

การโคลนและการแสดงออกของยีนอะมอร์ฟา-4,11-ไดอินซินเทสในชิงเฮา

นายธงชัย กุบ โภกกรวด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาชีวเวชเคมี ภาควิชาชีวเคมี

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2550

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย



4 6 7 6 9 7 0 0 3 3

CLONING AND EXPRESSION OF *AMORPHA-4,11-DIENE SYNTHASE* GENE
IN *ARTEMISIA ANNUA*

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Chemistry

Department of Biochemistry

Faculty of Pharmaceutical Sciences

Chulalongkorn University

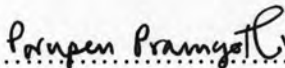
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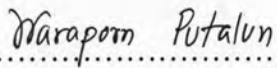
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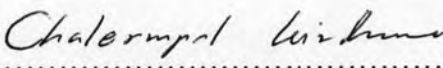
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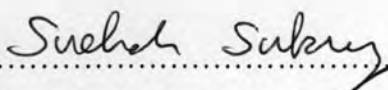
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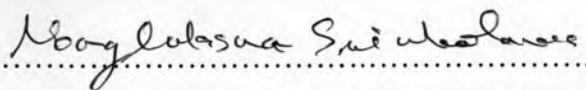
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ในชิงเฮา. (CLONING AND EXPRESSION OF AMORPHA-4,11-DIENE SYNTHASE
GENE IN *ARTEMISIA ANNUA*) อ.ที่ปรึกษา : รศ.ดร. วันชัย ดีเอกนามกุล, อ.ที่ปรึกษา
ร่วม : รศ.ดร. วราภรณ์ ภูตะถุน, 139 หน้า.

Artemisinin เป็นสารต้านโรคมาลาเรีย สกัดจากต้นชิงเฮา (*Artemisia annua* L.) ซึ่งสาร
นี้มีปริมาณน้อยมากในธรรมชาติ งานวิจัยนี้มีเป้าหมายที่จะเพิ่มปริมาณ artemisinin ในต้นชิงเฮา
ให้สูงขึ้นโดยวิธีการฉายรังสีแกมมา การเพาะเลี้ยงเนื้อเยื่อ และการใช้เทคนิคทางชีววิทยาโมเลกุล
โดยมุ่งเป้าที่เอนไซม์ amorpha-4,11-diene synthase (ADS) ซึ่งเกี่ยวข้องในขั้นตอนแรกของวิถี
ชีวสังเคราะห์ของ artemisinin จากการวัดกิจกรรมของเอนไซม์ดังกล่าวในต้นชิงเฮาที่ได้รับการ
ฉายรังสี พบว่ากิจกรรมของเอนไซม์มีความสัมพันธ์กับปริมาณสาร artemisinin ที่พืชสร้างขึ้น ซึ่ง
บ่งชี้ว่าการฉายรังสีแกมมามีผลกระทบต่อยีนที่สร้างเอนไซม์ ADS ในต้นชิงเฮาที่อยู่รอดจากการฉาย
รังสี ทำให้ทั้งเอนไซม์ ADS และ artemisinin มีปริมาณสูงขึ้น นอกจากนี้ยังพบว่า ต้นชิงเฮาที่
อยู่รอดจำนวนหนึ่ง ซึ่งได้รับการปรับสภาพจากหลอดทดลองไปสู่การปลูกในเรือนกระจก และใน
สภาพธรรมชาติ มีปริมาณ artemisinin ในระดับที่ใกล้เคียงกับปริมาณที่พบในแต่ละต้นที่เพาะเลี้ยง
เนื้อเยื่อเริ่มต้น ซึ่งแสดงให้เห็นถึงความคงตัวของการสร้างสาร artemisinin ในต้นชิงเฮาที่ได้รับ
การฉายรังสี นอกเหนือจากการฉายรังสี งานวิจัยนี้ยังได้พยายามเพิ่มปริมาณ artemisinin โดยการ
สร้างพืชดัดแปลงพันธุกรรมของต้นชิงเฮาให้มีการสร้างเอนไซม์ ADS ในระดับที่สูงขึ้น ในส่วนนี้ได้
ดำเนินการโดยการสังเคราะห์ และเพิ่มจำนวนยีน ADS ด้วยเทคนิค RT-PCR ตามด้วยการตัดต่อ
ยีนให้อยู่ภายใต้ plant expression vector แล้วจึงส่งถ่ายยีนเข้าไปในต้นชิงเฮาด้วยเชื้อ *Agrobac-
terium tumefaciens* จากนั้นทำการเหนี่ยวนำให้เซลล์เพาะเลี้ยงเป็นส่วนยอดโดยใช้ฮอร์โมนพืช
ในสัดส่วนจำเพาะ จากต้นชิงเฮาที่ได้รับการถ่ายยีนโดยกระบวนการดังกล่าว เมื่อนำมาตรวจสอบ
การเข้าไปและการคงอยู่ของ ADS ยีน ควบคู่กับการทำงานของเอนไซม์ ADS และปริมาณ arte-
misinin ที่ผลิตขึ้นในต้นดัดแปลงพันธุกรรม พบว่าสามารถตรวจพบ 35s promoter ซึ่งเป็น promoter
ใน plant expression vector ในต้นชิงเฮาที่ได้รับการถ่ายยีน อีกทั้งมีการทำงานของเอนไซม์ ADS
ในระดับที่สูงกว่าต้นชิงเฮาที่ไม่ได้รับการถ่ายยีนถึง 2-3 เท่า นอกจากนี้ การวิเคราะห์ปริมาณสาร
artemisinin ยังพบว่าในต้นชิงเฮาที่ได้รับการถ่ายยีนมีปริมาณ artemisinin เพิ่มขึ้นอยู่ที่ระดับที่
สูงถึง 0.8-1.0 % ของน้ำหนักแห้ง ซึ่งสูงกว่าปริมาณในต้นที่ไม่ได้รับการถ่ายยีนถึง 2-3 เท่าเช่นกัน
การศึกษาดังกล่าวชี้ให้เห็นว่า ทั้งเทคนิคการฉายรังสีแกมมา และการดัดแปลงพันธุกรรมต้นชิงเฮามีผลต่อ
การแสดงออกของยีน ADS ซึ่งทำให้สามารถผลิต artemisinin ในระดับสูงขึ้นในต้นชิงเฮาอันจะ
เป็นประโยชน์ต่อการใช้เป็นแหล่งของสารต้านมาลาเรียต่อไป

