Triterpenes Production of *Lagerstroemia speciosa* Tissue Culture and Transformation of Oxidosqualene Cyclase Gene in *Nicotiana tabacum*

Prapaporn Wititpanyawong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Herb Sciences (International Program)

Prince of Songkla University

2010

Copyright of Prince of Songkla University

Thesis Title	Triterpenes production of *Lagerstroemia speciosa* tissue culture and transformation of oxidosqualene cyclase gene in *Nicotiana tabacum*
Author	Miss Prapaporn Wititpanyawong
Major Program	Herb Sciences (International Program)

Major Advisor:

……

(Pimpimon Tansakul, Ph.D.)

Co-advisor:

…….....

(Orawan Chatchawankanphanich, Ph.D.)

Examining Committee:

…..……...…Chairperson

(Associate Professor Sompong Te-Chato, Ph.D.)

……

(Assistant Professor Worapan Sitthithaworn, Ph.D.)

…….

(Pimpimon Tansakul, Ph.D.)

…….

(Orawan Chatchawankanphanich, Ph.D.)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Herb Sciences (International Program)

……..……..

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์ การสร้างสารประกอบไตรเทอร์ปีนโดยเนื้อเยื่อเพาะเลี้ยงของอินทนิลน้ำ และการศึกษาการถ่ายยีนออกซิโดสควาลีนไซเคลสในยาสูบ
ผู้เขียน นางสาวประภาพร วิทิตปัญญาวงศ์
สาขาวิชา วิทยาศาสตร์สมุนไพร (หลักสูตร นานาชาติ)
ปีการศึกษา 2553

บทคัดย่อ

อินทนิลน้ำ เป็นไม้ยืนต้น จัดอยู่ในวงศ์ Lythraceae พบทั่วไปในประเทศเขตร้อน เช่น อินเดีย จีน ไทย ฟิลิปปินส์ มาเลเซีย และ ออสเตรเลีย เป็นพืชที่มีรายงานการศึกษาเกี่ยวกับฤทธิ์ทางชีวภาพอย่างกว้างขวาง โดยเฉพาะศักยภาพในการรักษาและบรรเทาอาการของโรคเบาหวาน โดยการนำใบมาใช้ในรูปของชาชงหรือรูปสารสกัดจากใบ เนื่องจากใบมีสารสำคัญคือกรดโคโรโซลิก ซึ่งเป็นสารที่มีฤทธิ์ลดน้ำตาลในเลือด เนื่องจากออกฤทธิ์คล้ายกับอินซูลิน และยับยั้งการเปลี่ยนแปลงของอะดีโพไซท์ ที่ถูกเหนี่ยวนำโดยอินซูลิน นอกจากนี้กรดโคโรโซลิก ยังมีฤทธิ์ต้านความดันโลหิตสูง ต้านการออกซิเดชั่นและยังมีฤทธิ์ต้านการอักเสบอีกด้วย กรดโคโรโซลิกเป็นสารประกอบไตรเทอร์ปีน ซึ่งคาดว่ามีชีวสังเคราะห์มาจาก 2,3-ออกซิโดสควาลีน ผ่านทาง อัลฟา-อะมัยริน และกรดเออร์โซลิก โดยปฏิกิริยาปิดวงแหวนด้วยเอนไซม์ ออกซิโดสควาลีนไซเคลส ซึ่งถูกควบคุมโดยยีน *osc* ซึ่งยีนในกลุ่มนี้มีเพียงยีนที่เรียกว่า ไตรเทอร์ปีนซินเทสแบบหลายหน้าที่ [Multifunctional triterpene synthase (MTS)] เท่านั้นที่สามารถสร้างผลิตภัณฑ์อัลฟา-อะมัยริน ซึ่งเป็นสารตั้งต้นของการสังเคราะห์กรดโคโรโซลิก ยีนกลุ่มนี้สร้างโปรตีน MTS ที่ไม่เพียงแต่สร้างสารอัลฟา-อะมัยรินเท่านั้น แต่ยังสร้างสารประกอบเบต้า-อะมัยรินร่วมด้วย และยังเชื่อว่ายีนนี้เป็นยีนที่ควบคุมการสังเคราะห์กรดโคโรโซลิกเช่นกัน และหนึ่งในยีนที่จัดอยู่ในกลุ่ม MTS คือ ยีน *oea* ซึ่งโคลนได้จากต้นมะกอกฝรั่ง (*Olea europaea*) เป็นอีกยีนที่สามารถสร้างผลิตภัณฑ์ได้ทั้งอัลฟา-อะมัยรินและเบต้า-อะมัยริน

การทดลองนี้ ในส่วนที่ 1 เป็นการชักนำเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอยจากใบของต้นอินทนิลน้ำ และทำการตรวจหาสารไตรเทอร์ปีนจากเนื้อเยื่อเพาะเลี้ยงดังกล่าว โดยเฉพาะอย่างยิ่งกรดโคโรโซลิกซึ่งเป็นสารที่พบมากในใบของต้นอินทนิลน้ำ ส่วนที่ 2 เป็นการถ่ายยีน *oea* เข้าสู่ต้นยาสูบ ซึ่งเป็นพืชต้นแบบที่นิยมใช้ในการถ่ายยีนและสามารถถ่ายยีนเข้าสู่เซลล์พืชได้ง่าย โดยใช้แบคทีเรียชนิดอะโกรแบคทีเรียม ซึ่งความสำเร็จในการถ่ายยีนจะนำไปสู่ความเข้าใจเกี่ยวกับชีวสังเคราะห์ของสารประกอบไตรเทอร์ปีนรวมทั้งกรดโคโรโซลิกในเซลล์พืชด้วย

จากการทดลองชักนำให้เกิดเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอย พบว่า ทั้งเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอย สามารถเจริญเติบโตได้ดีที่สุดในอาหารสูตร MS ที่เติม 2,4-ไดคลอโรฟีนอกซี อะซีติก เอซิด ปริมาณ 2 มิลลิกรัมต่อลิตร และ ไคเนติน 1 มิลลิกรัมต่อลิตร เมื่อนำผงแห้งของเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอยมาสกัดและตรวจหาการสร้างสารประกอบไตรเทอร์ปีน โดยเทคนิคโครมาโตกราฟีแบบแผ่นบางและโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่า ทั้งเนื้อเยื่อเพาะเลี้ยงและเซลล์แขวนลอยมีการสร้างกรดโคโรโซลิก รวมทั้งสารประกอบไตรเทอร์ปีนชนิดอื่นๆ อีกด้วย ในการถ่ายยีน *oea* เข้าสู่ใบยาสูบโดยใช้เวกเตอร์ pCAMBIA 2311 ที่มียีน *npt*II เป็นยีนคัดเลือก ด้วยเชื้อ *Agrobacterium tumefaciens* สายพันธุ์ C58 พบว่า มีต้นยาสูบที่ได้จากการถ่ายยีนจำนวน 61 ต้น และจากการตรวจสอบการมีอยู่ของยีน *oea* บนโครโมโซมของต้นยาสูบที่ได้รับการถ่ายยีนด้วยเทคนิคการเพิ่มปริมาณดีเอ็นเออย่างรวดเร็วและ Southern blot hybridization พบว่า มีต้นยาสูบที่ตรวจพบยีน *oea* ทั้งหมด 36 ต้น นอกจากนี้ยังพบว่าต้นยาสูบถ่ายยีนมีจำนวนชุดและตำแหน่งของยีนที่แตกต่างกันในแต่ละต้น โดยสามารถจัดกลุ่มต้นยาสูบที่มีรูปแบบชุดของยีนที่เหมือนกันให้อยู่ใน line เดียวกันได้เป็น 30 lines.

Thesis Title Triterpenes production of *Lagerstroemia speciosa* tissue culture and transformation of oxidosqualene cyclase gene in *Nicotiana tabacum*

Author Miss Prapaporn Wititpanyawong

Major Program Herb Sciences (International Program)

Academic Year 2010

ABSTRACT

Lagerstroemia speciosa is a deciduous tree found in tropical countries. It has been extensively studied for its different biological activities, particularly for its potency in curing diabetes. The tea from its leaves is used as a beverage, as well as, for alleviating symptoms of diabetes mellitus. The leaves contain corosolic acid, which has very good hypoglycemic activity, similar to insulin activity, and inhibits insulin-induced adipocyte differentiation. Moreover, corosolic acid has an antihypertensive effect together with a lipid-lowering effect, antioxidative, and anti-inflammatory activities. The corosolic acid is an ursane-type triterpene, which is proposed to be biosynthesized from 2,3-oxidosqualene via α-amyrin and ursolic acid to corosolic acid. Biosynthesis of α-amyrin from 2,3-oxidosqualene is catalysed by oxidosqualene synthase (OSC) under the control of *osc* gene. In fact, the known *osc* genes, which involved in the α-amyrin synthase, are reported as *psm* gene from *Pisum sativum* and *oea* gene from *Olea europaea*.

In this study, callus was induced from leaves of *L. speciosa* and determined triterpene production particularly, corosolic acid. The best growth medium for callus and cell suspension culture was Murashige and Skoog supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid and 1 mg/l kinetin. Dry powders of callus and cell suspension culture was extracted and screened for triterpene production by thin layer chromatography technique. Both callus and suspension cultures produced corosolic acid and other triterpene products in this medium analysed by high performance liquid chromatography. The *oea* gene was transformed into tobacco (*Nicotiana tabacum*), which is a model plant and can be transformed easily by *Agrobacterium tumefaciens*. It will lead to the understanding of triterpenoid biosynthesis in plant

cell. The *oea* gene was successfully transformed into tobacco leaves using *A. tumefaciens* C58 carrying pCAMBIA 2311-OEA plasmid containing *npt*II gene. Sixty-one transformed tobacco plants were obtained from transformation. The presence of *oea* gene was confirmed by PCR analysis and Southern blot analysis. Thirty-six transgenic tobaccos contained *oea* gene in their genome. Southern blot analysis revealed complex integration patterns in tobacco genome with different copy numbers. The 30 transgenic lines were obtained from classification of 36 transgenic tobaccos by pattern and copy numbers.

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation and grateful thanks to my thesis advisor, Dr. Pimpimon Tansakul for her helpful guidance, suggestion and encouragement throughout the course of this work.

My sincere thanks are expressed to my thesis co-advisor Dr. Orawan Chatchawankanphanich, for her kindness and helpful suggestions.

I would like to thank all staff of Faculty of Pharmaceutical Sciences, Prince of Songkla University for their kindness and help.

I would like to thank for all helpful and kindly support from staff at Plant Research Group, National Center for Genetic Engineering and Biotechnology (BIOTEC), Kasetsart University, Kamphang Saen campus.

I would like to thank Thailand Graduate Institute of Science and Technology (TGIST), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) for thesis grant and Prince of Songkla University for partial support on thesis grant.

Finally, I would to thank my family and my friends for their love and encouragement.

Prapaporn Wititpanyawong

CONTENTS

	Page
บทคัดย่อ	iii
ABSTRACT	v
ACKNOWLEDGEMENTS	vii
CONTENTS	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS AND SYMBOLS	xv
CHAPTER I INTRODUCTION	1
1.1 Background and rationale	1
1.2 Review of literature	3
1.2.1 Botanical characteristics of *Lagerstroemia speciosa*	3
1.2.2 Ethnopharmacology of *L. speciosa*	4
1.2.3 Chemical constituents of *L. speciosa*	5
1.2.4 Corosolic acid	15
1.2.5 Biological activity of corosolic acid	16
1.2.6 Plant tissue culture of *L. speciosa*	17
1.2.7 Oxidosqualene cyclases	17
1.2.8 Plant transformation	23
1.2.9 Objectives	24
CHAPTER II MATERIALS AND METHODS	25
2.1 Materials and equipments	25
2.1.1 Chemicals, kits and enzymes	25
2.1.2 Equipments	26
2.2 Plant tissue culture methods	27
2.2.1 Plant material	27

CONTENTS (Continued)

	Page
2.2.2 Solution preparations	27
2.2.3 Preparation of *L. speciosa* plant material and induction of callus and cell suspension	28
2.2.4 Medium preparations	28
2.3 Determination of triterpene	29
2.3.1 Harvest cell	29
2.3.2 Sample extraction	29
2.3.3 Thin layer chromatography (TLC) analysis	30
2.3.4 High performance liquid chromatography (HPLC) analysis	30
2.3.4.1 Sample preparation for HPLC analysis	30
2.3.4.2 Standard solution	30
2.4 Method for plant Transformation	31
2.4.1 Plant material	31
2.4.2 Bacterial strain	31
2.4.3 Media and solution	31
2.4.4 The *oea* gene construct for tobacco transformation	32
2.4.4.1 Preparation of *oea* gene	32
2.4.4.2 Cloning PCR product of *oea* gene into expression vector	32
2.4.4.3 Heat-shock transformation of *oea* gene constructs into *E.coli* strain DH5α	33
2.4.4.4 Plasmid extraction from *E. coli* by alkaline lysis method	33
2.4.5 Preparation of *A. tumefaciens* carrying pCAMBIA-OEA construct	34
2.4.5.1 Restriction enzyme digestion	34

CONTENTS (Continued)

	Page
2.4.5.2 Cloning of *oea* gene construct into plant expression vector	34
2.4.5.3 Preparation of *A. tumefaciens* strains C58 competent cell	35
2.4.5.4 Electroporation of *A. tumefaciens* competent cells	36
2.4.6 Transformation of *oea* gene into tobacco	37
2.4.6.1 The sensitivity to antibiotic for selection of transformed cells and plants	37
2.4.6.2 Transformation of *oea* gene into tobacco by *A. tumefaciens*	37
2.4.7 Molecular analysis of regenerated transgenic tobacco	38
2.4.7.1 Genomic DNA extraction from tobacco	38
2.4.7.2 PCR analysis of transformed tobacco	38
2.4.7.3 Detection of transgene copy number	40
2.4.7.3.1 Preparation of OEA DNA probe	40
2.4.7.3.2 Southern blot analysis	41
CHAPTER III RESULTS	43
3.1 Induction of callus and cell suspension culture and triterpene production	43
3.1.1 Callus induction	43
3.1.2 Screening of triterpene production by thin layer chromatography (TLC) analysis	45
3.1.3 Cell suspension culture	46
3.1.4 Thin layer chromatography (TLC) analysis	47
3.1.5 Determination of triterpene from callus and cell suspension culture	48
3.2 Expression of *oea* gene in tobacco	49

CONTENTS (Continued)

	Page
3.2.1 Cloning *oea* gene into expression vector	49
3.2.2 Transformation of *oea* gene into tobacco	52
3.2.2.1 The sensitivity to antibiotic for selection of transformed cells and plants	52
3.2.2.2 Transformation of *oea* gene into tobacco by *A. tumefaciens*	53
3.2.3 PCR analysis of transformed tobacco	54
3.2.4 Southern blot analysis	56
CHAPTER IV DISCUSSIONS	58
CHAPTER V CONCLUSIONS	63
REFERENCES	64
APPENDIX	79
VITAE	84

LIST OF TABLES

Table	Page
1-1 Chemical constituents of *L. speciosa*	6
1-2 Corosolic acid content of the different plant parts of *L. speciosa*	15
1-3 List of known oxidosqualene cyclase	20
2-1 Primers for amplification of *oea* and *npt*II genes	32
3-1 The copy numbers of *oea* gene in 36 transgenic tobacco classified by patterns and copy numbers	56
A-1 Inorganic salt and vitamin composition of Murashige and skoog medium (MS)	81
A-2 Inorganic salt and vitamin composition of Mc Cown Woody Plant medium (WPM)	82
A-3 Inorganic salt and vitamin composition of Gamborg's B5 medium (B5)	83

LIST OF FIGURES

Figure	Page
1-1 *Lagerstroemia speciosa* (L.) Pers. (Lythraceae)	4
1-2 Proposed biosynthetic pathway of triterpenes	22
1-3 Proposed biosynthetic pathway of corosolic acid	22
2-1 Plasmid pCAMBIA 2311-OEA	35
2-2 Diagram of *oea* gene and *npt*II gene cassette in plant expression vector pCAMBIA 2311	40
3-1 The characters of callus in MS medium supplemented with 2 mg/l 2,4-D and1 mg/l kinetin medum, WPM medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin, MS medium supplemented with 0.1 mg/l IBA and 2 mg/l BA and B5 medium supplemented with 0.1 mg/l IBA and 2 mg/l BA	44
3-2 Thin layer chromatography (TLC) patterns of ethyl acetate extracts from callus of *L. speciosa* in three solvent systems	46
3-3 Callus and cell suspension culture in MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin	46
3-4 Thin layer chromatography (TLC) patterns of methanol extracts from callus and cell suspension culture of *L. speciosa* in three solvent systems	47
3-5 HPLC-chromatogram of standard corosolic acid	48
3-6 HPLC-chromatogram of callus extracts compared with HPLC-chromatogram of callus extract spiking with corosolic acid	49
3-7 HPLC-chromatogram of cell suspension extracts (A) compared with HPLC-chromatogram of cell suspension extract spiking with corosolic acid	49
3-8 Analysis of pGEM® - T easy-OEA by digestion with *Xba*I and *Spe*I	50
3-9 Analysis of *oea* gene cloned into plant expression vector pCAMBIA 2311	51
3-10 Analysis of *oea* gene constructs transformed into *A. tumefaciens* strain C58 by colony direct PCR using OEA-*Xba*I and OEA-*Spe*I priners	51

LIST OF FIGURES (Continued)

Figure	Page
3-11 Analysis of *npt*II gene constructs transformed into *A. tumefaciens* strain C58 by colony direct PCR using NPTII-F and NPTII-R primers	52
3-12 Kanamycin sensitivity test for tobacco	53
3-13 Tobacco transformation process	54
3-14 Analysis of *oea* transgene in T lines of transgenic tobaccos by PCR analysis	55
3-15 Southern blot analysis of T lines of transgenic tobacco	57

LIST OF ABBREVIATIONS AND SYMBOLS

ATP	=	adenosine-5'-triphosphate
B5	=	Gamborg's B5 medium
BA	=	benzylanine
bp	=	base pair
BSA	=	bovine serum albumin
$CaCl_2$	=	calcium chloride
cAMP	=	cyclic adenosine monophosphate
CDP-*Star*	=	disodium-4-chloro-3-(methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricycle [3.3.1.1^{3,7}] decan}-4-yl)-1-phenyl phosphate
CH_3COOH	=	acetic acid
CH_3COOK	=	potassium acetate
$(CH_3COO)_2$ Mg	=	magnesium acetate
CH_3COONa	=	sodium acetate
cm	=	centimeter
CTAB	=	cetyl trimethyl ammonium bromide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
DTT	=	dithiothreitol
EDTA	=	ethylenediaminetetraacetic acid
g	=	gram
g	=	gravitational force
GLUT4	=	glucose transporter type 4
h	=	hour
HCl	=	hydrochloric acid
HPLC	=	high performance liquid chromatography
HPTLC	=	high performance thin layer chromatography
H_2SO_4	=	sulfuric acid
IBA	=	indolebutyric acid

IPTG	=	isopropyl-β-D-thiogalactoside
Kb	=	kilobase pair
KCl	=	potassium chloride
kg	=	kiligram
KV	=	kilovolt
l	=	liter
LB	=	Luria-Bertani medium
lb/in^2	=	pound per square inch
LS	=	Linsmaier and Skoog medium
m	=	meter
M	=	molar
mg	=	milligram
$MgCl_2$	=	magnesium chloride
min	=	minute
ml	=	mililiter
mM	=	millimolar
MS	=	Murashige and Skoog medium
msec	=	millisecond
N	=	normal
NAA	=	1-naphthalene acetic acid
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
ng	=	nanogram
$NH4^+$	=	ammonium ion
nm	=	nanometer
$NO3^-$	=	nitrate ion
OD	=	optical density
PCI	=	phenol, chloroform and isoamyl alcohol mixture (25:24:1, v/v)
PCR	=	polymerase chain reaction
pH	=	-log hydrogen ion concentration

RNase A	=	ribonuclease A
rpm	=	round per minute
SDS	=	sodium dodecyl sulphate
sec	=	second
SSC	=	standard saline citrate
TAE	=	Tris-acetate-EDTA
TLC	=	thin layer chromatography
Tris-HCl	=	Tris-hydrochloride
U	=	unit
UV	=	ultraviolet
V	=	volt
WPM	=	Mc Cown Woody plant medium
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µF	=	microfarad
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
v/v	=	volume by volume
w/v	=	weight by volume
2X-YT	=	double strength yeast extract tryptone medium
2,4-D	=	2,4-dichlorophenoxyacetic acid
°C	=	degree Celsius
Ω	=	ohm
%	=	percentage

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Triterpenes are one of the most abundant natural products mainly found in higher plants and regarded to be important as potential natural sources for medicinal compounds (Sparg *et al*., 2004). They exhibit a wide range of both structural diversity and biological activity. More than 100 different triterpenoid carbon skeletons from the plant kingdom are known and have been described (Xu *et al*., 2004).

Despite the increasing interest in the wide range of biological properties of plant triterpenoids and their derivatives for human health, pharmacological activities of triterpene compound are used in traditional and folk medicine that include antioxidant, antiallergic, anti-inflammatory, antitumor, antibacterial, gastroprotective and hepatoprotective effects (Ravokatra *et al*., 1974; Tanaka *et al*., 1992; Tabata *et al*., 1993; Yun *et al*., 1999; Ryu *et al*., 2000; Sunitha *et al*., 2001; Ukiya *et al*., 2002; Katerere *et al*., 2003). For example, corosolic acid, from *Lagerstremia speciosa* leaves, has good hypoglycemic activity and inhibits insulin-induced adipocyte differentiation (Murakami *et al*., 1993). Moreover, it has an antihypertensive effect together with a lipid-lowering effect, antioxidative, anti-inflammatory activities (Judy *et al*., 2003; Fukushima *et al*., 2006; Miura *et al*., 2006; Yamaguchi *et al*., 2006), and also effect on GLUT4 glucose transporter in mouse muscle, indicating the useful for treatment of Type II diabetes.

While, ursolic acid, from *Olea europaea*, was found to exhibit significant activities such as hepatoprotective, anti-inflammatory, antiviral, antitumor (Liu *et al*., 1995) and inhibit the growth of some of the food-associated bacteria and yeast (Collins and C1arles, 1987). It was also reported that ursolic acid decreases the cytopatholic effects *in vitro* cells exposed to Herpes simplex virus (Poehland *et al*., 1987).

