXR was located in the chloroplast (cTP 0.519) and the transit peptide contained 49 amino acid residues downstream from the NH_2-terminal. Thus, the mature MSDXR after cleavage from the chloroplast transit peptide consisted of 389 amino acids. Notably, MSDXR was shorter than others previously reported DXRs. However, MSDXR carried all important characteristics for DXR such as the chloroplast transit peptide (position 1-49) for plastid localization, extended proline rich region (position 54-60), NADPH binding motif (position 83-90) and invariant catalytic amino acid residues. Multiple alignment of MSDXR showed that MSDXR shared a high homology to other known plant DXRs (more than 80% identities). The *msdxr* gene was expressed in pseudomature form without the putative plastid-targeting sequence (position 1-49 of MSDXR) in a recombinant *Escherichia coli* strain. After induction with IPTG, the recombinant *E. coli* cells could produce the soluble protein and had an apparent molecular mass *ca.* 42 kDa on the SDS-PAGE. Notably, MSDXR was expressed mostly in insoluble fraction. After, MSDXR was purified by affinity chromatography on nickel-chelating column. Amount of MSDXR was not enough for further characterization. Optimization of protein expression should be investigated in terms of the recombinant plasmid construction and variation of expression host such as yeast system.

In this study, we also establish the plant model e.g. hairy root culture and *in vitro* culture for mitragynine biosynthesis. The transformed hairy roots of *M. speciosa* with *Agrobacterium rhizogenes* could accumulate compounds, which are triterpenoids; ursolic acid and oleanolic acid (with the ratio of 5:1) and phytosterols, β-sitosterol and stigmasterol (with the ratio of 1:1). Nevertheless, many studies reported that the alkaloids such as mitraphylline, rhynchophylline have been found in the root bark of *M. speciosa* (Houghton and Shellard, 1974; Shellard et al. 1978b). But none of the alkaloids were found in this culture. Intermediates of alkaloids were probably unstable and degraded during culture (Fig 4.1). Ursolic acid was produced in the late linear phase a yield of (3.468±0.034) mg/g dry wt. It can be noted that *M. speciosa* hairy roots could accumulate high yield of ursolic acid when compared to the *Uncaria*

tomentosa cell suspension culture (1.680±0.039 mg/g dry wt.) (Feria-Romero et al., 2005). This evidence concludes that the differentiated cells such as hairy roots preferred to produce higher amount of secondary metabolites.

For the biosynthetic point of view (Fig. 4.1), *M. speciosa* hairy roots could not produce any alkaloid, especially mitragynine. It may be caused by a lack of precursors and enzymes, which is involved in the terpenoid indole alkaloid, since primary metabolites need special cell compartments for storage and degradation (Luckner, 1984). The presence of ursolic acid, coexisting with oleanolic acid, indicated that at least isoprene units have been produced in this culture as shown in Fig. 4.1. These isoprene units are precursors for both triterpenoids and phytosterols (Luckner, 1984), which means their biosynthesis was active in this culture. Therefore, the *M. speciosa* hairy root culture is not only useful for ursolic acid production but also have a potential to be a model plant culture for triterpenoid biosynthesis.

Unexpectedly, plantlet regeneration of *M.speciosa* was obtained. Micropropagation of this plant was performed successfully transferring the plants to nature. From this study, TDZ was shown to be an efficient cytokinin for shoot multiplication. TDZ acts as a substitute for both auxin and cytokinin requirements to induce organogenesis and somatic embryogenesis in many species (He, 2007). From this study, the regenerated plantlets produced and accumulated the considerable amount of mitragynine, when compared to the *in vitro* plantlets (from seed germination). This plant can be used as plant model for mitragynine biosynthesis as shown in the study of *msdxs* expression profile.

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6. **Output** จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ และหน้า) พร้อมแจ้งสถานะของการตีพิมพ์ เช่น submitted, accepted, in press, published

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2. การนำผลการวิจัยไปใช้ประโยชน์ -

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   - การนำเสนอในที่ประชุมวิชาการนานาชาติ จำนวน 4 ครั้ง

   Wungsintaweekul, J. and Charoonratana, T. 2008. Mitragynine biosynthesis: cDNA cloning of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) genes from *Mitragyna speciosa* Korth. The 5th International conference on Plant Metabolomics, July 15-18, 2008, Pacifico Yokohama, Japan.