ภาควิชา.....ชีวเคมี.....

สาขาวิชา.....ชีวเวชเคมี.....

ปีการศึกษา.....2550.....

ลายมือชื่อนิสิต.....

ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4676970033: MAJOR BIOMEDICINAL CHEMISTRY

KEY WORD: ARTEMISININ / *ARTEMISIA ANNUA* L. / *EX VITRO* PLANT / AMORPHA-4,11-DIENE SYNTHASE / GENE CLONING / EXPRESSION / ENZYME ASSAY

THONGCHAI KOOBKOKKRUAD : CLONING AND EXPRESSION OF AMORPHA-4,11-DIENE SYNTHASE GENE IN *ARTEMISIA ANNUA*.
 THESIS ADVISOR : ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D.,
 THESIS CO-ADVISOR : ASSOC. PROF. WARAPORN PUTALUN, Ph.D.,
 139 pp.

Artemisinin is a new effective antimalarial drug present in the medical plant *Artemisia annua* L. Its content is generally low in cultivated plants. We, therefore, aimed to increase the yield of artemisinin in this plant by using techniques of gamma irradiation, plant tissue culture and molecular biology. Amorpha-4,11-diene synthase (ADS), the enzyme catalyzing the first step of the artemisinin biosynthetic pathway, was the target of this study and its activity was assayed in various γ -ray treated *A. annua* plantlets. Many of these irradiated plantlets showed a strong correlation between the enzyme activity of ADS and the artemisinin content, suggesting that gamma irradiation had significant effects on the ADS gene (*ads*) which is likely to be related to the enhancement of artemisinin content in *A. annua*. Moreover, when some of these selected plantlets were transferred from the *in vitro* culture to greenhouse or open-field, the mature plants obtained also contained artemisinin essentially in a similar content pattern and, interestingly, with observed individual correlation among the selected plantlets. These results suggested that stable high artemisinin-yielding plants of *A. annua* can be obtained by the technique of gamma irradiation. In addition, an attempt to enhance artemisinin content was also performed by creating transgenic plants of *A. annua*. For this approach, the full-length open reading frame of *ads* gene was first amplified by RT-PCR using specific primer and was then inserted into the plant expression vector in *A. annua* via *Agrobacterium tumefaciens*-mediate transformation. By using the process of plant regeneration through transgenic callus to shoot, the transgenic plants of *A. annua* were obtained. These transgenic plants were subsequently detected for the presence of the recombinant *ads* gene, its functional enzyme activity and finally the possible effect on the increase of artemisinin content. The results showed that the shoots of *A. annua* transferred with the 35s promoter were PCR positive. The ADS specific activity assayed by radioisotope method showed its activity in the transgenic plants 2-3 times higher than the untransgenic plant. Interestingly, the production level of artemisinin in the transgenic plant also showed 2-3 times higher than the untransgenic plant. These results indicated that transgenic plant of *A. annua* did contain *ads* gene and could be expressed by the expression vector leading to the apparent high enzyme activity of ADS which, in turn, caused the increase in the overall yield of artemisinin in the transgenic *A. annua* plants.