Several Thai medicinal plants contain triterpenoid compounds as their active constituents. Tirucaric acid from *Sapindus emarginatus* (Sapotaceae) is used for its anti-fungal activity to treat skin problem. Asiaticoside from *Centella asiatica* (Umbelliferae) is used as wound heeling (นันทวัน บุณยะประภัศร, 2541).

Our interest is focusing on a corosolic acid from *Lagerstroemia speciosa* (Lythraceae) which is a dietary supplement for lowering blood sugar (Judy *et al*., 2003). Increasing of this triterpene compound from tissue culture or by gene transformation might be alternative ways for improvement of the large scale production of corosolic acid in the future.

In the biosynthetic point of view, most of triterpenes are biosynthesized from a common precursor substrate, 2,3-oxidosqualene, with the participation of oxidosqualene cyclases (OSC) (Abe *et al*., 1993). The oxidosqualene cyclase is coded by *osc* gene. Several known *osc* gene were cloned and reported, including lanosterol synthase, cycloartenol synthase, β-amyrin synthase, lupeol synthase and also mixed amyrin synthase.

Unlike other OSCs which produced only sole products, the latter coded for multifunctional triterpene synthase (MTS) which converted 2,3-oxidosqualene to several cyclic triterpenes. Example of *mts* genes included *psm* gene from *Pisum sativum* (Morita *et al*., 2000), *oea* gene from *Olea europaea* (Saimaru *et al*., 2007). These two genes produced similar tritepene product as α-amyrin and β-amyrin in a ratio 2:1. Other *mts*, such as *CSOSC2* from *Costus speciosus*, produced lupeol, germanicol and β-amyrin (Kawano *et al*., 2002).

Several studies on the expression of *osc* gene were carried out in yeast system (Kushiro *et al*., 1998*a*; Shibuya *et al*., 1999; Sawai *et al*., 2006; Tansakul *et al*., 2006; Shibuya *et al*., 2009), but no such expression study has been reported in plant.

This study was divided into two parts. For tissue culture part, callus was induced from *L. speciosa* leaves and triterpene was detected for screening the possibility of corosolic acid production by plant tissue culture. The second part involved transformation of *oea* gene (Saimaru *et al*., 2007) into tobacco (*N. tabacum*). This process was carried out using *A. tumefaciens* C58 carrying pCAMBIA 2311-OEA plasmid. The scopes of this study are callus and cell suspension induction from leaves of *L. speciosa* then, determination of triterpene production from callus and cell suspension culture. In addition, transformation oxidosqualene cyclase gene into *N. tabacum*.

1.2 Review of literature

1.2.1 Botanical characteristics of *Lagerstroemia speciosa*

Lagerstroemia speciosa (L.) Pers. is a tree in family Lythraceae (Figure 1-1). It distributes in tropical countries such as India, China, Thailand, the Philippines, Vietnam, Malaysia and Australia (Kakuda *et al*., 1996; Suzuki *et al*., 1999; Liu *et al*., 2001; Judy *et al.,* 2003; Klein *et al*., 2007). In Thailand, this plant is called Inthanin, Inthanin num or Ta baek dum (Smitinand, 2001). Its English common name is Queen's flower and the Philippine's name is Banaba.

L. speciosa is a deciduous and tropical flowering tree that can reach 20 to 30 m heights. The trunks, highly valued as timber, are light colored and have a scaling bark. The leaves are simple, opposite or subopposite. The shape of the leaves is oval to elliptic, obovate and oblong. The leaves blade length is 2 - 4 inches. The margin of leaves is entire. The venation of leaves is pinnate and brachidodrome. The 4 - 7 cm diameter flowers, arranged in loose panicles 40 - 60 cm long, have twelve-ribbed, bell-shaped calyxes with six or occasionally seven lobes or teeth. The corollas have six crinkled, crape-like, pink, mauve or purple petals, and the flower centers are crowded with as many as 100 yellow stamens. The fruits are 2 - 3 cm in diameter. The shape of fruits is oval and round. The length of fruits is less than 0.5 inch. The fruits are brown in color with dry or hard cover (Gilman and Watson, 1998).

Figure 1-1 *Lagerstroemia speciosa* (L.) Pers. (Lythraceae)

1.2.2 Ethnopharmacology of *L. speciosa*

L. speciosa has been used as a traditional folk medicine for treatment of diabetes mellitus, kidney disease and also known to possess weight loss and antioxidant effects (Mazumder and Rahman, 2008). Folkloric used include diuretic and purgative action from leaf decoctions and the use of root for stomach ailments. The plant also has been studied for its application in the treatment of diabetes (Yamaguchi *et al*., 2006; Klein *et al*., 2007).

In 1996, Kakuda's research group studied antidiabetic activity of *L. speciosa* by preparing water and methanol extracts from this plant. After feeding food containing either 5% of water extract or 3% of methanol extract to dietary Type II diabetic KK-Ay mice, blood glucose and insulin levels were effectively reduced ($p<0.05$) (Kakuda *et al*., 1996). In a second study, obese female KK-Ay mice treated

with food containing 5% water extract had significantly reduced body weight (10%) compared with control mice fed with a regular diet (Klein *et al*., 2007).

In another study, obese diabetic rats was fed with a diet containing 5% of hot water extract for 12 weeks, their body weight gain and parametrical adipose tissue weight were lowered significantly in the *L. speciosa* diet group. Blood glucose levels were not suppressed in the *L. speciosa* diet group but hemoglobin A_{1C} was found to be suppressed at the end of the experiment (Suzuki *et al*., 1999).

Moreover, both hot water and methanol extracts of the leaves were shown to stimulate glucose uptake in 3T3-L1 cells in a similar manner to insulin, and inhibit adipocyte differentiation induced by insulin (Bai *et al*., 2008), isobutyl-methyl xanthine and dexamethasone, suggesting that plant extract may be useful for prevention and treatment of hyperglycemia and obesity in Type II diabetics (Liu *et al*., 2001).

The hypoglycemic effect has been elegantly demonstrated in rabbit and mouse models. Phase II/III clinical trials in the application of *L. speciosa* leaf decoctions to manage diabetes mellitus show promising data. Originally, because of its hypoglycemic activity, *L. speciosa* leaf is thought to contain an "insulin-like principle", a Type of plant-derived peptide hormone (Deocaris *et al*., 2005).

1.2.3 Chemical constituents of *L. speciosa*

Several groups of chemical constituents are found in *L. speciosa* including diterpenes, triterpenes, sterols, tannins, hydrocarbons and fatty acids. All chemical constituents were shown in Table 1-1.

Table 1-1 Chemical constituents of *L. speciosa*

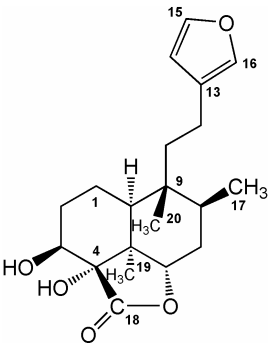
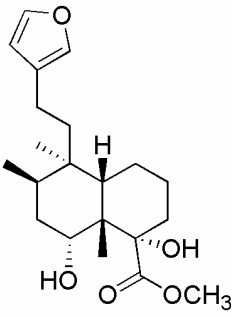
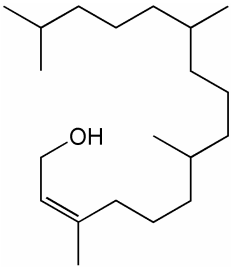
Chemical structure	Plant part	References
Diterpenes		
 Tinotufolin C	Leaf	Ragasa *et al*., 2005
 Tinotufolin D	Leaf	Ragasa *et al*., 2005
 Phytol	Leaf	Ragasa *et al*., 2005

Table 1-1 Chemical constituents of *L. speciosa* (continued)

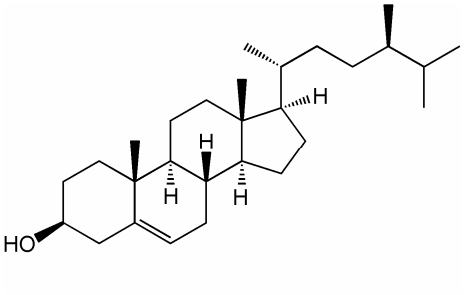
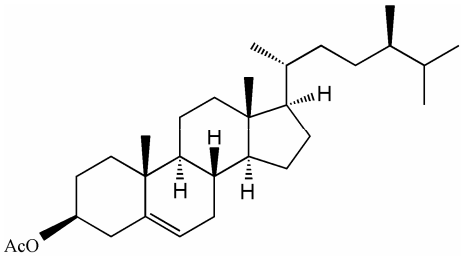
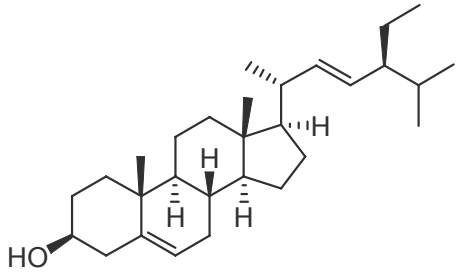
Chemical structure	Plant part	References
Triterpenes and steroids		
 β-sitosterol	Leaf	Faruk *et al*., 2002; Ragasa *et al*., 2005
 Sitosterol acetate	Leaf	Faruk *et al*., 2002; Ragasa *et al*., 2005
 Stigmasterol	Leaf and bark	Ragasa *et al*., 2005

Table 1-1 Chemical constituents of *L. speciosa* (continued)

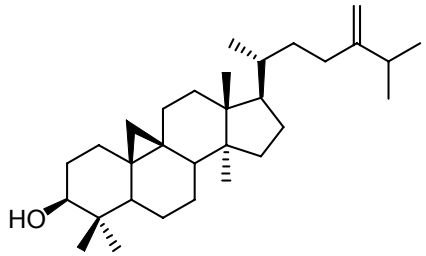
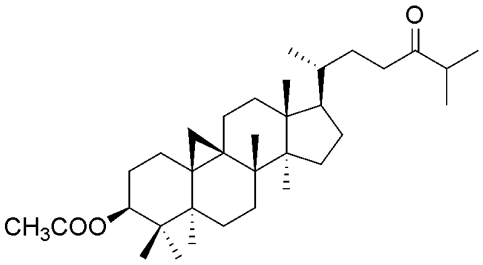
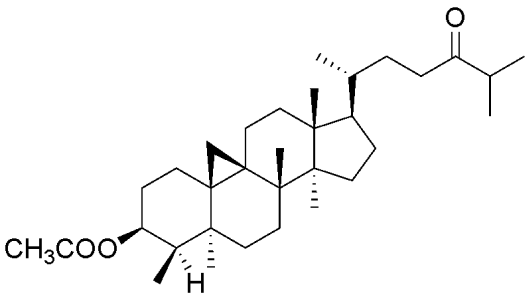
Chemical structure	Plant part	References
 24-methylene cycloartanol	Leaf	Ragasa *et al*., 2005
 Largerenol acetate	Leaf	Ragasa *et al*., 2005
 31-norlargerenol acetate	Leaf	Ragasa *et al*., 2005

Table 1-1 Chemical constituents of *L. speciosa* (continued)

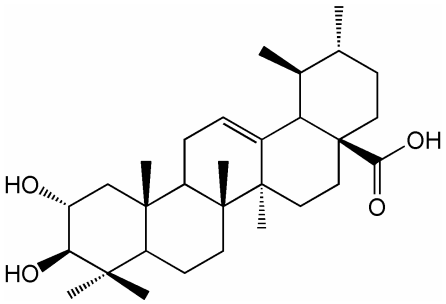
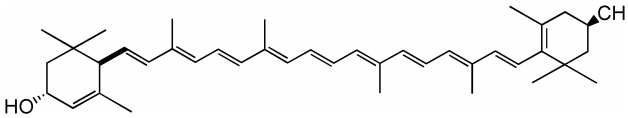
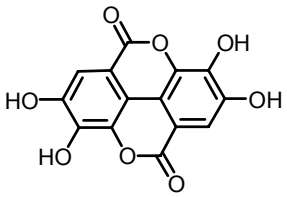
Chemical structure	Plant part	References
 Corosolic acid	Leaf	Ragasa *et al*., 2005
Tetraterpene		
 Lutein	Leaf	Ragasa *et al*., 2005
Tannins		
 Ellagic acid	Leaf and bark	Takahashi *et al*., 1977

Table 1-1 Chemical constituents of *L. speciosa* (continued)

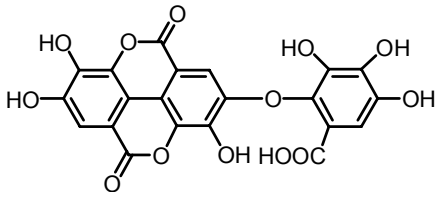
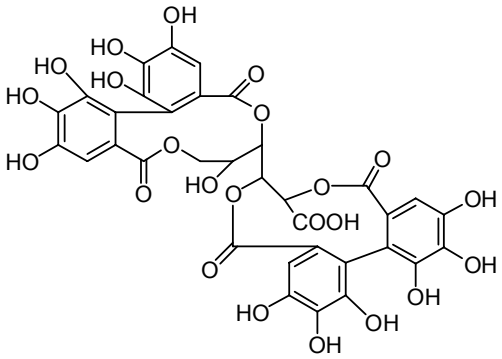
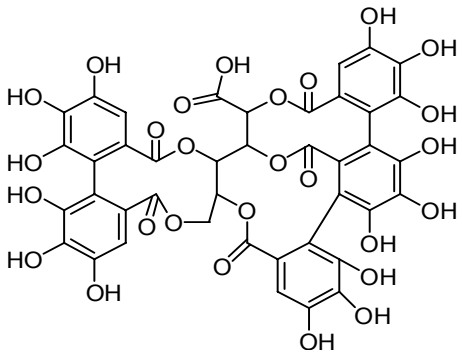
Chemical structure	Plant part	References
 Valoneic acid dilactone	Leaf	Hosoyama *et al*., 2003
 Lagerstannin A	Leaf and fruit	Tanaka *et al*., 1992
 Lagerstannin B	Leaf and fruit	Tanaka *et al*., 1992

Table 1-1 Chemical constituents of *L. speciosa* (continued)

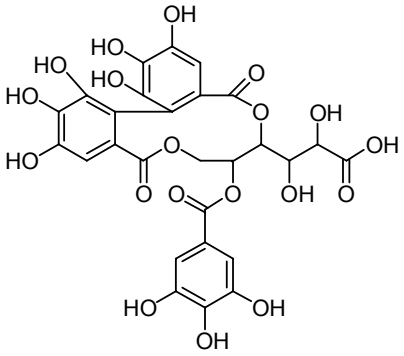
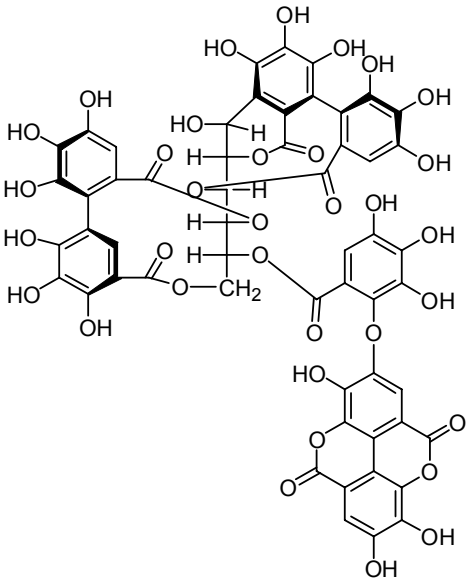
Chemical structure	Plant part	References
 Lagerstannin C	Leaf and fruit	Tanaka *et al*., 1992
 Lagerstroemin	Leaf and bark	Hayashi *et al*., 2002; Hosoyama *et al*., 2003

Table 1-1 Chemical constituents of *L. speciosa* (continued)

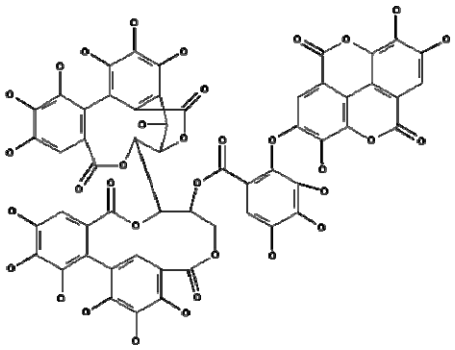
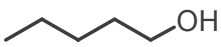
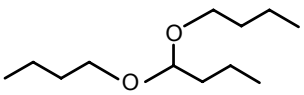
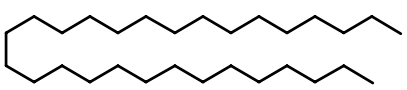
Chemical structure	Plant part	References
 Flosin B	Leaf and bark	Hayashi *et al*., 2002; Hosoyama *et al*., 2003
Hydrocarbons		
 1-pentanol	Leaf and bark	Egawa, 2001
 Lageracetal	Leaf and bark	Takahashi *et al*., 1976
 Nonacosane	Leaf and bark	Takahashi *et al*., 1979

Table 1-1 Chemical constituents of *L. speciosa* (continued)

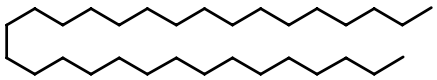
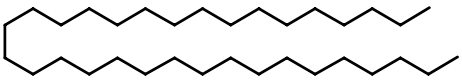
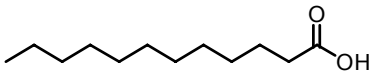
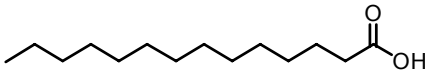
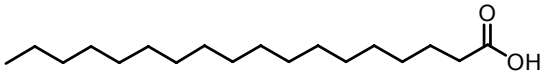
Chemical structure	Plant part	References
 Hentriacontane	Leaf and bark	Takahashi *et al*., 1979
 Tritriacontane	Leaf and bark	Takahashi *et al*., 1979
Fatty acids		
 Lauric acid	Seed	Badami *et al*., 1970
 Myristic acid	Seed	Badami *et al*., 1970
 Stearic acid	Leaf and bark	Takahashi *et al*., 1979

Table 1-1 Chemical constituents of *L. speciosa* (continued)

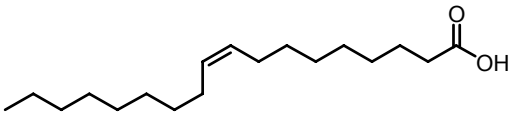
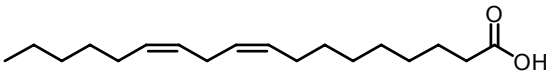
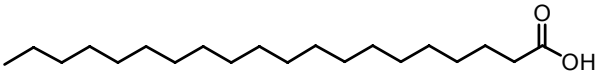
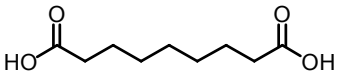
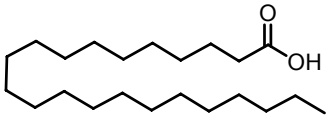
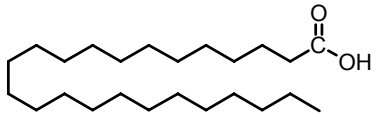
Chemical structure	Plant part	References
 Oleic acid	Seed	Badami *et al*., 1970
 Linoleic acid	Seed	Badami *et al*., 1970
 Arachidic acid	Leaf and bark	Badami *et al*., 1970; Takahashi *et al*., 1979
 Nonanedioic acid	Seed	Sinhababu *et al*., 1999
 Behenic acid	Leaf and bark	Badami *et al*., 1970; Takahashi *et al*., 1979

Table 1-1 Chemical constituents of *L. speciosa* (continued)

Chemical structure	Plant part	References
 Lignoceric acid	Seed	Badami *et al*., 1970

1.2.4 Corosolic acid

Corosolic acid is an ursane-type triterpene. The IUPAC name is (1S,2R,4aS,6aR,6aS,6bR,10R,11R,12aR,14bR)-10,11-dihydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid. Corosolic acid shows good hypoglycemic activity. The amount of corosolic acid from different plant part was determined by HPTLC method. The leaf, stem, stem bark, root, heartwood, calyx, and fruit were extracted with 80% ethanol. Amounts of corosolic acid were in the range 0.0225–0.8900%. The corosolic acid content was highest (0.89%) in the leaves (Mallavadhani *et al*., 2008) (Table 1-2).

Table 1-2 Corosolic acid content of the different plant parts of *L. speciosa* (Mallavadhani *et al*., 2008)

Plant part	Average amount (%)
Leaf	0.8900
Stem	0.0560
Stem bark	0.0840
Root	0.0225
Heartwood	0.0365
Calyx	0.0970
Fruit	Not detected

1.2.5 Biological activity of corosolic acid

Among those compounds, corosolic acid shows good hypoglycemic activity. Corosolic acid has similar activity to insulin and inhibits insulin-induced adipocyte differentiation (Murakami *et al*., 1993). Moreover, corosolic acid has an antihypertensive effect a lipid-lowering effect, antioxidative, and anti-inflammatory activities (Judy *et al*., 2003; Fukushima *et al*., 2006; Miura *et al*., 2006; Yamaguchi *et al*., 2006).

Aqueous extract from *L. speciosa* leaves and the pure compound, corosolic acid, decrease the blood glucose level of genetically diabetic (KK-Ay) in mice, and also effect on GLUT4 glucose transporter in mouse muscle, indicating the useful for treatment of Type II diabetes. Corosolic acid was also suggested to improve glucose metabolism by reducing insulin resistance (Hattori *et al*., 2003; Miura *et al*., 2004; Miura *et al*., 2006).

Recently, Takagi and his group reported that corosolic acid had a hypoglycemic effect in the small intestine in mice. Corosolic acid (10 mg/kg body weight) improved hyperglycemia after an oral administration of sucrose, and significantly reduced the hydrolysis of sucrose in the small intestine. These results suggested that the hypoglycemic activity of corosolic acid was derived, at least in part, due to the inhibition of the hydrolysis of sucrose (Takagi *et al*., 2008).

The mechanism of action of corosolic acid on gluconeogenesis in rat liver has been demonstrated to inhibit gluconeogenesis by increasing the production of fructose-2,6-bisphosphate, lowering the cAMP level and inhibiting protein kinase activity in isolated hepatocytes. Furthermore, corosolic acid increased glucokinase activity without affecting glucose-6-phosphatase activity, suggesting the promotion of glycolysis. These effects on hepatic glucose metabolism may underlie the various anti diabetic actions of corosolic acid (Yamada *et al*., 2008). Several clinical studies on corosolic acid have been demonstrated in a randomized clinical trial (Judy *et al*., 2003) and double blind controlled study (Fukushima *et al*., 2006) for significantly reducing blood glucose levels and for lowering effect on postchallenge plasma glucose level, respectively. *L. speciosa* leaf extract standardized to 1% corosolic acid (Glucosol™)

(Judy *et al*., 2003) and 3% corosolic acid (Vijaykumar *et al*., 2006) was widely used as dietary supplement for reduction blood sugar.

