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โครงการ: การสร้างเซลล์เพาะเลี้ยงแคลลัสตัดแต่งพันธุกรรมจากต้นกระท่อมเพื่อใช้ในการผลิตแอลคาลอยด์

(Establishment of transgenic *Mitragyna speciosa* callus culture for alkaloids production)

โดย

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บทคัดย่อ

รหัสโครงการ : TRG5880033

ชื่อโครงการ : การสร้างเซลล์เพาะเลี้ยงแคลลัสดัดแต่งพันธุกรรมจากต้นกระท่อมเพื่อใช้ในการผลิตแอลคาลอยด์

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การศึกษาครั้งนี้ได้เหนี่ยวนำชิ้นส่วนจากต้นกระท่อม (*Mitragyna speciosa*) ให้กลายเป็นเซลล์เพาะเลี้ยงแคลลัสแบบร่วน (friable callus) ด้วยฮอร์โมนชนิดต่าง ๆ พบว่าฮอร์โมน 2,4-D ให้แคลลัสในลักษณะที่ต้องการและมีการเจริญเติบโตดีกว่าการเหนี่ยวนำด้วยฮอร์โมนชนิดอื่น ทั้งนี้ตรวจพบแอลคาลอยด์ mitragynine เฉพาะในเซลล์เพาะเลี้ยงแคลลัสรุ่นที่หนึ่งทั้งจากการเหนี่ยวนำด้วยฮอร์โมน 2,4-D และ NAA แต่ตรวจไม่พบ *mitragynine* ในเซลล์เพาะเลี้ยงแคลลัสรุ่นถัดมาและในเซลล์แขวนลอย (cell suspension) ที่เลี้ยงด้วยฮอร์โมน 2,4-D ทั้งนี้อาจเกิดจากเซลล์เพาะเลี้ยงดังกล่าวขาดการแสดงออกของยีนในชีวสังเคราะห์ของ mitragynine ผู้วิจัยจึงทำการหาวิธีการถ่ายยีนเข้าไปในเซลล์เพาะเลี้ยงของต้นกระท่อม (genetic transformation) ซึ่งประสบความสำเร็จในการใช้เซลล์เพาะเลี้ยงแคลลัสเป็นชิ้นส่วนตั้งต้นในการถ่ายยีน tryptophan decarboxylase (*tdc*) และใช้การถ่ายยีนด้วย *Agrobacterium tumefaciens* สายพันธุ์ LBA4404 โดยมีอัตราความสำเร็จที่ 2% อย่างไรก็ตาม เมื่อวัดระดับการแสดงออกของ mRNA ของยีน *tdc* โดยวิธี quantitative real-time (qRT) PCR ของเซลล์เพาะเลี้ยงแคลลัสดัดแต่งพันธุกรรมเปรียบเทียบกับใบกระท่อม พบว่า mRNA ของ *tdc* ของเซลล์เพาะเลี้ยงแคลลัสดัดแต่งพันธุกรรม มีระดับการแสดงออกของยีนใกล้เคียงกับเซลล์เพาะเลี้ยงแคลลัสปกติและน้อยกว่าในใบกระท่อม และจากการวิเคราะห์หาปริมาณสารด้วย LC-MS ไม่พบ mitragynine ในเซลล์เพาะเลี้ยงทั้งสองชนิด จากผลการทดลองนี้สรุปได้ว่า เซลล์เพาะเลี้ยงแคลลัสดัดแต่งพันธุกรรมของต้นกระท่อมที่เพาะเลี้ยงในอาหาร WPM ด้วยฮอร์โมน 2,4-D 1 mg/L ไม่สามารถสร้างสาร *mitragynine* ได้ ทั้งนี้อาจเกิดจากขาดการแสดงออกของยีนในช่วงท้ายของชีวสังเคราะห์ mitragynine หรืออาจเกิดจากการขาดองค์ประกอบที่สำคัญภายในเนื้อเยื่อของเซลล์เพาะเลี้ยงเอง

คำหลัก : *Agrobacterium tumefaciens*, cell suspension, genetic transformation, *Mitragyna speciosa*, *mitragynine*

Abstract

Project Code: TRG5880033

Project Title: Establishment of transgenic *Mitragyna speciosa* callus culture for alkaloids production

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Project Peroid : 2 years (2015-2017)

In this study, the medium manipulation for *Mitragyna speciosa* friable callus induction was observed and it was found that the best callus formation was obtained from WPM supplemented with 1 mg/L 2,4-D. Mitragynine, an indole alkaloid, was detected in the first generation of both callus induced from 2,4-D and NAA. On the other hand, it was absence in the next generation calluses and in cell suspension grown in liquid WPM supplemented with 1 mg/L 2,4-D. Since the expression of genes involving in mitragynine biosynthesis of *M. speciosa* cell culture may be inadequate, the aim to study on genetic transformation was attended. A successful in genetic transformation allowed us to transform tryptophan decarboxylase (*tdc*) into *M. speciosa* callus culture for the first time using *Agrobacterium tumefaciens* strain LBA4404 with the transformation efficiency of 2%. Transcription profile of *tdc* mRNA from transgenic callus was measured by quantitative real-time (qRT) PCR in comparison with wild type callus. The results showed that the *tdc* mRNA of the transgenic callus was expressed as same as the wild type callus, and the expression was less than that of in *M. speciosa* leaf. In addition, the metabolite analysis using LC-MS revealed no mitragynine in both calluses. It was concluded that the *M. speciosa* transgenic callus cultured in WPM supplemented with 1 mg/L 2,4-D was unable to produce mitragynine. This may be due to lack of gene expression in late steps of mitragynine biosynthesis or the lack of differentiate tissues in callus culture.

Keywords: *Agrobacterium tumefaciens*, cell suspension, genetic transformation, *Mitragyna speciosa*, mitragynine

Executive Summary

1. Introduction to the research problem and its significance

Nowadays, a large number of patients are suffered from pain either by harsh diseases, or accident. Morphine which is used to relief the pain is insufficient in some cases, for the examples, late-stage cancer or HIV-infection patients, some post-operation patients, and etc. Undoubtedly, developing analgesic agents together with therapeutic agents for curing each specific disease is also necessary.

Based on the knowledge of pharmacognosy and pharmacology, mitragynine and 7-OH mitragynine, indole alkaloids, show analgesic activity through opioid receptor in which a latter one exhibits about 10 times more potent than that of morphine, thus the possibility is raised if one can develop new analgesic agents from these compounds. Regrettably, both valuable compounds can be found uniquely in *Mitragyna speciosa*, or kratom, which is an illegal plant and mainly being abused orally as a drink in Thailand. Because of the mentioned reason, it is improper growing *M. speciosa* in the open field utilizing it as a source of the indole alkaloids. On the other hand, one prospect to overcome this issue is to cultivate *M. speciosa* using plant tissue culture. A remarkable advantage of such a technique is a possibility to cultivate the plants in a small restricted area in which the distribution can hardly be made.

In this proposal, the investigators' goal is to establish the transgenic *M. speciosa* callus culture which has never been reported before. The transgenic callus culture development is required, considering two reasons; first, the callus culture of *M. speciosa* was found to possibly possess alkaloid production, and second, there are reports in limitation of mitragynine biosynthesis in *M. speciosa* which may be overcome by genetic engineering. The transgenic callus culture will be obtained using plant medium manipulation and *Agrobacterium tumefaciens* infection, the alkaloids level will be measured using HPLC. The obtained callus culture from this study can be further developed into cell suspension culture which was probably used as a source of mitragynine and 7-OH mitragynine in large scale production.

2. Objectives

- To establish alkaloid producing *M. speciosa* callus culture
- To transfer *as* and *tdc* into *M. speciosa* callus culture
- To determine mitragynine and 7-OH mitragynine in *M. speciosa* callus culture
- To determine mRNA expression of genes involved in alkaloid production in *M. speciosa* callus culture

3. Methodology

Plant preparation

According to the data from AOAC international, in Thailand, *M. speciosa* in Maeng Da variety, or potent red vein, contained the highest amount of alkaloids. *M. speciosa* seeds (potent red vein) were collected from the open field in Hat Yai district, Songkhla, Thailand. The seeds were surface sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox® for 5 min and finally rinsing with sterile distilled water. Sterilized seeds were germinated on Lloyd & McCown Woody Plant medium (WPM) and incubated at 25 °C under long-day conditions (16 h light/8 h dark).

Plasmid vector construction

The 35S-*gfp*-T_{nos} gene cassette was constructed in pTH2-Δ*EcoR*I vector. The *gfp* cassette was cut and ligated into pCAMBIA1300 with *Kpn*I and *Pst*I restriction enzymes. For pCAMBIA1300-*MsTDC* construction, the 35S-*MsTDC*-poly-A cassette from pRT101 was inserted into *Hind*III site of the *gfp* plasmid vector. For pCAMBIA1300-*MsAS* construction, the 35S-*MsAS*-poly-A cassette from pRT101 was inserted into *Sac*I site of the *gfp* plasmid vector. The 35S-*gfp-tdc-as*-T_{nos} gene cassette was constructed by cutting 35S-*MsAS*-poly-A cassette and inserted in pCAMBIA1300-*MsTDC*. The reaction mixture contained plasmid DNA, 10x Tango™ buffer, restriction enzyme, 500 µg/µl boiled RNase and distilled water and incubated at 37°C for 1 h. To analyze the DNA fragments, the solution was then mixed with sample buffer, loaded on 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide solution.

A. tumefaciens transformation

The *A. tumefaciens* cells were gently thawed on ice. The electroporation cuvette with 0.2-cm electrode gap was chilled on ice. Plasmid DNA was added to the competent cells suspension and mixed by tapping. The mixture was transferred to a prechilled cuvette and the electric pulse was applied at 2.5 kV, 25 µF and 200 Ohm. SOC medium was immediately added the mixture was transferred to a tube and incubated at 29°C for 1 h. Only 100 µl of the suspension was plated on the selective medium (50 µg/ml kanamycin for cells harboring pCAMBIA1300). The culture was incubated for 3 days at 29°C. Identification of the recombinant DNA from *A. tumefaciens* was also performed using plasmid extraction kit and gel electrophoresis.

M. speciosa callus induction

McCown Woody plant medium (WPM) pH 5.8 was selected. WPM medium was supplemented with a range of concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and zeatin. Cultures were grown under a 16 h light and 8 h dark photoperiod at 25±2 °C. The different explants were cultured on those various medium and were tested to determine which explants produced an optimal callus induction and alkaloid production. After 6 weeks, every sample were subcultured and grown further for 5 more generations, the alkaloid production was determined in all generations. Each experiment contained 10 replicates. The best alkaloid producing callus line was selected to use as a material for genetic transformation.

M. speciosa genetic transformation and cultivation

The explants were immerged in liquid medium with 100 mM acetosyringone in a steriled glass tube. The tube was sonicated for 10 min with an ultrasonicator. After sonication explants were added into sterilized flasks containing *A. tumefaciens* suspension and flasks were shaken gently for 30 min at 25 °C. Explants were blot-dried with sterile paper towels and transferred onto Petri dishes containing appropriate medium with 100 mM acetosyringone. The co-cultivation period was 3 days in the dark at 29 °C. After co-cultivation, the explants were transferred into appropriate callus induction medium supplemented with 250 µg/ml cefotaxime and 100 µg/ml vancomycin for *A. tumefaciens* elimination for 1-2 weeks. The explants were then transferred to appropriate medium for callus induction. For transgenic callus, the positive green fluorescence callus was selected using fluorescence stereomicroscope or using hygromycin B supplemented medium. After 6 weeks, every sample were subcultured and grown further for at least 5 more generations, the alkaloid production was determined in all generations. Each experiment contained 10 replicates.

