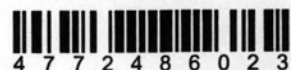


การลดระดับการแสดงออกของรีพอร์เทอร์ยีน โดยการสร้างแฮร์พินอาร์เอ็นเอ  
ในยาสูบ *Nicotiana tabacum* L.

นายวีรากร หอทิมาวรวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต  
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี  
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย  
ปีการศึกษา 2550  
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย



4 7 7 2 4 8 6 0 2 3

REPORTER GENE SILENCING BY PRODUCTION OF HAIRPIN RNA  
IN TOBACCO *Nicotiana tabacum* L.

Mr. Veerakorn Hotimavorakul

A Thesis Submitted in Partial Fulfillment of the Requirements  
for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

Faculty of Science

Chulalongkorn University

Academic Year 2007

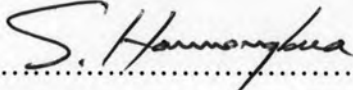
Copyright of Chulalongkorn University

502121


Thesis Title           REPORTER GENE SILENCING BY PRODUCTION OF HAIRPIN  
RNA IN TOBACCO *Nicotiana tabacum* L.  
By                       Veerakorn Hotimavorakul  
Field of Study        Biochemistry  
Thesis Advisor       Assistant Professor Teerapong Buaboocha, Ph.D.

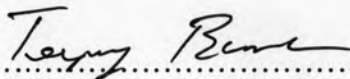
---


Accepted by the Faculty of Science, Chulalongkorn University in Partial  
Fulfillment of the Requirements for the Master's Degree

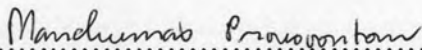
.....  ..... Dean of the Faculty of Science  
(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

.....  ..... Chairman  
(Professor Aran Incharoensakdi, Ph.D.)

.....  ..... Thesis Advisor  
(Assistant Professor Teerapong Buaboocha, Ph.D.)

.....  ..... Member  
(Associate Professor Siriporn Sittipraneed, Ph.D.)

.....  ..... Member  
(Assistant Professor Manchumas Prousoontorn, Ph.D.)

วีรากร หอทิมาวรกุล : การลดระดับการแสดงออกของรีพอร์เตอร์ยีนโดยการสร้างแฮร์พินอาร์เอ็นเอในยาสูบ *Nicotiana tabacum* L. (REPORTER GENE SILENCING BY PRODUCTION OF HAIRPIN RNA IN TOBACCO *Nicotiana tabacum* L.)  
 อ. ที่ปรึกษา : ผศ. ดร.ธีรพงษ์ บัวบูชา, 119 หน้า.

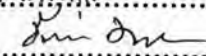
RNA interference (RNAi) เป็นกระบวนการลดระดับการแสดงออกของยีนที่เกิดขึ้นหลังจากการถอดรหัสดีเอ็นเอโดยมีอาร์เอ็นเอสายคู่ (dsRNA) เป็นตัวชักนำ อาร์เอ็นเอสายคู่จะมีลำดับเบสคู่สมกับลำดับเบส mRNA เป้าหมายทำให้เกิดการทำลาย mRNA นั้นในเวลาต่อมา การใช้ RNA interference เพื่อลดระดับการแสดงออกของยีนเป้าหมายเป็นวิธีการที่มีประสิทธิภาพในการศึกษาวิเคราะห์การทำงานของยีน ในการศึกษาครั้งนี้ได้สร้างยาสูบ (*Nicotiana tabacum* L. cv. Virginia Coker) แปลงพันธุ์ที่มีการแสดงออกของยีนเบต้ากลูคูโลนิเดส (*gus*) ลดลง โดยทำการส่งถ่ายยีนที่ให้แฮร์พินอาร์เอ็นเอของยีน *gus* (hpGUS) ภายใต้การควบคุมของโพรโมเตอร์ 35S CaMV โดยอาศัย *Agrobacterium* เข้าสู่ยาสูบทรานเจนิคส์ที่มียีน *gus* แสดงออก จากนั้นคัดเลือกยาสูบแปลงพันธุ์ที่มีความสามารถต้านทานต่อกานามัยซิน นำยาสูบแปลงพันธุ์ที่ได้มาตรวจสอบการแทรกตัวของชิ้นยีน hpGUS ด้วยวิธี PCR โดยใช้จีโนมิกดีเอ็นเอเป็นต้นแบบ พบว่ายาสูบแปลงพันธุ์ที่ได้จากการคัดเลือกมีชิ้นยีน hpGUS จากการส่งถ่ายแทรกอยู่ การตรวจสอบการแทรกตัวของชิ้นยีนในจีโนมยาสูบด้วยวิธี Southern blot analysis พบว่าได้ยาสูบแปลงพันธุ์ 3 สายพันธุ์ซึ่งได้รับการสอดแทรกด้วยชิ้นยีน จากการทำ northern blot analysis พบว่ายาสูบแปลงพันธุ์มีการแสดงออกของยีน *gus* ในระดับ RNA ลดลงเมื่อเปรียบเทียบกับยาสูบชุดควบคุม เมื่อทำการตรวจสอบการแสดงออกในระดับโปรตีนด้วยวิธีทาง histochemical และ spectrophotometric พบว่ายาสูบแปลงพันธุ์มีการแสดงออกของ GUS activity ต่ำกว่ายาสูบชุดควบคุมแสดงให้เห็นว่า hpGUS สามารถลดระดับการแสดงออกของยีน *gus* ในระดับโปรตีนได้