1.2.6 Plant tissue culture of *L. speciosa*

Previous works on tissue culture of *L. speciosa* were very rare. Recently, Kim and his group have invented the method for production of corosolic acid in suspension culture of plant cell. The callus was induced in MS medium or LS medium containing 10 μM NAA and 10 μM BA and cultured at 22-27°C. The cell suspension culture was then inoculated in B5 medium liquid medium with the same hormone for callus induction. Corosolic acid from cell suspension was found to be 56 times higher than natural leaves (Kim *et al*., 2007). In addition, study of crape myrtle on the effect of activated charcoal and BA on *in vitro* nitrogen uptake in *L. indica.* Explants grown in media supplemented with activated charcoal were able to uptakr both NO_3^- and NH_4^+, although NH_4^+ uptake was lower. However, NH_4^+ uptake was preferential in media without activated charcal (Eymar *et al.,* 2000). As *L. speciosa* is a tree plant that is difficult for callus induction or tissue culture construction. In general, application of tissue culture technique to woody plants is more difficult than those of herbaceous plants (Zimmerman, 1988). Woody plants often secrete polyphenols and tannins, which can inhibit development of explants and cause cell necrosis. In addition, contamination becomes a severe problem if the woody explants have systemic infections, since internal contamination can not be prevented by surface sterilization (Peternel *et al*., 2009). Successful callus induction from *L. speciosa* leaves will be an alternative way for large scale production and biosynthetic study of triterpene, especially corosolic acid.

1.2.7 Oxidosqualene cyclases

Triterpenes in higher plant such as cycloartenol, dammarenediol-II and β-amyrin, are derived from 2,3-oxidosqualene by oxidosqualene cyclase (OSC). In the formation of the triterpene skeleton reaction proceeds with (3*S*)-2,3-oxidosqualene adopting pre-chair-chair-chair-boat conformation giving dammrenyl cation. This

tetracyclic cationic intermediate then undergoes a deprotonation to afford various pentacyclic triterpenes, or is quenched by water molecule to afford dammarenediol. Purification of several OSC enzymes have been reported from vertebrate (Duriatti and Schuber, 1988; Kusano *et al*., 1991; Abe *et al*., 1992*a*; Moore and Schatzman, 1992), plant (Abe *et al*., 1988; Abe *et al*., 1989*a*; Abe *et al*., 1989*b*; Abe *et al*., 1992*b*) and yeast (Corey and Matsuda, 1991; Hoshino *et al*., 1991) sources. Up to now, more than forty OSC cDNAs involved in sterol and triterpenoid biosynthesis have been cloned from various plant species as showed in Table 1-3. The obtained thirty two plant OSCs consist of ten cycloartenol synthase clones (included three putative cycloartenol synthase clones), nine β-amyrin synthase clones, three lupeol synthase clones, four multifunctional triterpene synthaase clones, an isomultiflorenol synthase and two putative oxidosqualene cyclases.

Successful cloning and sequence comparison of several different triterpene synthase cDNAs have revealed the relation between reaction mechanism and the primary protein structure. In the reaction mechanism of cycloartenol synthase hydride and methyl shifts on protosteryl cation give lanosteryl cation. Cycloartenol snthase removes a proton of methyl group at C10 to form cyclopropane ring (Figure 1-2, route *a*). Formation of plant triterpenes can be rationalized on the basis of cyclization and rearrangement reactions (Figure 1-2). Butyrospermol and tirucalla-7,24-dien-3β-ol are produced from 2,3-oxidosqualene via dammrenyl cation by multifunctional triterpene synthases (PSM and YUP43) (Kushiro *et al*., 2000; Morita *et al*., 2000) through hydride and methyl shifts and deprotonation. Lupeol is formed from lupeol cation, an intermediate en route to oleanyl cation by lupeol synthases (OEW, TRW and BPW) (Shibuya *et al*., 1999 and Zhang *et al*., 2003) and multifunctional triterpene synthases (AtLUP1, CsOSC2, GFF) (Herrera *et al*., 1998; Kushiro *et al*., 2000; Kawano *et al*., 2002; Tsutsumi, 2003).

Two routes branch after lupenyl cation step as a methyl group shift from C20 (Figure 1-2, route *c*) would give the taraxasteryl cation, while a hydride shift from C18 (Figure 1-2, route *d*) would give oleanyl cation. Taraxasterol and Ψ-taraxasterol are produced from deprotonation at C30 and C21, respectively, of taraxasteryl cationn, while α-amyrin and bauernol are produced from deprotonation at

C12 and C7 protons, respectively, of ursanyl cation by multifunctional triterpene synthases (Kushiro *et al*., 2000; Morita *et al*., 2000).

β-amyrin is a product from deprotonation at C12 of oleanyl cation ny β-amyrin synthase obtained from many plant species (Kushiro *et al*., 1998*a*; Kushiro *et al*., 1998*b*; Morita *et al*., 2000; Haralampidis *et al*., 2001; Hayashi *et al*., 2001*a*; Suzuki, 2002; Iturbe *et al*., 2003; Zhang *et al*., 2003)

Table 1-3 List of known oxidosqualene cyclase

Enzyme	Plant species	Abbreviation	EMBL/Gene Bank AC	Reference
Cycloartenol Synthase	*Arabidopsis thaliana*	AtCAS1	U02555	Corey *et al*., 1993
Cycloartenol Synthase	*Pisum sativum*	PSX	D89619	Morita *et al*., 1997
Cycloartenol Synthase	*Panax ginseng*	PNX1	AB009029	Kushiro *et al*., 1998*a*
Cycloartenol Synthase	*Luffa cylindrica*	LcCAS1	AB033334	Hayashi *et al*., 1999
Cycloartenol Synthase	*Abies magnifica*	AbiCAS1	AF216755	Herrera *et al*., 1999
Cycloartenol Synthase	*Glycyrrhiza glabra*	GgCAS1	AB025968	Hayashi *et al*., 2000*b*
Cycloartenol Synthase	*Avena strigosa*	AsCS1	AJ311790	Haralampidis *et al*., 2001
Cycloartenol Synthase	*Costus speciosus*	CSOSC1	AB058507	Kawano *et al*., 2002
Cycloartenol Synthase	*Betula platyphylla*	CASBPX1	AB055509	Zhang *et al*., 2003
Cycloartenol Synthase	*Betula platyphylla*	CASBPX2	AB055510	Zhang *et al*., 2003
Putative CAS*	*Oryza sativa*	OsCAS1	AF169966	Darr *et al*., 1999
Putative CAS	*Allium macrostemon*	AllOSC1	AB025353	You *et al*., 1999
Putative CAS	*Olea europaea*	OEX***	AB024344	Shibuya *et al*., 1999
β-amyrin Synthase	*Panax ginseng*	PNY2	AB014057	Kushiro *et al*., 1998*b*
β-amyrin Synthase	*Panax ginseng*	PNY1	AB009030	Kushiro *et al*., 1998*a*
β-amyrin Synthase	*Pisum sativum*	PSY	AB034802	Morita *et al*., 2000
β-amyrin Synthase	*Glycyrrhiza glabra*	GgbAS1	AB037203	Hayashi *et al*., 2001*a*
β-amyrin Synthase	*Avena strigosa*	AsbAS1	AJ311789	Haralampidis *et al*., 2001
β-amyrin Synthase	*Medicago truncatula*	MtbAS	AJ430607	Suzuki *et al*., 2002
β-amyrin Synthase	*Betula platyphylla*	BPY	AB055512	Zhang *et al*., 2003
β-amyrin Synthase	*Lotus japonicus*	LjAmy2	AF478455	Iturbe *et al*., 2003
β-amyrin Synthase	*Glycine max*	GmAms	AY095999	Unpublished data
Lupeol Synthase	*Olea europaea*	OEW	AB025343	Shibuya *et al*., 1999
Lupeol Synthase	*Taraxacum officinale*	TRW	AB025345	Shibuya *et al*., 1999
Lupeol Synthase	*Betula platyphylla*	BPW	AB055511	Zhang *et al*., 2003
MTS**	*Arabidopsis thaliana*	AtLUP1	U49919	Herrera *et al*., 1998
MTS	*Pisum sativum*	PSM	AB034803	Morita *et al*., 2000
MTS	*Costus speciosus*	CSOSC2	AB058508	Kawano *et al*., 2002
MTS	*Arabidopsis thaliana*	YUP8H12R.43	AC002986	Kushiro *et al*., 2000
Isomultiflorenol Synthase	*Luffa cylindrica*	LcIMS1	AB058643	Hayashi *et al*., 2001*b*
Putative OSC	*Luffa cylindrica*	LcOSC2	AB033335	Hayashi *et al*., 2000*a*
Putative OSC	*Taraxacum officinale*	TRV	AB025346	Shibuya *et al*., 1999

* Putative CAS = Putative cycloartenol synthase ** MTS = Multifunctional triterpene synthase

*** OEX clone = Partial sequence

Corosolic acid is proposed to be biosynthesized from 2,3-oxidosqualene via α-amyrin and ursolic acid by cyclization reaction with enzyme oxidosqualene cyclase (OSC), under the control of *osc* gene (Kushiro, *et al*., 2000) (Figure 1-2 and Figure 1-3). Among several known *osc* genes, multifunctional triterpene synthase (MTS), which produce α-amyrin and β-amyrin as major products, is believed to be a gene that controls the biosynthesis of corosolic acid. The known *mts* genes have been cloned from *Pisum sativum* (*psm* gene) (Morita *et al*., 2000) and *Olea europea* (*oea* gene) (Saimaru *et al*., 2007).

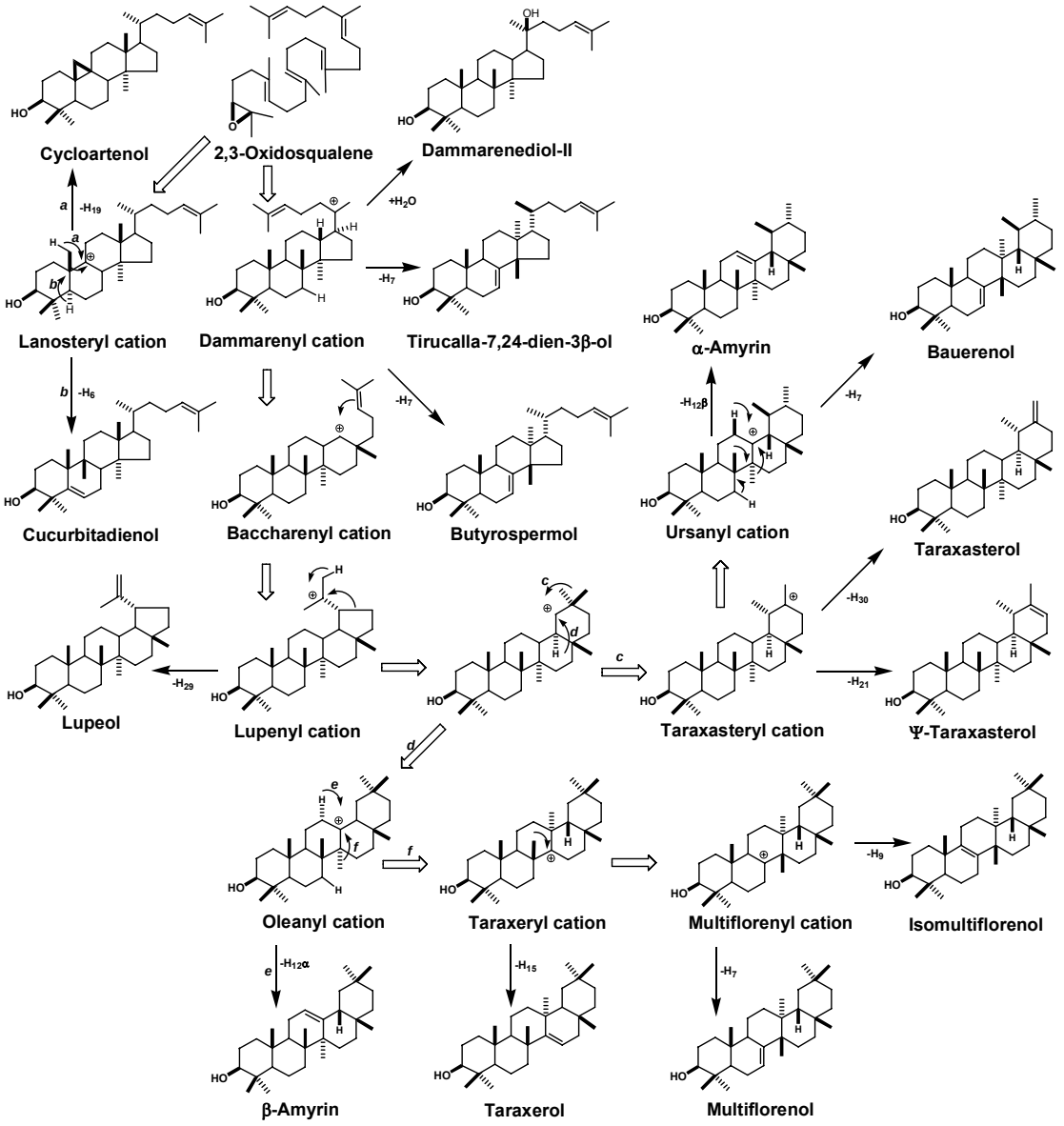


Figure 1-2 Proposed biosynthetic pathways of triterpenes (Tansakul, 2004)

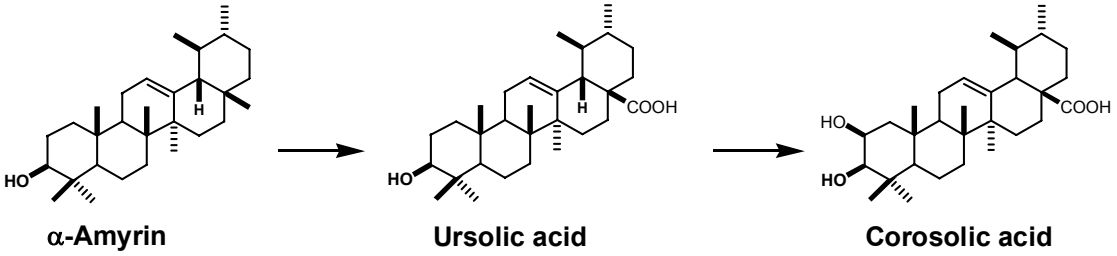


Figure 1-3 Proposed biosynthetic pathway of corosolic acid

1.2.8 Plant transformation

Several studies on the expression of *osc* gene (found in Table 1-3) were carried out in yeast system, but not in plant. Transformation of *osc* gene into plant cells, will lead to the understanding on biosynthesis of triterpene. However, transformation of *osc* gene into *L. speciosa* seems to be difficult, because of plant habitat. Model plant such as tobacco (*N. tabacum)* might be better to study *osc* gene expression because several well-established transformation protocols are available (Schlaman *et al*., 1997).

Tobacco is the widely used for gene transformation. Currently, several gene expressions in tobacco were studied. The gene betaine aldehyde dehydrogenase of hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f) (*HvBADH1* gene) was successfully transformed into tobacco (*N. tabacum* L.) by *A. tumefaciens* LBA4404 for study tolerance to salt stress in hulless barley (Zhao *et al*., 2008). The *CaRZFP1* gene, which encoded *Capsicum annuum* RING Zinc Finger Protein 1, was functional expressed in tobacco. The *CaRZFP1* gene, which was ligated into pBKS1-1 vector to construct pBKS1-1-*CaRZFP1* plasmid, was electroporated into the *A. tumefaciens* strain LBA4404 for tobacco (*N. tabacum* L. cv. Wisconsin 38) transformation. The transgenic tobacco exhibits enhanced growth such as larger primary root, more lateral root, and larger hypocotyls and bigger leaves size than non-transgenic tobacco (Zeba *et al*., 2009). The *Arabidopsis thaliana* metallothionein 2b (*AtMT2b*) gene has been transformed into tobacco leaf disc (*N. tabacum* Sr1.) by *A. tumefaciens* strain C58. The *AtMT2b* expression decreased arsenite tolerance and enhanced the arsenic root to shoot transport in transgenic lines compared to wild type (Grispen *et al*., 2009). The Rhizobium salt tolerance B (*rstB*) gene, which was identified and cloned from salt-tolerant *Sinorhizobium fredii* strain RT 19, was used as a selectable marker gene in the *A. tumefaciens* strain LBA4404 for transformation into tobacco (*N. tabacum* cv. Xanthi). The *rstB* transgenic tobacco showed improved salt tolerance and a normal phenotype compared to wild type (Zhang *et al*., 2009).

Successful transformation of *oea* gene into tobacco, a model plant, will bring the useful information of triterpene biosynthesis in plant system and also a

possibility of transformation of other *osc* gene into other medicinal plants including *L. speciosa* tissue culture.

1.2.9 Objectives

The objectives of the present study were

1. To induce callus from the leaves of *L. speciosa*
2. To determine triterpene production from the culture
3. To study the transformation of oxidosqualene cyclase gene in *N. tabacum*

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals, kits and enzymes

- Absolute ethanol (Lab-scan Asia, Thailand.)
- Acetonitrile, HLPC grade (Lab-scan Asia, Thailand)
- Agar (Bacto) (Himedia laboratories, India)
- Agarose gel (Research Organics, USA)
- Alpha-amyrin (kindly provided from Assist. Prof. Dr. Tetsuo Kushiro, University of Tokyo, Japan)
- Benzene, Analytical grade (J.T. Baker Inc., USA)
- Beta-amyrin (kindly provided from Assist. Prof. Dr. Tetsuo Kushiro, University of Tokyo, Japan)
- Cefotaxime sodium (UTOPIAN CO, Thailand)
- Chloroform, Analytical grade (Lab-scan Asia, Thailand)
- Clorox®(Clorox, Malaysia)
- Corosolic acid (kindly provided from Assist. Prof. Dr. Tetsuo Kushiro, University of Tokyo, Japan)
- DNA markers (Sib-enzyme, Russia)
- Ethanol (95 % (v/v)) (Lab-scan Asia, Thailand.)
- Ethidium bromide (Bio Basic INC, Canada)
- Ethyl acetate, Analytical grade (Lab-scan Asia, Thailand)
- Gamborg's (B5) medium (Duchefa Biochemie, The Netherlands)
- GeneClean kit (Promega, USA)
- Glacial acetic acid, Analytical grade (Lab-scan Asia, Thailand)
- Indolebutyric acid (IBA) (Fluka, Switzerland)
- Kinetin (Fluka, Switzerland)
- Ligation kit-pGEM®-T easy (Promega, USA)

- Mc Cown Woody plant medium (WPM) (Duchefa Biochemie, The Netherlands)
- Methanol, Analytical grade (RCI Labscan, Thailand)
- Methanol, HPLC grade (Lab-scan Asia, Thailand)
- Murashige-Skoog (MS) medium (Duchefa Biochemie, The Netherlands)
- Plant agar (Sigma, Germany)
- Sodium chlorite, Analytical grade (Univar, Astralia)
- Sodium hydroxide, Analytical grade (Univar, Astralia)
- Sucrose (Mitrpol, Thailand)
- TLC-plate silica gel GF_{254} (Merck, Germany)
- *Xba*I, *Spe*I, *BamH*I, *Kpn*I (Takara, Japan)
- Yeast extracts (Himedia laboratories, India)
- 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, The Netherlands)
- 6-benzylaminopurine (BA) (Fluka, Switzerland)

2.1.2 Equipments

- Autoclave Model HA-3D (Hirayama, Japan)
- Centrifuge Kubota model 5922 (Kubota Coporation, Tokyo)
- Digital water bath SB-1000 (EYELA, Japan)
- Electrophoresis chamber Mupid α-2 plus (Takara Bio Inc, Japan)
- High-speed Centrifuge Kubota 5922 (Kubota, Japan)
- Hot air oven (Memmert, Germay)
- Hot plate and stirrer (Fisher Scientific, USA)
- HPLC (Agilent, U.S.A) equipped with 1 pump, UV detector, recorder with agilent software (Agilent, Germany)
- HPLC (Agilent, U.S.A) equipped with 4 pumps, UV detector and photodiode-array detector, recorder with agilent software (Agilent, Germany)
- HPLC column: TSK-GEL™ C18, 2 µm, 4.6 x 200 mm (TOSOH, Japan)
- HPLC column: Vertisep™ Ges C18, 5 µm, 4.6 x 250 mm (Vertical®,

Thailand)

- Hybridization oven: Shel Lab (Sheldon Manufacturing, INC, U.S.A.)
- Innova 2300 shaker (New Brunswick Scientific, USA)
- Laminar air flow cabinet (Holten, Denmark)
- Micropipette 0.1-2 µL, 2-20 µL, 20-200 µL, 100-1,000 µL (Socorex, Switzerland)
- Microwave ovens (LG, China)
- pH meter (ORION Research, USA)
- Rotary evaporator (EYELA, Japan)
- Sonicator (Crest Ultrasonic Corp., USA)
- Takara PCR Thermal Cycler Dice (Takara, Japan)
- UV illuminator (Vilber Lourmat, France)
- Vacuum pump (EYELA, Japan)
- Vortex-Genie 2TM (Bohemia, NY, USA)

2.2 Plant tissue culture methods

2.2.1 Plant material

Young leaves of *L. speciosa* (L.) Pers. was collected from botanical garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

2.2.2 Solution preparations

- Anisaldehyde-sulphuric acid spray reagent

Anisaldehyde (0.5 ml) was mixed with glacial acetic acid (10 ml) and conc. H_2SO_4 (5 ml). Adjust the volume to 85 ml with methanol.

2.2.3 Preparation of *L. speciosa* plant material and induction of callus and cell suspension

L. speciosa leaves were surface sterilized by rinsing with 70% (v/v) ethanol for 10 sec, and immerging in 1.2% (v/v) sodium hypochlorite containing 0.1% Tween 20 for 20 min, then three times rinsing with sterile distilled water and cut into small pieces (0.5 x 0.5 cm^2 size). The explants were put on solid media. In the preliminary study, four culture media were used for the callus induction with 30 replications per treatment.

2.2.4 Medium preparations

MS medium (Murashige and Skoog, 1962), WPM medium (Lloyd and Mc Cown, 1981) and B5 medium (Gamborg *et al*., 1968) in powder form were used for culturing leaf explants. The composition of the MS, B5 and WPM media were shown in Table A-1, Table A-2 and Table A-3, respectively (see Appendix). The MS medium was prepared using 4.4 g MS, 30 g sucrose and plant hormones (as need). Distilled water was added up to a liter of medium. The pH was adjusted to pH 5.8 which was used a small volume of 1 N NaOH and 1 N HCl. For solid medium preparation, 0.8% (w/v) agar was added and the solution was gently heated with continuous stirring until the solution was clear. The culture medium was sterilized by autoclaving at 121°C, 15 lb/in^2, for 15 min. For WPM medium and B5 medium preparation, the media were prepared as the same procedure using 2.5 g and 3.2 g of each medium powder, respectively. The pH of solution was adjusted to 5.7 for WPM medium and to 5.5 for B5 medium.

The four general media used in the preliminary study include (1) MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin, which was reported for successful callus induction of *Croton stellatopilosus* (Kongduang *et al*., 2008), (2) WPM medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin, which is a medium for woody plant, (3) MS medium supplemented with 0.1 mg/l IBA and 2 mg/l BA, (4) B5 medium supplemented with 0.1 mg/l IBA and 2 mg/l BA.

Sterile explants were cultured at 25°C under 16-h photoperiod of fluorescent light and subcultured every four weeks.

Callus from the best medium, which grown well, was culture change (induction for suspension) to cell suspension in the best medium at 250 rpm in darkness and subculture every four weeks.

2.3 Determination of triterpene

2.3.1 Harvest cell

The callus and cell suspension were harvested after one month of subculture. Fresh weight of callus and cell suspension were determined and dried at 50°C overnight. The dry callus and cell suspension were ground into powder and dry weight was determined.

2.3.2 Sample extraction

The callus was extracted by two methods. The first method was modified from Dat *et al*., 2005. The dried callus powder (300 mg) was extracted in 30 ml ethanol by sonication for 1 h at room temperature, concentrated to yield crude extract by rotary eaporator. The crude extract was then partitioned with water and hexane. The aqueous part was partitioned again with ethyl acetate; the ethyl acetate fraction was concentrated to yield crude extract (Dat *et al*., 2005). The second method was the extraction of crude extract by methanol, which was modified after unsuccessful experiment using the first method. The dried callus powder (300 mg) was extracted in 30 ml methanol by sonication for 1 h at room temperature. The methanol extract was concentrated by rotary evaporator to yield crude extract (Vijaykumar *et al*., 2006). The triterpene product from crude extract was then determined by thin layer chromatography (TLC) analysis and high performance liquid chromatography (HPLC) analysis. As cell suspension was extracted by the second method and determined the triterpene production by TLC and HPLC analysis.

2.3.3 Thin layer chromatography (TLC) analysis

TLC plate silica gel GF_{254} (Merck, Germany) was used as stationary phase. Five microliter of sample was spotted on TLC plates, prior to analysis using 3 solvent systems, including chloroform and acetone (4:1), chloroform and methanol (9:1) and ethyl acetate and methylene chloride (8:2). After running on each solvent system, TLC plate was dried and sprayed by anisaldehyde-sulfuric acid spraying reagent and heated at 105°C for detection purple spots of triterpenoid compounds.

2.3.4 High performance liquid chromatography (HPLC) analysis

HPLC analysis was carried out using Agilent 1100 series equipped with UV detector and autosampler. Separation was performed using Agilent software and HPLC-C18 column (Vertisep Ges, 5 µm, 4.6 x 250 mm, Vertical® Thailand). Isocratic elution was carried out with acetonitrile and 0.1% phosphoric acid in water (80:20, v/v) at a flow rate of 1 ml/min and detection wavelength was at 210 nm (Vijaykumar *et al*., 2006).

2.3.4.1 Sample preparation for HPLC analysis

Three hundred milligrams of *L. speciosa* dry callus and cell suspension powder were resuspended in 30 ml methanol and sonicated at room temperature for 1 h. The extract was filtered and concentrated to yield crude extract. The 10 mg crude extracts was dissolved in methanol with a final volume of 5 ml and filtered through 0.2-µm membrane filter (Vijaykumar *et al*., 2006).

2.3.4.2 Standard solution

One milligram per liter of standard corosolic acid was dissolved in 1 ml methanol and used as standard solution for determination of corosolic acid in samples.

2.4 Method for plant transformation

2.4.1 Plant material

Seeds of *Nicotiana tabacum* cv. White Burley were kindly provided by Plant Research Group-BIOTEC, Kasetsart University, Kamphang Saen campus. Steriled seeds were sown on MS medium containing vitamin of B5 (MS-B5).

2.4.2 Bacterial strain

Escherichia coli strain DH5α and *Agrobacterium tumefaciens* strain C58 (Plant Research Group-BIOTEC, Kasetsart University, Kamphang Saen campus) was used for plant transformation.

2.4.3 Media and solution

- 2X-YT medium

2X-YT medium was prepared by mixing the tryptone (1.6 g), yeast extract (1 g), NaCl (0.5 g) and agar (6 g) in distilled water. The final volume was adjusted to 100 ml distilled. The medium was sterilized by autoclaving at 121°C, 15 lb/in^2, for 15 min.

- Cefotaxime stock solution

Stock solution of cefotaxime was prepared by dissolving the 1 g of cefotaxime in sterile distilled water up to 5 ml to make final concentration at 250 mg/ml.

- **Kanamycin stock solution**

Stock solution of kanamycin was prepared by dissolving the 500 mg kanamycin in sterile distilled water up to 10 ml to make final concentration at 50 mg/ml and filtered through 0.2-µm membrane filter.

2.4.4 The *oea* gene construct for tobacco transformation

2.4.4.1 Preparation of *oea* gene

The *oea* gene was constructed from plasmid pYES2-OEA. The *oea* gene was cloned from callus of *Olea europea* (Saimaru *et al*., 2007). The *oea* gene was amplified by PCR using 2 µg pYES2-OEA as a template, specific primers; OEA-*Xba*I and OEA-*Spe*I (0.4 µM each) (Table 2-1), 100 µM dNTP mix, 10x PCR buffer (500 mM KCl, 200 mM Tris HCl, pH 8.4) and 0.1 U *Taq* polymerase (RBC, Taiwan). The PCR was performed in a condition of initial step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 mim, extension at 72°C for 3 min, and 1 cycle of the final DNA extension at 72°C for 10 min. PCR product was analyzed by electrophoresis in 0.8% agarose gel and 1X TAE buffer (Tris-acetate-EDTA buffer, see Appendix).

Table 2-1 Primers for amplification of *oea* and *npt*II genes*

Primers	Sequences
OEA-*Xba*I	5'-GGTCTAGAATGTGGAAGCTTAAGATTGC-3'
OEA-*Spe*I	5'-TTACTAGTTTACAGGCTTTGAGATGACC-3'
35S-P	5'-TGAGACTTTTCAACAAAGGGTAAT-3'
OEA-300	5'-CACGGCAGTCGTGGCTGTTT-3'
NPTII-F	5'-GAAGATCTTCAGAAGAACTCGTCAAGAA-3'
NPTII-R	5'-GAAGATCTATGGGGATTGAACAAGATGG-3'

**npt*II gene = neomycin phosphotransferase II gene

2.4.4.2 Cloning PCR product of *oea* gene into expression vector

The PCR product of *oea* gene construct was cloned into pGEM®-T easy vector (Promega, USA.). The ligation mix contained 50 ng pGEM®-T easy vector, 2X ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA), 3 U T_4 DNA ligase and PCR product of *oea* gene in a total volume of 10 µl. The ligation mix was incubated at 16°C for 16 h, and transformed into *E. coli* strain DH5α by heat shock transformation.

2.4.4.3 Heat-shock transformation of *oea* gene construct into *E. coli* strain DH5α

The 2 µl of ligation mix from 2.4.4.2 was mixed with 100 µl competent cell of *E. coli* strain DH5α and chilled on ice for 20 min. The mixture was incubated at 42°C for 1 min and chilled on ice for 5 min, before addition of 1 ml 2X-YT medium into the mixture. Transformed bacterial cells were cultured on shaker at 250 rpm, 37°C for 1 h. Then spread on solid 2X-YT medium containing 100 mg/l ampicillin, 20 µl IPTG (100 mM) and 100 µl X-gal (20 mg/ml in dimethylformamide) and incubated at 37°C for 16 h.

2.4.4.4 Plasmid extraction from *E. coli* by alkaline lysis method

The pGEM®-T easy-OEA was extracted from bacterial cells by alkaline lysis method (Sambrook *et al*., 1989). Bacterial culture (1 ml) was collected by centrifugation at 19,200 *g* for 5 min. After removing of supernatant, The 200 µl Solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) was added. The pellet was resuspended by vortexing briefly. The cell suspension was chilled on ice for 5 min. The 200 µl Solution II (0.2 N NaOH, 1% SDS) was added and gently mixed by inverting tube several times. The sample was then chilled on ice for 5 min. The 150 µl Solution III (3 M

CH_3COOK, 0.2 M CH_3COOH) was added and gently mixed by inverting tube before chilled on ice for 5 min and centrifuged at 27,700 *g* for 5 min at room temperature. The supernatant was transferred into new microcentrifuge tube. One volume of phenol-chloroform-isoamyl alcohol mixture (PCI) (25:24:1, v/v) was added and mixed well by vortex mixture. The mixture was centrifuged at 27,700 *g* for 5 min at room temperature. The clear aqueous phase was transferred into new microcentrifuge tube before 2.5 of volumes absolute ethanol and 0.1 volumes of 3 M CH_3COONa (pH 5.2) were added and mixed well by inverting tube. The mixture was then incubated at -20°C for 10-15 min, centrifuged at 27,700 *g* for 5 min to pellet plasmid DNA. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and centrifuged at 27,700 *g* for 5 min. The ethanolic part was removed and the pellet was then dried under vacuum. The DNA was finally resuspended in 20 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, pH 8.0) containing 20 µg/ml RNase A.

2.4.5 Preparation of *A. tumefaciens* carrying pCAMBIA-OEA construct

2.4.5.1 Restriction enzyme digestion

Plasmid pGEM®-T easy-OEA was digested with *Xba*I (Takara, Japan) and *Spe*I (New England BioLabs, UK) enzymes to release *oea* gene from plasmid vector. For digestion with *Xba*I, the reaction contained 0.8 µl 10X M buffer [Tris-acetate (pH 7.5), CH_3COOK, $(CH_3COO)_2Mg$, DTT], 6 U *Xba*I and 8 µg plasmid. The reaction was incubated at 37°C for 3 h. The reaction was then digested with *Spe*I, in a reaction mixture containing 1.0 µl 10X NEBuffer 2 (1X NEBuffer, NaCl, Tris-HCl, $MgCl_2$, DTT, pH 7.9), 5 U *Spe*I. The reaction was incubated at 37°C for 2-3 h. Digested DNA fragment was checked by 0.8% agarose gel electrophoresis.

2.4.5.2 Cloning of *oea* gene construct into plant expression vector

The plant expression vector used in this study is pCAMBIA 2311-Rep. This plasmid is a pCAMBIA 2311 that carried mutant replication-associated protein (*Rep*) gene (kindly provided by Dr. Orawan Chatchawankanphanich Plant Research Group-BIOTEC, Kasetsart University, Kamphang Saen campus). This vector was digested with *Xba*I and *Spe*I to remove *Rep* gene from pCAMBIA 2311 with the same method as 2.4.5.1. The *oea* gene was ligated into pCAMBIA 2311 at the *Xba*I and *Spe*I sites and transformed into *E. coli* strain DH5α. The recombinant pCAMBIA 2311-OEA plasmid (Figure 2-1) was purified from overnight *E. coli* culture.

The present of recombinant DNA was analyzed by digestion with *Xba*I and *Spe*I enzymes. Construct plsasmid was then introduced into *A. tumefaciens* strain C58 by electroporation method (Walkerpeach and Velten, 1994).

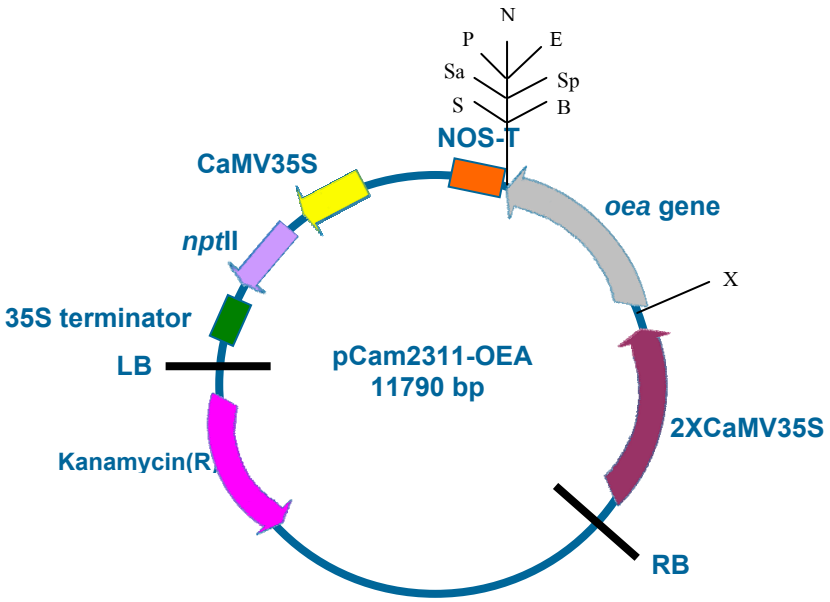


Figure 2-1 Plasmid pCAMBIA 2311-OEA

Abbreviation: B = *Bam*HI, E = *EcoR*I, S = *Sac*I, P = *Pst*I, Sa = *Sal*I, Sp = *Spe*I, X= *Xba*I, LB = left border, CaMV35S = 35S-promoter of Cauliflower mosaic virus, NOS-T = nopaline synthase terminator, *npt*II; neomycin phosphotransferaseII gene, R = resistance, RB = right border.

2.4.5.3 Preparation of *A. tumefaciens* strains C58 competent cell

A. tumefaciens strain C58 was obtained from the Plant Research Group, National Center for Genetic Engineering and Biotechnology (BIOTEC), Kasetsart University, Kamphang Saen campus. The single fresh colony of *A. tumefaciens* strain C58 was cultured in 10 ml 2X-YT medium on shaker at 200 rpm, 28°C for 16 h. One milliliter bacterial cell culture was transferred into 100 ml liquid 2X-YT medium and incubated at 28°C for 2-3 h until OD_{600} reached 0.4-0.6. The culture was then incubated on ice for 30 min. Bacterial cells were collected by centrifugation at 12,300 *g* for 10 min at 4°C. Then, 100 ml bacterial cell was resuspended with a volume of 10% cold steriled glycerol and centrifuged at 12,300 *g* for 10 min at 4°C. The supernatant was removed and the pellet was washed in 20 ml 10% cold steriled glycerol, and then centrifuged at 12,300 *g* for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 2 ml 10% cold steriled glycerol. The mixture was aliquot into 120 µl each in microcentrifuge tube before frozen in liquid nitrogen and kept at -80°C prior to use.

2.4.5.4 Electroporation of *A. tumefaciens* competent cells

The 100 µl of competent cells were thawed on ice for 20 min, mixed with 1 µg pCAMBIA 2311-OEA plasmid and transferred to electroporation cuvette gap (2 mm). Cells were transformed using a Gene Pulser™ (Bio-Rad, USA.) at 2.5 KV, resistor 400 Ω and capacitor 25 µF with the time constant at 8.0-9.0 msec delay. After electroporation, add the liquid 2X-YT medium 1 ml into the cuvette. Cells suspension were cultured on a shaker at 200 rpm, 28°C for 1 h. *Agrobacterium* cells were collected by centrifugation and the pellet was resuspended in 500 µl 2X-YT medium, the suspension was then spread on solid 2X-YT medium containing 50 mg/l kanamycin and incubated at 28°C for 2-3 days. Single colony of *Agrobacterium* was selected and the presence of *oea* and *npt*II gene constructs in cell was verified by PCR

using OEA-*Xba*I and OEA-*Spe*I primers for amplification of *oea* gene and NPTII-F and NPTII-R primers for *npt*II gene (Table 2-1).

2.4.6 Transformation of *oea* gene into tobacco

2.4.6.1 The sensitivity to antibiotic for selection of transformed cells and plants

The plasmid pCAMBIA 2311-OEA contains neomycin phosphotransferase II (*npt*II) gene as selectable marker for kanamycin (kanamycin resistant gene). The leaves of tobacco were, thus, studied under different levels of kanamycin to determine the sensitivity of explants and to optimize kanamycin concentration for the selection of transformed plants cell. Leaves were cultured on MS-B5 medium supplemented with various concentration of kanamycin (0, 50, 100 and 200 mg/l) as a selective medium for 4 weeks, before determination of shoot growth.

2.4.6.2 Transformation of *oea* gene into tobacco by *A. tumefaciens*

Agrobacterium cells containing *oea* gene constructs were grown in 2X-YT medium supplemented with 50 mg/l kanamycin at 28°C for 16-18 h until OD_{600} reaches 1.0-2.0. The bacterial cells were collected by centrifugation at 19,200 *g* for 5 min and resuspended in 1 ml liquid MS-B5 medium and 200 μM acetosyringone for tobacco transformation. Leaves of tobacco (*N. tabacum* cv. White Burley) were cut into 0.5 x 0.5 cm^2 size and inoculated into ten-fold diluted bacterial suspension for 20-30 min; Leaf explants were blotted with sterile filter paper to remove excess bacterial suspension. Explants were transferred to solid MS-B5 medium containing 1 mg/l BA and 200 μM acetosyringone and cultured for 3 days in the dark. After co-cultivation, the explants were transferred to MS-B5 medium containing 1 mg/l BA supplemented with 500 mg/l cefotaxime for the elimination of excess *Agrobacteria*. Two weeks after inoculation, the explants were transferred to

shoot regeneration medium [MS-B5 medium containing 1 mg/l BA supplemented with 500 mg/l cefotaxime and 50 mg/l kanamycin for selection of transformed cells]. After shoot forming, they were cut and transferred to root regeneration medium (MS-B5 medium without plant growth regulator. After one month, complete tobacco plantlets were transferred to sterile soil and kept in a plastic bag at 26-28°C for acclimatization for at least 1 week. After that, tobacco plantlets were kept in greenhouse at 28°C. A set of untransformed leaf was also cultured as control sample in regeneration medium without antibiotic selection.

2.4.7 Molecular analysis of regenerated transgenic tobacco

2.4.7.1 Genomic DNA extraction from tobacco

Genomic DNA was extracted from young leaves of transformed tobacco by a modified CTAB method (Fluton *et al*., 1995). Young leaves (1 g) were ground in 500 µl CTAB buffer [0.1 M Tris-HCl (pH 8.0), 0.02 M EDTA, 1.4 M NaCl, 2% CTAB] and incubated at 65°C for 30 min. An equal volume of PCI mixture was added and mixed by vortex mixture. The mixture was centrifuged at 27,700 *g* at 4°C for 10 min. The supernatant was collected and transferred to a new microtube. DNA was precipitated with 400 µl isopropanol and 40 µl 3 M CH_3COONa (pH 5.2). Genomic DNA was collected by centrifugation at 27,700 *g* for 5 min. DNA pellet was washed with 1 ml 70% ethanol and vacuum dried. The DNA pellet was resuspended in 30 µl TE buffer containing 20 µg/ml RNase A. Total DNA concentration was determined by UV spectrophotometer at wavelength 260 nm (OD_{260}).

2.4.7.2 PCR analysis of transformed tobacco

Genomic DNA of young leaves from transformed tobacco was extracted and used as DNA template for PCR analysis, which was performed using specific primers to detect *oea* gene (35S-P and OEA-300 primer:) (Table

2-1). The PCR mixture containing 2.5 µl 1X PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 0.1 mg/ml BSA, 10 mM $(NH_4)_2SO_4$) (RBC, Taiwan), 1 µl 100 µM dNTP mix (pH 7.9), 0.4 µM of each primer, 0.1 U *Taq* polymerase (RBC, Taiwan) and 1 µl genomic DNA from transformed tobacco leaves as template in a total volume of 25 µl. The PCR program consists of an initial step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min and extension at 72°C for 3 min and another 10 min of final DNA extension at 72° C. PCR products were analyzed with 0.8% agarose gel electrophoresis in 1X TAE buffer and ethidium bromide staining.

The transformed plantlets from *in vitro* culture were transferred to sterile soil and kept in greenhouse at 26-28°C. Genomic DNA from leaves of transformed tobacco was extracted by a modified CTAB method. The *oea* transgene in transformed tobacco was analyzed by PCR using OEA specific primers (Table 2-1). PCR was performed as described above to verify the presence of *oea* gene in tobacco genome.

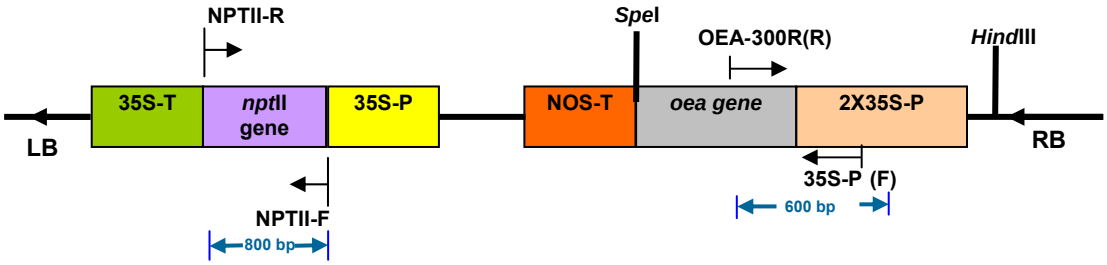


Figure 2-2 Diagram of *oea* gene and *npt*II gene cassette in plant expression vector pCAMBIA 2311

RB	= right border
2X35S-P	= a couple of CaMV35S promoters
oea gene	= oxidosqualene cyclase gene
NOS-T	= nopaline synthase terminator
35S-P	= CaMV35S promotersa
*npt*II gene	= neomycin phosphotransferase II gene
35S-T	= CaMV35S terminator
LB	= left border
35S-P(F)/OEA-300R(R)	= specific primers used for PCR analysis of *oea* transgene in transgenic tobacco
NPTII-F/ NPTII-R	= specific primers used for PCR analysis of *npt*II transgene in transgenic tobacco

2.4.7.3 Detection of transgene copy number

2.4.7.3.1 Preparation of OEA DNA probe

A digoxigenin (DIG)-labelled OEA DNA probe was synthesized by PCR using with digoxigenin-11-dUTP-labeling mixture (Roche, Germany). The PCR mixture contains 1X PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 0.1 mg/ml BSA, 10 mM $(NH_4)_2SO_4$) (RBC, Taiwan), 1X DIG-PCR labeling mix, 0.4 µM of OEA-*Xba*I and OEA-300R primer, 0.1 U *Taq* DNA polymerase (RBC,

Taiwan), and PCR product of *oea* gene as a template in a total volume of 50 µl. The PCR was performed in a condition of initial step at 95°C for 5 min, 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 50 sec, extension at 72°C for 50 sec, and the final extension at 72°C for 7 min. The PCR product was analyzed by 0.8% agarose gel electrophoresis and ethidium bromide staining. The OEA DNA probe was kept at -20°C until use.