Quantification of alkaloid content in *M. speciosa* callus culture

High performance liquid chromatography (HPLC) is used to determine TIAs contents, especially mitragynine and 7-OH mitragynine. Crude alkaloid extracts are prepared from transgenic and wild type *M. speciosa* callus cultures by reflux with methanol for 1 hour then washed with petroleum ether prior to analysis. Chromatographic separation is carried out on a reversed-phase C18 column with binary gradient mobile phase profile. The identification of the metabolites is based on the retention time and comparison of the UV absorption spectra with those standards. The amounts of intermediates are calculated based on those standards curves. Quantification is repeated for each culture with three replicates. The Pair t-Test was used to evaluate the data.

Total RNA extraction and relative expression by RT-qPCR

Extraction of total RNA was performed using an RNeasy Plant Mini Kit. The RT-qPCR was used to determine the transcription of *M. speciosa* anthranilate synthase isoform1 and isoform2 (*MsAS*1 and *MsAS*2; GenBank ID: JQ775867 and JQ775866), *M. speciosa* tryptophan decarboxylase (*MsTDC*; GenBank ID: JN643922.1), *M. speciosa* 1-deoxy-D-xylulose 5-phosphate synthase isoform1 (*MsDXS*1; GenBank ID: JQ038372.1), *M. speciosa* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*MsDXR*; GenBank ID: JQ038374.1) and *M. speciosa* strictosidine synthase (*MsSSS;* GenBank ID: EU288197.1) genes in callus. The RT-qPCR was performed using specific primers on an ABI PRISM® 7300 Sequence Detector with SYBR® Green to monitor double stranded DNA synthesis. The PCR reaction was carried out in 96-well plate. The PCR reaction contained 1x SYBR® GreenERTM qPCR Supermix for ABI PRISM®, 300 nM ROX reference dye, 0.2 µM forward primer, 0.2 µM reverse primer, 15 ng/µl cDNA and DEPC-treated water in total volume of 20 µl. Amplification involved 40 cycles of PCR reaction and the conditions were: 1 cycle (95°C, 10 min); 40 cycles (95°C, 30 s; 60°C, 1 min) and finally a dissociation stage at 95°C 15 s, 60°C 1 min, 95°C 15 s. Samples were run in triplicate and a negative control was performed in all runs.

4. Working plan

Plan	Months			
	1-6	6-12	12-18	18-24
Plasmid construction & transformation	✓	✓		
Callus induction & cultivation	✓	✓		
Plant transformation & cultivation		✓	✓	✓
Metabolite determination		✓	✓	✓
mRNA expression analysis		✓		
Report preparation				✓

5. Predictable publication

Title: *Mitragyna speciosa*: Transgenic callus culture for high alkaloids production

Journal: Applied Microbiology and Biotechnology

Impact factor (2013): 3.81

6. Budget

	Year 1	Year 2	Total
1. Honorarium -Honorarium for project leader (13,000 x 24 months)	156,000.-	156,000.-	312,000.-
2. Material (Type & price may be change or this budget may be shared with other expenses but overall must not exceed 288,000.-) - 2,4-Dichlorophenoxyacetic acid 1,500 Baht -Acetonitrile 4 L (HPLC grade) 14,720 Baht -Acetosyringone 1 g 1,773 Baht -Agarose 250 g 34,567 Baht -Ampicillin sodium salt 5 g 4,035 Baht -Bacteriological Agar 250 g 10,344 Baht -Calcium chloride 100 g 3,688 Baht -Casein hydrolysate 500 g 3,264 Baht -Cefotaxime sodium salt 500 mg 7,119 Baht -DNA marker (1 kb) 6,746 Baht -Ethanol 4 L 2,030 Baht -Ethidium bromide solution 10 mL 3,187 Baht -Ethyl acetate 1 L (HPLC) 3,829 Baht -Ethylenediaminetetraacetic acid 3,392 Baht -Formic acid 100 mL 2,776 Baht -Glycerol 100 mL 2,264 Baht -HEPES 100 g 4,742 Baht -High-speed Plasmid Mini Kit 8,000 Baht -*Hind*III 4,000 Baht -Hygromycin B 250 mg 9,907 Baht -HPLC vial 10 packs (Agilent) 8,000 Baht -Isopropanol 500 mL 2,074 Baht -Kinetin 1 g 3,238 Baht -McCown Woody plant medium 3,387 Baht -Methanol 4 L (B&J) 3,200 Baht	130,500.-	130,500.-	261,000.-

-Nylon membrane filter 1 pack 4,309 Baht -PCR Purification Kit 10,000 Baht -Petri dish polystyrene 2 packs 13,030 Baht -PIPES 25 g 1,647 Baht -Plant agar 100 g 4,176 Baht -RNeasy Plant Mini Kit 25,000 Baht -SacI 5,000 Baht -Sodium chloride 250 g 2,010 Baht -Sodium hypochlorite solution 2,236 Baht -Sucrose 1 kg 5,076 Baht -SYBR green® Brilliant II 10,662 Baht -Syringe filter nylon 5,000 Baht -Tris-(hydroxymethyl) amino 500 g 4,058 Baht -Tryptone 250 g 4,138 Baht -Vancomycin HCl 6,014 Baht -Yeast extract 250 g 1,928 Baht -Zeatin 10 mg 4,934 Baht			
3. Other expenses -Fluorescence microscope service 20,000 Baht -Electroporator machine service 7,000 Baht	13,500.-	13,500.-	27,000.-
Total budget	300,000.-	300,000.-	600,000.-

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Tossaton Charoonratana

Grantee no. TRG5880033

30 June 2017

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List of Abbreviations

2,4-D	=	2,4-Dichlorophenoxyacetic acid
ATP	=	adenosine 5'-triphosphate
cDNA	=	complementary deoxynucleic acid
CTP	=	cytidine 5'-triphosphate
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
EDTA	=	ethylenediaminetetraacetic acid
HPLC	=	High Performance Liquid Chromatography
IPP	=	isopentanyl diphosphate
IPTG	=	isopropyl-β-D-thiogalactopyranoside
LB	=	Luria Bertani
LC-MS	=	Liquid Chromatography Mass Spectrometry
MIA	=	monoterpenoid indole alkaloid
mRNA	=	messenger ribonucleic acid
MS	=	Murashige and Skoog
NAA	=	1-naphthaleneacetic acid
NADPH	=	nicotinamide adenine dinucleotidephosphate (reduced form)
PCR	=	Polymerase Chain Reaction
RNA	=	ribonucleic acid
TAE	=	Tris acetate EDTA
TDC	=	tryptophan decarboxylase
TDZ	=	thidiazuron
WPM	=	McCown woody plant medium

1. Introduction

Mitragyna speciosa (Roxb.) Korth. (Rubiaceae) is a tropical tree distributed in Southeast Asia, mainly in Thailand and Malaysia. The plant is generally 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. Owing to its anti-nociceptive as well as low addictive properties, mitragynine, a major monoterpenoid indole alkaloid (MIA) of *M. speciosa* leaves, has attracted much a scientific interest. Moreover, this molecule has potential for drug development, since 7-hydroxymitragynine was orally active, long-acting and found to be 10-fold more potent than morphine, the classical opiate (Kikura-Hanajiri et al., 2009).

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 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*. Among the enzymes in the early steps to be studied in the alkaloid biosynthesis were tryptophan decarboxylase and strictosidine synthase (TDC). Because these two enzymes link primary and secondary metabolism and were thought to be important regulatory enzymes in the biosynthesis. These studies resulted in the purification of TDC (Noe et al., 1984) and subsequent cloning of this gene (De Luca *et al.*, 1989). The *TDC* gene have been used in several studies to obtain transgenic plants and cell cultures with an increased TDC activity. Canel *et al.* (1998) obtained several transgenic *C. roseus* cell cultures with elevated TDC activities. In addition, high tryptamine accumulation, which is the precursor of MIAs, was increased after *TDC* cDNA driven by the strong Cauliflower Mosaic Virus 35S promoter was introduced into *C. roseus* (Goddijn *et al.*, 1995). To understand the regulatory role of *tdc*, plant tissue culture such as transgenic hairy root culture was established (Charoonratana et al., 2013a). The metabolic profile in that cultures was evaluated and used it as investigated materials for following the *tdc* expression in parallel with determination of mitragynine content.

In this study, the aim is to establish transgenic callus culture and study the regulation of *tdc*. The results obtained from this study can be further developed into cell suspension culture which was probably used as a source of mitragynine and 7-OH mitragynine in large scale production. It also 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. (Figure 1.1) belongs to the Rubiaceae family found generally in tropical Southeast Asia region. *Mitragyna* species are characterized by the globular flowering head each containing up to 120 florets. 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) which initially are closely oppressed and protect the apical bud.

The genus *Mitragyna* was given the name by Korthals because the shape of the stigmas in the species he examined resembled a bishop's mitre. 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). It was found that *Mitragyna* can be divided into two groups. First, Thai *Mitragyna* species and *M. inermis* from Sudanian regions showed close relation. Another group was the rest of African *Mitragyna* species.

1.2 Chemical constituents of *M. speciosa*

From the leaves of *M. speciosa*, found that Thai and Malay had in common the alkaloids mitragynine, speciogynine, speciociliatine, paynantheine and 7-hydroxymitragynine. In both samples, mitragynine was the most abundant alkaloid, but in the Thai kratom it made up 66% of the total alkaloid content, while it made up only 12% of the alkaloids from the Malay sample (Takayama, 2004). Reviews of the distribution of indole alkaloids in leaves, twigs, stem barks and root barks of *M. speciosa* are summarized in Table 1.1.