ภาควิชา.....ชีวเคมี...

สาขาวิชา.....ชีวเคมี...

ปีการศึกษา.....2550...

ลายมือชื่อนิสิต.....วีรากร.....หอทิมาวรกุล.....

ลายมือชื่ออาจารย์ที่ปรึกษา..........

# # 4772486023 : MAJOR OF BIOCHEMISTRY

KEY WORD: RNAi / Hairpin RNA /  $\beta$ -glucuronidase / *Nicotiana tabacum* L.

VEERAKORN HOTIMAVORAKUL: REPORTER GENE SILENCING BY  
PRODUCTION OF HAIRPIN RNA IN TOBACCO *Nicotiana tabacum* L.

THESIS ADVISOR: ASSIST. TEERAPONG BUABOOCHA, Ph.D., 119 pp.

RNA interference (RNAi) is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA (dsRNA) molecules that contain nucleotide sequences complementary to target mRNA sequences causing degradation of the latter. It is an effective approach in inhibiting expression of a target gene for gene function analysis. Here, transgenic tobacco plants in which a  $\beta$ -glucuronidase (*gus*) gene was silenced were generated by introducing a *gus* hairpin (hpGUS) sequence into a transgenic tobacco (*Nicotiana tabacum* L. cv. Virginia Coker) harboring a *gus* gene by *Agrobacterium*-mediated transformation. Then, transgenic tobaccos were selected on MS media containing kanamycin. All putative independent lines were confirmed by PCR amplification for the presence of the hpGUS construct using their genomic DNA as template. Three independent lines of the hpGUS-expressing double transformants were obtained from the regeneration of kanamycin-resistant cells and confirmed by Southern blot analysis. Northern blot analysis showed silencing of the *gus* transcript in all independent lines as compared with the control plants. The reduction of *gus* mRNA level was confirmed by histochemical and spectrophotometric GUS assay. GUS activity of the hpGUS-expressing double transformants was lower than the control double transformants indicating that hpGUS can silence expression of the *gus* gene at the protein level.

Department.....Biochemistry...

Field of study....Biochemistry...

Academic year...2007.....

Student's signature. *Veerakorn Hotimavorakul*.....

Advisor's signature. *Teerapong Buaboocha*.....

## ACKNOWLEDGEMENT

The completion of this thesis would not have been possible without the support and assistance of the people around me.

I thank my thesis advisor Assistant Professor Dr. Teerapong Buaboocha, for his support, guidance, and inspirations for excellence in science and great personality.

Sincere thanks and appreciation are due to Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Siriporn Sitthipraneed, Assistant Professor Dr. Manchumas Prousoontorn who serve as the members of the master thesis committees, for their helpful suggestion and comments.