Department.....Biochemistry.....
 Field of study....Biomedical Chemistry.....
 Academic year.....2007.....

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ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to my advisor, Associate Professor Dr. Wanchai De-Eknamkul of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for his kind, interest, helpful advices, guidance, excellent supervision and encouragement which enable me to carry out my thesis successfully.

I wish to express my grateful thank to my thesis co-advisor, Associate Professor Waraporn Putalun Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, for her kindness, helpful guidance, and encouragement throughout this study.

I wish to express my grateful thank to my thesis committee, Dr. Chalernpol Kirdmanee, Plant Biochemistry and Plant Physio-Biochemistry Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), for his kindness, helpful guidance, and encouragement throughout this study.

I also would like to acknowledge the members of my thesis committee for their valuable times and suggestions.

I wish to express my grateful thank to BIOTEC and the Graduate School, Chulalongkorn University for providing a Graduate Scholarship in Biotechnology. I am indebted to the Graduated School of Chulalongkorn University, and the Ministry of University Affairs, for giving financial supports during the course of my study.

I wish to thank all staff members of the R&D Unit for Herbs and Spices, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their kindness and helps.

I would like to thank all instructors of the Plant Biochemistry and Plant Physio-Biochemistry Laboratory, BIOTEC, NSTDA, for providing equipment, their kindness, and helpful suggestions.

I gratefully acknowledge to all instructors, classmates and personals in the Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their useful assistance.

Finally, I wish to express my infinite thanks and deepest gratitude to my parent for their endless love, care, understanding, supporting and encouragement.

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ABBREVIATIONS

%	= percent (part per 100), percentage
δ	= chemical shift
λ_{max}	= wavelength at maximum absorption
μCi	= microcurie (s)
μg	= microgram (s)
μl	= microliter (s)
μmol	= micromole (s)
$^{\circ}\text{C}$	= degree Celsius
$^{13}\text{C-NMR}$	= Carbon-13 nuclear magnetic resonance
$^1\text{H-NMR}$	= Proton- nuclear magnetic resonance
ADS	= amorphous-4,11-diene synthase
bp	= base-pair
BSA	= bovine serum albumin
cDNA	= complementary deoxyribonucleic acid
Ci	= curie (s)
cm	= centimeter (s)
cpm	= count per minute
DTT	= dithiothreitol
dw	= dry weight (s)
EC	= electrochemical detection
ELISA	= enzyme-linked immunosorbent assay
<i>et al</i>	= et alii
FPP	= farnesyl pyrophosphate
g	= centrifugal force (relative to gravity)
g	= gram (s)
GC	= gas chromatography
h	= hour (s)
HPLC	= high performance liquid chromatography
IPP	= isopentenyl pyrophosphate
L	= liter (s)
m	= meter (s)

mCi	= millicurie (s)
mg	= milligram (s)
mg	= milligram (s)
MgCl ₂	= magnesium chloride
min	= minute (s)
ml	= milliliter (s)
mm	= millimeter (s)
mM	= millimolar (concentration)
MoO ₄	= sodium molybdate
Mops	= 3-(N-morpholino)-propanesulfonic acid
MS medium	= Murashige and Skoog (1962) medium
MS	= mass spectrometry
Na ₂ HPO ₄ ·12H ₂ O	= dihydrogen orthophosphate
NADPH	= β-nicotinamide dinucleotide phosphate (reduced form), tetrasodium salt
NaH ₂ PO ₄ ·H ₂ O	= sodium dihydrogen orthophosphate
ng	= nanogram (s)
nm	= nanometer (s)
NMR	= nuclear magnetic resonance
pH	= The negative logarithm of the concentration of hydrogen ions
R _f	= distance spot moved/distance solvent moved (TLC)
TLC	= thin-layer chromatography
UV	= ultraviolet light
v/v	= volume/volume (concentration)
Vis	= visible light
w/v	= weight/volume (concentration)
w/w	= weigh/weigh (concentration)