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APPENDIX

Mitragyna speciosa: Hairy Root Culture for Triterpenoid Production and High Yield of Mitragynine by Regenerated Plants

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Hairy root cultures of *Mitragyna speciosa* were established by infection of *Agrobacterium rhizogenes* ATCC 15834 and maintained in McCown woody plant medium (WPM) supplemented with 0.5 mg/l naphthaleneacetic acid. The hairy roots were identified for the rooting genes loci of *rol*A and *rol*B by polymerase chain reaction. For studying the secondary metabolite production, the *n*-hexane extract of the hairy roots was prepared and the compounds were isolated by silica gel column chromatography, affording triterpenoids (ursolic acid and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol). The shoots from the hairy root cultures were regenerated and differentiated to the plantlets. For micropropagation, shoot multiplication was successfully induced from the axillary buds of the regenerated plantlets in WPM supplemented with 0.1 mg/l thidiazuron. The mitragynine contents of 5-month-old regenerated plants and *in vitro* plantlets (germinated from seeds) were determined using the TLC-densitometric method. The regenerated plants contained (14.25 ± 0.25) mg/g dry wt mitragynine, whereas the *in vitro* plantlets contained (4.45 ± 0.09) mg/g dry wt.

Key words: Mitragyna speciosa, Ursolic Acid, Mitragynine, Hairy Root Culture

Introduction

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is an endemic plant found in tropical Southeast Asia. It is of particular medicinal importance and known as "Kratom" in Thailand. In folklore medicine, Kratom has been used as an opium-substitute for pain relief and treatment of diarrhea (Jansen and Prast, 1988). Many studies reported the wide variety of indole alkaloids from Kratom's leaves including mitragynine (**1**) (Shellard, 1974) (Fig. 1). In addition, it also contains flavone, flavonol, flavonoid (Harvala and Hinou, 1988), and polyphenolic compounds (Hinou and Harvala, 1988), and triterpenoids such as ursolic acid (**2**) and oleanolic acid (**3**) (Said *et al.*, 1991). The pharmacological activities of mitragynine and its derivatives such as analgesic activity, antinociceptive activity (Watanabe *et al.*, 1997), antistress activity, muscle-relaxant activity (Aji *et al.*, 2001) and inhibition of gastric acid secretion (Tsuchiya *et al.*, 2002) have been reported from studies in tested animals. The mechanism of action of mitragynine as analgesic is binding to the opioid receptors, similar to morphine (Thongpraditchote *et al.*, 1998). Interestingly, mi-

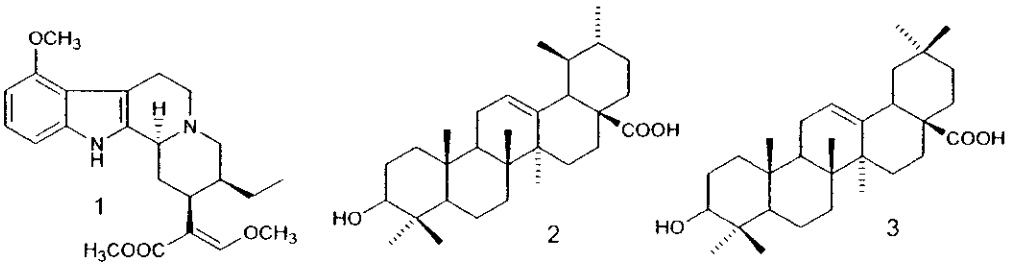

Fig. 1. Chemical structures of mitragynine (**1**), ursolic acid (**2**) and oleanolic acid (**3**).

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tragynine has a characteristic of less addiction than morphine. Therefore, it highlights the relevance to be an alternative to opioid analgesic drugs. Contrary to the potential of *M. speciosa*, it is recognized as an illegal plant, and culturing of this plant is prohibited in Thailand. As such, the basic knowledge of mitragynine biosynthesis is very little. From the biosynthesis point of view, the steps of mitragynine formation are still unknown.

In the present study, we aimed to induce the *in vitro* cultures of *M. speciosa* including the hairy root culture and to propagate plants containing high yield of mitragynine. The hairy root cultures were obtained from infection of *M. speciosa* with a wild-type *Agrobacterium rhizogenes* ATCC 15834. Secondary metabolites accumulated in the *M. speciosa* hairy roots were isolated and their structures elucidated by NMR spectroscopic methods. As we focused on the mitragynine biosynthesis in *M. speciosa*, the *in vitro* hairy root cultures and *in vitro* regenerated plantlets obtained from this study can serve as plant models for further biosynthetic studies.

Experimental

Bacterial strain and chemicals

Agrobacterium rhizogenes ATCC 15834 was obtained from the Microbiology Resource Center, Pathumtani, Thailand. Mitragynine was isolated from *M. speciosa* leaves (Keawpradub, 1990). Ursolic acid was purchased from Sigma-Aldrich Ptc., Ltd. (Singapore). McCown woody plant medium (WPM) and plant agar were from Duchefa Biochemie (Haarlem, The Netherlands). *N*-Benzyladenine (BA), 1-naphthaleneacetic acid (NAA) (95% GC), and 6-furfurylamino-purine (kinetin) were purchased from Fluka Chemie (Buchs, Switzerland). Thidiazuron (TDZ) was purchased from Supelco (Bellefonte, PA, USA). Bacterial media were purchased from Himedia Laboratories (Mumbai, India). All other chemicals were standard commercial products of analytical grade.

Plant materials

M. speciosa seeds were collected from Hat Yai District, Songkhla, Thailand. They were surface-sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox® (NaClO; Selangor, Malaysia) for 5 min and finally rinsing with sterile distilled water thoroughly. Sterile seeds were germinated on WPM supplemented with 1.0 mg/l BA and incubated at 25 °C under 16 h daily light. Two-month-old plantlets were used for bacterial infection.

Induction of M. speciosa hairy root cultures

A. rhizogenes ATCC 15834 were prepared freshly on yeast extract (YE) solid medium [containing 5.0 g/l beef extract, 1.0 g/l peptone, 5.0 g/l sucrose, 50 ml/l of 10% (w/v) $MgSO_4$ solution and 15 g/l agar] at 28 °C for 16 h. A single bacterial colony was inoculated into 5 ml YE broth medium, placed on a rotary shaker (218 rpm) and incubated at 28 °C for 16 h. The bacterial suspension was harvested by centrifugation at 4,000 rpm for 5 min and the pellet was re-suspended in sterile WPM. The OD_{600} value was adjusted to 0.5–0.6. The explants (stems and leaves) were wounded by a needle and submerged in bacterial suspension for 30 min. The infected explants were thoroughly washed with sterile water and transferred to WPM containing 0.7% (w/v) plant agar. After 3 d of infection, explants were transferred to solid WPM containing 500 mg/l cefotaxime disodium (M&H, Bangkok, Thailand) and, for further culture, the cefotaxime disodium concentrations were decreased to 250, 100 and 50 mg/l each week. Cultures free of *A. rhizogenes* were transferred to hormone-free solid WPM. Hairy roots were initiated within 10 d after infection, at (25 ± 2) °C under darkness. Hairy roots were excised from explants and maintained in liquid WPM. For untransformed roots, the seedling roots were cut and cultured in liquid WPM. Both types of culture were kept at (25 ± 2) °C in the darkness and rotary-shaken at 80 rpm.

Identification of transformed hairy roots by PCR analysis

Genomic DNA was isolated from untransformed roots and hairy roots using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The transformed genes in hairy roots were identified by PCR analysis for the rooting locus genes *rol*A and *rol*B. For *rol*A, forward primer 5′-CAGAATGGAATTAGCCGGACTA-3′ and reverse primer 5′-CGTATTAATCCCGTAGGTTTGTTT-3′ were used for amplification of a 300-bp fragment. For *rol*B, forward primer 5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′ and reverse primer 5′-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′ were used for amplification of a 780-bp

fragment. The PCR reactions were performed in a total volume of 50 μl, containing 50 pg of genomic DNA, 0.5 μM of each primer, 200 μM dNTP, 2.5 U of Taq DNA polymerase (New England Biolab, MA, USA), and 1x ThermolPol buffer (New England Biolab). PCR conditions were 94 °C for 2 min, 24 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min for *rol*A and 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min for *rol*B and a final extension at 72 °C for 10 min. PCR fragments were analyzed by 1.2% (w/v) agarose gel electrophoresis and visualized using UV transluminator (312 nm) after ethidium bromide staining.

Isolation of ursolic acid and phytosterols

Dried hairy roots of *M. speciosa* (37.4 g) were macerated with 300 ml methanol for 3 d and filtered. The marc was re-macerated and the methanol fractions were pooled and evaporated to dryness. The residue (8.52 g) was dissolved in 100 ml methanol, partitioned with 100 ml *n*-hexane for three times, and evaporated. The crude *n*-hexane extract (403 mg) was further purified by loading on the top of a silica gel column (3 × 18 cm; Scharlau, La Jota, Spain) and eluting with *n*-hexane/ethyl acetate (9:1, 8:2, 7:3, 5:5, v/v), ethyl acetate, ethyl acetate/methanol (8:2, 5:5, v/v) and methanol. From TLC analysis, 11 fractions were obtained. Fractions F6 and F10 were further purified. F6 was re-crystallized using chloroform/methanol (7:3, v/v). White needle crystals were obtained, affording MSF1 (6.1 mg). F10 was washed with *n*-hexane. A white amorphous solid was obtained, affording MSF2 (10.11 mg).

Concerning the structure of MSF2, the ^{1}H NMR spectra exhibited signals typical for the structure of triterpenoid compounds. The NMR data were obtained as follows.

^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$): 3.20 (t, 1H, J = 7.5 Hz, H-3), 5.24 (m, J = 3.5 Hz, 1H, H-12), 2.20 (d, J = 11.0 Hz, 1H, H-18), 2.02–1.15 (m, H-22), 0.98 (s, 3H, Me-23), 0.78 (s, 3H, Me-24), 0.93 (s, 3H, Me-25), 0.82 (s, 3H, Me-26), 1.10 (s, 3H, Me-27), 0.86 (d, J = 6.5 Hz, 3H, Me-29), 0.95 (d, J = 6.5 Hz, 3H, Me-30).

^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$): 38.4 (C-1), 26.5 (C-2), 78.5 (C-3), 39.2 (C-4), 55.0 (C-5), 18.0 (C-6), 32.8 (C-7), 39.2 (C-8), 47.3 (C-9), 36.7 (C-10), 23.9 (C-11), 125.2 (C-12), 137.9 (C-13), 41.8 (C-14), 23.9 (C-15), 23.0 (C-16), 47.6 (C-17), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20), 30.4 (C-21), 36.6 (C-22), 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 180.5 (C-28), 16.7 (C-29), 20.9 (C-30).

DEPT 90 experiment CH ($CDCl_3/CD_3OD$): 78.5 (C-3), 55.0 (C-5), 47.3 (C-9), 125.2 (C-12), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20).

DEPT 135 experiment ($CDCl_3/CD_3OD$) CH_2: 38.4 (C-1), 26.5 (C-2), 18.0 (C-6), 32.8 (C-7), 23.9 (C-11), 23.9 (C-15), 23.0 (C-16), 30.4 (C-21), 36.6 (C-22); CH_3: 27.7 (C-23), 15.3 (C-24), 15.1 (C-25), 16.6 (C-26), 23.2 (C-27), 16.7 (C-29), 20.9 (C-30).

Quantification of ursolic acid content

The ursolic acid content was determined using the HPLC method as described by Chen *et al.* (2003). Dried hairy root powder (100 mg) was refluxed thrice with 50 ml of *n*-hexane for 1 h at 50 °C and filtered. Pooled *n*-hexane fractions were evaporated to dryness. For HPLC analysis, the residue was dissolved in 5 ml of acetonitrile, filtered through a 0.45 μm membrane prior to HPLC injection. An HPLC system (Agilent 1100 Series LC System, Agilent Technologies, Wilmington, USA) was equipped with a C18 reverse phase column (3.9 × 300 mm, 10 μm, Bondapak, Waters, USA) and a UV detector (photodiode array) set at 206 nm. Ursolic acid was eluted isocratically with acetonitrile/0.1% (v/v) H_3PO_4 in water (70:30, v/v) with a flow rate of 1 ml/min at 15.5 min. A calibration curve of authentic ursolic acid (Sigma-Aldrich) was established. Linearity of the calibration curve was observed in the range 15–120 μg/ml with r^2 of 0.9999 (% RSD of 0.09–0.45%). Each calibration point was established in triplicate.

Quantification of mitragynine content

The TLC-densitometric method was developed by Keawpradub (1990) for determination of the mitragynine content. The regenerated plants were harvested, dried at 50 °C for 12 h, ground and used as material for preparation of the crude alkaloid extract. The dried powder (200 mg) was refluxed with 50 ml methanol three times at 60 °C for 1 h and filtered. The filtrates were pooled and evaporated to dryness. The dried residue was re-dissolved in 30 ml of 7% (v/v) acetic acid/H_2O and filtered. The acidic filtrate was washed with petroleum ether, and then the solution was basified with 25% (v/v) ammonia solution to pH 9. The filtrate was partitioned with 50 ml chloroform three times.

The chloroform fractions were pooled and evaporated to dryness. The crude alkaloid extract was dissolved in 5 ml chloroform and the solution (5 μl) was subjected to a TLC plate (Silica gel F_{254}, Merck). The mobile phase was chloroform/methanol (9:1). The R_F-value of mitragynine was 0.7. After removing from the tank, the TLC plate was dried and placed in a CAMAG TLC scanner (Muttenz, Switzerland) equipped with Cats version 4.01 software. The UV detector was set at 254 nm, and peak areas were integrated and converted to concentrations using a calibration curve. The linearity of the calibration curve of authentic mitragynine was in the range of 0.3–5.0 mg/ml with R^2 of 0.9984. The peak identity was performed by scanning the UV absorption at 200–600 nm.

Spectroscopy

^1H (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Unity Inova NMR spectrophotometer (Varian, Darmstadt, Germany). A mixture of $CDCl_3$ and CD_3OD (1:1) was used as solvent and tetramethylsilane (TMS) was used as the internal standard.

Results

Hairy root cultures of M. speciosa

The *M. speciosa* hairy root culture was established by infection of 2-month-old plantlets with *A. rhizogenes* ATCC 15834. Optimization of the site of infection, leaf veins and stems was performed for the explants. The result suggested that an appropriate explant was from the leaf vein with a percentage of hairy root induction of 85%, while a percentage of hairy root induction of 67% was found when using the stems as the explants. The hairy roots obtained from leaf veins appeared as thin roots and contained small pubescent. In contrast, the hairy roots obtained from the stems contained nodules (undeveloped hairy roots) and thick roots. Genotypes of the hairy roots were identified for the rooting locus genes *rol*A and *rol*B by polymerase chain reaction. The presence of *rol*A and *rol*B genes of *A. rhizogenes* ATCC 15834 in the transformed hairy roots was accounted by 4/6 clones (Fig. 2).

Due to the slow growth rate of the hairy roots in WPM, other types of media were manipulated.

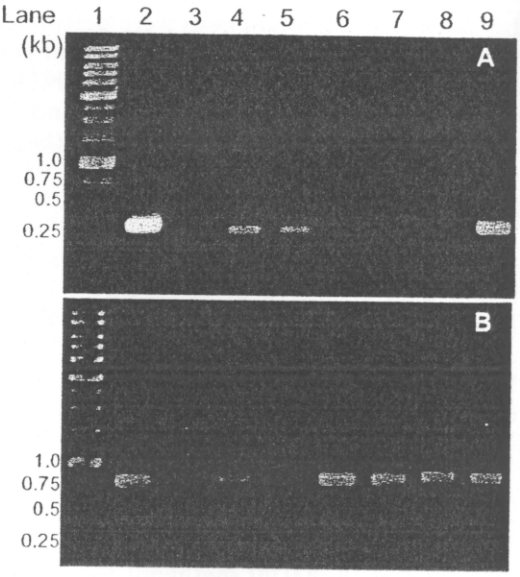

Fig. 2. PCR analyses of the hairy roots. PCR was performed with primers for the rooting locus genes (A) *rol*A (300 bp) and (B) *rol*B (780 bp). Lane 1, marker (10 kb DNA ladder); lane 2, *A. rhizogenes* ATCC 15834; lane 3, untransformed roots; lanes 4–9, transformed hairy roots (lines 1–6).

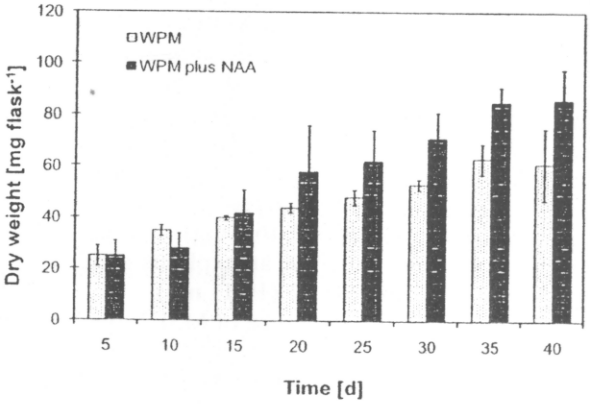

Fig. 3. Effect of NAA (0.5 mg/l) in WPM on the growth of the *M. speciosa* hairy root culture. Values are means of triplicate determinations. Error bars present standard deviation.

Hairy roots in half-strength WPM, B5 and MS media grew badly (data not shown). Comparison of the growth curves of the hairy roots in WPM and WPM plus NAA revealed that the addition of NAA (0.5 mg/l) to WPM stimulated the growth of hairy roots. As shown in Fig. 3, doubling time of the hairy roots was decreased from about 2

months of culture to about 30 d. The hairy roots were, therefore, maintained in WPM supplemented with NAA (0.5 mg/l) and used as materials for evaluation of secondary metabolites production.

Accumulation of triterpenoids and phytosterols in M. speciosa hairy roots

Preliminary screening by TLC after detection with Dragendorff's reagent showed that *M. speciosa* hairy roots were unable to produce alkaloids. To identify the secondary metabolites present in the hairy roots, an *n*-hexane extract was prepared and isolated by silica gel column chromatography. NMR data of MSF1 and MSF2 were accomplished by ^{1}H, 1D and 2D ^{13}C NMR analysis. Concerning the structure of MSF1, analysis of ^{1}H NMR data was in agreement with NMR data of published by Subhadhirasakul and Pechpongs (2005). From ^{13}C NMR spectra, peaks at 138.32 (C-22) and 129.21 (C-23) were observed. Both signals corresponded to the double bond in the structure of stigmasterol. Integration of the allylic protons H-22 and H-23 indicated that MSF1 was a mixture of β-sitosterol and stigmasterol, present in the ratio of 1:1.

The peaks at of δ 78.5 (C-3; COH), 125.2 (C-12), 137.9 (C-13), 180.5 (C-28; C=C) suggested that MSF2 contained ursolic acid (**2**). 2D-NMR: COSY, HMQC and HMBC indicated the correlation of ^{13}C-^{13}C, ^{1}H-^{13}C and long-length coupling ^{1}H-^{13}C, respectively. Analysis of NMR data of MSF2 was in agreement with a previous report on triterpenoids (Güvenalp *et al.*, 2006). The presence of the signal at δ 5.28 in the ^{1}H NMR spectrum indicated that MSF2 contained the isomer of ursolic acid, oleanolic acid. Integration of the H-12 signal in ^{1}H NMR spectrum indicated that MSF2 contained a mixture of ursolic acid and oleanolic acid in the ratio of 5:1.

To construct the growth curve and production curve, samples were taken every 5 d over a period of 40 d of culture. The ursolic acid content was determined from the hairy roots in comparison with the untransformed roots. The HPLC chromatograms of authentic ursolic acid, and the extracts from the untransformed roots and the hairy roots are shown in Fig. 4. The ursolic acid contents are summarized in Table I. The results indicated that both types of cultures were able to produce ursolic acid. It can be noted that the hairy roots, at 30 d, accumulated ursolic acid with the yield of (3.47 ± 0.03) mg/g dry weight (DW), whereas the untransformed roots produced (2.41 ± 0.01) mg/g DW.

Table I. Ursolic acid contents in transformed hairy roots and untransformed roots (n = 4).

Time [d]	Ursolic acid content [mg/g DW ± SD]	
	Transformed hairy roots	Untransformed roots
5	1.52 ± 0.00	1.58 ± 0.01
10	1.90 ± 0.02	1.74 ± 0.01
15	2.49 ± 0.02	1.89 ± 0.01
20	1.65 ± 0.01	1.69 ± 0.01
25	2.00 ± 0.04	2.09 ± 0.01
30	3.47 ± 0.03	2.41 ± 0.01
35	1.98 ± 0.03	1.74 ± 0.02
40	2.40 ± 0.01	2.01 ± 0.00

M. speciosa plant regeneration containing high yields of mitragynine

During the establishment of *M. speciosa* hairy root culture, shoot regeneration was observed when the hairy roots contained part of the stem (cut from the original explant). After 2 months, the regenerated shoots were removed and placed in the hormone-free solid WPM. The roots were then initiated after culture for 10 d to complete the plantlets. The regenerated plantlets were then used as materials for micropropagation. Axillary buds were excised from the stems and used as explants for shoot multiplication. Since the hairy roots have been obtained from infection with *A. rhizogenes* ATCC 15834, therefore, the regenerated plantlets were determined for the presence of the *rol*A and the *rol*B genes. However, the data indicated that the regenerated plantlets did not carry the genes from *A. rhizogenes*.