I am most grateful to all members in Dr. Teerapong's laboratory, Biochemistry department for their assistance and encouragement, especially Srivilai Phean-o-pas, for her patience and guidance in carrying out various experiment. Thanks also to Santhana Nakapong, Bongkoj Boonburapong and Mayura Thongchuang for their constant support and advice.

Last but not least, I would like to extend my appreciation and sincere thanks to all that had helped me in one way or another during the course of the research

## CONTENTS

	<b>Page</b>
ABSTRACT (Thai).....	iv
ABSTRACT (English).....	v
ACKNOWLEDGMENT.....	vi
CONTENTS.....	vii
LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xiv
LIST OF ABBREVIATIONS.....	xvii
CHAPTER I INTRODUCTION.....	1
RNA interference (RNAi) in plants.....	1
Mechanism of RNA interference.....	1
Different classes of RNAs.....	2
Hairpin RNA (hpRNA).....	4
Dicer.....	6
RNA-induced silencing complex (RISC).....	6
RNAi as tool for gene function analysis.....	6
Use of transgenic plants for characterizing gene function.....	11
Reporter gene.....	15
$\beta$ -glucuronidase gene (GUS).....	16
Objective of this research.....	17
CHAPTER II MATERIALS AND METHODS.....	20
2.1 Materials.....	20
2.1.1 Plant.....	20
2.1.2 Instruments.....	20

	<b>Page</b>
2.1.3 Materials.....	21
2.1.4 Chemicals and reagent.....	22
2.1.5 Enzymes.....	25
2.1.6 Kit and plasmids.....	25
2.1.7 Radioactive.....	25
2.1.8 Antibiotics.....	25
2.1.9 Oligonucleotide primers.....	26
2.1.10 Microorganisms.....	27
2.2 Bacterial growth medium.....	26
2.3 Methods.....	27
2.3.1 Preparation of <i>gus</i> fragment .....	27
2.3.1.1 Primer design.....	27
2.3.1.2 PCR amplification.....	27
2.3.1.3 Agarose gel electrophoresis.....	30
2.3.1.4 Extraction of DNA fragment from agarose gel.....	31
2.3.2 Cloning of <i>gus</i> fragment into pGEM <sup>®</sup> -T vector.....	32
2.3.2.1 Ligation of <i>gus</i> fragment to pGEM <sup>®</sup> -T vector.....	32
2.3.2.2 Transformation of <i>E. coli</i> host cells with the ligated products by electroporation .....	32
a) Preparation of <i>E. coli</i> strain Top10 competent cells.....	32
b) Electroporation of recombinant DNA.....	33
2.3.2.3 Plasmid DNA isolation by alkaline lysis method.....	34
2.3.2.4 Restriction enzyme analysis.....	35
2.3.3 Construction of <i>gus</i> inverted repeat.....	35



	<b>Page</b>
2.3.3.1 The first construction step.....	35
a) Preparation of <i>gus</i> fragment for the sense cloning	
Orientation.....	35
b) Preparation of pFGC5941 for the sense cloning	
Orientation.....	37
c) Ligation of the first <i>gus</i> sequence into pFGC5941.....	37
d) Transformation of <i>E. coli</i> host cells with the ligation	
mixture by electroporation.....	38
2.3.3.2 The second construction step.....	38
a) Preparation of <i>gus</i> fragment for the antisense cloning	
Orientation.....	38
b) Preparation of pFGC5941 for the antisense cloning	
Orientation.....	39
c) Ligation of the second <i>gus</i> sequence into pFGC5941....	40
d) Transformation of <i>E. coli</i> host cells with the ligation	
mixture by electroporation.....	40
2.3.3.3 Restriction analysis of pFGC5941-hpGUS.....	40
2.3.3.4 Sequence analysis of pFGC5941-hpGUS.....	41
2.3.4 Construction of plant expression vector for silencing <i>gus</i> gene.....	41
2.3.5 Plant transformation.....	42
2.3.5.1 Preparation of Tobacco leaf discs.....	42
2.3.5.2 Transformation of <i>A. tumefaciens</i> EHA105 host cells	
with pCAMBIA2300 and pCAMBIA2300-hpGUS by	
electroporation.....	43