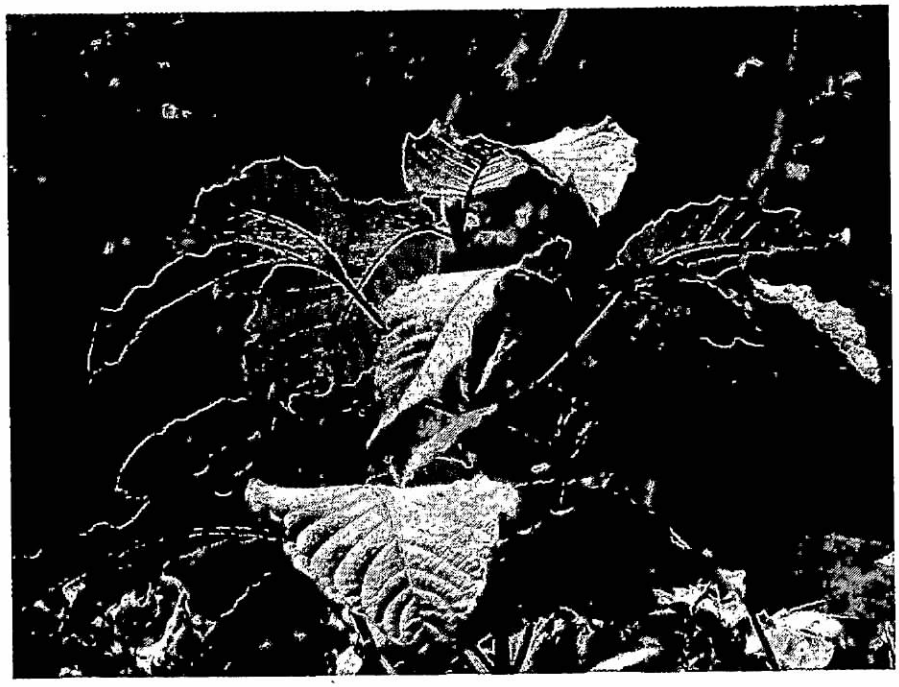
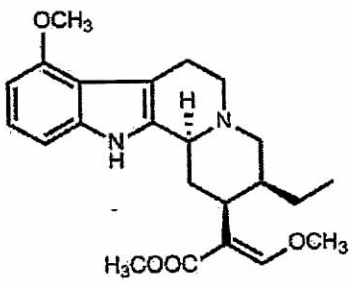


Fig. 1.1 *Mitragyna speciosa* (Roxb.) Korth. (Rubiaceae)

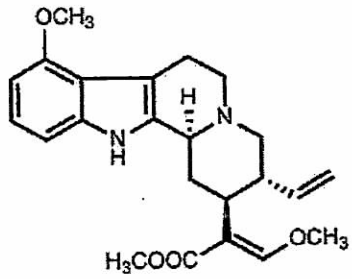
Table 1.1 Chemical constituents of *M. speciosa* (Roxb.) Korth.

Plant part	Category/chemical substances	Reference
leaves	Alkaloid: ajmalicine, akuammigine, angustine, corynantheidine, corynantheidaline, corynantheidalinic acid, corynoxeine, corynoxine, corynoxine B, hirsutine, hirsuteine, isocorynoxeine, isomitraphylline, 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-	Phillipson et al., 1973; Phillipson et al., 1973; Shellard et al., 1966; Shellard et al., 1978a; Shellard et al., 1978b Takayama, 2004

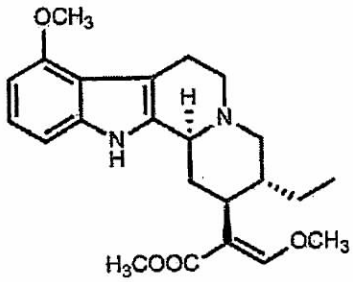
	tetradehydromitragynine, 7α-hydroxy-7*H*-mitragynine	
	flavones:	Hinou and Harvala, 1988
	apigenin, apigenin-7-O-rhamnoglucoside, cosmosiin	Hinou and Harvala, 1988
	flavonol: astragalin, hyperoside, kaempferol, quercetin, quercitrin, quercetin-3-galactoside-7-rhamnoside, quercitrin, rutin	
leaves	Phenylpropanoid: caffeic acid, chlorogenic acid	Hinou and Harvala, 1988
	Flavonoid: (-)-epicatechin	Houghton and Said, 1986
	Lignin: (+)-pinoresinol	Takayama et al., 1998
	Triterpene: ursolic acid	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



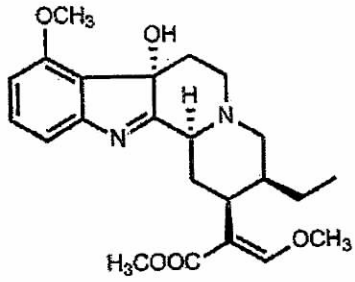
mitragynine



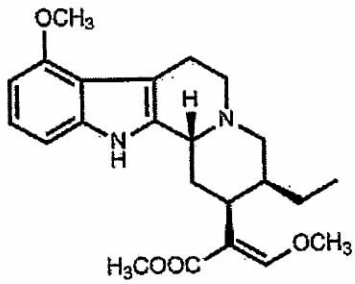
paynantheine



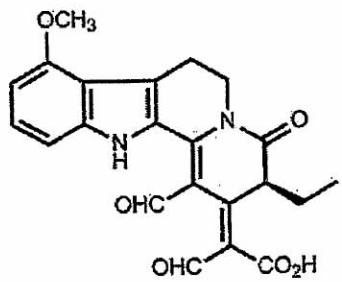
speciogynine



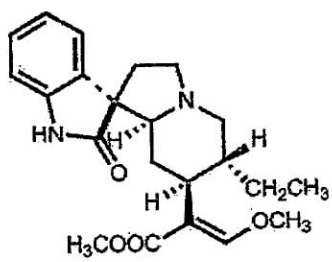
7-hydroxymitragynine



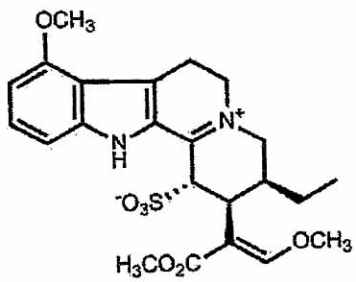
speciociliatine



mitragynalinic acid

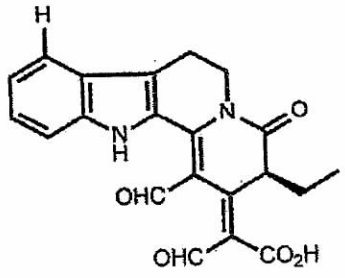


rhynchophylline

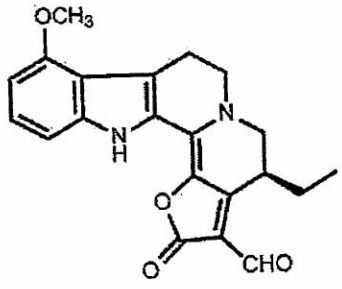


mitrasulgynine

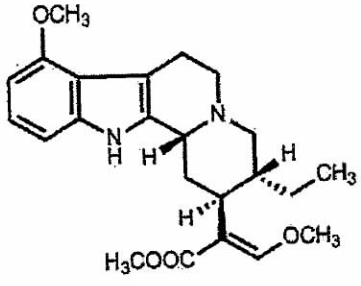
Figure 1.2 The chemical structures of indole alkaloids found in *M. speciosa.*



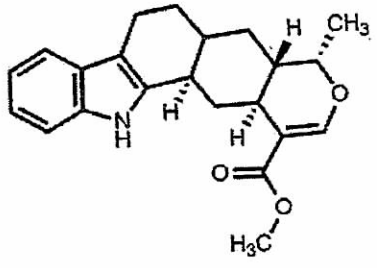
corynantheidalinic acid



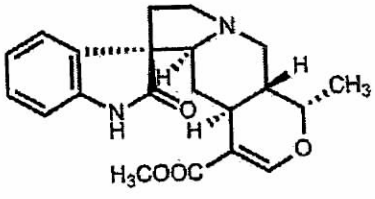
mitralactonal



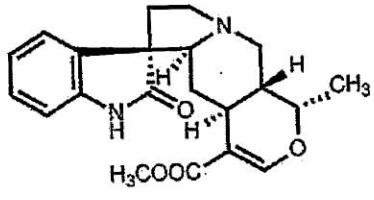
mitraciliatine



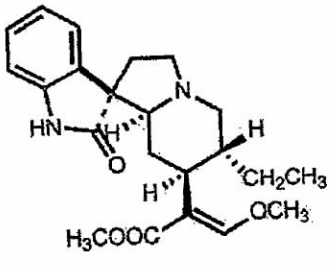
ajmalicine



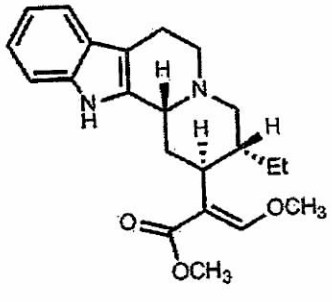
mitraphylline



isomitraphylline

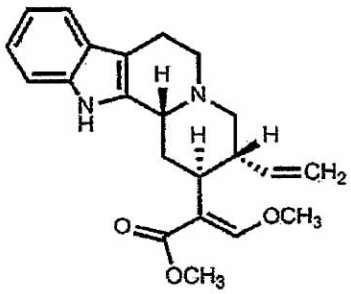


isorhynchophylline

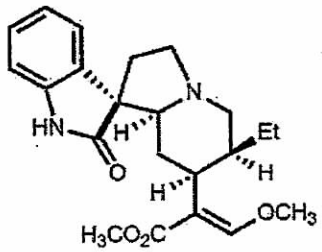


hirsutine

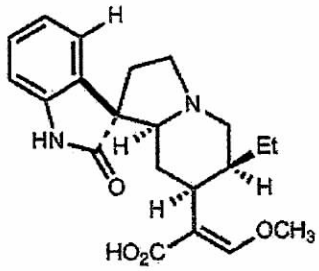
Figure 1.2 (Continued).



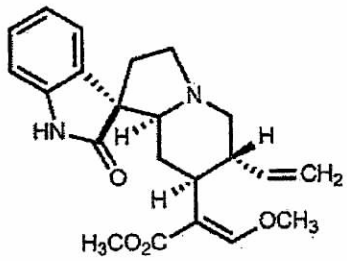
hirsuteine



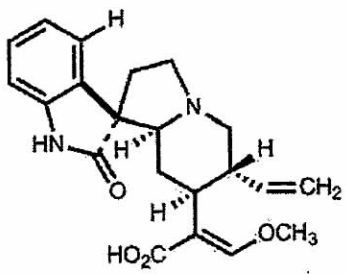
corynoxine B



corynoxine



corynoxeine



isocorynoxeine

Figure 1.2 (Continued).

1.3 Biological activities of mitragynine

As mentioned earlier, mitragynine is a major component that presents in leaves of *M. speciosa*. Traditionally usages of its leaves are for opium-substitute, in the treatment of diarrhea and coughing (Suwanlert, 1975). In 1996, Japanese group investigated the antinociception of mitragynine in mice. It was suggested that mitragynine acted as opioid receptor agonist in the brain (Matsumoto *et al.*, 1996a). Later, they found that the mechanisms for antinociceptive effects varied from those of morphine in mice (Matsumoto *et al.*, 1996b). Mitragynine preferred to bind *Mu* and δ opioid receptors, unlike morphine that binds only to *Mu* 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). The analgesic activity of mitragynine was again examined in the hot-plate and tail-pinch tests resulting in antinociceptive activity which was totally abolished by naloxone, non-selective opioid receptor antagonist (Matsumoto *et al.*, 2005a). Moreover, they indicated the contribution of descending noradrenergic and serotonergic systems in the analgesic activities of mitragynine (Matsumoto *et al.*, 2005b). This is like what is known with the actions of morphine (Matsumoto *et al.*, 2006). 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). The preliminary study of the effect of mitragynine on working memory was performed and it was found that mitragynine has impaired the cognitive function in rat (Apryani *et al.*, 2010). The mitragynine also showed antidepressant effect in animal swim test and hypothalamic-pituitary-adrenal test (Idayu *et al.*, 2011). Moreover, it was discovered that mitragynine suppresses PGE_2 production by inhibiting COX-2 expression in which maybe useful for inflammation treatment (Utar *et al.*, 2011). *M. speciosa* use is illegal in Thailand and neighborhoods such as, Malaysia, Myanmar, Australia (EMCDDA, 2012) since it possesses additive effect. In Thailand, *M. speciosa* is classified as Category V narcotic agent.

According to the abuse, policy to control its availability has been conducted (Saingam *et al.*, 2013).

1.4 Synthesis of mitragynine

The molecular formula of mitragynine is $C_{23}H_{30}O_4N_2$ and molecular mass is 398. Recently, there are two research groups studying in total synthesis of mitragynine. Takayama *et al.* (1995) firstly reported the chiral total synthesis of this compound by starting with an optically active pure *R*-(+)-alcohol. On the other hands, Ma *et al.* (2007) used a diester compound, a Michael adduct of *n*-butanol with alkylidene malonate, as an intermediate to synthesize mitragynine. The overall yield of mitragynine is not reported from both studies, nevertheless at present, neither synthesis is capable to be an alternative for extraction and purification of the natural product to obtain mitragynine. This is in part due to the difficulty in synthesizing the 4-methoxy indole derivatives.

1.5 Mitragynine biosynthesis

The biosynthesis of mitragynine and the other MIAs are though to involve over twenty enzymatic steps, which distribute among several subcellular compartments. Biosynthetically, mitragynine was supplied from strictosidine as suggested by feeding experiment of strictosidine in *M. speciosa* (Rueffer *et al.*, 1978) (Figure 1.3). Generally, the indole nucleus is obtained from the condensation of one molecule of tryptamine, decarboxylation of trytophan and one molecule of secologanin, iridoid moiety. Tryptamine is supplied from the shikimate pathway, where as secologanin is originated from terpenoids pathway. Therefore, the alkaloid group is named as "monoterpenoid-indole alkaloids" (MIAs). Up to date, the full-length of strictosidine synthase (*sss*), 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*), tryptophan decarboxylase (*tdc*) and anthranilate synthase (*as*) were cloned and studied by Wungsintaweekul's group. Moreover, comparing the DNA sequences of known plants from NCBI database, the partial sequences of 9 genes involving in MIAs biosynthesis were cloned from *M. speciosa*. This result guided us the existence of non-mevalonate pathway since *dxs*, *dxr*, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (*mct*), 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (*cmk*), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*mds*), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (*hds*), 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (*hdr*) were tracked in *M. speciosa*. For the other

biosynthetic genes, the partial fragments of geraniol 10-hydroxylase (*g10h*), 10-hydroxygeraniol oxidoreductase (*10hgo*), secologanin synthase (*sls*) and strictosidine β-D-glucosidase (*sgd*) were also cloned from *M. speciosa*. However, the distance between strictosidine and mitragynine is still unknown.

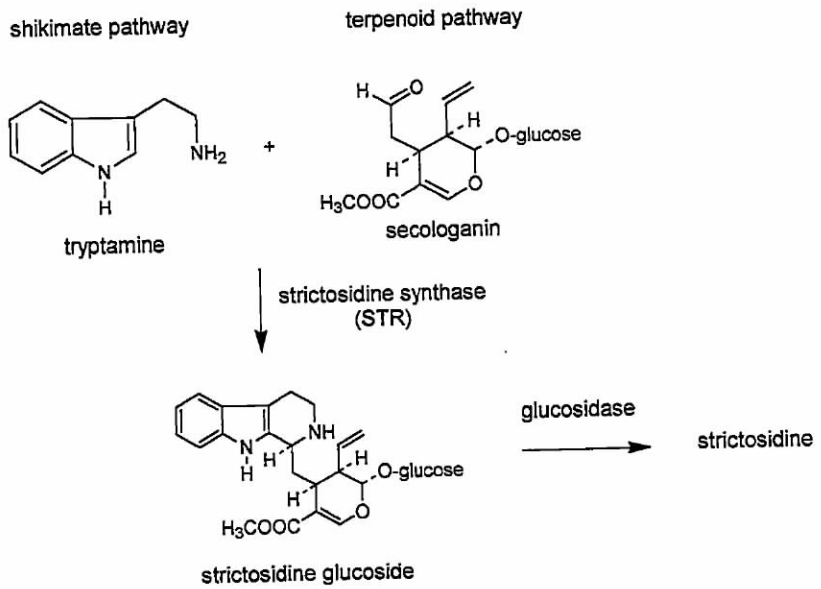


Figure 1.3 Formation of strictosidine, a common precursor of MIAs.

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 1.2 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 Braga, 2002
Uncaria rhynchopylla	hirsuteine, hirsutine,3α-dihydrocadambine, ursolic acid	callus culture :	Kohda et al., 1996
U. tomentosa	ursolic acid, oleanolic acid	suspension culture	Feria-Romero et al., 2005

1.7 *M. speciosa* tissue culture

Various attempts were evaluated to find a suitable system in *M. speciosa* producing alkaloid tissue culture. First, *M. speciosa* shoot culture was successfully established and it was found to have a capability for mitragynine production (Wungsintaweekul *et al.*, 2012). Nevertheless, there is a limitation of this technique for the development into large scale production. The drawback was also considering to difficulty for the genetic engineering since the shoots obtained from direct genetic transformation was found to be chimera (Charoonratana, unpublished). Second, it was found that both normal and transgenic *M. speciosa* hairy root cultures were found not to possess alkaloid production (Phongprueksapattana *et al.*, 2008; Charoonratana *et al.*, 2013a). Third, *M. speciosa* callus and suspension culture were reported to contain alkaloids such as mitragynine but the amount is not stable and lesser than that of in shoot culture (Wungsintaweekul, unpublish; Zuldin *et al.*, 2013). To boost the mitragynine or 7-OH mitragynine production in *M. speciosa* cell culture, stable line of callus culture need to be established.

Recently, metabolites in *M. speciosa* were clarified by NMR and HPLC analysis. The most remarkable outcome confirmed from both techniques was low tryptamine level in contrast to the excess amounts of secologanin found in all plant tissues. Additionally, high level of epigallocatechin suggested that the flux of primary metabolites goes through tyrosine and phenylalanine, which might compete with the biosynthesis of tryptophan (Charoonratana *et al.*, 2013b). To overcome the limitation of tryptophan or tryptamine availability in *M. speciosa*, genetic transformation of genes involved in shikimate pathway, such as *tdc*, may be possible to push more metabolite flux into mitragynine biosynthesis resulting in more mitragynine accumulation.

1.8 Tryptophan decarboxylase

Tryptophan decarboxylase (TDC) which catalyses the conversion of tryptophan into tryptamine, operates at the interface between primary and secondary metabolism. It therefore is regarded as a putative site for regulatory control of alkaloid biosynthesis. TDC has been significantly studied in a variety of plants. Tryptophan decarboxylase cDNA has been cloned from several plants such as *C. roseus* (De Luca *et al.*, 1989), *Camptotheca acuminata* (Lopez-Meyer & Nessler, 1997), *Ophiorrhiza pumila* (Yamazaki *et al.*, 2003), and *M. speciosa* (Charoonratana et al., 2013a). In *C. roseus* hairy root cultures, increased alkaloid production following jasmonate elicitation had been linked to enhanced *TDC* transcript levels (Goklany *et al.*, 2009). The transformation of different plant species with homologous or heterologous *TDC* has also shown the different responses which is species-dependent. Berlin *et al.* (1993, 1994) introduced heterologous *TDC* into transformed callus and root cultures of *Peganum harmala*. The accumulation of the harmane-type β-carboline alkaloids and serotonin, products derived from tryptamine. Expression of 35S-*TDC* in transgenic potato resulted in a drastic alteration in the balance of key substrate and product pools involved in the shikimate and phenylpropanoid pathways Yao *et al.* (1995). In *O. pumila*, the *TDC* mRNA expression was tissue-specificity related with the amount of camptothecin and may be regulated by a different mechanism from that affecting the expression of this gene in *C. roseus* (Yamazaki *et al.*, 2003).

2. Materials and methods

2.1 Equipments

Autoclave	Autoclave FD36R Zealway, USA
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)
Electroporator	Micropulser™ Electroporator, USA
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)
LC-MS	Dionex Ultimate 3000 equipped Bruker Amazon SL
Micropipettes	Socorex: 0.1-2.0 µl, 2-20 µl, 20-200 µl, 100-1000 µl (Switzerland)
Microscope	Nikon Eclipse TE 2000-S fluorescence microscope
pH meter	Benchtop, pH meter Model 710A (Germany)
Power supply	Model EPS301 (Amersham Biosciences, USA)
Real Time PCR	Applied Biosystems ABI PRISM® 7300 Real-time PCR system with
Sequence	Detection Software version 1.4, Foster City, USA
Refrigerator	Sanden Intercool (4 °C); Whirlpool (-20 °C); Deep-freezer (-80 °C) (Thailand), Forma Scientific (USA)
Rotary evaporator	Eyela N 1000, Tokyo, Japan
Shaking incubator	BIOER Technology Co., Ltd. (Tokyo, Japan)
Spectrophotometer	Labomed, Inc. (USA)

Transilluminator	Vilber Lourmat ECX, Marne-la-Vallee, France
Ultrasonicator	High intensity ultrasonic processor 1500-watt model with high volume flow cell (CT., USA)
Vortex	Vortex-Genie 2™, New York, USA
Water bath	Memmert WB-14, Schwabach, Germany

2.2 Materials

2.2.1 Plant materials

M. speciosa seeds were collected from the open field in Hat Yai district, Songkhla, Thailand. The seeds were surface sterilized by rinsing with 70% (v/v) ethanol for 5 min, rinsing with 20% (v/v) Clorox® for 5 min and finally rinsing with sterile distilled water. Sterilized seeds were germinated on Lloyd & McCown Woody Plant medium (WPM) and incubated at 25 °C under long-day conditions (16 h light/8 h dark). After 2 months, the plantlets were transferred into freshly WPM medium and subcultured every month.

2.2.2 Chemicals

Solvents used in this study were analytical grades, excepted for HPLC grade acetonitrile and water. The reagents used for molecular biology were biotechnological grade. All solvents were purchased from Lab-Scan Asia Co., Ltd., Bangkok, Thailand, but acetonitrile and water were purchased from B&J, Korea. Culture media are supplied from Himedia Laboratories, India. Chemicals for buffer preparation were from Biobasic Inc., Canada. All compounds were pure grade and for biotechnological purposes.

2.2.3 Standard

The authentic mitragynine was kindly provided by Dr. Supattra Limsuwanchote, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

2.2.4 Molecular biology kits and enzymes

PCR purification kit, Gel extraction kit, RNeasy plant Mini kit, and Plasmid isolation kit were purchased from Qiagen, Germany. Luna® one step RT-qPCR was from NEB, USA. GFX micro-palsmid prep kit was from Amersham Biosciences, USA. The restriction endonucleases

were purchased from TaKaRa, Japan; Qiagen, Germany; Toyobo, Japan and NEB (New England Biolabs), New England, USA.

2.2.5 Media and 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. All solutions were prepared as listed:

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.

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.

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.

SOC medium containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

Ty(Ca) medium containing 5 g tryptone, 3 g yeast extract and 1.3 g $CaCl_2.6H_2O$ in distilled water 1000 ml.

WPM medium containing 2.12 g WPM, 20 g sucrose and 2 g gellan gum in 1,000 ml distilled water. The pH was adjusted to 5.7. The solution was aliquot in the culture bottles and sterilized using autoclave.

2.2.6 Primers

The oligonucleotides used in this study were purchased from Operon, Germany.

Table 2.1 List of primers used in RT-qPCR.

Primer name	Primer efficiency [a]	Nucleotide sequence (5' – 3')	Fragment size
18S rRNAf	0.88	CAAAGCAAGCCTACGCTCTG	530 bp
18S rRNAr		CGCTCCACCAACTAAGAACG	
AS1f	0.97	CTCTCTCCAGTCTTCATCTCC	316 bp
AS1r		CACAACCATCTCCTTCAACTTCC	
AS2f	0.97	CCACAGTTTCTCCTCCGTCA	311 bp
AS2r		CTGTTCCACTCTTCGTCCTTG	
TDC147S	0.98	GCTTTTTCGTCAAGACTTGCAAATTGGCTG	361 bp
TDC508A		GTAACAAAGTAGCCACTTATGAGGGCTC	
SSS808S	0.92	GGAATTACTGTTACGCCTAGAGC	245 bp
SSS1053A		AGAAGAAGCCACTCCATTCAAAG	

[a] Primer efficiency was calculated from REST 2009 software V. 2.0.13 (Qiagen)

Table 2.2 List of primers used in genetic transformation.

Primer name	Nucleotide sequence (5' – 3')	T_m (°C)
TDCf	TCTCCGGTACCATGGGCAGCATTGATACGAGTG	71.1
TDCr	TAGCAGATCTTCAGTCTAAAACATTTTCTTTAAGCACAG	68.4

2.2.7 Plasmid vectors

The pRT101 vector is supplied in a circular form. This vector allows ampicillin selection. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids. This vector, carrying the 35S promoter and the polyadenylation signal of CaMV strain Cabb B-D (corresponding to bp 7016-7434 and 7436-7639 of CM 1841) were constructed in modified polylinkers of pUC18/19. The pRT101 used for 35s-*MsTDC* cassette construction was treated with restriction endonuclease before ligation. In this study, restriction endonuclease (*Kpn*I and *Xba*I) were used.

The pCAMBIA1300 vector is supplied in a circular form. This vector allows kanamycin selection in bacteria and hygromycin B selection in plants. An adequate restriction sites were designed for introducing DNA of interest within a T-border region. It contained pVS1 replicon for high stability in *Agrobacterium*. The pCAMBIA1300 used for the plant transformation was treated with restriction endonuclease before ligation. In this study, restriction endonuclease (*Hind*III, *Kpn*I, *Pst*I and *Sac*I) were used.

2.2.8 Bacterial strains

E. coli strains were used for gene storage. *Agrobacterium* spp. strains were used for plant transformation. The characteristics of these strains are shown below.

Table 2.3 Bacterial strains used in this study.

Strain	Characteristic	Source
TOP10	F⁻, *mcrA*, □(*mrr-hsd* RMS-*mcrBC*), Φ80*lacZ*, □*M15*, □*lacX74*, *recA1*, *araD139*, □(*ara-leu*)7697, *galU*, *galK*, *rps*L(Str^R), *endA1*, *nupG*	Invitrogen, USA
A. tumefaciens LBA4404	*Ach5, pTiAch5* Sm/Sp (R)	Invitrogen, USA

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 Full-length *MsTDC* amplification

For the construction of 35S-*MsTDC*-poly-A cassette, TDC primers were designed to amplify full-length *MsTDC* with *KpnI*/*XbaI* restriction sites. The diluted cDNA (2.5-500 ng) was used in a 50 µl PCR reaction [10x Taq buffer, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25 U/µl *Taq* DNA polymerase (Fermentas)]. The general PCR conditions were performed as follows: 3 min initial denaturation at 95 °C for one cycle, then 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 2 min, followed by a 10 min extension at 72°C and cooling to 4°C.

2.3.3 Purification of DNA fragment

DNA fragments obtained from the PCR reactions were purified using PCR purification kit (Qiagen) and using gel extraction kit (Qiagen) following the manufacturer's instruction. PCR

purification kit was used to purify the PCR product that will be used as template for nested PCR. Whereas, gel extraction kit was used to purify the PCR product that will be separated on agarose gel and was used for further gene cloning. The principle of both kits is selective binding properties of a uniquely-designed silica-gel membrane. All buffers are optimized for efficient recovery of DNA and removal of contaminants. The DNA adsorbs to the silica-membrane in the presence of high salt and pH less than 7.5 while contaminants pass through the column. Salts are quantitatively washed away by the ethanol-containing buffer. The pure DNA is eluted with basic buffer and low salt concentrations.

Purification of the DNA fragments using the PCR purification kit began with dilution of the PCR reaction with 3 volumes of buffer PB. The mixture was loaded into the mini column, allowed to stand at room temperature for 1 min and centrifuged at 13,000 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 13,000 rpm for 1 min, a mini column was transferred to a new microcentrifuge tube. The DNA fragment was then eluted with buffer EB (30 µl) (10 mM Tris-HCl, pH 8.5), left at room temperature for 1 min and centrifuged.

For purification of the DNA fragment purification using the gel extraction kit (Qiagen). The PCR reaction was loaded on agarose gel and separated under electric field. After staining and visualized under the transluminator, 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 buffer PE (0.75 ml) 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 min to dry the matrix. The column was then placed in a new microcentrifuge tube, buffer EB (30 µl) was added into this column and left to stand at room temperature for 1 min and centrifuged for 1 min to elute the DNA.

2.3.4 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.

2.4 Genetic transformation method

2.4.1 Plasmid and bacteria preparation

2.4.1.1 Plasmid vector construction

The 35S-*gfp*-Tnos gene cassette was constructed in pTH2-Δ*EcoR*I vector. The *gfp* cassette was cut and ligated into pCAMBIA1300 with *Kpn*I and *Pst*I restriction enzymes. The reaction mixture contained plasmid DNA (2 µl), 10x TangoTM buffer (1 µl), *Kpn*I (0.5 µl), *Pst*I (0.5 µl), 500 µg/µl boiled RNase (1 µl) and distilled water (5 µl) and incubated at 37°C for 1 h. To analyze the DNA fragments, the solution was then mixed with sample buffer, loaded on 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide solution. This *gfp* plasmid vector was used to optimize the plant transformation efficiency of *A. tumefaciens*.

gfp cassette was cut from pTH2 plasmid and inserted into pCAMBIA1300 by *Pst*I/*Kpn*I as described in 2.7.1. For the construction of 35S-*MsTDC*-poly-A cassette, the pRT101 vector and *MsTDC* were treated with restriction endonuclease before ligation. The reaction mixture contained plasmid DNA (2 µl), 10x Tango^{TM} buffer (1 µl), *Kpn*I (0.5 µl), *Xba*I (0.5 µl), 500 µg/µl boiled RNase (1 µl) and distilled water (5 µl) and incubated at 37 °C for 1 h. The ligation mixture composed of pRT101 (E/N) (1 µl), DNA fragment (E/N) (4 µl), 10x ligase buffer (1 µl), T4 DNA ligase (0.5 µl) and the volume was adjusted to 10 µl by distilled water. The ligation mixture was incubated at 4°C for overnight. The ligation mixture was transformed into the XL-1 blue host strain by the method described in 2.4.4.4. For pCAMBIA1300-*MsTDC* construction, the 35S-*MsTDC*-poly-A cassette from pRT101 was inserted into *Hind*III site of the *gfp* plasmid vector. The pCAMBIA1300 vector and *MsTDC* cassette were treated with restriction endonuclease before ligation. The reaction mixture contained plasmid DNA (2 µl), 10x buffer R (1 µl), *Hind*III (0.5 µl), 500 µg/µl boiled RNase (1 µl) and distilled water (5.5 µl) and incubated at 37°C for 1 h. Only the pCAMBIA1300 was dephosphorylated by adding 1 M Tris-HCl pH 8.0 (10 µl), 1 U/µl alkaline phosphatase (3 µl), distilled water (77 µl) and incubated at 37°C for 30 min. The phenol/$CHCl_3$ extraction was performed twice by adding phenol (100 µl) (saturated with 0.1 M Tris-HCl pH 8.0) and $CHCl_3$ (100 µl). The mixture was vortexed for 30 s and

centrifuged at 13,000 rpm for 2 min. The upper phase was transferred to a new tube. The DNA precipitation was occurred by addition of 3 M sodium acetate (20 µl) and absolute ethanol (chilled, 550 µl). The solution was mixed and centrifuged at 13,000 rpm for 5 min to harvest the DNA. The pellet was washed with 70% (v/v) ethanol (400 µl), mixed and centrifuged at 13,000 rpm for 5 min. The pellet was dried completely at 60°C and resuspended in 40 µl TE buffer.

2.4.1.2 Bacterial strains and preparation of the competent cells

Agrobacterium tumefaciens LBA4404 was maintained on Ty(Ca) liquid medium supplemented with 50 µg/ml rifampicin. *A. tumefaciens* was activated by culturing on Ty(Ca) agar plate supplemented with 50 µg/ml rifampicin at 29°C for 72 h. A single bacterial colony was picked up and used for inoculated into 2 ml liquid Ty(Ca) medium supplemented with 50 µg/ml rifampicin. The culture was then placed on rotary shaker (150 rpm) at 29°C for 6 h. After that, the preculture (100 µl) was inoculated in Ty(Ca) medium (100 ml) supplemented with 50 µg/ml rifampicin and 0.1% (w/v) glucose and shaken overnight at 29°C to an OD660 of 1.0-1.5. The culture was chilled on ice for 15 min and harvested by centrifugation in a cold rotor (4 °C) at 4000g for 20 min. The pellet was resuspended in 1 mM HEPES pH 7 (10 ml), centrifuged and repeated the washing step for three times. The pellet was washed in 10% (v/v) glycerol (chilled), resuspended in a final volume of 10% (v/v) glycerol (500-750 µl). The suspension was divided in 40-µl aliquots, frozen in liquid nitrogen and stored at -80 °C..

2.4.1.3 Electroporation

The *A. tumefaciens* cells were gently thawed on ice. The electroporation cuvette with 0.2-cm electrode gap was chilled on ice. Ten ng of plasmid DNA was added to the competent cells suspension and mixed by tapping. The mixture was transferred to a prechilled cuvette and the electric pulse was applied at 2.5 kV, 25 µF and 200 Ohm. This should result in a pulse of 12.5 kV/cm with a time constant of approximately 4.7 ms. One ml of SOC medium was immediately added, the mixture was transferred to a tube and incubated at 29°C for 1-1.5 h. The suspension (100 µl) was put onto the selective medium (50 µg/ml kanamycin for cells habouring pCAMBIA1300). The culture was incubated for 72 h at 29°C.

2.4.1.4 Isolation and identification of the recombinant DNA

A single bacterial colony was obtained and used to inoculate into 2 ml liquid Ty(Ca) medium supplemented with 50 µg/ml rifampicin and kanamycin. The culture was then placed on rotary shaker (150 rpm) at 29°C for 18 h. The overnight liquid culture was centrifuged, the pellet was resuspended in solution 1 (200 µl) (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH8, 4 mg/ml lysozyme) was added and incubated for 10 min at room temperature. The solution 2 (400 µl) (1% (w/v) SDS, 0.2 M NaOH) was added and incubated

for 10 min at room temperature. Alkaline phenol (60 µl) was added and mixed by inversion, the solution 3 (300 µl) (3 M Na-Acetate pH 4.8) was immediately added and incubated for 20 min on ice. The sample was spin at 13,000 rpm for 5 min and the supernatant was poured in eppendorf tube, filled with ethanol. The sample was spin at 13,000 rpm for 5 min. The DNA pellet was washed with 70% (v/v) ethanol, dried and resuspended in T10E1 (30 µl). The DNA (10 µl) was used for a restriction enzyme digestion with *BamHI*.

2.4.2 Induction of friable callus

2.4.2.1 Medium preparation

The hormone-free McCown Woody Plant (WPM) medium (Phytotechnology Laboratories) and Murashige and Skoog medium (MS) was prepared by dissolve 2.5 g of WPM powder, 2% (w/v) sucrose and adjusted the volume with distilled water. The pH was adjusted with 1 N sodium hydroxide to 5.7. For solid medium preparation, 0.2% (w/v) gellan gum was added and the solution was heated gently with continuous stirring until the clear solution was obtained. The culture medium was sterilized by autoclaving at 121^{o}C, 15 lb/inch^{2}, for 15 min.

2.4.2.2 *M. speciosa* callus induction

Mccown Woody Plant medium (WPM) and Murashige and Skoog medium (MS) pH 5.8 was supplemented with a range of concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), or 1-naphthaleneacetic acid (NAA), or Kinetin. Cultures were grown under dark condition at 25±2 °C. The different explants, leaf and stem, were cultured on those various medium and were tested to determine which explants produced an optimal callus induction. After 3 weeks, every sample were subcultured and grown further for 3 more generations. Each experiment contained 20 replicates.

2.4.3 *M. speciosa* genetic transformation and cultivation

The explants, callus, was immerged in liquid medium with 100 mM acetosyringone in a steriled glass tube. The tube was sonicated for 10 min with an ultrasonicator. After sonication explants were added into sterilized flasks containing *A. tumefaciens* suspension and flasks were shaken gently for 30 min at 25 °C. Explants were blot-dried with sterile paper towels and transferred onto Petri dishes containing appropriate medium with 100 mM acetosyringone. The co-cultivation period was 3 days in the dark at 29 °C. After co-cultivation, the callus was transferred into appropriate callus induction medium supplemented with 250 µg/ml cefotaxime and 100 µg/ml vancomycin for *A. tumefaciens* elimination for 1-2 weeks. The callus then transferred to appropriate medium for callus induction. For transgenic callus, the positive callus was selected using hygromycin B supplemented medium.

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

The RT-qPCR was used to determine the transcription of *M. speciosa* anthranilate synthase (*MsAS*; GenBank ID: JQ775867), *M. speciosa* tryptophan decarboxylase (*MsTDC*; GenBank ID: JN643922.1), and *M. speciosa* strictosidine synthase (*MsSSS;* GenBank ID: EU288197.1) genes in callus. The RT-qPCR was performed using specific primers (Table 2.2) on an ABI PRISM® 7300 Sequence Detector (Applied Biosystems) with Luna® one step RT-qPCR to monitor double stranded DNA synthesis. The PCR reaction was carried out in 96-well plate. The PCR reaction contained Luna® one step RT-qPCR Reaction Mix (2x), Luna WarmStart® RT Enzyme Mix (20x), 10 µM forward primer, 10 µM reverse primer, 15 ng/µl cDNA and Nuclease-free water in total volume of 20 µl. Amplification involved 40 cycles of PCR reaction and the conditions were: 1 cycle (95°C, 10 min); 40 cycles (95°C, 30 s; 60°C, 1 min) and finally a dissociation stage at 95°C 15 s, 60°C 1 min, 95°C 15 s. Samples were run in triplicate and a negative control of the Master Mix with primers was performed in all runs.

2.6 Secondary metabolites determination of *M. speciosa* callus culture

Liquid chromatography mass spectrometry (LC-MS) was used to determine secondary metabolites in *M. speciosa* callus culture. Crude extract was prepared by sonication with methanol for 30 min 2 times. Chromatographic separation is carried out on a reversed-phase C18 column with binary gradient mobile phase profile [0.15 ml/min, acetonitrile: 0.1% formic acid in water (10:90 to 90:10, v:v within 15 min), injection volume 5 µl]. The identification of the metabolites is based on the retention time and the mass spectra.

2.7 Statistical analysis

Values are expressed as mean $\pm$ S.D. Data were analyzed by student *t*-test. The level of statistical significance was taken at $P < 0.05$.

3. Results

3.1 Cloning of the full-length cDNA for *MsTDC* gene

The full-length gene was amplified using MSFTC and MSRTC for forward primer and reverse primer, respectively. The full-length PCR product was purified ligated to pDrive and transformed into *E. coli* TOP10 competent cells. The recombinant plasmid was isolated and the double strand DNA was sequenced. The full-length cDNA sequence of *MsTDC* was deduced and subsequently confirmed by sequencing, which is 1,615 bp and has an open reading frame of 1,518 bp (Figure 3.1) starting with an initiation codon ATG and ending with a termination codon TGA.

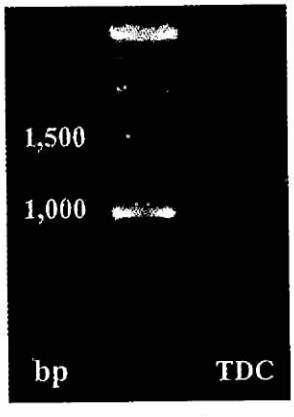


Figure 3.1 1.2% (w/v) Agarose gel electrophoresis of the PCR products of full-length *MsTDC*.

3.2 Genetic transformation

3.2.1 Plasmid vector construction

The 35S-*gfp*-T_{nos} gene cassette was ligated into the pCAMBIA1300 vector with *Kpn*I and *Pst*I restriction enzymes to use as control (Fig. 3.2). The pCAMBIA1300-*MsTDC* was constructed to study the effect of gene to metabolites of *M. speciosa* callus.

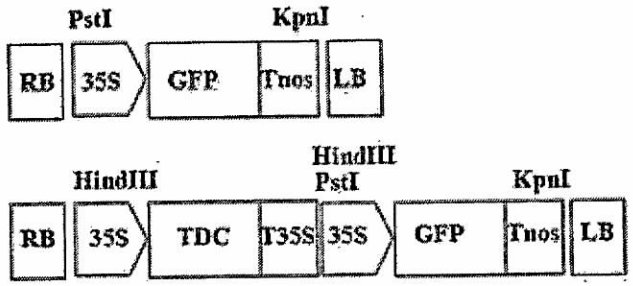


Figure 3.2 Constructions of pCAMBIA1300-*gfp*, pCAMBIA1300-*gfp-MsTDC* plasmid

3.2.2 *Agrobacterium* transformation and identification

All pCAMBIA1300 plasmid was transformed into competent *Agrobacterium* by electroporation. After selection, the recombinant plasmids were isolated and analyzed for restriction site analysis. The DNA fragment was analyzed by 1.2% (w/v) agarose gel electrophoresis. As shown, the 35S promoter of *gfp* insert product (400 bp) was obtained (Fig. 3.3). For *MsTDC* construction, *BamH*I was also used to verify and confirm correctly orientation. As described in Fig. 3.3, the bands around 400 bp, 600 bp and pCAMBIA1300 vector must appeared in the stained gel.

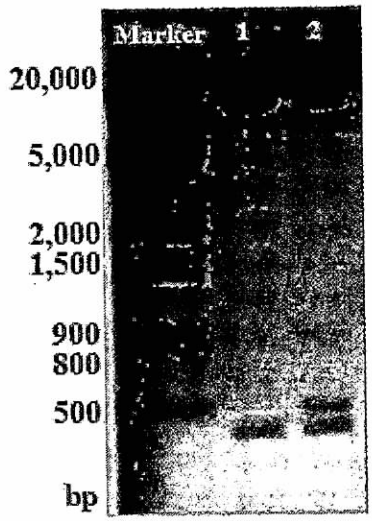


Figure 3.3 1.2% (w/v) agarose gel electrophoresis of pCAMBIA1300-*gfp* and pCAMBIA1300-*gfp -MsTDC* staining with ethidium bromide, 1: pCAMBIA1300-*gfp* cut with *BamH*I, 2: pCAMBIA1300-*gfp-MsTDC* cut with *BamH*I

3.2.3 *M. speciosa* callus induction

The medium manipulation for callus induction was observed. The best callus formation was obtained from stem in WPM medium supplemented with 1 mg/L 2,4-D. The percentage of explants that produced callus was 95% and it was shown in figure 3.4. The percentage of stem that produced callus was also 95% in MS medium but the color was a bit brown (figure 3.5). The detail of percentage of explants that produced callus was shown in table 3.1.

Figure 3.4 The third-generation friable callus from *M. speciosa* stem in WPM medium

Figure 3.5 The third-generation friable callus from *M. speciosa* stem in MS medium

Table 3.1 The percentage of stem that produced callus

Medium	Hormone	Concentration (mg/L)	Callus / Explant	Percentage
WPM	Control	-	0/20	0
	2,4-D	1	19/20	95
		3	18/20	90
		5	19/20	95
	NAA	1	10/20	50
		3	11/20	55
		5	13/20	65
	kinetin	5	0/20	0
		50	0/20	0
		100	0/20	0
MS	Control	-	0/20	0
	2,4-D	1	19/20	95
		3	15/20	75
		5	16/20	80
	NAA	1	8/20	40
		3	12/20	60
		5	10/20	50
	kinetin	5	0/20	0
		50	0/20	0
		100	0/20	0

3.2.4 Callus transformation by *Agrobacterium tumefaciens*

The callus of *M. speciosa* were infected with *A. tumefaciens* (LBA4404) harboring pCAMBIA1300-*gfp* with and without *tdc* gene by the method described above. Initiation of the transgenic callus was occurred after 1 week from the callus and the size was depended on each line (Fig. 3.6). After 1 month, the *gfp* positive callus which had appropriate size (around 1 cm) was transferred into WPM medium supplemented with 1 mg/mL 2,4-D. The callus was grown further for 2-3 months (subculture every month) to obtain sufficient materials for futher experiment (Fig. 3.7).

Figure 3.6 GFP positive cell was detected under Nikon Eclipse TE 2000-S fluorescence microscope

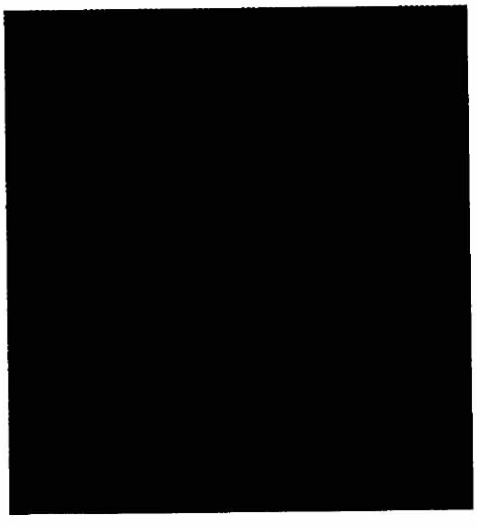


Figure 3.7 GFP positive 3-month-old *M. speciosa* callus under Nikon Eclipse TE 2000-S

3.2.5 Transgenic *M. speciosa* suspension culture

The transformation efficiency of the transgenic callus of *M. speciosa* harboring pCAMBIA1300-*gfp-tdc* was 2%. The transgenic callus was further transferred to liquid WPM medium supplemented with 1 mg/mL 2,4-D (Figure 3.8). Growth cycles of the suspension was within 20 days. It composed of lag phase (5 days), exponential phase (15 days) and gradually enter to the stationary phase (Fig. 3.9).

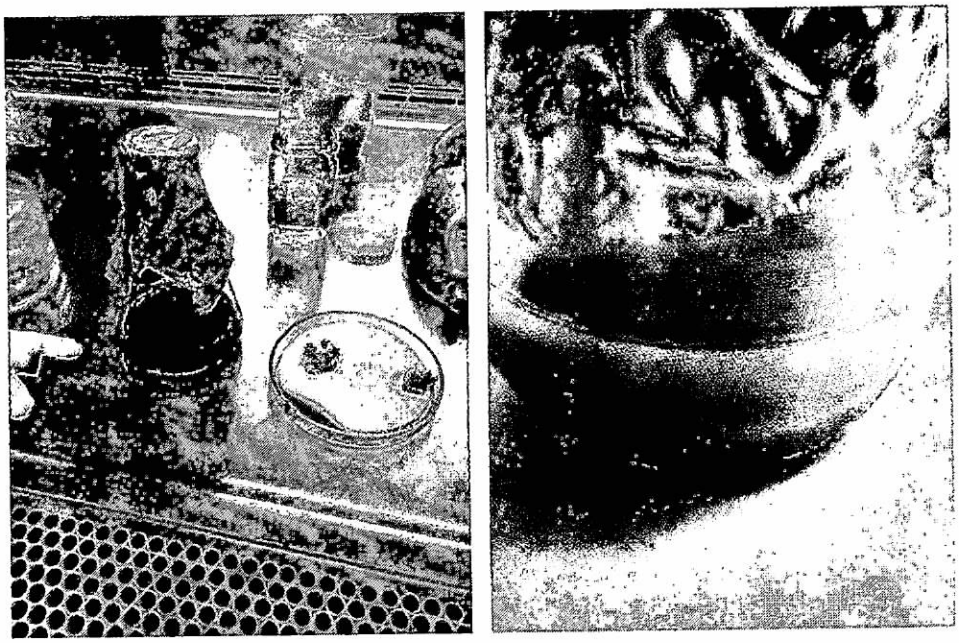


Figure 3.8 *M. speciosa* suspension culture.

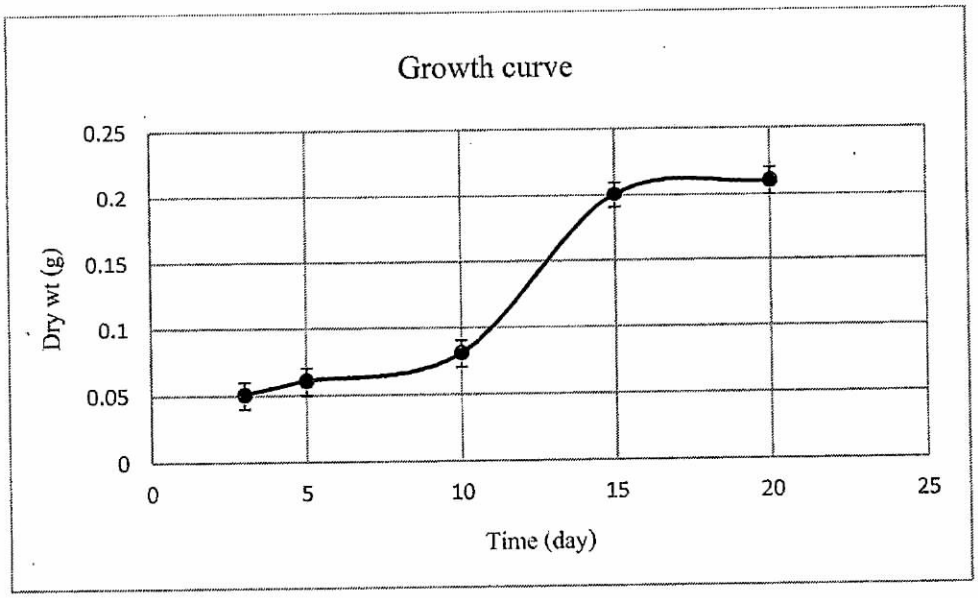


Figure 3.9 Growth curves of suspension culture in media. Error bars represent SEM. (n=4)

3.3 mRNA expression in *M. speciosa* callus

To confirm the expression of *MsTDC* in mRNA level, total RNA of the callus was extracted and subjected to RT-qPCR. The relative expression of *TDC* in pCAMBIA1300-*gfp*-*MsTDC* callus lines were compared to control (*M. speciosa* leaf). In Fig. 3.10, it was shown that *MsTDC* of transgenic callus lines were expressed as same as in wild type callus root line 2,4-D. The control showed the highest *MsTDC* mRNA expression. For *MsAS* and *MsSTR* expression levels were almost the same in all samples.

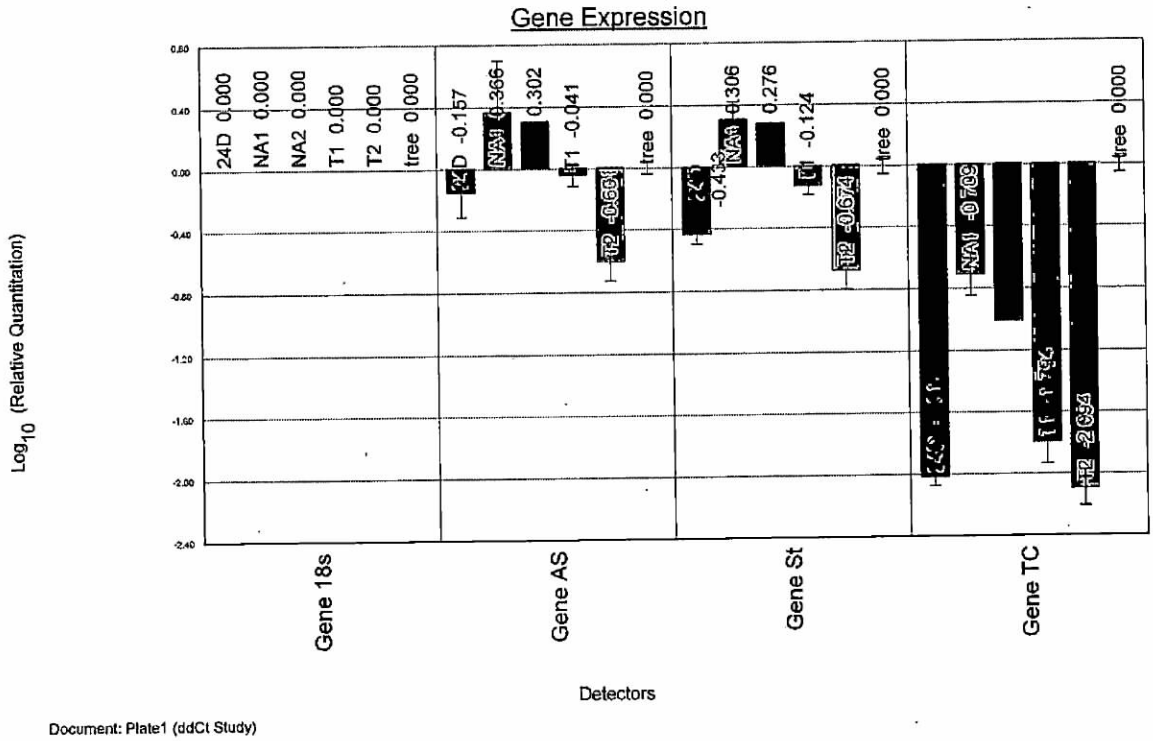


Figure 3.10 Relative expression of *MsTDC* in transgenic MIAs genes callus lines compared to control. 2,4-D = wild type callus in WPM supplemented with 2,4-D; NA = wild type callus in WPM supplemented with NAA; T = transgenic *tdc* callus; tree = *M. speciosa* leaf.

3.4 Metabolites quantification in *M. speciosa* callus

Liquid chromatography mass spectrometry (LC-MS) was used to determine secondary metabolites in *M. speciosa* callus culture. Crude extract was prepared by sonication with methanol for 30 min 2 times. Chromatographic separation is carried out on a reversed-phase C18 column with binary gradient mobile phase profile. The identification of the metabolites is based on the retention time and the mass spectra. There are no mitragynine in all samples but the extracted ion chromatogram showed mH+ at 415.3 m/z indicated the possibility of 7-OH mitragynine accumulation in callus but confirmation with reference standard is needed.

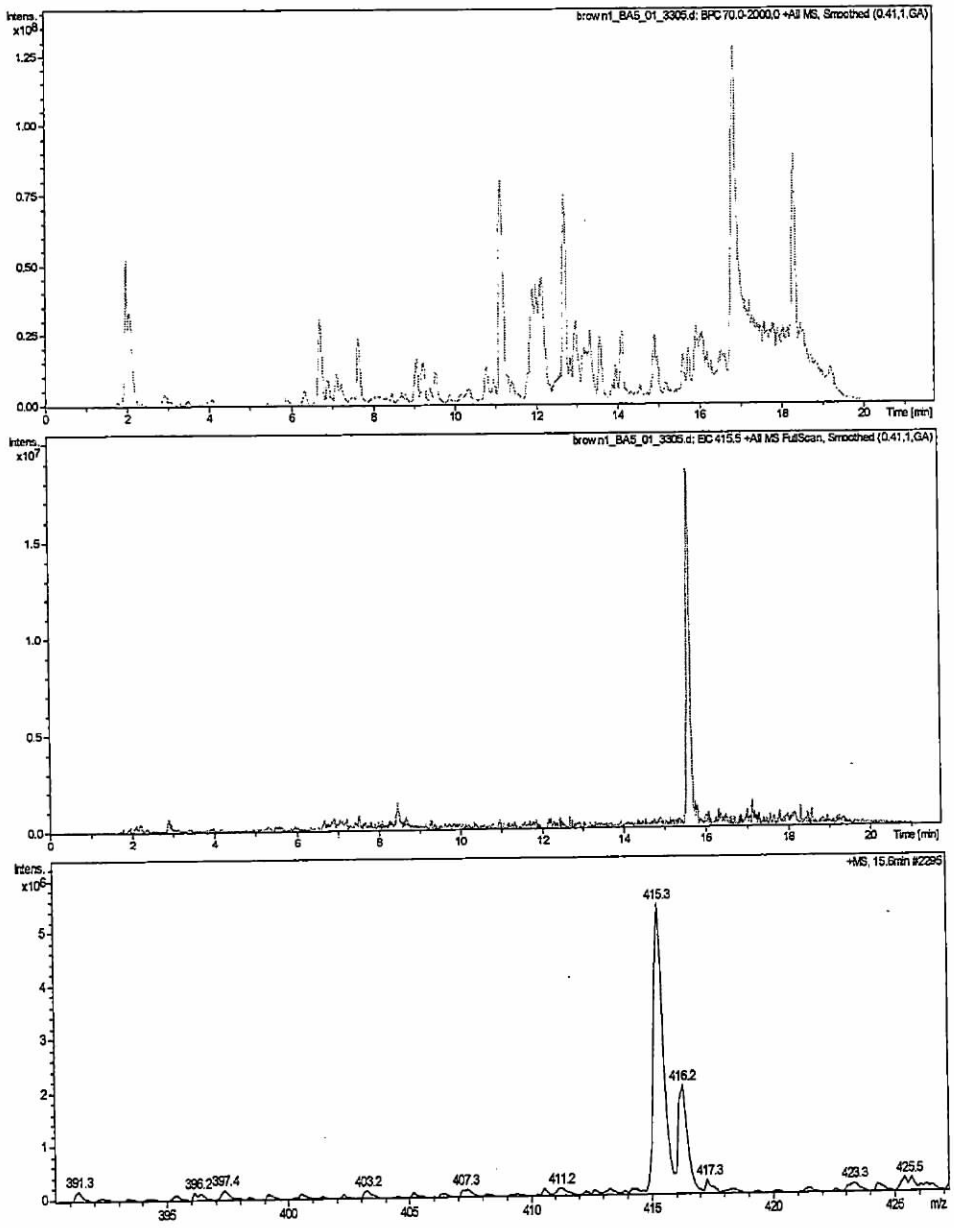


Figure 3.11 Chromatogram of *M. speciosa* callus culture.

4. Discussion

Mitragyna speciosa is a plant species known for its production of mitragynine and 7-hydroxymitragynine which own analgesic activity. Since these alkaloids occur only in low amounts in *M. speciosa* plant, this research has focused on metabolic engineering to raise production. For this, a thorough knowledge is necessary of the biosynthetic pathway of mitragynine and its regulation. To increase the production of a desired product one can think of increasing enzyme activities of rate-limiting step in the biosynthesis by introducing or over-expressing the gene involved. In this study, metabolite profiling is firstly used to identify the rate-limiting step, followed by the cloning of the gene coding for the enzyme. The enzyme is characterized and verified for the activity. Moreover, under the control of a strong promoter one can introduce and express the gene in the plant to overcome the bottleneck of the biosynthesis. Another possibility to raise production would be to inhibit unwanted side-branches in the pathway, or to recover regulation mechanisms which control the complete pathway.

Recently, metabolites in *M. speciosa* were clarified by NMR and HPLC analysis. The most remarkable outcome confirmed from both techniques was low tryptamine level in contrast to the excess amounts of secologanin found in all plant tissues. Additionally, high level of epigallocatechin suggested that the flux of primary metabolites goes through tyrosine and phenylalanine, which might compete with the biosynthesis of tryptophan (Charoonratana *et al.*, 2013b). To overcome the limitation of tryptophan or tryptamine availability in *M. speciosa*, genetic transformation of genes involved in shikimate pathway, such as *tdc*, may be possible to push more metabolite flux into mitragynine biosynthesis resulting in more mitragynine accumulation.

The role of the shikimate pathway in mitragynine biosynthesis is summarized in Fig. 4.1. Since the metabolome represents the endpoint of gene expression, transcriptome and proteome should also be studied in detail. From the metabolite profiles, the availability of tryptamine is an important target for further study. Therefore, the gene in shikimate pathway such as *MsTDC* was cloned since its enzyme has an important role in fluxing the tryptophan to tryptamine, the intermediate in TIAs biosynthesis. The gene information will be continuously used for further studies such as mRNA expression of *M. speciosa* cultures, genetic transformation etc.

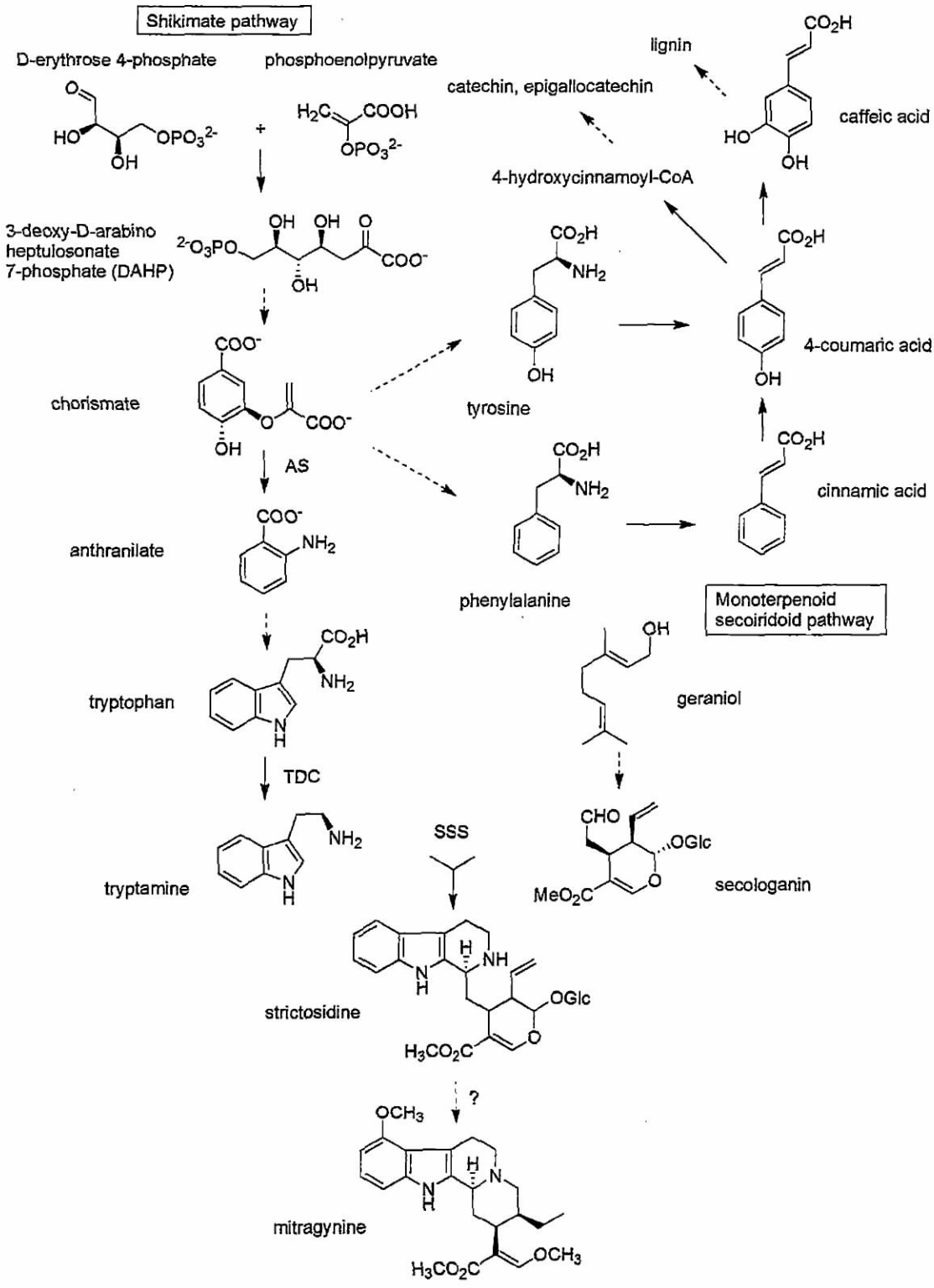


Fig. 4.1 Mitragynine and related metabolites biosynthesis.

Various requirements are needed for the introduction of genes into the *M. speciosa* to develop the transgenic *in vitro* plant. They consist of efficient DNA delivery process, efficient tissue culture process for shoot regeneration, selection strategy for transgenic tissue, strategy for the expression of transgenes (Birch, 1997). *Agrobacteriums* are the preferred method for transformation of a wide range of plant species (Stachel and Zambryski, 1989). Commonly, the genes to be transferred are cloned between the left and right T-DNA borders of so-called binary T-DNA vectors that can replicate both in plants and microbes (Clough and Bent, 1998). Avoiding the effect of fusion protein which may alter the native enzyme expression (Schornack *et al.*, 2009; Carnarius *et al.*, 2012), in this study, pCAMBIA1300 vector was used in plant transformation. The desirable genes, such as *gfp* or *MsTDC* were then constructed into a plant expression cassette individually. Since the *gfp* is used as a reporter gene, the transgenic tissues were capable to detect by green fluorescence protein (GFP) under microscope. All transgenes were expressed by driving of a strong plant 35S promoter (Fraley *et al.*, 1994). Due to a capability of large scale production, friable callus was chosen to be the model for metabolic engineering.

Transgenic *M. speciosa* friable callus was obtained by supplemented WPM with 2,4-D. In this study, 2-month old plantlet of *M. speciosa* stem was used successfully for callus induction. Once callus was occurred, genetic transformation using *A. tumefaciens* was performed to obtain transgenic callus. Fortunately, as *gfp* was also constructed in between T_i region of pCAMBIA1300. The feasible identification method of transgenic callus was *in vivo* fluorescence GFP signal under microscope and the transformation rate was about 2%. Instead of higher *MsTDC* mRNA expression, anyway, the result from RT-qPCR showed that low *MsTDC* mRNA expression was observed in transgenic callus lines. This may be due to the different in the presence of *MsTDC* mRNA expression in normal callus lines and transgenic callus lines.

Transgenic *MsTDC* callus in WPM supplemented with 1 mg/L 2,4-D was evaluated for secondary metabolites production by LC-MS technique. It was found that transgenic *M. speciosa* callus was unable to produce mitragynine after detection. Therefore, up-regulation of *MsTDC* alone is evidently not sufficient to produce mitragynine in this plant tissue culture. This implies that steps in MIAs biosynthesis other than conversion of tryptophan into tryptamine by MsTDC must be yield-limiting in transgenic *M. speciosa* callus. From biosynthetic point of view,

M. speciosa callus could not produce mitragynine. It may cause of lacking precursors and enzymes that involved in the terpenoid indole alkaloid. Since primary metabolites need special cell compartments for storage and degradation (Luckner, 1984). In addition, the late step genes may be not expressed in callus. Experiments for detection of crucial genes, enzymes and determination of substrate production may need to evaluate the potential of mitragynine production in *M. speciosa* callus culture.

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6. **Output** จากโครงการวิจัยที่ได้รับทุนจาก สกว.

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

   - กำลังเตรียมผลงานเพื่อยื่นตีพิมพ์ จำนวน 1 เรื่อง คือ

   Genetic transformation of *M. speciosa* callus culture and the metabolite analysis.

2. การนำผลการวิจัยไปใช้ประโยชน์ -
3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

   - การนำเสนอในที่ประชุมวิชาการนานาชาติ จำนวน 1 ครั้ง

   Charoonratana, T. and Wungsintaweekul, J. 2017. Metabolite analysis of hygromycin-resisted *Mitragyna speciosa* callus culture. The 16th The Thailand Research Fund conference, January 11-13, 2017, The Regent Cha-am, Thailand.