2.4.7.3.2 Southern blot analysis

Transgene copy number was determined by Southern blot analysis. Thirty micrograms of genomic DNA was digested with 90 U *Xba*I at 37°C for 18-20 h to cut at 5' region of *oea* gene construct. DNA was analyzed on 0.8% agarose gel in TAE buffer, and electrophoresis at 60 V for 2 h. Gel was stained with ethidium bromide and photographed before proceeding to the next steps. DNA was depurinated by soaking gel in 250 mM hydrochloric acid with agitation for 10 min at room temperature. The gel was rinsed briefly with sterile distilled water. DNA was denatured in a solution containing 0.5 N NaOH and 1.5 M NaCl by twice gentle agitation for 15 min each at room temperature. DNA was then neutralized with a solution containing 0.5 M Tris-HCl (pH 8.0) and 1.5 M NaCl by twice gentle agitation for 15 min each at room temperature. DNA was then transferred from gel to nylon membrane (Roche, Germany) in 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate) by capillary force for 18-20 h. Transferred DNA was fixed by baking the membrane at 120°C for 30 min. The membrane was incubated in pre-hybridization buffer [5X SSC, 0.1% N-lauroyl sarcosine, 1% blocking reagent (Roche, Germany)] at 65°C for 1 h, while the labeled DIG-OEA DNA probe was incubated at 65°C for 5-10 min and chilled on ice immediately. Denatured DIG-OEA DNA probe was added to the pre-hybridized membrane. The hybridization was performed at 65°C for 18-20 h. After hybridization, the membrane

was washed with washing solution I (2X SSC, 0.1% SDS) twice on shaker for 10 min at room temperature, and washed with washing solution II (0.5% SSC, 0.1% SDS) twice on shaker for 15 min at 65°C.

The hybridized DNA was detected by chemiluminescent technique (Roche, Germany). The membrane was soaked briefly in washing solution [100 mM Maleic acid (pH 7.5), 150 mM NaCl, 0.3% Tween 20], and incubated in blocking solution [1% blocking reagent (Roche, Germany), 100 mM Maleic acid (pH 7.5), 150 mM NaCl, 0.3% Tween 20] by shaking for 1 h at room temperature. The Anti-Digoxygenin-AP (Anti-Digoxigenin conjugated alkaline phosphatase) (Roche, Germany) at 1:20,000 dilution was added in the blocking solution, and the membrane was incubated with Anti-Digoxigenin-AP by shaking for 30 min at room temperature. The membrane was washed twice for 15 min in washing solution and then soaked in detection buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl] for 5 min. The CDP-*Star* substrate (Roche, Germany) diluted to 1:200 in detection buffer was added to the membrane. The DNA was visualized by exposing membrane on the X-ray film (KODAK, USA.).

CHAPTER 3

RESULTS

3.1 Induction of callus and cell suspension culture and triterpene production

3.1.1 Callus induction

After cultured the *L. speciosa* leaf explant on 4 different media as described in chapter 2 (2.2.4), the calli were induced after 5 days of culture. The fastest callus induction medium was MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin. This medium also gave the highest growth of the callus. The characters of callus were white-green and dense with high growth. The callus growth was stable even after several subcultures (Figure 3-1 A).

Callus in other 3 media (WPM medium supplemented with 2 mg/l of 2,4-D and 1 mg/l of kinetin, MS medium supplemented with 0.1 mg/l IBA and 2 mg/l BA and B5 medium supplemented with 0.1 mg/l IBA and 2 mg/l BA) were also green. However, the growth decreased after first subculture. Finally, the callus turned brown and died (Figure 3-1 B-D). MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin was selected as the best medium for callus induction.

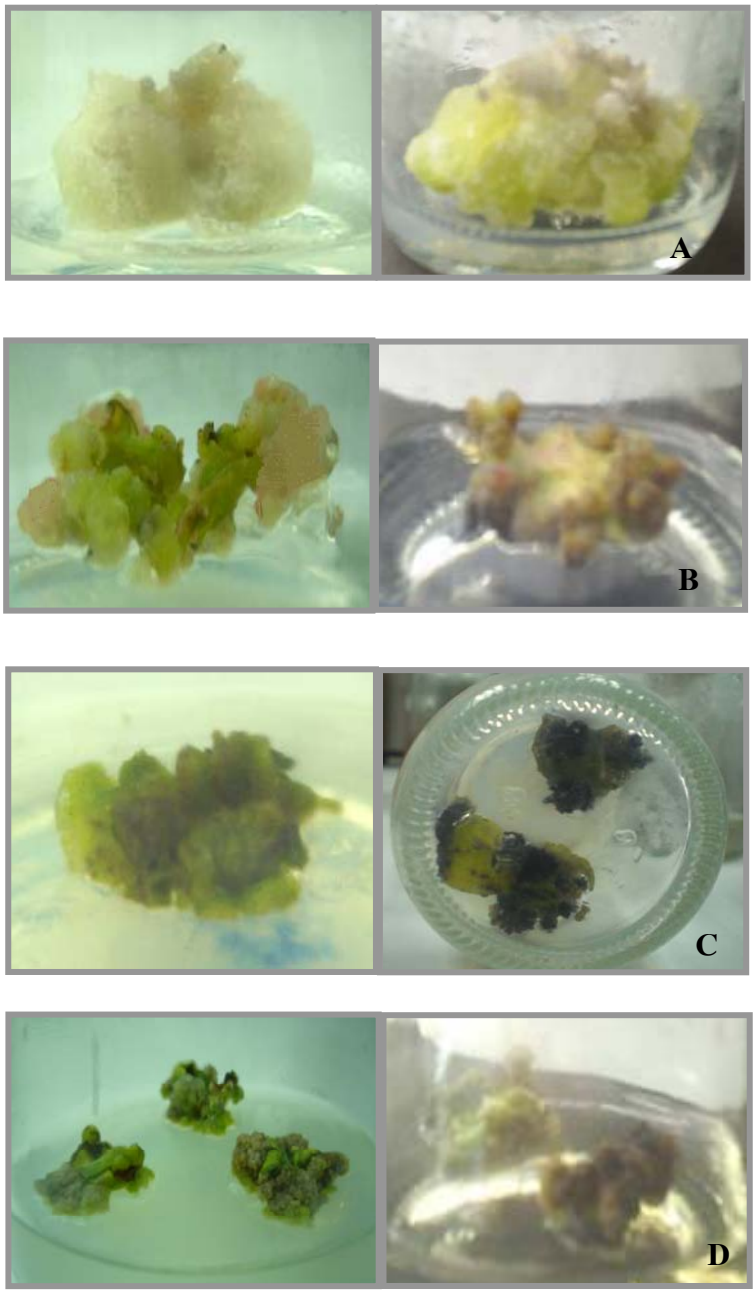


Figure 3-1 The characters of callus in MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin medum (A), WPM medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin (B), MS medium supplemented with 0.1 mg/l IBA and 2 mg/l BA (C) and B5 medium supplemented with 0.1 mg/l IBA and 2 mg/l BA (D)

3.1.2 Screening of triterpene production by thin layer chromatography (TLC) analysis

Two months old callus was harvested and dried in a hot air oven at 50°C overnight and ground into powder. The extraction process was modified from Dat *et al*. (2005). The callus powder was extracted with ethanol by sonication at room temperature, concentrated to yield crude extract. The crude extract was then partitioned with water and hexane. The aqueous part was partitioned again with ethyl acetate; the ethyl acetate fraction was concentrated to yield ethyl acetate extract. The ethyl acetate extract was then analyzed by TLC (Dat *et al*., 2005). TLC plates were sprayed with anisaldehyde spray reagent Corosolic acid and ursolic acid were used as authentic compounds.

Separation of ethyl acetate extract on TLC with three solvent systems was shown in Figure 3-2.

The separation of standard corosoliac acid and ursolic acid in chloroform and methanol (9:1) system showed the best separation and R_f at 0.45 and 0.21, respectively. The chloroform and acetone (4:1) system gave R_f at 0.61 and 0.28 while benzene and acetone (19:1) system gave R_f at 0.21 and 0.02 of corosolic acid and ursolic acid, respectively (Figure 3-2).

The TLC pattern in three solvent systems showed pale spots indicating that this extraction process may not suitable for triterpene analysis of callus.

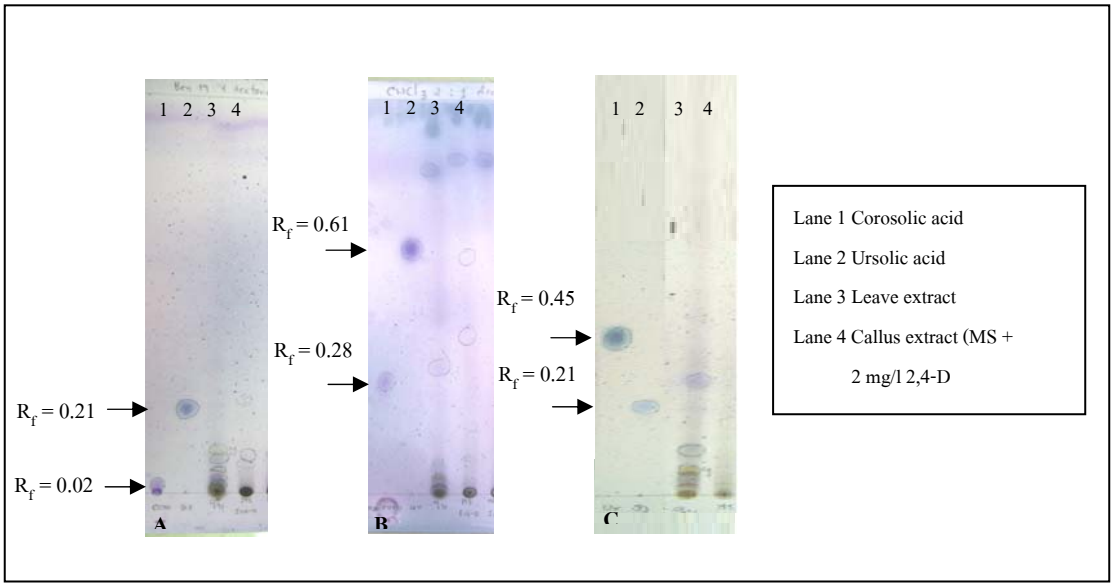


Figure 3-2 Thin layer chromatography (TLC) patterns of ethyl acetate extracts from callus of *L. speciosa* in three solvent systems:

A. Benzene and Acetone (19:1)

B. Chloroform and Acetone (4:1)

C. Chloroform and Methanol (9:1)

3.1.3 Cell suspension culture

Two-month old cell suspension culture was generated from callus and cultured in MS liquid media supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin. The cells had brown-green color (Figure 3-3). Cells were transferred to culture in new culture medium every week for two weeks.

Figure 3-3 Callus and cell suspension culture in MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin

3.1.4 Thin layer chromatography (TLC) analysis

To improve the separation of triterpene from cell culture, separation method of Vijaykumar was used (Vijaykumar *et al*., 2006). The cell suspension culture was harvested and dried at 50°C overnight and ground into powder. The dry powder was extracted with 30 ml methanol by sonication for 1 h at room temperature, concentrated to yield crude extract. The crude extract was directly subjected on TLC plates and developed in three different solvent systems chloroform and acetone (4:1), ethyl acetate and methylene chloride (8:2) and chloroform and methanol (9:1). Corosolic acid was used as authentic standard with R_f = 0.18, 0.36 and 0.47 respectively. Anisaldehyde sulfuric acid was used as spraying reagent.

Analysis of triterpenes from cell suspension and callus culture on TLC showed several separate spots (Figure 3-4). The spots at the same R_f as that of corosolic acid could be seen in both callus and cell suspension culture. Moreover, it can be seen in all 3 solvent systems. This confirmed the presence of corosolic acid in both callus and cell suspension culture which gave the better yield of corosolic acid. The further analysis will be determined by HPLC technique.

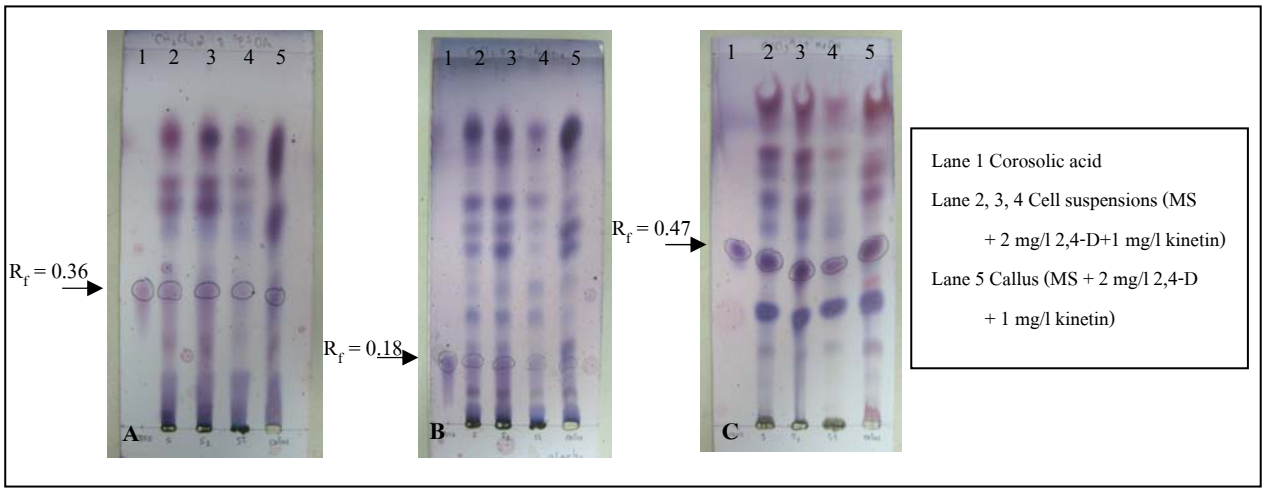


Figure 3-4 Thin layer chromatography (TLC) patterns of methanol extracts from callus and cell suspension culture of *L. speciosa* in three solvent systems:

A. Ethyl acetate and Methylene chloride (8:2)

B. Chloroform and Acetone (4:1)

C. Chloroform and Methanol (9:1)

3.1.5 Determination of triterpene from callus and cell suspension culture

The callus and cell suspension cultured in MS medium supplemented with 2 mg/l of 2,4-D and 1 mg/l of kinetin, produced compounds which showed the same TLC pattern with corosolic acid. Therefore, dry callus and cell suspension (300 mg each) were extracted and analyzed for corosolic acid content by HPLC. The retention time of standard corosolic acid was at 9.61 min under HPLC condition (2.3.3) (Figure 3-5).

HPLC chromatograms of *L. speciosa* callus and cell suspension methanol extracted showed peaks of compound at 9.50 and 9.51 min, respectively (Figure 3-6 A and 3-7 A). The retention time of these compounds seems a little different from that of corosolic acid (9.61 min). However, the spiking technique was introduced to confirm these compounds. Corosolic acid was added to methanol extract of callus and cell suspension and was analyzed by HPLC. The results were shown in Figure 3-6 B and 3-7 B while only peak at 9.50 and 9.51 min increased. This indicated that compound at 9.50 and 9.51 min were corosolic acid from callus and cell suspension, respectively.

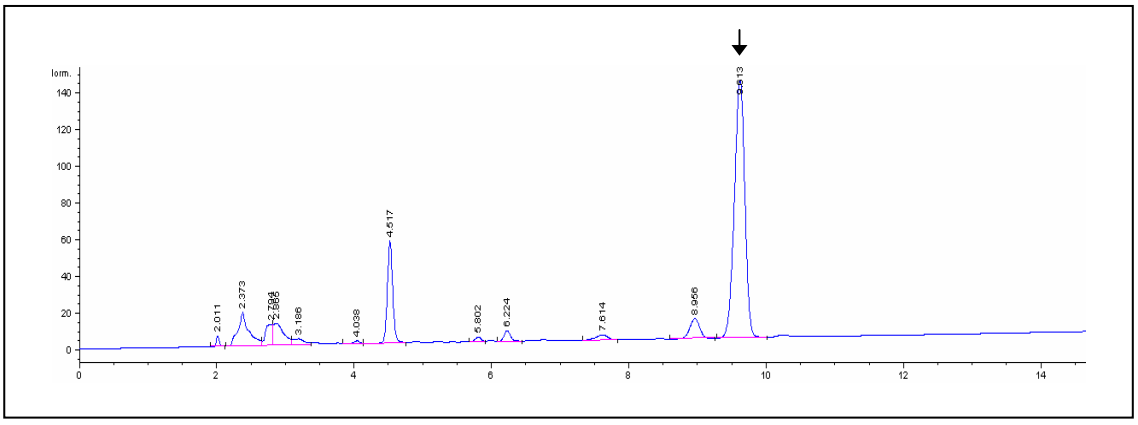


Figure 3-5 HPLC-chromatogram of standard corosolic acid (The arrow indicated corosolic acid peak.)

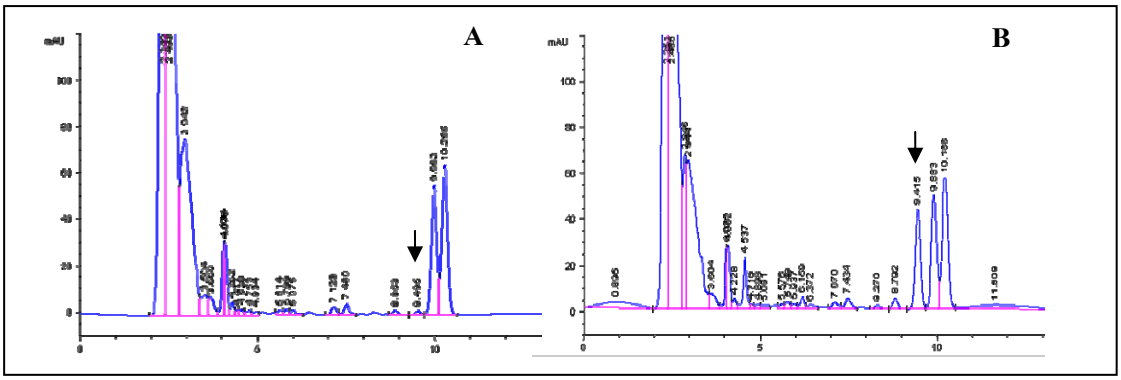


Figure 3-6 HPLC-chromatogram of callus extracts (A) compared with HPLC-chromatogram of callus extract spiking with corosolic acid (B) (The arrow indicated corosolic acid peak.)

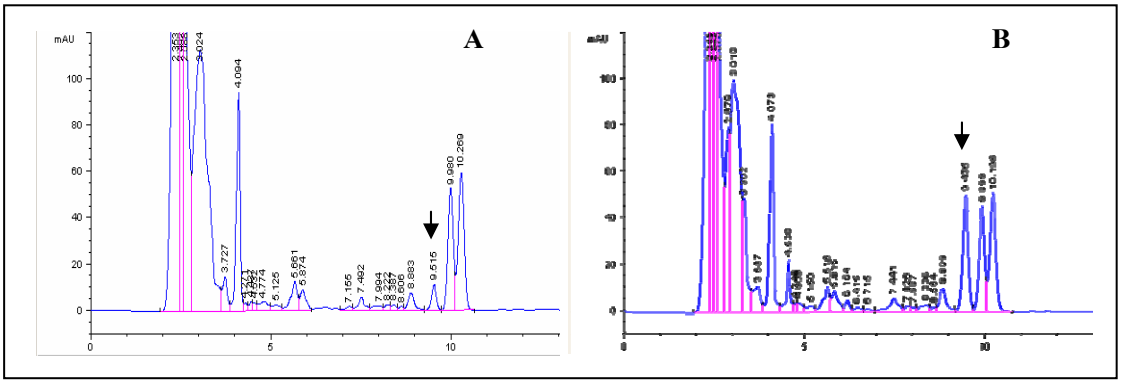


Figure 3-7 HPLC-chromatogram of cell suspension extracts (A) compared with HPLC-chromatogram of cell suspension extract spiking with corosolic acid (B) (The arrow indicated corosolic acid peak.)

3.2 Expression of *oea* gene in tobacco

3.2.1 Cloning *oea* gene into expression vector

The gene *oea* was amplified from pYES2-OEA (Saimaru *et al*., 2007) by PCR using a specific primers; OEA-*Xba*I and OEA-*Spe*I primers. The PCR product yielded *Spe*I site for ligating into pGEM®-T easy and pCAMBIA 2311 vectors. Products were cloned into pGEM®-T easy vector and recombinant plasmid was then digested with *Xba*I and *Spe*I enzymes to release 2.3 kb *oea* gene (Figure 3-8).

The *oea* gene (from pGEM®-T easy-OEA) was ligated into pCAMBIA 2311, at *Xba*I and *Spe*I sites. The pCAMBIA 2311 vector containing double CaMV 35S promoter (2X CaMV 35S), NOS terminator and *npt*II gene for kanamycin resistance. The pCAMBIA 2311-OEA construct was first transformed into *E. coli* strain DH5α. The pCAMBIA 2311-OEA plasmid was checked by digestion with *Xba*I and *Spe*I (Figure 3-9).

The *oea* gene construct was then transformed into *A. tumefaciens* strain C58 by electroporation method. The recombinant DNA was analyzed by PCR using OEA-*Xba*I and OEA-*Spe*I primers for *oea* gene detection (Figure 3-10) and NPTII-F and NPTII-R primers for *npt*II gene detection (Figure 3-11).

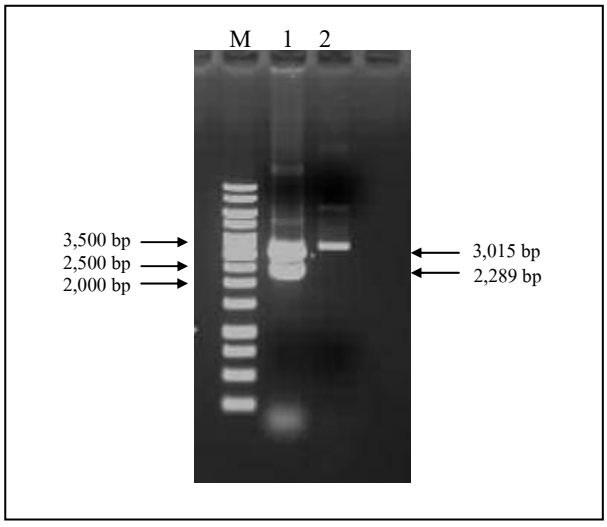


Figure 3-8 Analysis of pGEM® - T easy-OEA by digestion with *Xba*I and *Spe*I.
M = 1-kb DNA ladder (Fermentas, Lithunia), Lane 1 = pGEM® - T easy-OEA digested with *Xba*I and *Spe*I, Lane 2 = uncut pGEM® - T easy-OEA

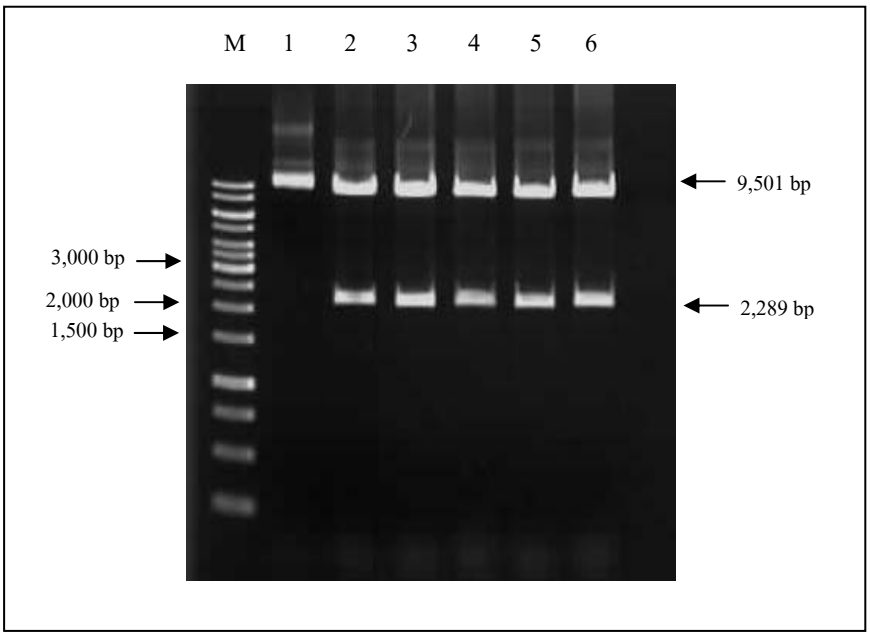


Figure 3-9 Analysis of *oea* gene cloned into plant expression vector pCAMBIA 2311. The recombinants DNA were digested with *Xba*I and *Spe*I enzymes. Lane M = 1-kb DNA ladder (Fermentas, Lithunia), Lane 1 = uncut plasmid DNA, Lane 2-6 = pCAMBIA 2311-OEA digestion with *Xba*I and *Spe*I enzymes

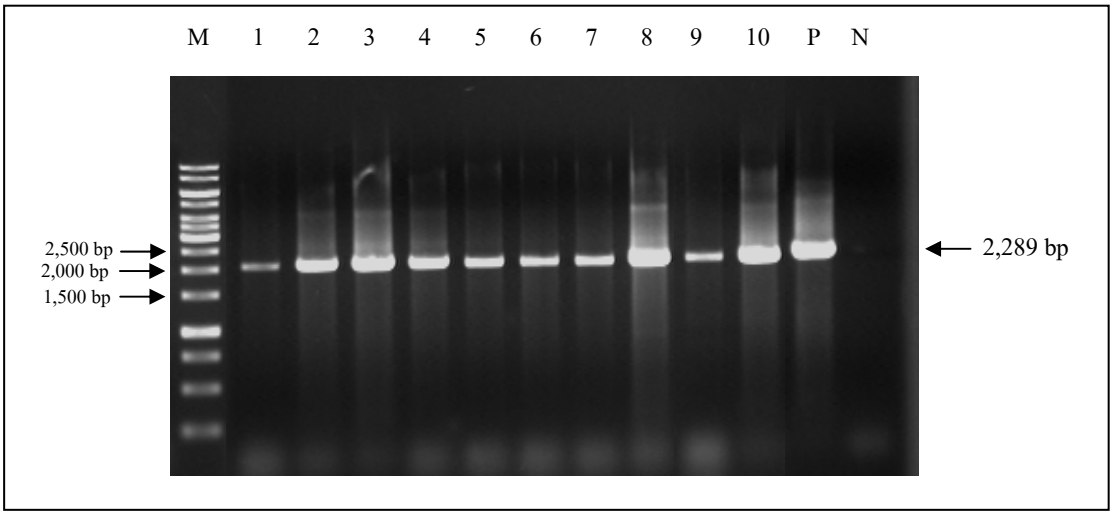


Figure 3-10 Analysis of *oea* gene constructs transformed into *A. tumefaciens* strain C58 by colony direct PCR using OEA-*Xba*I and OEA-*Spe*I priners. Lane M = 1-kb DNA ladder (Fermentas, Lithunia), Lane 1-10 = colony of *A. tumefaciens* harboring pCAMBIA 2311-OEA, P = plasmid pCAMBIA2311-OEA as positive control, N = sterile water as negative control

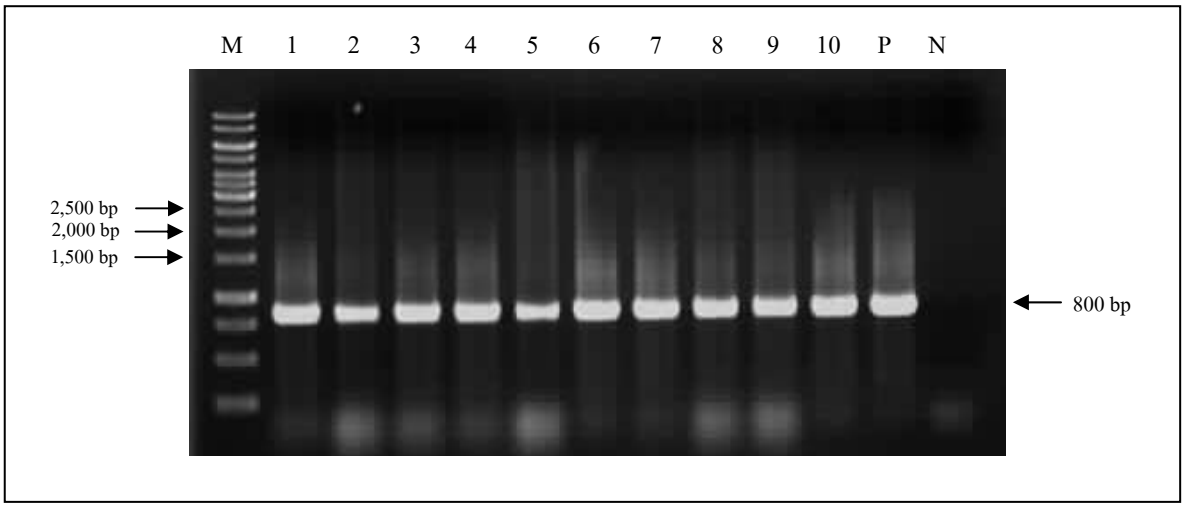


Figure 3-11 Analysis of *npt*II gene constructs transformed into *A. tumefaciens* strain C58 by colony direct PCR using NPTII-F and NPTII-R primers. Lane M = 1-kb DNA ladder (Fermentas, Lithunia), Lane 1-10 = colony of *A. tumefaciens* harboring pCAMBIA 2311-OEA, P = plasmid pCAMBIA2311-OEA as positive control, N = sterile water as negative control

3.2.2 Transformation of *oea* gene into tobacco

3.2.2.1 The sensitivity to antibiotic for selection of transformed cells and plants

Before transformation of *oea* gene into tobacco, the normal tobacco was tested for its sensitivity to kanamycin by culturing the explant in medium containing several concentration of kanamycin. The result showed that kanamycin at the concentration of 50 mg/l was effective enough to completely inhibit growth of wild type leaves (Figure 3-12). This concentration was used entire the transformation experiment.

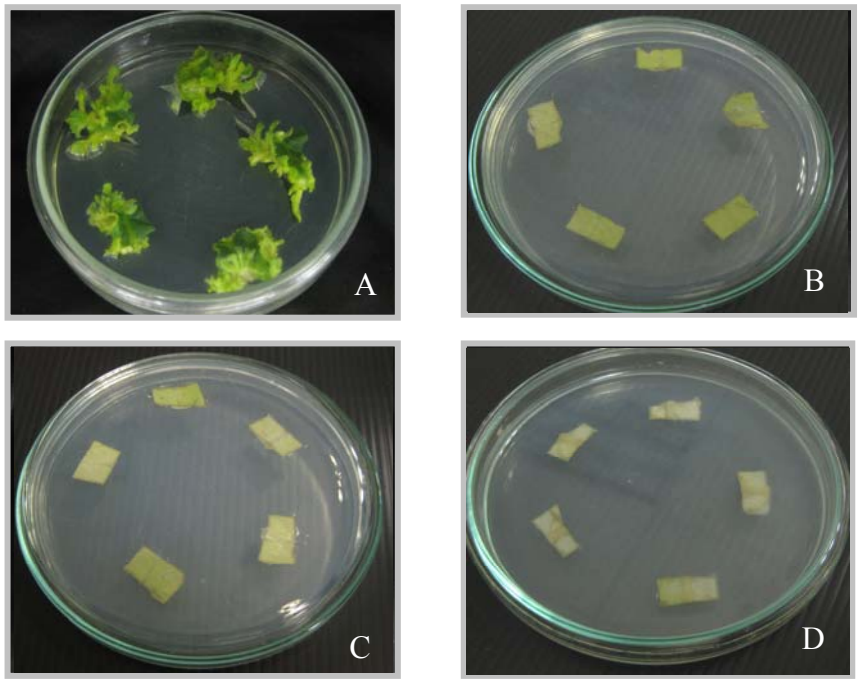


Figure 3-12 Kanamycin sensitivity test for tobacco. Tobacco leaf explants were cultured for 4 weeks on MS-B5 medium supplemented with kanamycin at (A) 0 mg/l, (B) 50 mg/l, (C) 100 mg/l and (D) 200 mg/l kanamycin.

3.2.2.2 Transformation of *oea* gene into tobacco by *A. tumefaciens*

The plasmid pCAMBIA 2311-OEA was transformed into *A. tumefaciens* strain C58 by electroporation method. The *A. tumefaciens* C58 containing pCAMBIA 2311-OEA was cocultivated with tobacco leaf explant for 20-30 min. The infected leaves were then cultured on MS-B5 medium containing 1 mg/l BA and 200 µM acetosyringone (Figure 3-13 A-C). After co-cultivation, infected leaves were transferred to MS-B5 medium containing 1 mg/l BA supplemented with 500 mg/l cefotaxime for suppression of *Agrobacterium* growth. Two weeks after inoculation, the explants were transferred to shoot regeneration medium with 50 mg/ml kanamycin. The cefotaxime was then removed from the media after *A. tumefaciens* was completely suppressed.

The shoot was regenerated from explants after 15 days of culture (Figure 3-13 D-F). The shoots were transferred to rooting medium and cultured for

1 month. Complete tobacco plantlets were acclimatized and kept in greenhouse at 28°C. Young leaves of transformed tobacco were harvest for genomic DNA extraction by CTAB method.

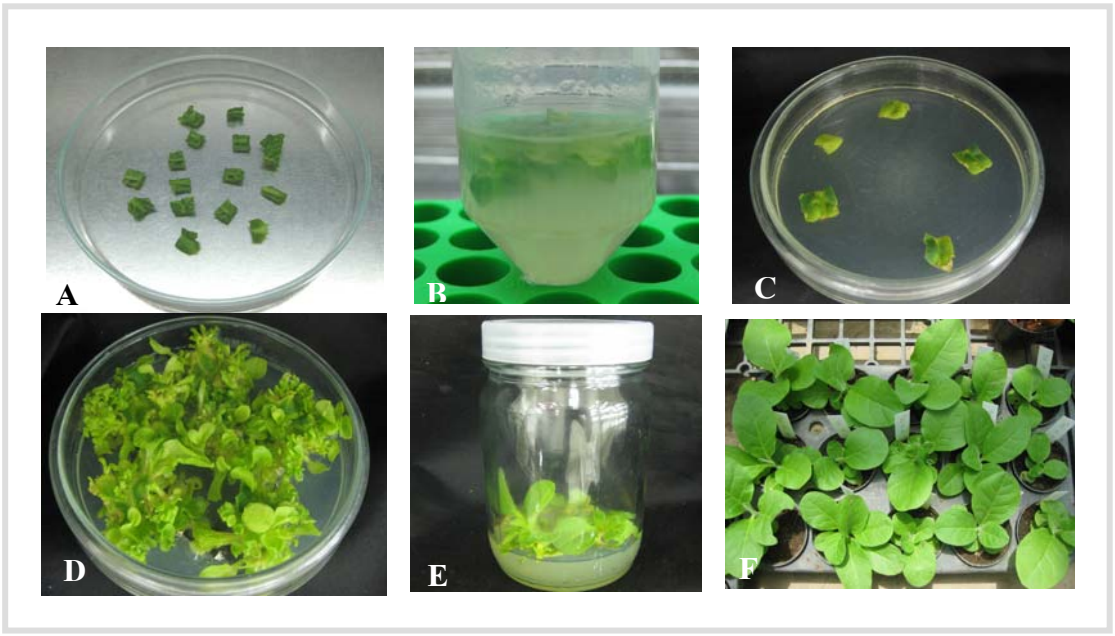


Figure 3-13 Tobacco transformation process: A. Tobacco leaf explants, B. Co-cultivation of tobacco leaves with *A. tumefaciens* haboring *oea* gene, C. Infected leaf explants on MS-B5 medium containing 1 mg/l of BA and 200 µM of acetosyringone, D. Transformed tobacco shoots in shoot regeneration medium, E. Transformed shoots in root regeneration medium, F. Transformed tobacco in sterile soil

3.2.3 PCR analysis of transformed tobacco

The presence of *oea* transgene and 35S-promotor genes in transformed tobacco was investigated by PCR analysis. From 61 transformed tobaccos, thirty-six (line numbers 1-15, 20-21, 23, 25-27, 32-35, 37-40, 43, 53-56, 58 and 60) transgenic lines contained *oea* gene and 35S-promotor (Figure 3-14).

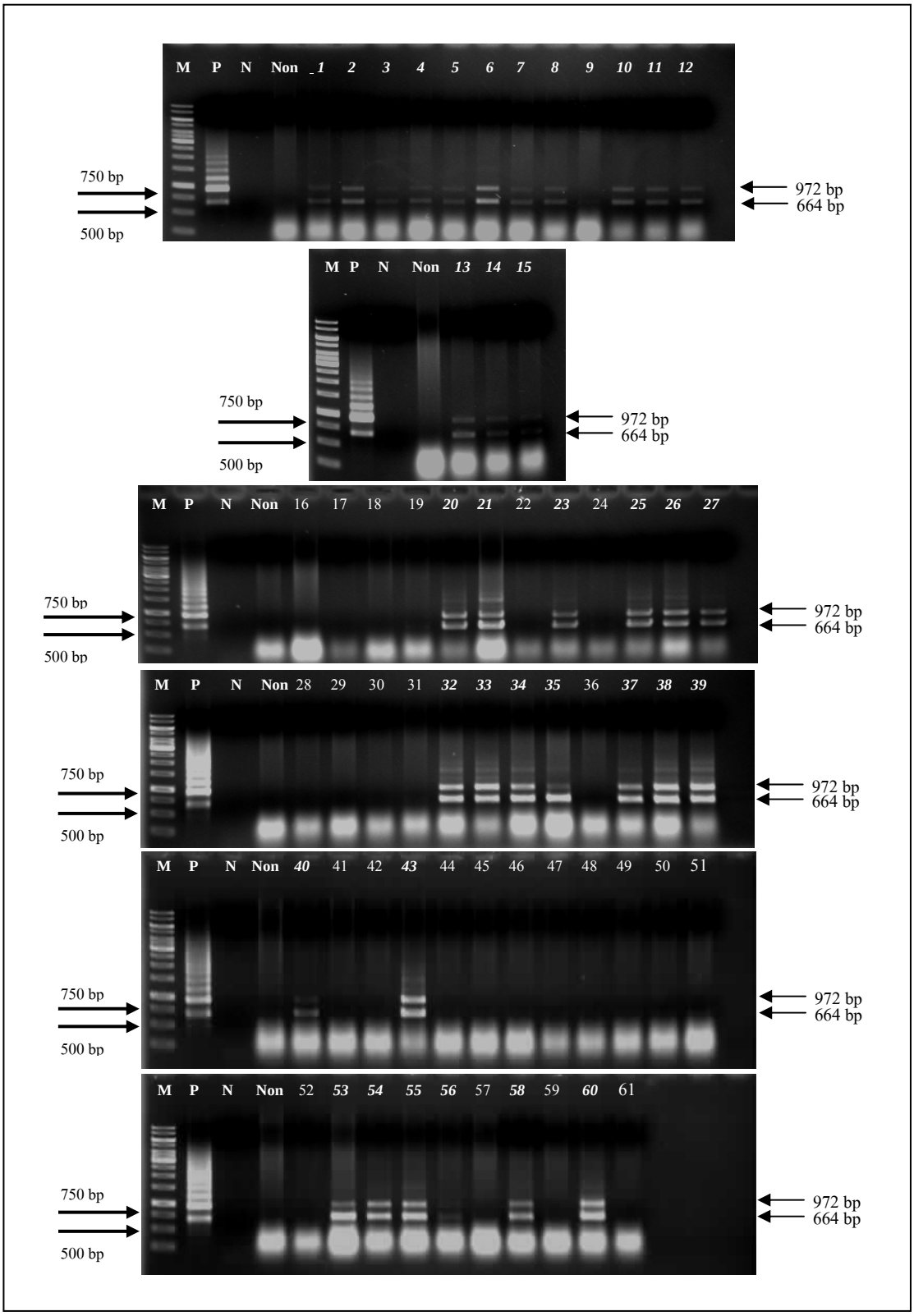


Figure 3-14 Analysis of 35S promoter and *oea* transgene in transformed tobaccos by PCR analysis. M = 1-kb DNA ladder, P = plasmid pCAMBIA 2311-OEA as positive control, N = sterile water as negative control, Non = non-transformed tobacco, 1-61 = transformed tobacco

3.2.4 Southern blot analysis

Southern blot analysis was preformed to determine the copy number of *oea* transgene in tobacco genome. Genomic DNA from each PCR positive transformed tobacco was digested with *Xba*I at the 5′ region of *oea* transgene construct. The copy number of *oea* transgene was determined by hybridization with OEA-DNA probe and by chemiluminescent detection technique useing the CDP-*Star* kit. The DNA was visualized by exposing membrane on the X-ray film. The results shown in Figure 3-15 indicated variable copy numbers of *oea* transgene in each transgenic line ranging from 1 to more than 4 copy numbers. The 30 transgenic lines were obtained from classification of 36 transgenic tobaccos by pattern and copy numbers (Table 3-1).

Table 3-1 The copy numbers of *oea* gene in 36 transgenic tobaccos classified by patterns and copy numbers

Patterns	**Copy number of transgenic lines**				
	1	**2**	**3**	**4**	**≥ 4**
1	14	23	5	1	2
2	21	32	7, 8, 35	3, 4	6
3	54	33	12, 13, 34	9	10
4	-	39	34	11	25
5	-	40	35	15	-
6	-	58	37, 38, 55, 56	20	-
7	-	60	53	26	-
8	-	-	-	27	-
9	-	-	-	43	-

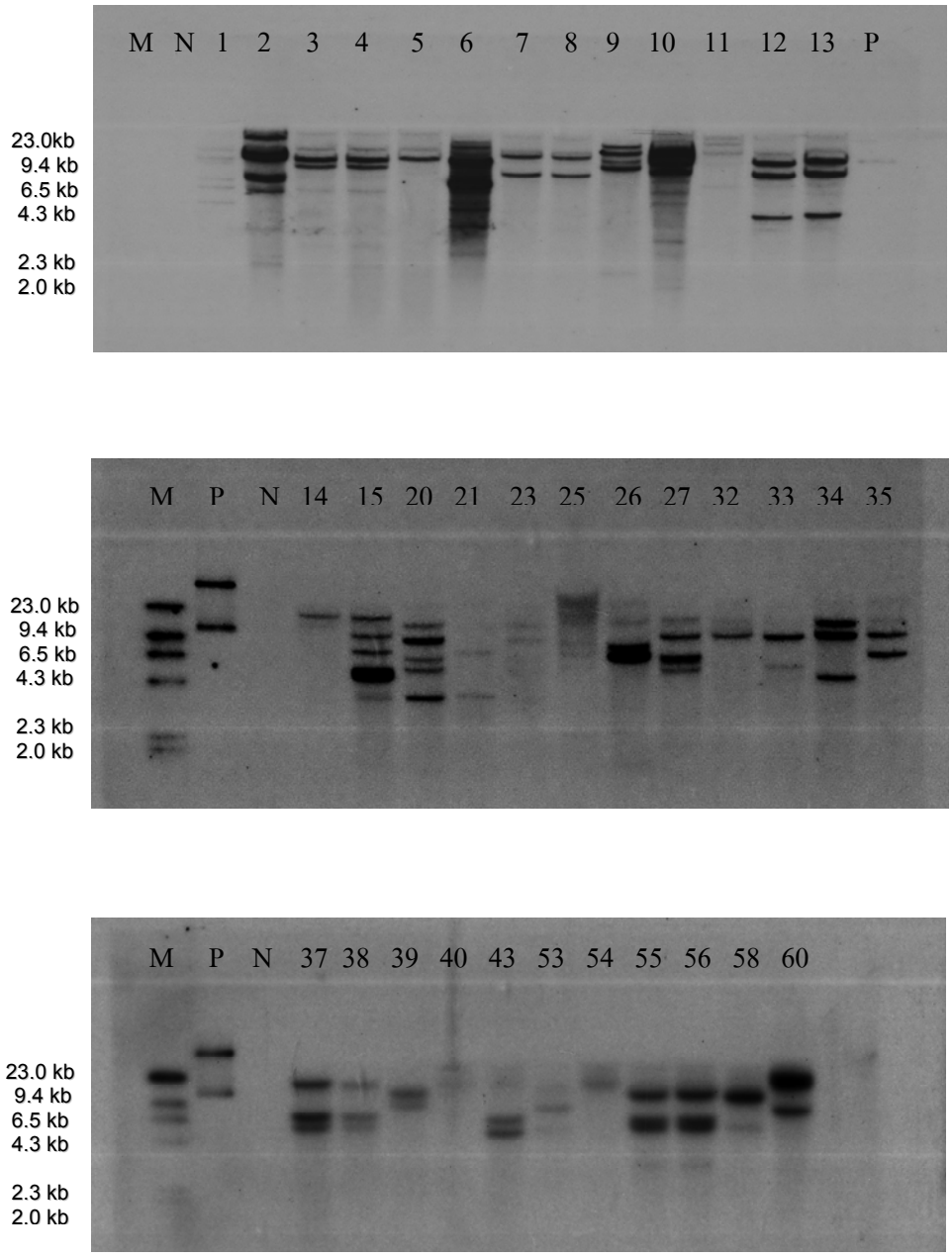


Figure 3-15 Southern blot analysis of transgenic tobacco. M = Dig-labeled molecular marker, P = plasmid pCAMBIA 2311-OEA as positive control, N = Non-transformed tobacco as negative Control, 1-15, 20-21, 23, 25-27, 32-35, 37-40, 43, 53-56, 58, 60 = transgenic lines

CHAPTER 4

DISCUSSIONS

This study consists of two parts involving in the triterpene biosynthesis. In first part (4.1), tissue cultures of *L. speciosa* were established in both callus and cell suspension culture for preliminary study of *in vitro* corosolic acid production. The second part (4.2) focused on transformation of olive oxidosqualene cyclase (*oea*) gene into tobacco. Both parts focused on enhancement of triterpene production in the appropriate sources, either tissue culture or transgenic plants. The corosolic acid is an ursane type triterpene. It is biosynthesized from α-amyrin via ursolic acid. The *oea* gene from olive encoded oxidosqualene cyclase which converted 2,3-oxidosqualene to α- and β-amyrin in a ratio of 2:1 (Saimaru *et al*., 2007). Thus, some *osc* genes in *L. speciosa* might have similar enzyme activity as *oea* gene. Transformation of *oea* gene into *L. speciosa* might reveal biosynthesis of triterpene in this plant. However, transformation into tobacco was carried out in this study instead of transformation in to *L. speciosa*, because of the available of transformation protocol.

4.1 Induction of callus and cell suspension culture and triterpene production

L. speciosa is one of main sources of corosolic acid in nature. The highest accumulation of corosolic acid is in the leaves of this plant (Mallavadhani *et al*., 2008), so the callus induction was carried out using *L. speciosa* leaf explant. From preliminary study, four general media were used for induction of callus. Callus and cell suspension culture of *L. speciosa* grew well in MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin. Corosolic acid production could be observed by TLC analysis and was determined by HPLC anaysis.

In vitro plant cell cultures have provided an alternative for the production of a number of high-value secondary metabolites. So far, hundreds of plant species have been explored for this purpose (Jianfeng *et al*., 1998). Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves or stems. Medium optimizations can lead to an enhancement in secondary metabolite production. However, most trials with plant cell cultures fail to produce the desired products. In such cases, strategies to

improve the production of secondary metabolites must be considered. Information concerning the factors regulating secondary metabolism is as important as the selection of high-producing cell lines in increasing the production of secondary metabolites. A number of physical and chemical factors that could influence secondary metabolism in plant cell cultures have been found. Optimization of the hormone concentration and combinations are often effective; high auxin levels, although good for cell growth, are often deleterious. Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and a condensed biosynthetic cycle. This is the most important advantage of plant cell cultures as model systems for the study of biosynthetic pathways, as secondary metabolite formation can take place within a short cultivation time (Dörnenburg and Knorr, 1995).

The cell suspension culture of *L. speciosa* has been induced for production of corosolic acid. The callus was induced in MS or LS medium containing 10 μM NAA and 10 μM BA (Kim *et al*., 2007). The cell suspension culture was then transferred to B5 liquid medium containing 10 μM NAA and 10 μM BA. Corosolic acid from cell suspension was found to be 56 times higher than natural leaves. In addition, *in vitro* culture of crape myrtle (*L. indica* L.) was studied on the effect of activated charcoal and BA *in vitro* nitrogen uptake in *L. indica.* Explants grown in media supplemented with activated charcoal were able to take up both NO_3^- and NH_4^+, although NH_4^+ uptake was lower. While NH_4^+ uptake was preferential for explants grown in media without activated charcoal (Eymar *et al.,* 2000). As *L. speciosa* is a woody tree species, it is difficult for callus induction or establishment of tissue culture. In general, application of tissue culture technique to woody plants is more difficult than herbaceous plants. Woody plants often secrete polyphenols and tannins, which inhibit growth and development of explants and cause cell necrosis. In addition, contamination becomes a severe problem if the woody explants have systemic infections, since internal contamination cannot be resolved by surface sterilization (Peternel *et al*., 2009).

Moreover, it has been reported on the triterpene production in cell culture. Cell culture of olive (*Olea europaea*) was induced and its triterpene constituents were studied. Six ursane type triterpene acids; ursolic acid, pomolic acid, rotundic acid, tormentic acid, 2α-hydroxyursolic acid and 19α-hydroxyasiatic acid, and two oleanane type acids; oleanolic acid and maslinic acid, have been isolated. Quantity of ursane type triterpene acids produced by cell

cultures was larger than that of oleanane type (Saimaru *et al*., 2007). *Eriobotrya japonica* Lindl is a traditional Chinese medicinal plant that contains triterpenes. They investigated various different culture conditions for cultured cells of loquat to produce triterpenes. When cultured on 2.5 mg/l of BA, 1 mg/l of NAA and 30 g/l of sucrose at 25±2 °C in the dark for 30 days, the nutrient composition significantly regulated the cell growth and triterpene production. Supplied with the MS medium reached higher level of dry weight (1.27±0.09 g per flask) and total triterpene production (151.54±12.58 mg/g of cultured cells), and the B5 medium produced high corosolic acid (Ho *et al*., 2010).

In this study, the induction of callus and cell suspension culture from leaves of *L. speciosa* was successful and they can produce corosolic acid.

4.2 Expression of *oea* gene in tobacco and triterene production

Transformation of olive *oea* gene into *N. tabacum* mediated by *A. tumefaciens* strain C58 has been successful for the first time. Sixty-one transformed tobaccos were obtained from transformation. PCR analysis and Southern blotting analysis showed 36 transgenic tobaccos contained *oea* gene in their genomes. This study is the first report on *osc* (*oea*) gene transformation into tobacco and the method can be applied for transformation of other *osc* genes into other plants including *L. speciosa* for study on gene functions in those plants.

The functional expression of *oea* gene in *Saccharomyces cerevisiae* GIL77 revealed α-amyrin and β-amyrin, in a ratio of 2:1, as two main products of this gene. This related to the presence of ursolic acid (α-amyrin derived triterpene), oleanolic acid (β-amyrin derived triterpene) in olive cell culture (Saimaru *et al*., 2007). Moreover, several *mts* genes have been cloned and functional by expressed in this mutant yeast. They are *psm* from *Pisum sativum*, (Morita *et al*., 2000) At1g78960 (Kushiro *et al*., 2000) and At1g78500 (Ebizuka *et al*., 2003; Shibuya *et al*., 2007) from *Arabidopsis thaliana*, and *KcMS* from *Kandelia candel*, (Basyuni *et al*., 2006). The characteristic of *oea* was similar to *psm* and *KcMS*, which α-amyrin was one of main product from these clones together with other triterpenes monoalcohols. So far, no *osc* yielding α-amyrin as a sole product has been reported.

For the plant system, *A. thaliana* and *N. tabacum* are two model plants used for gene transformation. Several *osc* gene clones from *A. thaliana* have been reported (Ebizuka *et al*.,

2003). The *osc* products from *A. thaliana* could interfere in the presence of triterpenes produced by *osc* of transgene. *N. tabacum,* on the other hands, has no report on the *osc* gene. The latter plant was then chosen as expression host in this study.

The *oea* gene was transformed into *N. tabacum* by *A. tumefaciens* strain C58. Sixty-one transformed tobaccos were obtained from selection on media containing kanamycin. PCR analysis and Southern blotting analysis showed the presence of *oea* gene in genome of 36 transgenic tobaccos with different patterns of copy number in their genome, from 1 to more than 4 copy numbers. The 30 transgenic lines were obtained from classification of 36 transgenic tobaccos by pattern and copy numbers. As size of T-DNA region in tobacco genome approximates 4.3 kb. Then, we regarded only band that has size ranged from 4.3 kb to more than this. For band of T-DNA region, which has small size, might be deletion of T-DNA during inserted into genome.

There are several cases of successful plant transformation. The expression of *Capsicum annuum* RING Zinc Finger Protein 1 (*CaRZFP1*) gene into tobacco was studied by Zeba and his group. The *CaRZFP1* gene, which was ligated into pBKS1-1 vector to construct pBKS1-1-*CaRZFP1* plasmid, was electroporated into the *A. tumefaciens* strain LBA4404 for tobacco (*N. tabacum* L. cv. Wisconsin 38) transformation. The transgenic tobacco exhibits enhanced growth such as larger primary root, more lateral roots and larger hypocotyls and bigger leaves size than the empty vector line (Zeba *et al*., 2009). Several studies were reported on the Southern blot hybridization in transgenic plant. This technique allows investigators to determine the molecular weight of a restriction fragment, measure relative amounts of a DNA sequence in samples and locate a particular sequence within a complex mixture. Normally, in *Agrobacterium*-mediated transformation, T-DNA was inserted randomly into the plant genome (Chilton and Que, 2003). Therefore, we cannot predict the number of T-DNA and positions where they integrated. In the transformation of farnesyl diphosphate synthase (*FDS*) gene into *Artemisia annua* L. via *A. tumefaciens* strain LB4404, Southern blot analysis showed one to two copy numbers of T-DNA (Chen *et al*., 2000). The transformation of β-glucuronidase (*gus*) genes into *Ruta graveolens* L. leaves by *A. tumefaciens* strain C58C1Rif^{R}. Southern blot analysis of transformed plant showed the number of T-DNA ranging from one to three copies (Liévre *et al*., 2005). Embryogenic suspension cultures of *Ipomoea batatas* (L.) Lam. was transformed with the *A. tumefaciens* strain EHA105 harboring a binary vector pCAMBIA1301 with *gus* gene. The

copy number of integrated DNA was analyzed by Southern blot. This result displayed different patterns and the number of integrated T-DNA copies varied from one to four copies (Yu *et al*., 2007). Moreover, *Helianthus annuus* L. embryos were bombarded by microparticles and then co-cultured with disarmed *A. tumefaciens* strain EHA105 bearing a binary vector carrying *gus* gene. Southern blot analysis revealed complex integration T-DNA in transformed plants from two to more than ten copies numbers (Lucas *et al*., 2000).

The transform protocol used in this study show effective transformation of *oea* gene into tobacco. The 36 transgenic tobaccos were obtained from 61 transformed tobaccos. Highly transformed plants were shown in *Ruta graveolens* L. which obtaining 25 transgenic plants from 32 transformed plants (Lievre *et al*., 2005). Etiolated petiole segments of *Cyclamen persicum* Mill. were infected with *A. tumefaciens* strain AGL0 or LBA4404. These strains have a binary vector plasmid, pIG121Hm, which includes the *gus* gene. They obtained 60 regenerates from *Agrobaterium* strain AGL0 and it was examined for GUS activity. The assay showed that 38 plants were GUS-positive. While, transformations by *Agrobaterium* strain LBA4404, they obtained 3 regenerates. The result from GUS assay showed 2 plants were GUS-positive. Therefore, transformation efficiency was higher with AGL0 than with LBA4404 (Aida *et al*., 1999).

CHAPTER 5

CONCLUSIONS

L. speciosa callus and cell suspension cultures were successfully induced from leaves. The best medium for callus induction and cell suspension culture was MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin. The corosolic acid accumulation was determined by TLC and HPLC analysis.

Transformation of olive *oea* gene into *N. tabacum* by *Agrobacterium* has been successful for the first time. Sixty-one transformed tobaccos were obtained from transformation. PCR analysis and Southern blotting analysis showed the presence of *oea* gene in genome of 36 transgenic tobaccos. The 30 transgenic lines were obtained from classification of 36 transgenic tobaccos by pattern and copy numbers. This method can be applied for transformation of other *osc* genes into other plants for study on their gene functions.

REFERENCES

นันทวัน บุณยะประภัศร. 2541. *สมุนไพรไม้พื้นบ้าน เล่ม 2*. กรุงเทพมหานคร: โรงพิมพ์ประชาชน.

Abe, I., Ebizuka, Y. and Sankawa, U. 1988. Purification of 2,3-oxidosqualene: cycloartenol cyclase from pea seedlings. *Chemical and Pharmaceutical Bulletin.* 36: 5031-5034.

Abe, I., Sankawa, U. and Ebizuka, Y. 1989*a*. Purification of 2,3-oxidosqualene: β-amyrin cyclase from pea seedling. *Chemical and Pharmaceutical Bulletin.* 37: 536-538.

Abe, I., Ebizuka, Y., Seo, S. and Sankawa, U. 1989*b*. Purification of 2,3-epoxide cyclases from cell suspension cultures of *Rabdosia japonica* Hara. *FEBS Letters*. 249: 100-104.

Abe, I., Bai, M., Xiao, X.Y. and Prestwich, G.D. 1992*a*. Affinity labeling of vertebrate oxidosqualene cyclases with a tritiated suicide substrate. *Biochemical and Biophysical Research Communications*. 187: 32-38.

Abe. I., Sankawa, U. and Ebizuka, Y. 1992*b*. Purification and properties of squalene-2,3-epoxide cyclases from pea seedlings. *Chemical and Pharmaceutical Bulletin.* 40: 1755-1760.

Abe, I., Rohmer, M. and Prestwich, G.D. 1993. Enzymatic cyclization of squalene and oxidos-qualene to sterols and triterpenes. *Chemical Reviews*. 93: 2189-2206.

Aida, R., Hirose, Y., Kishimoto, S. And Shibata, M. 1999. *Agrobacterium tumefaciens*–mediated transformation of *Cyclamen persicum* Mill. *Journal of Plant Science*. 148: 1-7.

Badami, R. and Kudari, S.M. 1970. Component acids of *Lagerstroemia speciosa* seed oil. *Indian Journal of Applied Chemistry*. 33: 213-215.

Bai, N., He, K., Roller, M., Zheng, B., Chen, X., Shao, Z., Peng, T. and Zheng, O. 2008. Active compounds from *Lagerstroemia speciosa*, insulin-like glucose uptake-stimulatory/

inhibitory and adipocyte differentiation-inhibitory activities in 3T3-L1 Cells. *Journal of Agricultural and Food Chemistry*. 56: 11668-11674.

Basyuni, M., Oku, H., Inafuku, M., Baba, S., Iwasaki, H., Oshiro, K., Okabe, T., Shibuya, M., and Ebizuka, Y. 2006. Molecular cloning and functional expression of a multifunctional triterpene synthase cDNA from a mangrove species *Kandelia candel* (L.) Druce. *Phytochemistry*. 67: 2517-2524.

Chen, D.H., Ye, H.C. and Li, G.F. 2000. Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. *Journal of Plant Science.* 155: 179-185.

Chilton, M.D.M. and Que, Q. 2003. Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: New insights on the mechanism of T-DNA integration. *Plant Physiology*. 133: 956-965.

Collins, M.A. and Charles, H.P. 1987. Antimicrobial activity of carnosol and ursolic acid: Two antioxidant constituents of *Rosmarinus officinalis* L. *Journal of Food Microbiology*. 4: 311-315.

Corey, E.J. and Matsuda, S.P.T. 1991. Purification of 2,3-oxidosqualene-lanosterol cyclase from *Sacchromyces cereviceae*. *Journal of the American Chemical Society USA*. 113: 8172-8174.

Corey, E.J., Matsuda, S.P.T. and Bartel, B. 1993. Isolation of an *Arabidopsis thaliana* gene encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromatographic screen. *Proceedings of the National Academy of Sciences.* 90: 11628-11632.

Darr, L.B., Godzina, S.M. and Matsuda, S.P.T. 1999. A rice cDNA (Accession No. AF169966) similar tocycloartenol synthase. *Journal of Plant Physiology*. 121: 686.

Dat, N.T., Cai, X.F., Rho, M-C., Lee, H.S., Bae, K. and Kim, Y.H. 2005. The inhibition of diacylglycerol acyltransferase by terpeno from *Youngia koidzumiana*. *Archives of Pharmacal Research*. 28: 164-168.

Deocaris, C.C., Aguinaldo, R.R., Ysla, J.L., Asencion, A.S. and Mojica, E.E. 2005. Hypoglycemic activity of irradiated banaba (*Lagerstroemia speciosa* Linn.) leaves. *Journal of Applied Science Research*. 1: 95-98.

Dörnenburg, H. and Knorr, D. 1995. Strategies secondary for the improvement of metabolite production in plant cell cultures. *Enzyme and Microbial Technology*. 17: 674-684.

Duriatti, A. and Schuber, F. 1988. Partial-purification of 2,3-oxidosqualene lanosterol cyclase from hog-liver: Evidence for a functional thiol residue. *Biochemical and Biophysical Research Communications*. 151(3): 1378-1385.

Ebizuka, Y., Katsube, Y., Tsutsumi, T., Kushiro, T. and Shibuya, M. 2003. Functional genomics approach to the study of triterpene biosynthesis. *Pure Applied Chemistry*. 75: 369-374.

Egawa, K. 2001. *Lagerstroemia speciosa* (Banaba). Ota M, *Shokuhin KS II*, Tokyo, Japan. pp. 237-241.

Eymar, E., Alegre, J., Toribio, M. and Lopez-Vela, D. 2000. Effect of activated charcoal and 6-benzyladenine on *in vitro* nitrogen uptake by *Lagerstroemia indica*. *Plant Cell Tissue and Organ Culture.* 63: 57-65.

Faruk, M.J.A., Nahar, N., Aziz, M.A., Mosihuzzaman, M. and Rashid, M.A. 2002. Two new ellagic acids from *Lagerstroemia speciosa* Linn. plant. *Journal of the Bangladesh Chemical Society.* 15: 73-78.

Fluton, T.M., Chunwongse, J. and Tanksley, S.D. 1995. Miniprep protocol for extraction of DNA from tomato and herbaceous plant. *Plant Molecular Report*. 13: 207-209.

Fukushima, M., Matsuyama, F., Ueda, N., Egawa, K., Takemoto, J., Kajimoto, Y., Yonaha, N., Miura, T., Kaneko, T., Nishi, Y., Mitsui, R., Fujita, Y., Yamada, Y. and Seino, Y. 2006. Effect of corosolic acid on postchallenge plasma glucose levels. *Diabetes Research and Clinical Practice*. 73: 174-177.

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experiment Cell Research.* 50: 151-158.

Gilman, E.F. and Watson, D.G. 1998. *Lagerstroemia speciosa* queens crape-myrtle. US Forest Service, Department of Agriculture. Available at: http://hort.ifas.ufl.edu/trees/LAGSP-EA.pdf . Accessed August on 10, 2010.

Grispen, V.M.J., Irtelli, B., Hakvoort, H.W.J., Vooijs, R., Bliek, T., Bookum, W.M., Verkleij, J.A.C. and Schat, H. 2009. Expression of the *Arabidopsis metallothionein* 2b enhances arsenite sensitivity and root to shoot translocation in tobacco. *Environmental and Experimental Botany*. 66: 69-73.

Haralampidis, K., Bryan, G., Qi, X., Papadopoulou, K., Bakht, S., Melton, R. and Osbourn, A. 2001. A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots. *Journal of the American Chemical Society USA*. 98: 13431-13436.

Hattori, K., Sukenobu, N., Sasaki, T., Takasuga, S., Hayashi, T., Kasai, R., Yamasaki, K. and Hazeki, O. 2003. Activation of insulin receptors by lagerstroemin. *Journal of Pharmacological Science*. 93: 69-73.

Hayashi, H.,Hiraoka, N., Ikeshiro, Y., Yazaki, K., Tanaka, S., Kushiro, T., Shibuya, M. and Ebizuka, Y. 1999. Molecular cloning of a cDNA encoding cycloartenol synthase from *Luffa cylindrical* (Accession No. AB033334). *Journal of Plant Physiology*. 121: 1384.

Hayashi, H., Hiraoka, N., Ikeshiro, Y., Yazaki, K., Tanaka, S., Kushiro, T., Shibuya, M. and Ebizuka, Y. 2000*a*. Nucleotide sequence of a cDNA encoding putative oxidosqualene cyclase from *Luffa cylindrica* (Accession No. AB033335) (PGR00-046). *Journal of Plant Physiology*. 122.1459.

Hayashi, H., Hiraoka, N., Ikeshiro, Y., Kushiro, T., Morita, M., Shibuya, M. and Ebizuka, Y. 2000*b*. Molecular cloning and characterization of a cDNA for *Glycyrrhiza glabra* cycloartenol synthase. *Biological and Pharmaceutical Bulletin*. 23: 231-234.

Hayashi, H., Huang, P., Kirakosyan, A., Inoue, K., Hiraoka, N., Ikeshiro, Y., Kushiro, T., Shibuya, M. and Ebizuka, Y. 2001*a*. Cloning and characterization of a cDNA encoding β-amyrin synthase involved in glycyrrhizin and soyasaponin biosyntheses in licorice. *Biological and Pharmaceutical Bulletin*. 24: 912-916.

Hayashi, H., Huang, P., Inoue, K., Hiraoka, N., Ikeshiro, Y., Yazaki. K., Tanaka, S., Kushiro, T., Shibuya, M. and Ebizuka, Y. 2001*b*. Molecular cloning and characterization of isomultiflorenol synthase, a new triterpene synthase from *Luffa cylindrical*, involved in biosynthesis of bryonolic acid. *Europeae Journal of Biochemistry*. 268: 6311-6317.