	<b>Page</b>
a) Making competent <i>A. tumefaciens</i> strain EHA105	
Cells.....	43
b) Electrotransformation of <i>A. tumefaciens</i> EHA105.....	43
2.3.5.3 Co-cultivation of tobacco leaf discs.....	44
2.3.5.4 Selection and regeneration of transformed tobacco.....	44
2.3.6 Molecular analysis of transformed plants.....	45
2.3.6.1 Analysis of genomic DNA of the transformed tobacco	
plants by PCR.....	45
a) Genomic DNA extraction.....	45
b) PCR amplification of <i>gus</i> and <i>hpGUS</i> in the transformed	
tobacco plants.....	46
2.3.6.2 Southern Blot Analysis.....	47
a) Digestion of genomic DNA with restriction enzymes.....	47
b) Agarose gel electrophoresis.....	47
c) Southern blotting.....	48
d) Preparation of probes.....	49
e) Non-radioactive DIG hybridization.....	50
2.3.6.3 Northern blot analysis.....	51
a) Total RNA extraction.....	51
b) Formaldehyde-agarose gel electrophoresis.....	52
c) Northern blotting.....	53
d) Preparation of probes.....	53
e) Northern blot hybridization and autoradiography.....	54
2.3.7 Assays of $\beta$ -glucuronidase (GUS) activity.....	56

	<b>Page</b>
2.3.7.1 Histochemical analysis for $\beta$ -Glucuronidase (GUS) activity.....	56
2.3.7.2 Spectrophotometric analysis for $\beta$ -glucuronidase (GUS) activity.....	56
a) Crude enzyme extraction.....	56
b) Protein measurement.....	56
c) Determination of $\beta$ -glucuronidase activity.....	57
CHAPTER III RESULTS AND DISCUSSION.....	58
3.1 Preparation of <i>gus</i> fragment.....	58
3.2 Construction of <i>gus</i> inverted repeat.....	60
3.3 Construction of plant expression vector for silencing a <i>gus</i> gene.....	70
3.4 Plant transformation.....	74
3.5 PCR analyses of transgenic tobacco plants.....	76
3.6 Southern blot analysis of hpGUS-expressing double transformants.....	78
3.7 Northern blot analysis of transgenic tobacco plants.....	83
3.8 $\beta$ -glucuronidase (GUS) activity of transgenic tobacco plants.....	86
3.8.1 Histochemical analysis of GUS expression in double transformants..	86
3.8.2 GUS spectrophotometric analysis in double transformants.....	87
CHAPTER IV CONCLUSION.....	95
REFERENCES.....	97
APPENDICES.....	104
APPENDIX A.....	105
APPENDIX B.....	108
APPENDIX C.....	110

	<b>Page</b>
APPENDIX D.....	116
APPENDIX E.....	117
APPENDIX F.....	118
BIOGRAPHY.....	119

## LIST OF TABLES

Table	Page
3.1 GUS activity from crude extract of the control and the hpGUS-expressing double transformants.....	91

## LIST OF FIGURES

Figure	Page
1.1 The mechanism of RNA interference.....	3
1.2 Diagram processing of ssRNA into hpRNA.....	5
1.3 Diagram representing DNA-directed RNAi.....	7
1.4 Diagram processing of dsRNA into siRNA by Dicer.....	8
1.5 Structure of RNA-induced silencing complex (RISC).....	9
1.6 Basic step in the transformation of plants cells by <i>A. tumefaciens</i> .....	13
1.7 $\beta$ -glucuronidase specifically hydrolyzes $\beta$ -linked D-glucuronides to D-glucuronic acid and aglycone.....	18
2.1 The coding sequence and the deduced amino acid sequence of <i>gus</i> gene.....	28
2.2 Production of the hpGUS RNAi cassette.....	36
3.1 Agarose gel electrophoresis of the amplified fragment of the <i>gus</i> gene.....	59
3.2 Agarose gel electrophoresis of recombinant plasmid pGEM- <i>gus</i> digested with <i>NcoI</i> and <i>SpeI</i> .....	61
3.3 Agarose gel electrophoresis of recombinant plasmid pGEM- <i>gus</i> digested with <i>AscI</i> and <i>SwaI</i> .....	62
3.4 Purified products of the <i>gus</i> fragment and the pFGC5941.....	63
3.5 Agarose gel electrophoresis of recombinant plasmid pFGC5941-sGUS digested with <i>AscI</i> and <i>SwaI</i> .....	64
3.6 Agarose gel electrophoresis of recombinant plasmid pGEM- <i>gus</i> digested with <i>BamHI</i> and <i>XbaI</i> .....	66
3.7 Purified products of the <i>gus</i> fragment and the pFGC5941-sGUS.....	67