To increase the number of shoots in the *M. speciosa* plantlet, the axillary buds were excised and manipulated in solid WPM supplemented with three kinds of cytokinin. In this study, BA (1 mg/l) and TDZ (0.1 and 0.5 mg/l) were used for the shoot inductions. After 4 weeks, the shoot numbers were counted and calculated as the shoots number per explant. The results indicated that the shoot numbers of (2.8 ± 1.5), (6.3 ± 1.6) and (6.3 ± 1.3) per explants were obtained from WPM supplemented with 1 mg/l of BA, 0.1 mg/l of TDZ and 0.5 mg/l of TDZ, respectively. These results showed that TDZ was an appropriate plant growth

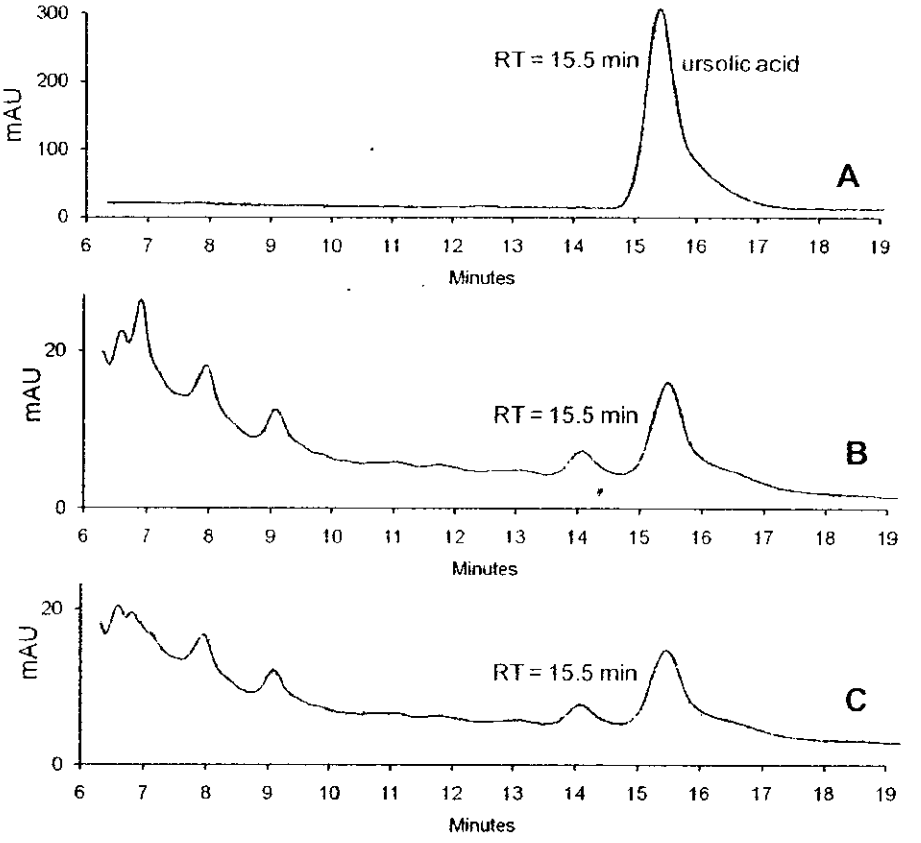

Fig. 4. HPLC chromatograms of (A) authentic ursolic acid, (B) the *n*-hexane extracts of the transformed hairy roots and (C) the untransformed hairy roots.

regulator for shoot multiplication. Root initiation of the regenerated plantlet of *M. speciosa* was simply performed. The shoots (containing 2–4 leaves) were cut from the explants and placed on hormone-free WPM. Roots were initiated after 1 week of culture.

To evaluate the mitragynine production in the regenerated plantlets, the *in vitro* plants, obtained from seeds germination in WPM supplemented with 1 mg/l BA and grown under controlled conditions, were used as control. Mitragynine contents of *in vitro* plantlets and regenerated plantlets were determined. A simple and rapid method of mitragynine quantification was established using the TLC-densitometric method. The extract from the whole plant of the *in vitro* and the regenerated plantlets (5 months old) were prepared for alkaloid extracts and determined for mitragynine contents. As shown in Table II, the mitragynine content in the regenerated plantlet was about 3.2 times higher than that in the *in vitro* plantlet. From this experiment it can be concluded that mitragy-

Table II. Mitragynine content determined from 5-month-old plants.

Sample	Mitragynine content[a] [mg/g DW ± SD]
In vitro plantlet[b]	4.45 ± 0.09
Regenerated plant[c]	14.25 ± 0.25
Leaves from regenerated plant	12.5 ± 0.25

[a] Samples were determined in triplicate.
[b] From plantlets that germinated from seeds and grew in WPM supplemented with BA (1 mg/l).
[c] From regenerated plants that were maintained in the hormone-free WPM.

nine was produced and accumulated mostly in leaves.

Discussion

The main objective of this investigation was to establish model plants for mitragynine biosynthesis in *M. speciosa*, a monoterpenoid indole alkaloid. Hairy root cultures were performed by infection with the wild-type *A. rhizogenes*. Since the susceptibility of plant cells to *A. rhizogenes* is dependent upon age and differentiation status of plant tissue (Sevón and Oksman-Caldentey, 2002), the hairy root culture used in this study was induced from the meristematic tissues such as stems and leaves due to ease of differentiation. Theoretically, the hairy root culture should grow relatively fast and in plant growth regulator-free medium (Guillon *et al.*, 2006). However, from this study, the hairy roots could grow only slowly and required a low concentration of NAA (0.5 mg/l) for promoting their growth. This evidence is unusual for a normal hairy root culture (Sevón and Oksman-Caldentey, 2002). Concerning case of other Rubiaceous plants, an *Ophiorrhiza pumila* hairy root culture in B5 medium [containing 2% (w/v) sucrose] could grow fast and produce camptothecin (Saito *et al.*, 2007). It can be suggested that the hormonal balance in *M. speciosa* hairy roots was not appropriate for root proliferation (Guillon *et al.*, 2006).

The hairy roots of *M. speciosa* could accumulate triterpenoids (ursolic acid and oleanolic acid) and phytosterols (β-sitosterol and stigmasterol) but none of the alkaloids were found in this culture. Nevertheless, many studies have reported that alkaloids such as mitraphylline and rhynchophylline have been found in the root bark of *M. speciosa* (Houghton and Shellard, 1974; Shellard *et al.*, 1978). It can be suggested that the intermediates of alkaloids were probably unstable and degraded during culture. However, the hairy roots accumulated ursolic acid, which is pharmacologically important for antibacterial, anti-inflammatory, antiviral activities, etc. (Liu, 1995). It is produced in the late linear phase at a yield of (3.47 ± 0.03) mg/g DW. It can be noted that *M. speciosa* hairy roots could accumulate high yields of ursolic acid when compared to a *Uncaria tomentosa* cell suspension culture [(1.68 ± 0.04) mg/g DW] (Feria-Romero *et al.*, 2005). From this evidence, it can be concluded that the organ culture such as hairy roots preferred to produce a higher amount of triterpenoids.

From the biosynthetic point of view, *M. speciosa* hairy roots could not produce any alkaloid, especially mitragynine. It may be caused by a lack of precursors and enzymes that are involved in the synthesis of monoterpenoid indole alkaloids, since primary metabolites need special cell compartments for storage and degradation (Luckner, 1990). The presence of ursolic acid, coexisting with oleanolic acid, indicated that at least isoprene units have been produced in this culture. These isoprene units are precursors for both triterpenoids and phytosterols (Luckner, 1990), which means that their biosynthesis was active in this culture. Therefore, the *M. speciosa* hairy root culture is not only useful for ursolic acid production but may also have the potential to be a model plant culture for triterpenoid biosynthesis. Unexpectedly, plantlet regeneration of *M. speciosa* was obtained. In this study, TDZ was shown to be an efficient cytokinin for shoot multiplication. TDZ acts as a substitute for both auxin and cytokinin, requirements to induce organogenesis and somatic embryogenesis in many plant species (He *et al.*, 2007). The regenerated plantlets produced and accumulated a considerable amount of mitragynine, when compared to the *in vitro* plantlets. It was postulated that this may be caused by the effect of TDZ. Nevertheless, it is still unknown.

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