Hayashi, T., Maruyama, H., Kasai, R., Hattori, K., Takasuga, S., Hazeki, O., Yamasaki, K. and Tanaka, T. 2002. Ellagitannins from *Lagerstroemia speciosa* as activators of glucose transport in fat cells. *Planta Medica.* 68: 173-175.

Herrera, J.B., Bartel, B., Wilson, W. and Matsuda, S.P.T. 1998. Cloning and characterization of the *Arabidopsis thaliana* lupeol synthase gene. *Phytochemistry*. 49: 1905-1911.

Herrera, J.B., Godzina, S.M. and Matsuda, S.P.T. 1999. Cloning and characterization of an *Abies magnifica* cycloartenol synthase cDNA. *EMBJ*, *GenBank*.

Ho, H.Y., Liang, K.Y., Lin, W.C., Kitanaka, S. and Wu, J.B. 2010. Regulation and improvement of triterpene formation in plant cultured cells of *Eriobotrya japonica* Lindl. *Journal of Bioscience and Bioengineering*. 110: 588-592.

Hoshino, T., Williams, H.J., Chung, Y.S. and Scott, A.I. 1991. Partial purification and characterization of oxidosqualene-lanosterol cyclase from bakers-yeast. *Tetrahedron*. 47: 5925-5932.

Hosoyama, H., Sugimoto, A., Suzuki, Y., Sakane, I. and Kakuda, T. 2003. Isolation and quantitative analysis of the α-amylase inhibitor in *Lagerstroemia speciosa* (L.) Pers. (Banaba). *Journal of Pharmacological Science: The Japanese Pharmacological Society.* 123: 599-605.

Iturbe-Ormaetxe, I., Haralampidis, K., Papadopoulou, K. and Osbourn, A.E. 2003. Molecular cloning and characterization of triterpene synthases from *Medicago truncatula* and *Lotus japonicus*. *Plant Molecular Biology*. 51: 731-743.

Jianfeng, X., Zhiguo, S. and Pusun, F. 1998. Suspension culture of compact callus aggregate of *Rhodiola sachalinensis* for improved salidroside production. *Enzyme and Microbial Technology*. 23: 20-27.

Judy, W.V., Hari, S.P., Stogsdill, W.W., Judy, J.S., Naguib, Y.M.A. and Passwater, R. 2003. Antidiabetic activity of a standardized extract (Glucosol™) from *Lagerstroemia speciosa* leaves in Type II diabetics: A dose-dependence study. *Journal of Ethnopharmacology*. 87: 115-117.

Kakuda, T., Sakane, I., Takihara, T., Ozaki, Y., Takeuchi, H. and Kuroyanagi, M. 1996. Hypoglycemic effect of *Lagerstroemia speciosa* L. leaves in genetically diabetic KK-A^Y mice. *Journal of Bioscience Biotechnology and Biochemistry*. 60: 204-208.

Katerere, D.R., Grev, A.I., Nash, R.J. and Waigh, R.D. 2003. Antimicrobial activity of pentacyclic triterpenes isolated from African combretaceae. *Phytochemistry*. 63: 81-88.

Kawano, N., Ichinose, K. and Ebizuka, Y. 2002. Molecular cloning and functional expression of cDNAs encoding oxidosqualene cyclases from *Costus speciosus*. *Biological and Pharmaceutical Bulletin.* 25(4): 477-482.

Kim, C.H., Kim, J.H., Song, J.Y. And Choi, H.J. 2007. The method for production of corosolic acid in suspension culture of plant cells. *Korea Paten No. KR 20050058377 20050630*. 11 Jan 2007.

Klein, G., Kim, J., Himmeldirk, K., Cao, Y. and Chen, X. 2007. Antidiabetes and anti-obesity activity of *Lagerstroemia speciosa*. *Evidence-based Complemetary and Alternative Medicine.* 4: 401-407.

Kongduang, D., Wungsintaweekul, J. and De-Eknamkul, W. 2008. Biosynthesis of β-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspension cultures of *Croton stellatopilosus*. *Tetrahedron Letters.* 49: 4067-4072.

Kusano, M., Abe, I., Ebizuka, Y. and Sankawa,U. 1991. Purification and some properties of squalene-2,3-epoxide: Lanosterol cyclase from rat liver. *Chemical and Pharmaceutical Bulletin.* 18: 195-197.

Kushiro, T., Shibuya, M. and Ebizuka, Y. 1998*a*. β-Amyrin synthase: Cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants. *European Journal of Biochemistry*. 256: 238-244.

Kushiro, T., Shibuya, M. and Ebizuka, Y. 1998*b*. Molecular cloning of oxidosqualene cyclase cDNA from *Panax ginseng*: The isogene that encodes β-Amyrin synthase. In *Towards Natural Medicine Research in the 21st Century*; Ageta, N., Aimi, N., Ebizuka, Y., Fujita, T. and Honda, G., Eds.; Oxford: Elsevier Science. pp 421-427.

Kushiro, T., Shibuya, M., Masuda, K. and Ebizuka, Y. 2000. A novel multifunctional triterpene synthase from *Arabidopsis thaliana*. *Tetrahedron Lett*. 41: 7705-7710.

Liévre, K., Hehn, A., Tran, T.L.M., Gravot, A., Thomasset, B., Bourgauda, F. and Gontier, E. 2005. Genetic transformation of the medicinal plant *Ruta graveolens* L. by an *Agrobacterium tumefaciens*-mediated method. *Journal of Plant Science*. 168: 883-888.

Liu, J. 1995. Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology.* 49: 57-68.

Liu, F., Kim, J., Li, Y., Liu, X., Li, J. and Chen, X. 2001. An extract of *Lagerstroemia speciosa* L. has insulin-like glucose uptake stimulatory and adipocyte differentiation inhibitory activities in 3T3-L1 cells. *Journal of Nutrition.* 131: 2242-2247.

Lloyd, G.B. and Mc Cown, B.H. 1980. Commercially feasible micropropogation of mountain laurel (*Kalmia latilia*) by use of shoot tip culture. *Proc. Int. Plant Prop. Soc*. 30: 421-437.

Lucas, O., Kallerhoff, J. and Alibert, G. 2000. Production of stable transgenic sunflowers (*Helianthus annuus* L.) from wounded immature embryos by particle bombardment and co-cultivation with *Agrobacterium tumefaciens*. *Molecular Breeding*. 6: 479-487.

Mallavadhani, U.V., Mohapatra, S. And Mahapatra, A. 2008. Quantitative analysis of corosolic acid, a Type-II antidiabetic agent, in different parts of *Lagerstroemia speciosa* Linn. *Journal of Planar Chromatography.* 21: 461-464.

Mazumder, M.E.H. and Rahman, S. 2008. Pharmacological evaluation of Bangladeshi medicinal plants for antioxidant activity. *Pharmaceutical Biology.* 46: 704-709.

Miura, T., Itoh, Y., Kaneko, T., Ueda, N., Ishida, T., Fukushima, M., Matsuyama, F. and Seino, Y. 2004. Corosolic acid induces GLUT4 translocation in genetically Type 2 diabetic mice. *Biological and Pharmaceutical Bulletin.* 27: 1103-1105.

Miura, T., Ueda, N., Yamada, K., Fukushima, M., Ishida, T., Kaneko, T., Matsuyama, F. and Seino, Y. 2006. Antidiabetic effects of corosolic acid in KK-Ay diabetic mice. *Biological and Pharmaceutical Bulletin*. 29: 585-587.

Moore, W.R. and Schatzman, G.L. 1992. Purification of 2,3-oxidosqualene cyclase from rat-liver. *Journal of Biological Chemistry*. 267: 22003-22006.

Morita, M., Shibuya, M., lee, M.S., Sangawa, U. And Ebizuka, Y. 1997. Molecular cloning of pea cDNA encoding cycloartenol synthase and its functional expression in yeast. *Biological and Pharmaceutical Bulletin*. 20(7): 770-775.

Morita, M., Shibuya, M., Kushiro, T., Masuda, K. and Ebizuka, Y. 2000. Molecular cloning and functional expression of triterpene synthases from pea (*Pisum sativum*). *European Journal of Biochemistry*. 267: 3453-3460.

Murakami, C., Myoga, K., Kasai, R., Ohtani, K., Kurokawa, T., Ishibashi, S., Dayrit, F., Padolina, W.G. and Yamasaki, K. 1993. Screening of plant constituents for effect on glucose transport activity on Ehrlich ascites tumor cells. *Chemical and Pharmaceutical Bulletin.* 41: 2129-2131.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. 15: 473-497.

Okada,Y., Omae, A. and Okuyama, T. 2003. "A new triterpenoid isolated from *Lagerstroemia speciosa* (L.) Pers.". *Chemical and Pharmaceutical Bulletin.* 51: 452-454.

Peternel, S., Gabrovsek, K., Gogala, N. and Regvar, M. 2009. In vitro propagation of European aspen (*Populus tremula* L.) from axillary buds via organogenesis. *Scientia Horticulturae.* 121: 109-112.

Poehland, B.L., Carto, B.K., Francis, T.A., Hyland, L.J., Allaudeen, H.S. and Troupe, N. 1987. In vitro antiviral activity of dammar resin triterpenoids. *Journal of Natural Product*. 50: 706-713.

Ragasa, C.Y., Ngo, H.T. and Rideout, J.A. 2005. Terpenoids and sterols from *Lagerstroemia speciosa* (L.) Pers. *Chemical and Pharmaceutical Bulletin*. 51: 7-12.

Ravokatra, A., Nigeon-Dureuil, M. and Ratsimamanga, A.R. 1974. Action of a pentacyclic triterpenoid, asiaticoside, isolated from *Hydrocotyle masagascariensis* or *Centella asiatica*, against gastric ulceration in the Wistar rat exposed to cold at 2°C. *CR Hebd Seances Acad Sci D*. 278: 1743-1746.

Ryu, S.Y., Oak, M.H., Yoon, S.K., Cho, D.I., Yoo, G.S., Kim, T.S. and Kim, K.M. 2000. Anti-allergic and anti-inflammatory triterpenes from the herb of *Prunella vulgaris*. *Planta Medica*. 66: 358-360.

Saimaru, H., Orihara, Y., Tansakul, P., Kang, Y-H., Shibuya, M. and Ebizuka, Y. 2007. Production of triterpene acids by cell suspension cultures of *Olea europeae*. *Chemical and Pharmaceutical Bulletin*. 55: 784-788.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Sawai, S., Shindo, T., Sato, S., Kaneko, T., Tabata, S., Ayabe, S. and Aoki, T. 2006. Functional and structural analysis of genes encoding oxidosqualene cyclases of *Lotus japonicus*. *Journal of Plant Science.* 170: 247-257.

Schlaman, H.R.M. and Hooykaas, P.J.J. 1997. Effectiveness of the bacterial gene *codA* encoding cytosine deaminase as a negative selectable marker in *Agrobacterium*-mediated plant transformation. *Plant Journal.* 11: 1377-1385.

Shibuya, M., Zhang, H., Endo, A., Shishikura, K., Kushiro, T. and Ebizuka, Y. 1999. Two branches of the lupeol synthase gene in the molecular evolution of plant oxidosqualene cyclases. *European Journal of Biochemistry*. 266: 302-307.

Shibuya, M., Xiang, T., Katsube, Y., Otsuka, M., Zhang, H. and Ebizuka, Y. 2007. Origin of structural diversity in natural triterpenes: Direct synthesis of *seco*-triterpene skeletons by oxidosqualene cyclase. *Journal of the American Chemical Society*. 129: 1450-1455

Shibuya, M., Katsube, Y., Otsuka, M., Zhang, H., Tansakul, P., Xiang, T. and Ebizuka, Y. 2009. Identication of a product specific β-amyrin synthase from *Arabidopsis thaliana*. *Plant Physiology and Biochemistry*. 47: 26-30.

Sinhababu, A., Das, S., Laskar, S., Thakur, S. And Sen, S.K. 1999. Characterization and identification of antibacterial components in extracts of seeds from *Lagerstroemia speciosa*. *Advance Journal of Food Science and Technology.* 21: 19-22.

Smitinand, T. 2001. *Thai Plant Names*. The Forest Herbarium, Royal Forest Department. Bangkok, Thailand. p. 310.

Sparg, S.G., Light, M.E. and van Staden, J. 2004. Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology*. 94: 219-243.

Sunitha, S., Nagaraj, M. and Varalakshmi, P. 2001. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defense system in cadmium-induced hepatotoxicity in rats. *Fitoterapia*. 72: 516-523.

Suzuki, Y., Unno, T., Ushitani, M., Hayashi, K. and Kakuda, T. 1999. Antiobesity activity of extracts from *Lagerstroemia speciosa* L. leaves on female KK-Ay mice. *Journal of Nutritional Science and Vitaminology (Tokyo)*. 45: 791-795.

Suzuki, H., Achnine, L., Xu, R., Matsuda, S.P.T. and Dixon, R.A. 2002. A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. *Plant Journal*. 32: 1033-1048.

Tabata, M., Tanaka, S., Cho, H.J., Uno, C., Shimakura, J. and Ito, M. 1993. Production of an antiallergic triterpene bryonolic acid, by plant cell cultures. *Journal of Natural Products*. 56: 165-174.

Takahashi, M., Osawa, K., Ueda, J., Yamamoto, F. and Tsai, C.T. 1976. The components of the plants of *Lagertroemia* genus III On the structure of the new tannin “lagertannin” from the leaves of *Lagerstroemia speciosa* (L) Pers. *Yakugaku Zasshi Journal of the Pharmaceutical Society of Japan*. 96: 984-987.

Takahashi, M., Ueda, J. and Sasaki, J.I. 1977. The components of the plants of *Lagerstroemia* genus. IV On the presence of the ellagic acid derivatives from the leaves of *Lagestroemia subcostata* Koehne and *L. speciosa* (L) Pers and synthesis of 3,4-di-*o*-methylellagic acid. *Yakugaku Zasshi Journal of the Pharmaceutical Society of Japan*. 97: 880-882.

Takahashi, M., Osawa, K., Ueda, J. and Yamazaki, J. 1979. The components of the plants of *Lagerstroemia speciosa* (L.) Pers. *Annual Report of the Tohoku College of Pharmacy*. 26: 65-68.

Takagi, S., Miura, T., Ishibashi, C., Kawata, T., Ishihara, E., Gu, Y. And Ishida, T. 2008. Effect of corosolic acid on the hydrolisis of disaccharides. *Journal of Nutritional Science and Vitaminology.* 54: 266-268.

Tanaka, T., Tong, H.H., Xu, Y., Ishimaru, K., Nonaka, G. and Nishioka, I. 1992. Tannins and related compounds. CXVII. Isolation and characterization of three new ellagitannins, lagerstannins A, B, and C, having a gluconic acid core, from *Lagerstroemia speciosa* (L.) Pers. *Chemical and Pharmaceutical Bulletin.* 40: 2975-2980.

Tansakul, P. 2004. Study on triterpene biosynthesis in *Panax ginseng*: cDNA cloning and functional analysis of dammarenediol-II synthase. *Ph.D thesis*. The University of Toyko, Japan.

Tansakul, P., Shibuya, M., Kushiro, T. and Ebizuka, Y. 2006. "Dammarenediol-II synthase, the first dedecated enzyme for ginsenoside biosynthesis, in *Panex ginseng*". *FEBS Letters*. 580: 5143-5149.

Tsutsumi, T. 2003. Functional analysis of triterpene synthase from *Taraxacum officinale*. *Master thesis*. The University of Tokyo, Japan.

Ukiya, M., Akihisa, T., Tokuda, H., Suzuki, H., Mukainaka, T., Ichiishi, E., Yasukawa, K., Kasahara, Y. and Nishino, H. 2002. Constituents of compositae plants: III. Antitumor promoting effects and cytotoxic activity against human cancer lines of triterpene diols and triols from edible *Chrysanthemum* flowers. *Cancer Letters*. 177: 7-12.

Vijaykumar, K., Murthy, P.B., Kannababu, S., Syamasundar, B. and Subbaraju, G.V. 2006. Quantitative determination of corosolic acid in *Lagerstroemia speciosa* leaves, extracts and dosage forms. *International Journal of Applied Science and Engineering*. 4: 103-114.

Walkerpeach, C.R. and Velten, J. 1994. *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. *In: Plant Molecular Biology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 1-19

Xu, R., Fazio, G.C., and Matsuda, S.P.T. 2004. On the origins of triterpenoid skeletal diversity. *Phytochemistry*. 65: 261-291.

Yamada, K., Hosokawa, M., Fujiwara, H., Fujimoto, S., Fujita, Y., Harada, N., Yamada, C., Fukushima, M., Kaneko, T., Matsuyama, F., Yamada, Y., Ueda, N., Seino, Y. and Inagaki, N. 2008. Effect of corosolic acid on gluconeogenesis in rat liver. *Journal of Diabetes Research and Clinical Practice.* 80: 48-55.

Yamaguchi, Y., Yamada, K., Yoshikawa, N., Nakamura, K., Haginaka, J. and Kunitomo, M. 2006. Corosolic acid prevents oxidative stress, inflammation and hypertension in SHR/NDmcr-cp rats, a model of metabolic syndrome. *Journal of Life Sciences*. 79: 2474-2479.

You, S., Kawano, N., Ichinose, K., Yao, X.S. and Ebizuka, Y. 1999. Molecular cloning and sequencing of an *Allium macrostemon* cDNA probably encoding oxidosqualene cyclase. *Plant Biotechnology*. 16: 311-314.

Yu, B., Zhai, H., Wang, Y., Zang, N., He, S. and Liu, Q. 2007. Efficient *Agrobacterium tumefaciens*-mediated transformation using embryogenic suspension cultures in sweetpotato, *Ipomoea batatas* (L.) Lam. *Plant Cell Tissue and Organ Culture*. 90: 265-273

Yun, B.S., Ryoo, I.J., Lee, I.K., Park, K.H., Choung, D.H., Han, K.H. and Yoo, I.D. 1999. Two bioactive pentacyclic triterpene esters from the root bark of *Hibiscus syriacu*s. *Journal of Natural Products*. 62: 764-766.

Zeba, N., Isbat, M., Kwon, N.J, Lee, M.O., Kim, S.R. and Hong, C.B. 2009. Heat-inducible C3HC4 type *RING zinc finger protein* gene from *Capsicum annuum* enhances growth of transgenic tobacco. *Planta.* 229: 861-871.

Zhang, H., Shibuya, M., Yokota, S. and Ebizuka, and Ebizuka, Y. 2003. Oxidosqualene cyclases from cell suspension cultures of *Betula platyphylla* var. *japonica*: Molecular evolution of oxidosqualene cyclases in higher plants. *Biological and Pharmaceutical Bulletin*. 26: 642-650.

Zhang, W.J., Yang, S.S., Shen, X.Y., Jin, Y.S., Zhao, H.J. and Wang, T. 2009. The salt-tolerance gene *rstB* can be used as a selectable marker in plant genetic transformation. *Molecular Breeding.* 23: 269-277.

Zhao, Y.W., Hao, J.G., Bu, H.Y., Wamg, Y.J. and Jia, J.F. 2008. Cloning of *HvBADH1* gene from Hulless Barley and its transformation to tobacco. *Acta Agronomica Sinica.* 34: 1153-1159.

APPENDIX

APPENDIX

- Preparation of Luria-Bertani (LB) medium

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

- dH_2O q.s. 1 L
- Sterile by autoclaving

- Preparation of 50X TAE (Tris-acetate-EDTA)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA	100 ml

- dH_2O q.s. 1 L
- Adjust pH to 8.5

- Preparation of 1X TAE (Tris-acetate-EDTA buffer)

50X TAE	20 ml
dH_2O	980 ml

Table A-1 Inorganic salt and vitamin composition of Murashige and skoog medium (MS) (DUCHEFA BIOCHEMIE B.V.) (Murashige and Skoog, 1962)

Constituent	**Concentration (mg/liter)**
Macronutrients:	
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1,900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Micronutrients:	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_2BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Sucrose	30,000
Vitamins:	
Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine hydrochloride	0.50
Thiamine hydrochloride	0.10
pH	5.8

To prepare the MS medium using the mixed powder 4.4 g/l

Table A-2 Inorganic salt and vitamin composition of Mc Cown Woody Plant medium (WPM) (DUCHEFA BIOCHEMIE B.V.) (Lloyd and Mc Cown, 1980)

Constituent	**Concentration (mg/liter)**
Macronutrients:	
$CaCl_2$	72.50
$Ca(NO_3)_2.4H_2O$	471.26
KH_2PO_4	170.00
K_2SO_4	990.00
$MgSO_4$	180.54
NH_4NO_3	400.00
Micronutrients:	
$CuSO_4.5H_2O$	0.25
FeNaEDTA	36.70
H_3BO_3	6.20
$MnSO_4.H_2O$	22.30
$NaMoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Sucrose	20,000
Vitamins:	
Glycine	2.00
myo-Inositol	100.00
Nicotinic acid	0.50
Pyridoxine hydrochloride	0.50
Thiamine hydrochloride	1.00
pH	5.7

To prepare the WPM medium using the mixed powder 2.5 g/l

Table A-3 Inorganic salt and vitamin composition of Gamborg's B5 medium (B5) (DUCHEFA BIOCHEMIE B.V.) (Gamborg *et al*., 1968)

Constituent	**Concentration (mg/liter)**
Macronutrients:	
$CaCl_2$	113.23
KNO_3	2500.00
$MgSO_4$	121.56
NaH_4PO_4	130.44
$(NH_4)_2SO_4$	134.00
Micronutrients:	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Disodium EDTA	36.70
H_3BO_3	3.00
KI	0.75
$MnSO_4.H_2O$	10.00
$NaMoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	2.00
Sucrose	20,000
Vitamins:	
myo-Inositol	100.00
Nicotinic acid	1.00
Pyridoxine hydrochloride	1.00
Thiamine hydrochloride	10.00
pH	5.5

To prepare the B5 medium using the mixed powder 3.2 g/l

VITAE

Name Miss Prapaporn Witítpanyawong

Student ID 5010720020

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	2005

List of Publication and Proceedings

Proceedings

Prapaporn Witítpanyawong, Orawan Chatchawankanphanich and Pimpimon Tansakul. Expression of oxidosqualene cyclase gene in *Nicotiana tabacum*. Proceeding of the 1st Current Drug Development International Conferrence 2010. Woraburi Resort and Spa, Phuket, Thailand, May 6-8, 2010.