<b>Figure</b>	<b>Page</b>
3.8 Agarose gel electrophoresis of recombinant plasmid pFGC5941-hpGUS digested with <i>Bam</i> HI and <i>Xba</i> I.....	68
3.9 Agarose gel electrophoresis of recombinant plasmid pFGC5941-hpGUS digested with <i>Eco</i> RI and <i>Pst</i> I .....	69
3.10 Nucleotide sequence of hpGUS in the pFGC5941-hpGUS plasmid....	71
3.11 Schematic diagram of the hpGUS cassette of pFGC5941-hpGUS vector	72
3.12 Purified products of the hpGUS cassette and the pCAMBIA2300-hpGUS	73
3.13 Agarose gel electrophoresis of recombinant plasmid pCAMBIA2300-hpGUS digested with <i>Eco</i> RI and <i>Pst</i> I.....	75
3.14 Leaf discs transformation and regeneration of <i>Nicotiana tabacum</i> L. Virginia Coker.....	77
3.15 PCR analysis of <i>gus</i> gene and hpGUS construct insertion in the genome of six putative independent lines of the hpGUS-expressing and the control double transformants.....	79
3.16 Agarose gel electrophoresis of the amplified fragments of the <i>npt</i> II gene.....	81
3.17 Genomic DNA blot analysis of three putative independent lines of the hpGUS -expressing double transformants.....	82
3.18 RNA blot analysis of <i>gus</i> gene in three putative independent lines of the control double transformant and three independent lines of the hpGUS-expressing double transformants.....	85
3.19 Histochemical analysis of GUS activity in leaf and root of the hpGUS-expressing double transformants.....	88

<b>Figure</b>	<b>Page</b>
3.20 Graphs between absorbance at 415 nm monitoring the release of <i>p</i> -nitrophenol and the incubation time of the extract from each of six transgenic tobacco leaves .....	90
3.21 Histograms showing GUS activity levels of all six transgenic tobacco plants.....	92
3.22 Histograms showing the comparison of the average specific activity of GUS between the control plants and each line of hpGUS-expressing plants.....	94



## LIST OF ABBREVIATIONS

A	absorbance, 2'-deoxyadenosine (in a DNA sequence)
bp	base pairs
C	2'-deoxycytidine (in a DNA sequence)
°C	degree Celsius
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
G	2'-deoxyguanosine (in a DNA sequence)
g	gram
GUS	β-glucuronidase
hpGUS	hairpin GUS
hr	hour
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-stranded nucleic acid
KCl	potassium chloride
kDa	kiloDalton
KOH	potassium hydroxide
l	liter
LB	Luria-Bertani
Mg <sup>2+</sup>	magnesium ion
μg	microgram
μl	microliter
μM	micromolar
M	mole per liter (molar)
mA	milliampere
mg	milligram
min	minute
ml	milliliter

mM	millimolar
MW	molecular weight
N	normal
ng	nanogram
NH <sub>4</sub> Cl	ammonium chloride
NH <sub>4</sub> OH	ammonium hydroxide
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
pmol	picomole
PNPG	<i>p</i> -nitrophenyl- $\beta$ -D-glucuronide
RNA	ribonucleic acid
RNase	ribonuclease
Rpm	revolution per minute
SDS	sodium dodecyl sulfate
T	2'-deoxythymidine (in a DNA sequence)
Tris	tris (hydroxyl methyl) aminomethane
UV	ultraviolet
V	voltage
v/v	volume by volume
vir	virulence
w/w	weight by weight
X-Gluc	5-Bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid