FUNCTIONAL GENOMIC OF RICE (*Oryza sativa* L.)

β-GLUCOSIDASE

Wipaporn Wanthanalert

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2009

บทบาทหน้าที่ของยีนเบต้ากลูโคซิเดสในข้าว

นางสาววิภาภรณ์ วรรณธนาเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2552

FUNCTIONAL GENOMIC OF RICE (*Oryza sativa* L.)

β-GLUCOSIDASE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

_____

(Asst. Prof. Dr. Chokchai Wanapu)

Chairperson

_____

(Asst. Prof. Dr. Mariena Ketudat-Cairns)

Member (Thesis Advisor)

_____

(Assoc. Prof. Dr. James Ketudat-Cairns)

Member

_____

(Asst. Prof. Dr. Rodjana Opassiri)

Member

_____

(Dr. Porntip Atichart)

Member

_____

(Prof. Dr. Pairote Sattayatham)

Vice Rector for Academic Affairs

_____

(Asst. Prof. Dr. Suwayd Ningsanond)

Dean of Institute of Agricultural Technology

วิภาภรณ์ วรรณธนาเลิศ : บทบาทหน้าที่ของยีนเบต้ากลูโคซิเดสในข้าว (FUNCTIONAL GENOMIC OF RICE (*Oryza sativa* L.) β-GLUCOSIDASE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 162 หน้า

การวิจัยนี้มีเป้าหมายเพื่อศึกษาหน้าที่ของยีนเบต้ากลูโคซิเดสจำนวนห้ายีน คือ *Os1bglu1 Os3bglu7 Os3bglu8 Os7bglu26* และ *Os12bglu38* ด้วยเทคนิค RNAi ในการทดลองนี้ได้แบ่งการยับยั้งการแสดงออกของยีนเบต้ากลูโคซิเดสเป็นสองกลุ่ม กลุ่มแรกเป็นการยับยั้งการแสดงออกของเบต้ากลูโคซิเดสทั้งห้ายีนพร้อมกันโดยใช้ส่วนของ coding region ของ *Os3bglu7* มีความคล้ายกับยีนอื่นมากที่สุด กลุ่มที่สองเป็นการยับยั้งการแสดงออกของแต่ละยีนโดยใช้ส่วนของ 3'UTR ซึ่งมีความเฉพาะเจาะจงกับยีนเบต้ากลูโคซิเดสแต่ละตัว ยีนเป้าหมายถูกใส่เข้าไปในเวคเตอร์ pHELLSGATE8 และถูกส่งเข้าไปในแคลลัสของข้าวโดยใช้อะโกรแบคทีเรียมเพื่อสร้างอาร์เอ็นเอสายคู่ในข้าว

การชักนำการเกิดแคลลัสบนอาหาร N6D เป็นระยะเวลา 4 ถึง 6 สัปดาห์ ที่อุณหภูมิ 28 องศาเซลเซียส มีประสิทธิภาพสูงที่สุดในการชักนำให้เกิดแคลลัสของข้าวขาวดอกมะลิ 105 และโคชิฮิคาริ ที่ 94.5 และ 93.4 เปอร์เซนต์ ตามลำดับ แคลลัสของข้าวขาวดอกมะลิ 105 มีขนาดเล็ก ผิวขรุขระ และเจริญเติบโตช้าเมื่อเปรียบเทียบกับโคชิฮิคาริ สภาวะที่เหมาะสมสำหรับการถ่ายยีนเข้าสู่แคลลัสของข้าว คือ การบ่มแคลลัสร่วมกับอะโกรแบคทีเรียมที่ความเข้มข้น OD_{600} เท่ากับ 0.02 ในอาหาร infection medium และซับแบคทีเรียส่วนเกินออกบนกระดาษปลอดเชื้อก่อนย้ายลงบนอาหาร co-cultivation (พีเอช 5.2) ที่เติม acetosyringone 200 ไมโครโมลาร์ และบ่มเป็นระยะเวลาสามวันที่อุณหภูมิ 25 องศาเซลเซียส สารปฏิชีวนะที่มีประสิทธิภาพในการกำจัดอะโกรแบคทีเรียมและทำความเสียหายต่อแคลลัสน้อยที่สุดคือ timentin ที่ความเข้มข้น 300 มก./ล. การคัดเลือกแคลลัสที่ได้รับการถ่ายยีนบนอาหารคัดเลือก N6D ที่ใส่สารปฏิชีวนะ timentin และ paromomycin ที่ความเข้มข้น 300 และ 100 มก./ล. ตามลำดับ เป็นระยะเวลาสองเดือนพบว่าสามารถส่งถ่ายยีนเข้าสู่แคลลัสของข้าวได้โดยพบแถบของดีเอนเอเป้าหมายของ *npt*II ในแคลลัสจากการทำการเพิ่มสายดีเอนเอด้วยปฏิกิริยา PCR แคลลัสที่ได้รับการถ่ายยีนเวคเตอร์ควบคุมที่ไม่มียีนแทรกอยู่มีประสิทธิภาพในการถ่ายยีนมากที่สุดที่ 19.7 เปอร์เซนต์ ส่วนแคลลัสที่ได้รับการถ่ายยีนจากเวคเตอร์อื่นมีค่าอยู่ระหว่าง 15.3 ถึง 15.9 เปอร์เซนต์ แคลลัสกลุ่มที่หนึ่งซึ่งได้รับการถ่ายยีนที่ยับยั้งการแสดงออกของทั้งห้ายีนพร้อมกันพบว่ามีการปนเปื้อนของอะโกรแบคทีเรียมในข้าวทั้งสองสายพันธุ์ทำให้แคลลัสไม่สามารถมีชีวิตรอดบนอาหารคัดเลือกได้ แคลลัสกลุ่มที่สองซึ่งได้รับการถ่ายยีนที่ยับยั้งการแสดงออกของแต่ละยีนบนอาหารคัดเลือก พบการปนเปื้อนของอะโกรแบคทีเรียมเพียงเล็กน้อยและไม่พบความแตกต่างทางสรีรวิทยา

ผลการตรวจสอบระดับการแสดงออกของ mRNA ในแคลลัสที่ผ่านการยับยั้งการแสดงออกของแต่ละยีน พบว่าไม่มีการแสดงออกของ mRNA ของยีนนั้น ๆ ในการยับยั้งยีน *Os1bglu1* *Os3bglu8* และ *Os7bglu26* แต่ในการยับยั้ง *Os3bglu7* ยังพบการแสดงออกของ mRNA ในระดับที่ต่างกันในแคลลัส ส่วน *Os12bglu38* นั้นปกติจะไม่พบการแสดงออกของ mRNA ในแคลลัส การตรวจสอบกระบวนการ RNAi ที่เกิดขึ้นในแคลลัส โดยใช้ Northern blot เพื่อตรวจหา siRNA พบว่า *Os1bglu1* *Os3bglu8* *Os7bglu26* และ *Os3bglu7* สามารถตรวจพบ siRNA ได้ในระดับที่แตกต่างกันแต่ไม่พบในแคลลัสที่ผ่านการถ่ายยีนด้วยเวคเตอร์ควบคุม

การชักนำให้เกิดต้นของแคลลัสบนอาหารสูตรชักนำให้เกิดต้น พบว่าเฉพาะแคลลัสที่ได้รับการถ่ายยีนด้วยเวคเตอร์ควบคุมและ *Os12bglu38* สามารถชักนำให้เกิดต้นได้ 5.5 และ 3.1 เปอร์เซนต์ ตามลำดับ และพบแถบของดีเอนเอเป้าหมายของ *npt*II แต่ไม่พบความแตกต่างทางสรีระวิทยาระหว่างต้นข้าวที่ได้รับถ่ายยีนด้วยเวคเตอร์ควบคุมและ *Os12bglu38*

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษา_____

WIPAPORN WANTHANALERT : FUNCTIONAL GENOMIC OF RICE (*Oryza sativa* L.) β-GLUCOSIDASE. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 162 PP.

β-GLUCOSIDASE/RICE/*AGROBACTERIUM*/RNA INTERFERENCE/TRANSFORMATION

This research attempted to study the function of 5 β-glucosidase genes (*Os1bglu1*, *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os12bglu38*) by RNAi technique. The suppression of β-glucosidase genes was divided into 2 parts. In the first part, 5 β-glucosidase genes were knocked down with one construct containing a highly conserved coding region that matched all 5 genes which were based on the *Os3bglu7* sequence. In the second part, individual β-glucosidase genes were knocked down with 3'UTR sequences of each gene. The target genes fragments were cloned into the pHELLSGATE8 vector, then transferred into rice calli via *Agrobacterium* to produce dsRNA in rice.

High percentages of effective callus induction of 94.5% and 93.4%, respectively, were obtained when seeds of rice cv. KDML105 and Koshihikari were cultured on N6D medium for 4-6 weeks at 28^{o}C. KDML105 calli were small, with rough surfaces and their growth rate was slower than Koshihikari. The suitable conditions for rice transformation were incubation of the calli with *Agrobacterium* (OD_{600} = 0.02) in infection medium and blotted dry on sterile tissue paper to remove excess bacteria cells, then transferred to co-cultivation media (pH 5.2) with 200 μM acetosyringone and incubated for 3 days at 25^{o}C. Timentin at 300 mg/L was able to eliminate *Agrobacterium* with little damage to the calli. The calli were grown on N6D selection medium supplemented with 300 mg/L timentin and 100 mg/L paromomycin

for 2 months, then checked for the presence of the *npt*II gene to confirm the integration of T-DNA fragment in the calli. High transformation efficiency of 19.7% was obtained from the calli transformed with control plasmid while the individual gene knock down constructs had transformation efficiencies of about 15.3-15.9%. However, the transformation of knock down 5 genes construct into the calli of KDML105 and Koshihikari were contaminated with *Agrobacterium* and all calli died after being transferred onto a selection medium. The knock down individual genes in calli had low *Agrobacterium* contamination and did not show any different phenotypes when compared to the control calli.

Expression of *Os1bglu1*, *Os3bglu8* and *Os7bglu26* mRNA was not found in calli transformed with their respective knock down constructs. However, *Os3bglu7* still shows mRNA expression at different levels after transformation with its knock down construct. The mRNA expression of *Os12bglu38* was not found in a nontransformed callus. Northern blot analysis was performed to check the presence of siRNA to confirm the RNAi mechanism in calli. The results indicated that different siRNA levels were found in the calli with the *Os1bglu1*, *Os3bglu8*, *Os7bglu26* and *Os3bglu7* constructs but no siRNA could be detected in the control transformed calli.

The plantlet regeneration efficiencies at 5.5% and 3.1% were obtained from the calli transformed with the control and *Os12bglu38* contructs, respectively. All transformed plantlets contained the *npt*II gene from the T-DNA integration. Plantlets transformed with the control and *Os12bglu38* contructs did not show any differences in the phenotype.

School of Biotechnology Student's Signature_____

Academic Year 2009 Advisor's Signature_____

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my family for all the love, encourage and support they gave during my study in Suranaree University of Technology.

I would like to express my deepest gratitude to Assistant Professor Dr. Mariena Ketudat-Cairns, my advisor for work instruction, guidance and support chemicals and Associate Professor Dr. James Ketudat-Cairns, coadvisor for kindly providing me the opportunity to study in Ph.D under the National Center for Genetic Enginnering and Biotechnology (BIOTEC) scholarship in the School of Biotechnology, SUT. I would like to thank Assistant Professor Dr. Rodjana Opassiri for all kindness in guidance and suggestion.

I would like to thank Mr. Akkawat Tharapreuksapong, who always supported me for several equipments and giving encouragement from God during my study. Special thanks to Dr. Jirapha Phetsom who teach and suggest me for a basic technique in molecular biology in the lab. I would like to thank for all members of Biotechnology, SUT for their friendship, especially Assistant Professor Dr. Mariena Ketudat-Cairns's students.

Finally, I would like to give my special thanks to my love Richard Mateman whose patient love, smile, support, understanding, help with English and enabled me to complete this thesis.

Wipaporn Wanthanalert

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

°C	=	degree celcius
µg	=	microgram
µL	=	microlitre
bp	=	base pair
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
dNTP	=	deoxyribonucleotide 5’ phosphate
g	=	gram
hr	=	hour
L	=	liter
mg	=	milligram
min	=	minute
mL	=	milliliter
mM	=	millimolar
ng	=	nanogram
PCR	=	polymerase chain reaction
w/v	=	weight per volume
µg	=	microgram
MS	=	Murashige and Skook medium
V	=	voltage

LIST OF ABBREVIATIONS (Continued)

U	=	Unit, μmol/min
TEMED	=	tetramethylenediamine
RNase	=	ribonuclease
RNAi	=	RNA interference
PAGE	=	polyacrylamide gel electrophoresis
OD	=	optical density
mRNA	=	messenger ribonucleic acid
LB	=	luria-berani
2, 4-D	=	2, 4-dichlorophenoxyacetic acid
NAA	=	α-naphthaleneacetic acid
Kinetin	=	6-furfurylaminopurine
L-proline	=	(S)-2-pyrrolidinecarboxylic acid
Timentin	=	ticarcillin disodium salt
Tris	=	tri-(hydroxymethyl)-aminoethane
dH_2O	=	Distilled water

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is a special type of grass that is used as cereal. It is one of the world's most important food crops for humans, especially in tropical Latin America, East, South and Southeast Asia. Rice cultivation is suitable in high rainfall regions, such as Thailand, Indonesia, Spain and Brazil, as well as many other countries. There are over 7,000 varieties of rice grown around the world (Jahn et al., 2007). The two species of domesticated rice are *O. sativa* (Asian) and *O. glaberrima* (African).

O. sativa contains two major subspecies (Crawford and Shen, 1998). The first subspecies is japonica or sinica variety, which is sticky and short-grained. Koshihikari falls in this japonica subspecies. It is one of the most popular strains of rice cultivated in Japan and other part of the world because it has high cooking quality and taste. Other rice cultivars close to Koshihikari, such as Akitakomachi, Hitomebore, and Hinohikari have been created by cross-breeding Koshihikari with other Japanese rice varieties (Tian et al., 2006, Wakasa et al., 2007). The second subspecies is indica variety which is non-sticky and long-grained. Khao Dawk Mali 105 (KDML 105) is one of the most famous strains of indica rice. It was bread and grown in Thailand. The quality of this strain is odorous and good for cooking (Kupkanchanakul, T. 1998, Pisithkul et al., 2006, Pongtongkam et al., 2004).

In the past few decades, rice has been the model system for the study of

monocots. It is one of the smallest cereal genomes with an estimated size of 430 megabase pairs (Mbp) (Feng et al., 2002). The genome database is available with up to 100% coverage (Syngenta Company, 2006). But many gene functions in rice are still unknown. Therefore, the study of gene functions is needed to increase the understanding in order to improve rice varieties in the future. Rice production is still insufficient for the population's demand. Although some varieties can produce high yields most of them cannot give the optimum yield due to biotic and abiotic stresses, such as flood, drought, insects and disease infestation, which cause yield loss. Therefore, the study of factors affecting the growth of rice and its response to stress can increase the yield in normal and stress conditions.

One set of proteins that are involved in rice growth and responses to stress is the β-glucosidases. β-glucosidases are glycosyl hydrolases which hydrolyzed the β-O-glycosidic bond at the anomeric carbon of glucose moiety at the nonreducing end of a carbohydrate or glycoside moelcule and release of glucose. These enzymes act in cell wall remodeling, response to abiotic and biotic stress, defense against herbivores and activation of hormones, for example abscisic acid and gibberelin. The β-glucosidases are involved in plant germination, growth and development in every stage (Rodo et al., 2008, Cicek and Esen. 1998, Cicek et al., 2000). The presence of many β-glucosidases in plants suggests that they may also have other functions that are yet unknown. Rice contains 40 β-glucosidase genes, of which 34 are actively expressed (Opassiri et al., 2006). The phylogenetic tree analysis shows that these genes are closely related. Therefore, the increased understanding of these enzymes should lead to better strategies for breeding rice strains for higher yields and increase the quality of rice production.

Nowadays, RNA interference (RNAi) is one of the most preferable gene knock down methods of high power to do the functional analysis of genes in many organisms. RNAi is a mechanism to protect against RNA viral infection and transposable elements in many eukaryotic organisms, for example *Drosophila*, protozoa, plant and animal. Double strand RNA (dsRNA) is the major activator of the RNAi mechanism. Dicer, the enzyme that cuts dsRNA, will generate small interfering RNAs (siRNA) of 21-25 bp. The RNA induced silencing complex (RISC) will combine with siRNA to unwind the siRNA from double strand RNA to single strand RNA. The siRNA-RISC complex will search and destroy the mRNA target has the sequence complementary with the siRNA. Recently, the function of many genes in rice has been studied by RNAi. The dsRNA can be produced from a special vector that can be transformed into rice via *Agrobacterium* or other methods. The RNAi mechanism will be activated automatically in a cell after the dsRNA is produced in the cell, which leads to the knock down of the target gene by destroying the target mRNA before it can be translated to protein. RNAi is an accessible technique for scientists to apply in a lab. It has become a high powerful tool and a widely used technique in functional genomic research.

CHAPTER II

LITERATURE REVIEW

2.1 Rice

The two famous consumed rice subspecies in the world are *O. sativa* var. indica, which is mainly found in the lowlands of tropical Asia and *O. sativa* var. japonica, which is found in upland and dry fields in China, Japan and other parts of the world (Londo et al., 2006). Further analysis of the genetic material of various types of rice indicates that japonica was the cultivar that first emerged followed by indica (Garris et al., 2005). Rice has been the main staple in Thailand and many other countries. Rice is also the main export product of Thailand. The main focus of Thai research has been on the rice variety KDML 105 or jasmine rice because of its aromatic nature and good cooking quality. This rice is very famous all over the world (Tulyathan and Leeharatanaluk, 2007). Koshihikari (*O. sativa* var. japonica) is a famous variety widely cultivated in Japan. It was first created in 1956, by combining the Nourin no. 1 and Nourin no. 22 strains at the Fukui Prefectural Agricultural Research Facility. Koshihikari has good eating quality and has become the leading variety of nonglutinous rice in Japan. However, Koshihikari has the disadvantage that it is difficult to achieve high yields because of its low resistance and high susceptibility to disease and insects that cannot be controlled without chemical application (http://en.wikipedia.org/wiki/Koshihikari).

In the past 30 years, the production of rice with high yielding varieties has

taken place and improved cultivation practices have been performed. However, it is still insufficient to meet the population's demand. It is necessary to develop high yielding varieties and also minimizing yield loss due to biotic and abiotic stresses, such as disease, insect infestation, drought, flood, salinity and weed competition. Engineering plants to resist various stresses, both biotic and abiotic, requires a thorough understanding of the cellular and functional aspects of the plant's genes (Tyagi, 2004). Rice is an excellent model plant among cereals for genomics studies. In addition, it is considered to be a model plant for molecular biological studies (Somerville and Somerville, 1999). The International Rice Genome Sequencing Project (IRGSP) was launched in 1998 with ten countries participating (Sasaki and Burr, 2000). The IRGSP released the rice genomic sequencing data in public database such as the DNA Data Bank of Japan (DDBJ), GenBank, the European Molecular Biology Laboratory (EMBL) and the RGP database (http://rgp.dna.affrc.go.jp /index.html). The sequencing data, rice linkage map, and physical map are integrated in the RGP database, the Integrated Rice Genome Explorer (INE: http:/ /rgp.dna.affrc.go.jp/giot/INE.html) (Sakata et al., 2000). In 2000, a private company, Monsanto, made 60% of the rice genome sequences public (http://www.rice-research.org/). They also released about 7,000 sequence data including microsatellites (http://www.rice-research.org/rice_ssr.html). This data was imcorporated in a coordinated effort to determine a high quality draft sequence of the rice genome by 2002 (accomplished) and a “finished” sequence by 2004 (Data from The Cooperative State Research, Education, and Extension Service, http://www.csrees.usda.gov/).

Since the recent sequencing of the rice genome, the functional identification of rice genes has become increasingly important. Various tagged lines have been

generated; however, the numbers of tagged genes available are not sufficient for extensive study of the genes function. Therefore, a high powerful technique for study the gene function in rice has been developed through gene silencing or RNAi. This technique is very specific with high efficiency to study the function of target gene.

2.2 RNA interference (RNAi)

In recent years, sequence-specific gene silencing has become more popular because of its powerful effect to inhibit the expression of a homologous endogenous gene. RNAi is one of these means to induce a gene-specific block of translation (Wang et al., 2004). RNAi has been a highly powerful technique to study functional genomics in many organisms (Cottrell and Doering, 2003).

Eukaryotes have evolved many different systems to resist virus infection and transposable elements. As long double-stranded (ds) RNAs do not occur in the cytoplasm of eukaryotic cells, the accumulation of ds, replication intermediates of RNA viruses, activates the antiviral responses of the RNA interference (RNAi) or translation inhibition and apoptosis. RNAi is an ancient defense mechanism that degrades dsRNAs and cognate mRNAs in a sequence-specific manner (Lichner, 2003). RNAi has been shown to act as an efficient antiviral system for other double-strand RNAs in plant and insect cells and might also play an antiviral role in mammalian cells (Waterhouse and Wang, 2001). One key feature of RNAi is the production of double-stranded RNA (dsRNA) homologous to the gene being targeted for silencing (Waterhouse et al., 1998). This dsRNA is degraded by the enzyme Dicer into approximately 21-25 nucleotide RNAs, known as small interfering RNAs (siRNAs). These siRNAs then provide specificity to the endonuclease-containing,

RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation (Hannon, 2002) (Figure 1).

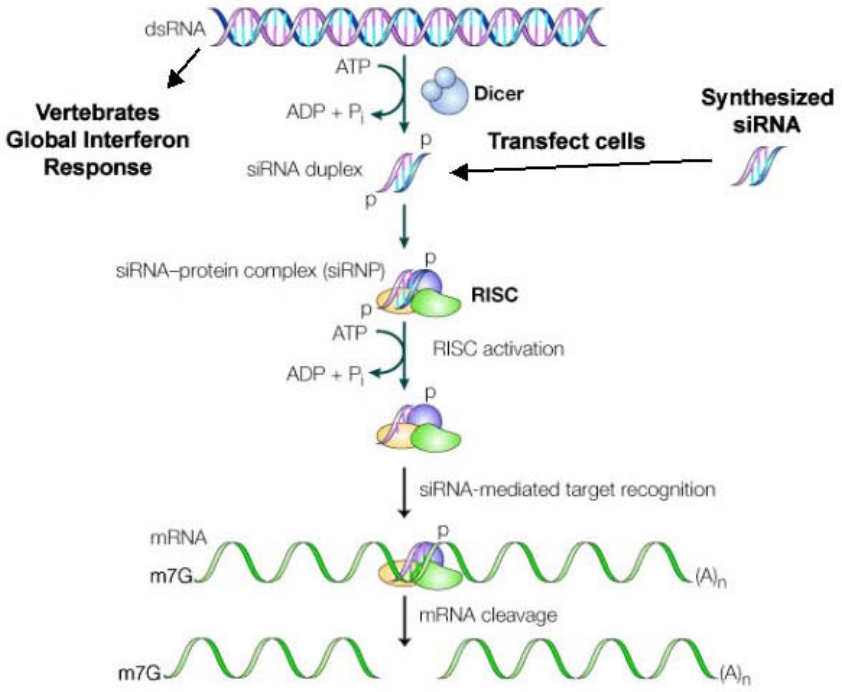


Figure 1 Schematic of the Pathway of RNAi (Modified from Dykxhoorn, 2003).

In plants, RNAi also known as post-transcriptional gene silencing (PTGS) or co-suppression which was first discovered in petunia by Napoli et al. (1990). They attempted to overexpress chalcone synthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS gene. CHS is the key enzyme in the anthocyanin biosynthesis pathway. They tried to deepen the purple color but the result showed that the flowers appeared totally white or irregularly colored. In 1994, Macino et al. found the cosuppression phenomena in fungus. They wanted to enhance orange pigment in the fungi by introducing extra copies of carotenoid pigment genes. The results were similar to the petunias. The fungus showed white color rather than orange color.

They called this process is quelling. Four years later in 1998, Fire and Sun introduced the sense and antisense strands RNA into nematodes by feeding the nematodes with *Escherichia coli* expressing dsRNA homologous to the *Caenorhabditis elegans unc-22* gene. The result showed that the *unc-22* gene of the nematode was silenced and this silent was heritable to the first generation offspring. Moreover Richard et al. (1999) studied gene silencing that involved homology-dependent degradation of mRNAs in *Drosophila*. They suppressed the genes *bicoid* and *hunchback,* by initially injecting the double-stranded RNA into fertilized eggs. They found that *bicoid* dsRNA reduced the expression of Bicoid protein and induced a *bicoid* loss-of-function phenotype in which embryos have partial transformation of anterior structures to posterior identities.

Currently, the RNAi technique is widely used to study the function of many genes in rice. In 2003, the functions of the rice *PI*/*GLO* paralogs, *OsMADS2* and *OsMADS4*, that share substantial sequence similarity were examined (Prasad and Vijayaraghavan, 2003). This research demonstrated that these genes play an essential role in lodicule development and implicated that the second *PI*/*GLO* paralog, *OsMADS4*, is sufficient for stamen specification.

Xiao et al. (2003) studied the rice *OsMADS16* gene. This gene is phylogenetically related to the angiosperm B-function MADS-box genes. They isolated its genomic sequences and characterized its functions in plants by RNAi. Transgenic lines from the introduced gene expressing double-stranded RNA with the *OsMADS16* cDNA fragment were male-sterile and displayed alternations of lodicules and stamens, occasionally depressed palea and overgrown glume. Further investigations of the transcription of the *OsMADS16* gene in these transgenic lines by

RT-PCR revealed that its transcript was significantly reduced. Their results demonstrated that *OsMADS16* is an AP3/DEF orthologue that specifies the identities of lodicules and stamens in rice flower and also supported the finding that *OsMADS4* is the PI orthologue.

Wang et al. (2004) tested the efficiency of the RNAi vector pTCK303. The rice gene *OsGAS1* was used, and its RNAi construct was introduced into rice calli. Southern blot analysis of the transgenic rice confirmed the presence of the *OsGAS1* RNAi construct. RNA expression level in RNAi transgenic rice was decreased approximately 85%. They concluded that the RNAi vector pTCK303 based on the homology-dependent gene-silencing mechanisms facilitated the inhibition of endogenous genes in a monocot and was proven to be a practical and efficient platform for silencing rice genes.

Islam et al. (2005) developed the versatile-type of RNAi vectors, which are driven by constitutive promoters, and GATEWAY^{TM} cloning technology which made it easy to construct the RNAi vectors with trigger sequences and to analyze the function of a target gene. They suggested that *OsGAMYB* has an essential function in endosperm formation through embryo development at the early stage of seed development in rice.

Miki et al. (2005) studied the single and multiple members of the *OsRac* gene family in rice. This family encodes a Rac/Rop-type GTPases, which have multiple functions in various cellular activities, including defense, cell polarity, development, and hormone signaling. They designed invert repeat (IR) constructs transcribing dsRNA fragments from the gene specific region (3' UTR) of each *OsRac* gene and introduced them into rice. Each of the seven members of the *OsRac* gene family was

specifically suppressed by its respective IR construct. They also examined IR constructs in which multiple 3'UTR regions were fused and showed that three members of the *OsRac* gene family were effectively suppressed by a single construct. Using highly conserved regions of two members of the *OsRac* gene family, they also suppressed the expression of all members of the gene family with variable efficiencies.

Lin et al. (2005) studied a water deficit-suppressed gene, *OsARD* encoding an acid-reductone-dioxygenase-like protein, identified from rice. *OsARD* is mainly expressed in roots under flood conditions and suppressed by abiotic stresses including water deficit, high salinity and low temperature. The genes for S-adenosylmethionine (SAM) synthase and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase were upregulated in RNAi transgenic rice plants with a significant reduction of *OsARD* expression. Furthermore, the expression of the two genes for ethylene signal transduction, *ETR2* and *EIN3*, increased in these RNAi transgenic plants, whereas the expression of *ERF3* was suppressed. These results suggest that *OsARD* may play a role in the metabolism of methionine and ethylene in response to abiotic stresses.

Luo et al. (2005) found a rice mutant with reduced heading date (designated *rhd1*) and found that in a transgenic line of cultivar Teqing 2 (*Oryza sativa* L. ssp. indica) used to identify the genes related to rice heading and thereby study its molecular mechanism. Sequence analysis showed that *rhd1* shared 99% similarity to the *OsGRF1* (*O. sativa* growth-regulating factor 1) gene. RNAi results revealed that transgenic lines with reduced *OsGRF1* transcript displayed delayed growth and development, developed small leaves, and had delayed heading. The extent of the phenotypes that developed was well-correlated with the level of *OsGRF1* gene

transcript. The results clearly demonstrate that the *OsGRF1* gene is not only involved in regulating growth at the juvenile stage, but that it may also be involved in the regulation of heading in rice.

The isolation and characterization of a somatic embryogenesis receptor-like kinase (*OsSERK1*) gene in rice have been reported (Hu et al., 2005). The suppression of *OsSERK1* expression in transgenic calli by RNAi resulted in significant reduction of shoot regeneration rate (from 72% to 14% in the japonica rice Zhonghua11). The *OsSERK1* gene was activated by the rice blast fungus and associated with host cell death in Sekigushi lesion mimic mutants. This gene is also inducible by defense signaling molecules, such as salicylic acid, jasmonic acid and abscisic acid. Furthermore, constitutive overexpression of *OsSERK1* in two rice cultivars led to an increase in host resistance to the blast fungus. The data suggest that *OsSERK1* may partially mediate defense signal transduction in addition to its basic role in somatic embryogenesis.

Penga et al. (2007) studied the gene regulation and environment signal in flowering time in mutant rice. Genotype W378 is a late-flowering mutant that codes *OsLFL1* in T-DNA. *OsLFL1* is expressed exclusively in spikes and young embryos, while in the mutant W378, it is ectopically expressed. The insertion of *OsLFL1*-RNAi into mutant W378 showed the down-regulation of *OsLFL1* and recovers the flowering to normal time while the overexpression of *OsLFL1* showed late flowering, as in mutant W378.

Zhong et al. (2007) designed a plant universal binary vector pCRiRRD3. The vector contains two multiple cloning sites to integrate sense and antisense target sequences, which is separated by a 200 bp intron fragment. Their results indicated

that the vector pCRiRRD3 can effectively silence genes in both monocot and dicot plants.

Xu et al. (2008) studied the *OsHXK10* gene in rice. This gene has been reported to be related to the sugar signaling and metabolism. The hexokinase gene family contains 10 members in rice and one of the members, the *OsHXK10* gene, is expressed in stamen. The anthers of some flowers in RNAi lines (suppressed *OsHXK10* expression) were unable to dehisce, which is needed to inhibit the cell wall thickening of the anthers. The mRNA level of *OsHXK10* was significantly reduced in anther wall when compared to the normal flower. The pollen from RNAi lines showed the same morphology with the wild type. However, the germination capacity of *OsHXK10* RNAi lines was clearly decreased and the proportion of empty seed increased compared to the wild type. So, the *OsHXK10* gene is important in anther dehiscence, pollen germination and hence grain filling in rice.

Yang et al. (2008) reported the role of a member of CBL (Calcineurin B-Like) interacting protein kinase (CIPK) family, *OsCIPK23* in pollination and stress responses in rice. *OsCIPK23* is expressed in pistil and anther and is up regulated by pollination. RNAi mediated suppression of *OsCIPK23* decreased seed set and conferred a hypersensitive response to drought stress. These results indicate that *OsCIPK23* is important in pollination and drought stress.

Wang et al. (2009) reported the interactions of gibberellins (GAs) and brassinosteroids (BRs), two growth-promoting phytohormones, which regulate many common physiological processes. *OsGSR*1, a member of the GAST (GA-stimulated transcript) gene family, is induced by GA and repressed by BR. RNA interference (RNAi) transgenic rice plants with reduced *OsGSR*1 expression show phenotypes

similar to plants deficient in BR, including short primary roots, erect leaves and reduced fertility. The *OsGSR*1 RNAi transgenic rice shows a reduced level of endogenous BR, and the dwarf phenotype could be rescued by the application of brassinolide. These results suggest that *OsGSR*1 activates BR synthesis by directly regulating a BR biosynthetic enzyme at the post-translational level. *OsGSR*1 RNAi plants show a reduced sensitivity to GA treatment. *OsGSR*1 is a positive regulator of GA signaling and plays important roles in both BR and GA pathways.

Li et al. (2009) studied the *RACK*1 gene which plays an important role in the regulation of plant growth and development. Transgenic rice plants in which *RACK*1 was inhibited by RNAi were studied to elucidate the possible functions of *RACK*1 in responses to drought stress in rice. The expression of *RACK*1 in transgenic rice plants was inhibited by more than 50%. The peroxidation of membrane and the production of malondialdehyde were significantly lower, and the superoxide dismutase activity in transgenic rice plants was significantly higher than in nontrangenic rice plants. It is suggested that *RACK*1 negatively regulated the redox system-related tolerance to drought stress of rice plants.

RNAi is one of the most powerful technologies so far with specific and potent gene knock down has proven and a highly successful approach for loss-of-function studies, discovering important trait genes and identification of the functions of novel genes in many organisms. Researchers can cause a decrease in the expression of a targeted gene and see its effects on physiological changes. Several appropriate tools to induce RNAi include synthetic siRNA, RNAi vectors, and *in vitro* dicing, depending on the model system, the length of time to require knockdown and other experimental parameters. RNAi has been used for applications in biotechnological

engineering of food plants which are possible to produce lower levels of natural plant toxins, for example gossypol, linamarin or allergens (Sunilkumar et al., 2006, Siritunga et al., 2003, Le et al., 2006). It maybe possible to use RNAi in therapy to destroy viruses, tumors and cancer cells in humans in future.

2.3 Gateway cloning technology

The key of Gateway cloning technology is the lambda recombination phage system, which is used to move the DNA fragment via the specific recombination site or attachment site (att site). Enterobacteria phage λ (lambda phage) is a temperate bacteriophage that infects *E. coli*. It is a virus particle composed of a head, genetic material (double strand linear DNA) and tail. Lambda phage, which was discovered by Esther Lederberg in 1950, has two different life cycles called lytic and lysogenic. The life cycle of phage is usually entering the lytic pathway. The phage particle injects its DNA into a host through the tail and replicates its DNA. The host DNA will be degraded and it allows the transcription and translation mechanisms to produce many phage particles. When cell resources are depleted, the phages will lyse or break the host cell, releasing the new phage particles. However, under certain conditions, the phage DNA may integrate itself into the host cell chromosome in the lysogenic pathway. The λ DNA is called a prophage and stays resident within the host's genome without apparent harm to the host, which can be termed a lysogen. The prophage is duplicated with every subsequent cell division of the host. The phage genes expressed in this dormant state code for proteins that repress expression of other phage genes. These proteins are broken down when the host cell is under stress, resulting in the expression of the repressed phage genes. Stress can be from starvation, poisons (like

antibiotics), or other factors that can damage or destroy the host. In response to stress, the activated prophage is excised from the DNA of the host cell by one of the newly expressed gene products and enters its lytic pathway.

The normal cloning method works through many steps, including restriction enzyme digestion, ligation, purification and clean-up steps, which required a lot of time. Gateway cloning technology is a universal cloning technique that is developed by Invitrogen Life Technology (www.invitrogen.com). Gateway cloning technology transfers the DNA fragments between a cloning vector and expression vector without restriction enzyme digestion and ligation while maintaining the reading frame (Figure 2). This fast step can deliver the clone with more than 99% efficiency within an hour and eliminates the re-sequencing of the same clone from target identification to validation.

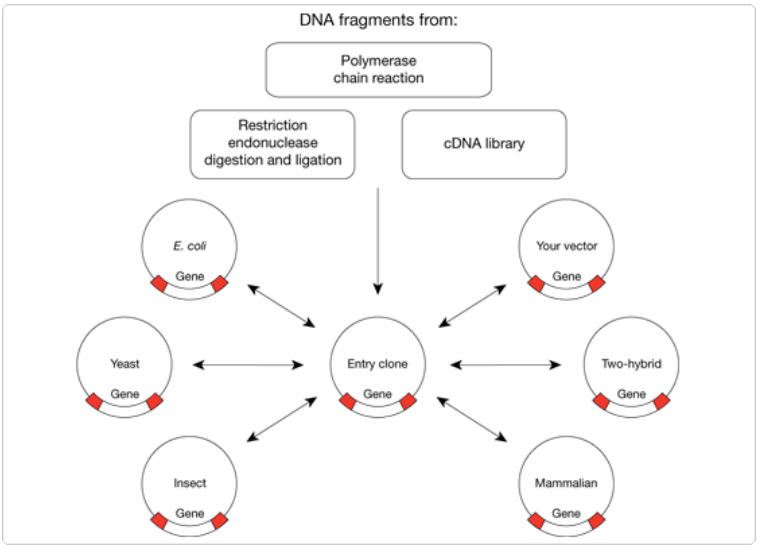


Figure 2 Gateway® technology facilitates cloning of genes into and back out of multiple vectors via site-specific recombination. Once a gene is cloned into an entry clone, it moves the DNA fragments into one or more destination vectors simultaneously (http://www.invitrogen.com).

The att (L, R, B, P) sites are necessary in the Gateway system. The recombinational cloning consists of two reactions. First, in the LR reaction, the attL site on entry clone will be cut to form a sticky end by Gateway recombination proteins (Integrase (Int), Integration Host Factor (IHF) and excisionase (Xis)) and it will match up with the sticky end on a destination vector that contain attR site. The two products are an expression vector that contains the target gene and the entry vector that containa the ccdB gene.

The destination vector contains the ccdB gene, which is a killer protein that is a natural analogue of the quinolone antibiotics (ciprofloxacin, enoxacin, etc.). It will bind to the DNA *gyrase* subunit A, the product of the *gyrA* gene, turning it into a cellular poison (Bahassi et al., 1999). The *E. coli* strain DB3.1 contains the *gyr*A462 allele which renders the strain that is resistant to the toxic effects of the *ccd*B gene. The entry vector and destination vector have to contain the different antibiotics for selection in the host cell. In the second reaction, the BP reaction, the attB site will flank a DNA fragment and the attP site will flank the insertion site on a donor vector. The attB and attP sites will match up with each other with Int and IHF proteins. The two products of the BP reaction are the entry clone and the fragment of ccdB gene that is flanked by attR sites (Figure 3). The host cells for selecting the clone after transformation of the vector with LR and BP reaction are *E.coli* strains DH5α, which strains are susceptible to ccdB genes. The cells that contain the entry clone and expression vector, in which the target gene has replaced the ccdB gene, can grow on the selection medium.

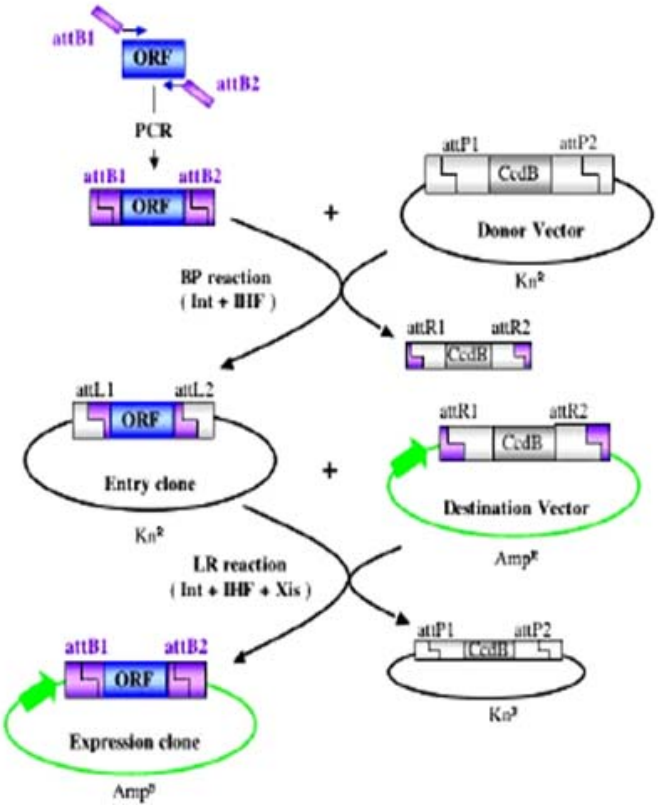


Figure 3 The BP and LR reactions (http://www.pasteur.fr/ip/easysite/go/03b-000011031/principe_gateway_en.html).

2.4 *Agrobacterium* transformation

The method of transformation is very important for gene transfer process in plants. Researchers have been trying to find out the best method many years. Thirty-two years ago, Chilton et al. (1977) used *Agrobacterium* to transport new genes into plant cells. Although the procedure is now routine and has been used for many types of crops, there is still work for further improvement. The transformation methods based on the use of *Agrobacterium* are preferred in many instances due to the following properties: (i) it is easy to handle, (ii) high efficiency, (iii) more predictable pattern of foreign DNA integration, and (iv) low copy number of integration

(Ignacimuthu et al., 2006, Datta and Datta, 2006). Thus, *Agrobacterium* biology and biotechnology have been the establishment of many *Agrobacterium* strains, plasmids and protocols uniquely adapted for the genetic transformation of various plant species (Draper et al., 1988).

Agrobacterium is a genus of Gram-negative bacteria that causes tumors (crown-gall disease) in plants (Quintela et al., 1995). Recent taxonomic studies have reclassified all of the *Agrobacterium* species into new genera, such as *Ruegeria*, *Pseudorhodobacter* and *Stappia*, but most species have been reclassified as *Rhizobium* species (Pulawskaa et al., 2006). *A. tumefaciens* have flagella that allow them to swim through the soil towards photoassimilates that accumulate in the rhizosphere around roots or move towards chemicals that indicate a wounded plant cell, such as acetosyringone. The bacterium contains a tumour-inducing plasmid (Ti plasmid or pTi), which contains the T-DNA (transfer DNA). It also contains all the genes necessary to transfer T-DNA to the plant cell and incorporate it at a semi-random location into the plant genome. The integration of the T-DNA into the plant genome enables the genetic modification.

In nature, the transferred DNA (T-DNA) carries a set of oncogenes (such as gene in the biosynthesis pathway of plant hormones, auxin and cytokinin) (Gaudin et al., 1994) and opine-catabolism genes. The expression of these genes in plant cells can produce opines; amino acid derivatives that are used by bacteria as a nitrogen source and leads to neoplastic growth of the transformed tissue. Because of the altering of the hormone balance in the plant cell, tumors formation occurs in those cells because cell division cannot be controlled by the plant (Montoya et al., 1977). The mechanism of T-DNA transfer is shown in Figure 4 (www.mcdb.lsa.umich.edu/labs/ttzfira/).

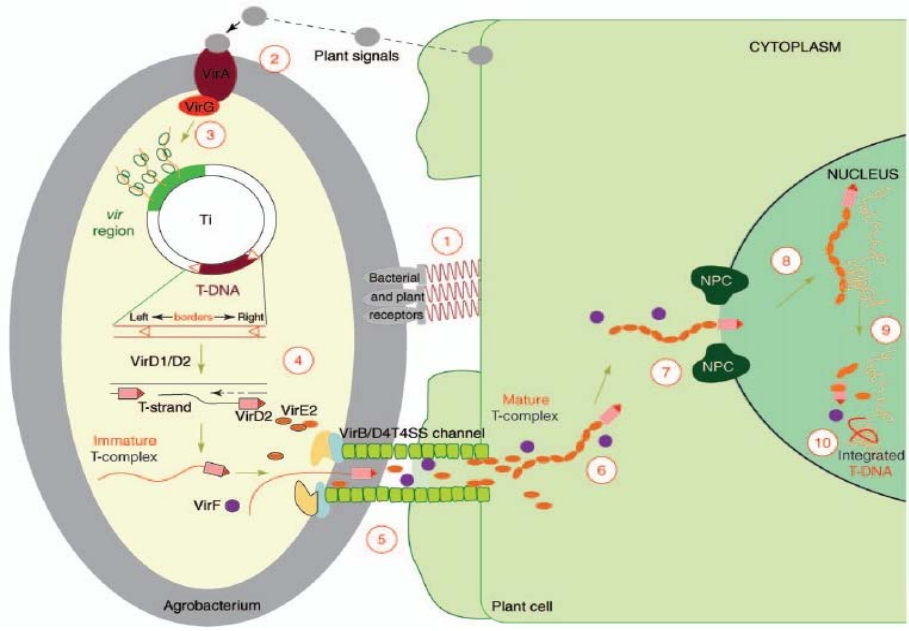


Figure 4 A model for the *Agrobacterium*-mediated genetic transformation. The transformation process comprises 10 major steps. In the begining, it starts with recognition and attachment of the *Agrobacterium* to the host cells (1) and then sensing of phenolic compounds from the wounded plant cell induces the VirA/VirG in *Agrobacterium* (2) followed by the activation of the *vir* gene region (3). A mobile copy of the T-DNA is generated by the VirD1, which is a Topiomerase that helps VirD2 to recognize and cleave within the 25 bp border sequence. Then the VirD2 which is the endonuclease will cut the T-DNA at the right border to initiate T-strand synthesis (4) and deliverit as a VirD-DNA complex (immature T-complex), together with several other Vir proteins (5). Following the association of VirE2 that binds with the T-strand and protects it from nuclease attack and intercalates with lipids to form channels in the plant

membranes through the host-cell cytoplasm (6) and actively imports it into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), it is stripped of its escorting proteins (9) and is integrated into the host genome (10).

Recombinant *Agrobacterium* strains, in which the native T-DNA has been replaced with genes of interest, are the most efficient vehicles used today for the introduction of foreign genes into plants and for the production of transgenic plant species. Marc Van Montagu and Jozef Schell at the University of Ghent (Belgium) discovered the gene transfer mechanism between *Agrobacterium* and plants, which resulted in the development of methods to alter *Agrobacterium* into an efficient delivery system for gene engineering in plants (Schell and Van Montagu, 1977). The genes to be introduced into the plant were cloned into a plant transformation vector that contains the T-DNA region of a disarmed plasmid, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed. Plants were then grown on media containing antibiotics following transformation, and those that do not have the T-DNA integrated into their genome will die (Gelvin, 2003).

Agrobacterium-mediated transformation is routinely used for the transformation of many dicots and monocots, including rice. *Agrobacterium*-mediated transformation of rice is now used in many laboratories worldwide. Several protocols have been developed to use either mature seeds or immature embryos as target tissue for *Agrobacterium* infection. In rice, tissue browning and necrosis following *Agrobacterium* infection is still a major problem in genetic transformation.

Moreover, the strain of rice, *Agrobacterium* strain, binary vector, selectable marker gene and promoter, inoculation and co-culture conditions, inoculation and co-culture medium, osmotic treatment, desiccation, *Agrobacterium* density and surfactants, tissue culture, and regeneration medium may influence the recovery of stable plant cells after *Agrobacterium* infection (Cheng et al., 2004, Shrawat and Lörz, 2006)

‘‘Super-virulent’’ *Agrobacterium* strain EHA105 has high effectiveness for transformation and use in rice transformation (Cheng et al., 1998, He et al., 2008, Cao et al., 1998). *Agrobacterium* strain EHA105 is a Km(S) derivative of EHA101. The helper Ti plasmid for *Agrobacterium* strain EHA105 are derived from three different *Agrobacterium* Ti plasmids, the octopine plasmid pTiB6, the nopaline plasmid pTiC58, and the L, L-succinamopine plasmid pTiBo542. The T-DNA regions of these plasmids were deleted using site-directed mutagenesis to yield replicons carrying the *vir* genes (Hood et al., 1993). Several researchers have reported the successful genetic transformation in japonica and indica rice cultivars by *Agrobacterium* strain EHA105 (Ma et al., 2009, Pipatpanukul et al., 2004, Cheng et al., 1998, Rongtian et al., 2002, Cao et al., 1998, Saharan et al., 2004, Zhang et al., 2007, Amin et al., 2004, Torregrosa et al., 2002). Therefore, *Agrobacterium* strain EHA105 was used to infect rice callus in this research.

2.5 β-glucosidase

Carbohydrate-active enzymes including glycosidases, transglycosidases, glycosyltransferases, polysaccharide lyases and carbohydrate esterases are responsible for the enzymatic processing of carbohydrates in plants (Coutinho and Henrissat**,** 1999, Bellincampi et al., 2004). A glycoside is a kind of secondary metabolite linked

with a sugar group, such as glucose or glucuronide. The type of sugar and its position can play an important role in its biological function. For some glycosides, partly or totally removing sugars (deglycosylation) will improve its biological activity or change its physical, chemical or active properties completely (Aharoni et al., 2003). β-Glucosidases are enzymes that catalyze the hydrolysis of terminal, non-reducing β-D-glucose residues with release of β-D-glucose cleavage of β-linked sugars from glycosides. The catalytic mechanism of β-glucosidase is shown in Figure 5 (Davies and Henrissat, 1995). Two conserved glutamic acid residues serve as a catalytic nucleophile and a general acid/base catalyst. The catalytic glutamic acid residues are situated on opposite sides of the β-glycosidic bond of the substrate. As the initial step in catalysis, the nucleophile performs a nucleophilic attack at the anomeric carbon, which results in formation of a glucose–enzyme intermediate. In this process, aglycone departure is facilitated by protonation of the glucosidic oxygen by the acid catalyst. During the second catalytic step (deglycosylation) a water molecule is activated by the catalytic base to serve as a nucleophile for hydrolysis of the glycosidic bond causing the release of the glucose (Davies and Henrissat, 1995, http://www.cazy.org/fam/ghf_INV_RET.html). Under suitable conditions, β-glucosidases can perform a transglucosylation, in which the covalently bound glucose in the enzyme–glucose intermediate is transferred to an alcohol or a second sugar group (Morant et al., 2008).

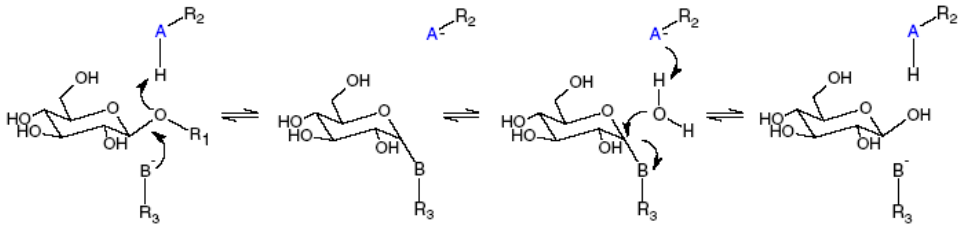


Figure 5 Reaction mechanism of the retaining β-glucosidases. A glutamic acid residue in the conserved TFNEP motif serves as a general acid/base catalyst (AH) while a glutamic acid residue in the I/VTENG motif serves as a nucleophile (B) (Davies and Henrissat, 1995).

Most plant β-glucosidases belong to the glycosyl hydrolase family 1 (GH1) (Henrissat and Bairoch, 1996, http://www.cazy.org/fam/GH1.html). The other commonly found activities in the GH1 are β-galactosidases, myrosinases, 6-phospho-β-glucosidase and 6-phospho-β-galactosidase, β-mannosidase, β-fucosidase and β-glucuronidase (He and Withers, 1997). The β-glucosidases are a heterogeneous group of enzymes, present in eukaryotic and prokaryotic organisms (Faure et al., 2001). β-glucosidase expression shows that the enzyme is exclusively found in actively growing plant parts (primordial leaves, shoot apex, mesocotyl and primary roots) of young seedlings or female reproductive organs (ovule, silk, and husk). Plant parts that are terminally differentiated and matured (endosperm, ear axis, and rachis) are devoid of this enzyme activity (Nikus and Lisbeth, 1999). The compartmentalization predictions of rice β-glucosidases by the PSORT program indicated that they may be found at many cellular locations such as chloroplast, cytoplasm, endoplasmic reticulum membrane, endoplasmic reticulum lumen, mitochondria inner membrane,

intermembrane space, matrix, and outer membrane, peroxisome, plasma membrane and vacuole (Opassiri et al., 2006).

Furthermore, glucosidases are distributed widely in soils and are important enzymes in the soil carbon cycle. β-glucosidase has the low concentration in the soil, but possesses the high activity and catalyzing efficiency (Klose and Tabatabai, 2002). Busto and Perez-Mateos (1995) demonstrated that extracellular β-glucosidase is stabilized in soil by association with humic materials. This enzyme, which is active in the final steps of degradation of cellulose into sugar, is fundamental in the carbon cycle and for soil in general. The β-glucosidase activity has been recovered and shows the highest activities in the pyrophosphate extract from natural forest soil in Tuscany (Italy) and Murcia (South east of Spain) (Masciandaro et al., 2008).

β-glucosidase functions in plant

Plants are, unlike animals, cannot move or escape their predators, disease or stress conditions. By evolution, plants enhanced and produced the nature's organic chemical and secondary metabolites to defend themselves against herbivores and microorganism and adapts to different types of abiotic environmental stresses. Many plant defense compounds, hormones and other molecules that are hydrolyzed by β-glucosidases are stored in a nonactive glycosylated or glycosidic conjugated form to chemically stabilize and increase the solubility of the defense compound. It is a suitable form for storage in vacuole and protects the plant from the toxic effects of its own defense system (Jones and Vogt, 2001, Vasella et al., 2002, Nematollahi and Roux, 1999). The β-glucosidases are stored separately from the substrate and after cell disruption, such as chewing by insect, the defense compounds are activated via

hydrolysis of the glycosidic linkage catalyzed by β-glucosidases and immediately defends against attacking herbivores and pathogens (Figure 6) (Morant et al., 2008). Plant β-glucosidases show differences in biochemical activity, localization or expression, which are important for bio-activation of defense compounds in response to diverse biotic stresses at different stages of plant development. β-glucosidases are receiving increased attention in biotechnological and industrial applications such as aroma formation in tea, wine and fruit juice and the engineering of microorganisms for use in the biomass conversion. (Mizutani et al., 2002, Maicas and Mateo, 2005, Fia et al., 2005)

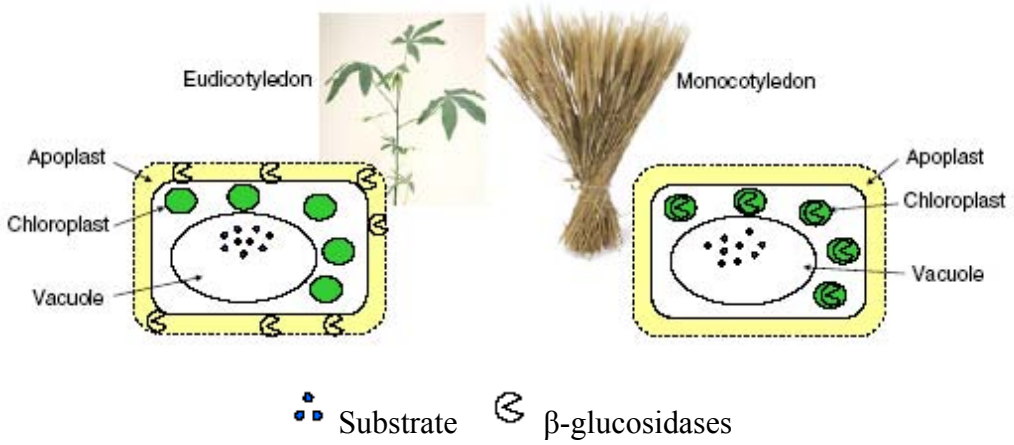


Figure 6 Compartmentalization of β-glucosidases and their substrates in eudicotyledenous and monocotyledenous plants. In eudicotyledenous and monocotyledonous plants, some of the plant secondary metabolites are classified as phytoanticipins which are stored in the vacuole. The bioactivating β-glucosidases in eudicotyledons are glycoproteins that are stored apoplastically or intracellularly in protein bodies (exemplified here by an apoplast location which is most often reported). In

monocotyledons, the β-glucosidases are unglycosylated and plastid-localized (exemplified here by chloroplasts) (Morant et al., 2008).

The purification, production and some properties of β-glucosidase in the intracellular fraction and cell wall of *Acremonium persicinum* have been examined (Pitson et al., 1999). Anion exchange chromatography showed the presence of two β-glucosidases in intracellular extracts of *A. persicinum*. The remaining β-glucosidase appeared to occur only intracellularly and possessed different properties from its extracellular counterpart and its synthesis relatively insensitive to glucose in the medium. Similar anion exchange chromatographic analysis of the cell wall associated enzyme activities extracted by 4 M LiCl shows the β-glucosidase. Possible roles for these enzymes are proposed in light of their location, action patterns and apparent regulation of syntheses.

In white lupin (*Lupinus albus* L.) plants, isoflavones and their glucosides were localized in cell walls where the high constitutive activity of β-glucosidase was also identified (Pislewska et al., 2002). The enzyme was partially purified from root cell walls. The enzyme is an exoglucosidase, preferentially hydrolysing conjugates of phenolic compounds with β anomers of glucose. The specific β-glucosidase activity varies in different tissues with the highest one in roots, and always higher in cell walls than in protoplast. The cell wall location of the enzyme was confirmed biochemically by its activity in intercellular washing fluids. Both aglycones and glycosides were also present in these fluids. The specific β-glucosidase activity correlated well with the isoflavonoid aglycone/glycoside ratios in various tissues.

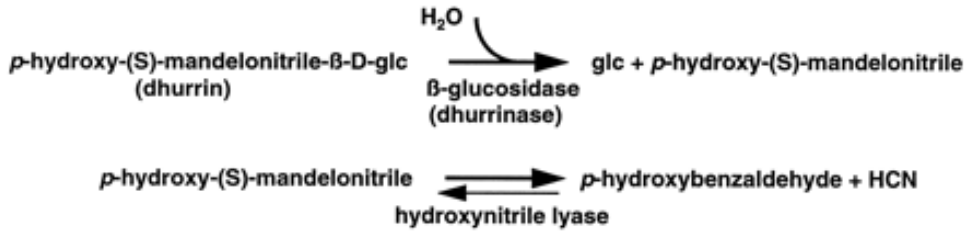
The β-glucosidases appeared to be ionically bound to cell walls. A large

portion of β-glucosidase (EC 3.2.1.21) in germinating rice seeds can be solubilized with 1 M NaCl and its activity increased more than eight-fold within five days of germination (Akiyama et al., 1998). Its N-terminal amino acid sequence (44 residues) exhibited high homology to those of β-glucosidases from other plants such as barley and white clover. β-glucosidase purified by Akiyama et al. (1998) is probably involved not only in hydrolysis but also in modification of oligosaccharides in cell walls of germinating rice seeds (Opassiri et al., 2004).

The activity of β-glucosidase (EC 3.2.1.21) in the protein fraction solubilized with 3 M LiCl from cell walls of carrot cell cultures was found to be much higher than those of the other glycan-hydrolases. The enzyme also acted on *p*NP-β-cellobioside, lichenan and laminarin, but was not capable of hydrolysing the glucose-containing polymers isolated from cell walls of carrot cell cultures (Konno et al., 1996). Crude cell wall preparations from *Cicer arietinum* L. cell suspension cultures show high activity for the hydrolysis of coniferyl alcohol β-D-glucoside (coniferin) (Hösel et al., 1978). Various β-glucosidase activities could be solubilized from these preparations by 0.5 M NaCl treatment and one of these could be shown to possess a high activity for the hydrolysis of coniferin. Mazzuca et al. (2006) studied the β-glucosidase involved in degradation of oleuropein. In situ hybridization of β-glucosidase shows the activity in mesocarp tissue of olive during fruit ripening. The variations of oleuropein-degradative-β-glucosidases activity during ripening are due to changes in the competence of single mesocarp cells to synthesize the enzyme isoforms β-glucosidase encoded by the *Zm-p60.1* gene that was transiently expressed was capable of hydrolyzing exogenous inactive cytokinin, zeatin-O-glycoside, to zeatin, the active form, enabling the tobacco protoplasts to undergo cell division (Mok and Mok, 2001).

The role of β-glucosidase in zeatin metabolism was studied in *Zea mays* root tips during cold-stress and subsequent recovery. A dramatic increase in β-glucosidase activity was observed following a 24-hour recovery period. The dramatic increase in β-glucosidase enzyme activity in the 24-hour recovery period may be due to a change in its activation or localization (Keri et al., 2004).

Cyanogenic glucosides are amino acid-derived phytoanticipins found in plants (Bak et al., 2006). The cyanogenic glucosides are hydrolyzed to an unstable aglycone, which either spontaneously or enzymatically degrades in to a ketone or aldehyde and toxic hydrogen cyanide when the tissue is disrupted (Conn, 1980, Poulton, 1990, Morant et al., 2003). Cyanogenic β-glucosidases have been characterized from a wide variety of cyanogenic plants including sorghum (Hösel et al., 1987), cassava (Mkpong et al., 1990), white clover (Pocsi et al., 1989), rubber tree (*Hevea brasiliensis*), black cherry (*Prunus serotina*) (Kuroki and Poulton, 1986), flax (*Linum ussitatissimum*) (Fan and Conn, 1985) and *Lotus japonicus* (Selmar et al., 1987). The cyanogenic glucoside dhurrin in seedling of the monocotyledonous crop sorghum is stored in the epidermal cell layer and the dhurrinases are stored in the chloroplasts in the bundle sheath cells. The disruptions of the tissue cause the glucosides to come into contact with the degrading β-glucosidase, which results in immediate release of toxic defense compounds. The highest amount of the two components is found in seedling and young plant parts in order to protect the plant from herbivore and pathogen attack (Sue et al., 2000, Forslund et al., 2004). The hydrolysis of dhurrin by β-glucosidase (dhurrinase) and the production of HCN are shown below (Cicek and Esen, 1998).



Cabbage (*Brassica oleracea* L.) responds to *Pieris brassicae* by releasing a mixture of volatiles that make them highly attractive to the *Cotesia glomerata* that attacks the *P. brassicae*. The presence of β-glucosidase in *P. brassicae* regurgitant shows similar release of a volatile blend as with commercial β-glucosidase from almonds. The treated leaves with almond β-glucosidase are highly attractive to the *C. glomerata*. It could not distinguish between the leaves that treated with β-glucosidase from regurgitant or almond. The β-glucosidase was recorded in cabbage leaf extract and it is a *P. brassicae* secreted elicitor of the defense response of cabbage plants to herbivores injury (Mattiacci et al., 1995).

The defence response in olive fruits injured by pathogens and mechanical damage is associated with the β-glucosidase. The histochemical assay and biochemical analyses of β-glucosidase after fruit injury showed strong β-glucosidase activity within 20 min and it increased rapidly in olive fruits tissues. The oleuropein contents following puncture did not change significantly in the high susceptibility cultivar, whereas it rapidly decreased in the cultivar showing low susceptibility. The results showed that olive fruits susceptible towards fly infestation could be related to the ability of oleuropein-degrading-β-glucosidase to produce the highly reactive molecules in the damaged tissue. So it could suggest that this enzyme could play a role in the defense response against insect injuries (Spadafora et al., 2008).

The benzoxazinoids DIBOA (2, 4-dihydroxy-1, 4-benzoxazin-3-one) and DIMBOA (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-1) are defense chemicals widely distributed within Poaceae and have been implicated in resistance of maize against insects, fungi and bacteria (Niemeyer, 1988). The benzoxazinoids are found in their glycosylated form (BxGlc) and are hydrolyzed by β-glucosidase to release the toxic aglycones. DIMBOA specifically blocks growth of *A. tumefaciens* and the presence of benzoxazinoids have been suggested as one of the main factors explaining the recalcitrant behavior of monocotyledons to *Agrobacterium*-mediated transformation (Sahi et al., 1990).

Oat accumulates saponins which are widespread defense compounds found in many plant species, although oat represents the only saponin accumulating cereal (Osbourn, 2003). Saponins are mainly characterized as antimicrobial compounds. Saponins are found either in their active monodesmosidic form (glycosylated at the C3 position) or in their non-active bisdesmosidic forms (glycosylated at the C3 and the C26 or C28 positions) (Osbourn, 1996). Upon tissue disruption, this β-glucosidic bond is immediately hydrolyzed by a specific β-glucosidase to yield the active 26-desglucoavenacoside, which has been shown to possess antifungal activity (Nisius, 1988). Phylogenetic analysis of β-glucosidases involved in plant defense summarized by Morant et al. (2008) is shown in Figure 7.

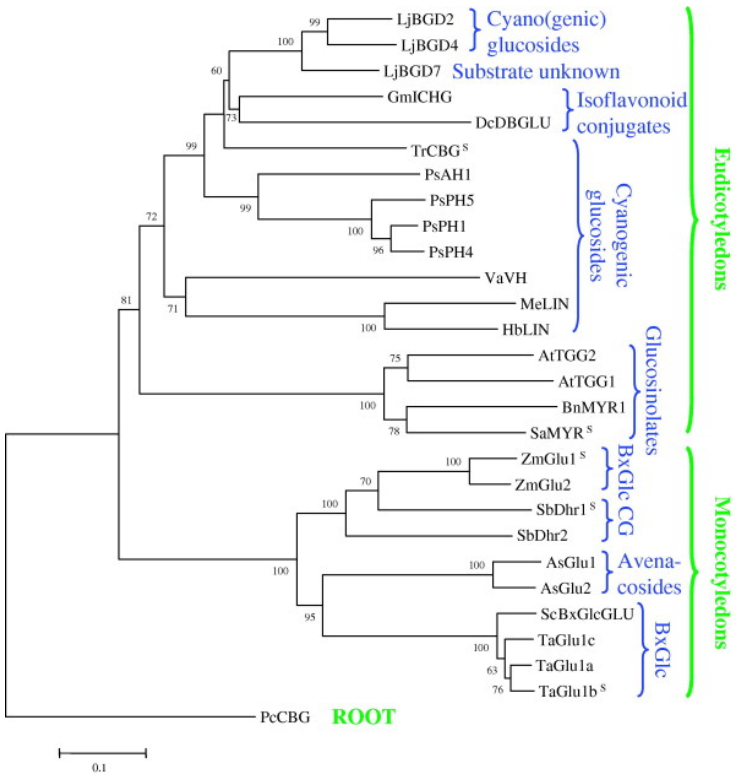


Figure 7 Phylogenetic analysis and structural conservation of β-glucosidases involved in plant defense. Neighbor-Joining phylogenetic tree including a selection of cyanogenic glucoside and isoflavonoid conjugate hydrolyzing β-glucosidases from eudicotyledons, glucosinolate-degrading myrosinases (Capparales) and β-glucosidases involved in bio-activation of defense compounds in monocotyledons. The defense compounds bio-activated by the β-glucosidases are indicated in blue (CG = cyanogenic glucosides). "S" indicates enzymes for which the crystal structures have been solved. LjBGD2, LjBGD4 and LjBGD7: *L. japonicus* β-glucosidases. TrCBG: White clover (*Trifolium repens*) linamarase. GmICHG: Soybean (*Glycine max*) isoflavone conjugate-hydrolyzing β-glucosidase. DcBDGLU: Thai rosewood (*Dalbergia cochinchinensis* Pierre) dalcochinase. PsAH1, PH1, PH4 and PH5:

Black cherry (*Prunus serotina*) amygdalin hydrolase and prunasin hydrolase isozymes. VaVH: *Vicia angustifolia* vicianin hydrolase. MeLIN: Cassava (*Manihot esculenta* Crantz) linamarase. HbLIN: Rubber tree (*Hevea brasiliensis*) linamarase. AtTGG1 and AtTGG2: *Arabidopsis thaliana* myrosinases. SaMYR: White mustard (*Sinapis alba*) myrosinase. BnMYR1: oilseed rape (*Brassica napus*) myrosinase. ZmGlu1 and ZmGlu2: Maize (*Zea mays*) DIMBOAGlc β-glucosidases. SbDhr1 and SbDhr2: Sorghum (*Sorghum bicolor* Moench) dhurrinases. AsGlu1 and AsGlu2: Oat (*Avena sativa*) avenacosidases. ScBxGlcGLU: Rye (*Secale cereale*) DIBOAGlc β-glucosidase. TaGlu1a, b and c: Wheat (*Triticum aestivum* L.) DIMBOAGlc β-glucosidases. PcCBG: Lodgepole pine (*Pinus contorta*) coniferin β-glucosidase. The phylogenetic tree was rooted using PcCBG as the outgroup. The bootstrapped Neighbor-Joining tree was built in MEGA4.0. The tree was bootstrapped with 1000 iterations (node cutoff value 50%). The underlying amino acid sequences in fastA format and the multiple alignment can be assessed at and http://www.p 450.kvl.dk/VintherMora nt_etal_Figure3A_Alignment.pdf. For the bootstrap analysis 1000 trials were performed, and the bootstrap values are shown as in %. Bootstrap node values below 50% are not shown (Morant et al., 2008).

Coniferin is a glucoside of the monolignol coniferyl alcohol that highly accumulates in gymnosperms during spring-cambial reactivation. A cinnamyl alcohol glucoside/β-glucosidase system plays a role in lignification by releasing the

monolignol aglycones. The enzyme system in xylem of *Pinus contorta*, β-glucosidases hydrolyzed the coniferin and was active against synthetic glucosides. The result reported that the coniferin β-glucosidase has high homology to known plant β-glucosidases and the coniferin, syringin, and a synthetic coniferin analog were preferred substrates for the coniferin β-glucosidase. In situ localization using the chromogenic coniferin analog showed the presence of β-glucosidase activity in the xylem (Dharmawardhana et al., 1995). The coniferin from *Cicer arietinum* L. cell suspension cultures have been found to be one of the best substrates for β-glucosidase isoenzymes. The data suggest that β-glucosidase-catalyzed reaction might be involved in lignification of these plant cell cultures (Hösel et al., 1978).

Kaisa et al. (2003) examined the relationship between β-glucosidase and peroxidase activities and xylem lignification in the stems of Scots pine (Pinus sylvestris L.), Norway spruce (*Picea abies* (L.) Karst) and silver birch (*Betula pendula* Roth) during the 1999 growing season. Hydrolysis of the synthetic β-glucosidase substrate *p*NP-β-O-D-glucopyranoside was correlated with radial growth and lignification in the xylem of both conifers, but the relationship between lignification and the hydrolysis of coniferin by β-glucosidase was not obvious.

β-glucosidase activity releases abscisic acid (ABA) from the physiologically inactive ABA-glucose conjugate pool in the leaf apoplast. ABA induces rapid stomatal closure as a short-term response to water deficit. ABA glucose ester (ABA-GE) is one of the major inactive forms of ABA and is widespread in the plant kingdom (Hartung et al., 2002). ABA-GE is located in intracellular storage organelles (vacuoles), in xylem sap, and probably in the cytosol and cell wall as well (Dietz et al., 2000). ABA-GE can be hydrolyzed in response to stress by the β-glucosidase

AtBG1 during stress responses. Thus, it leads to an increase of the active ABA concentration. In addition to its role in stress responses, ABA also affects many developmental processes, including induction and maintenance of seed dormancy, thereby inhibiting seed germination. Lee et al. (2006) report that loss of function of the *atbg1* gene (that encodes a β-glucosidase) causes a reduction in the ABA concentration in seeds and concomitantly an earlier germination of seeds.

Glycosides with monoterpene alcohols and aryl alcohols as aglycone are abundant in fresh tea leaves. The alcoholic tea aroma is generated by enzymatic hydrolysis of this kind of glucoside during the manufacture of tea, which especially important for the quality of black tea, Oolong tea and green tea (Ogawa et al., 1995). Many published studies have focused on the activity assay of the β-glucosidase enzyme and its activity variations during the development of tea plants and in the process of manufacturing tea (Jiang et al., 1999, Liu et al., 2003)

Opassiri et al. (2003) reported the β-glucosidase genes in rice *bglu1* (identified new name in 2006 was *Os3bglu7*) and *bglu2*, that were highly expressed in the shoot during germination. They suggested that *bglu1* acts in hydrolysis and recycling of oligosaccharides generated from rapid cell wall expansion during seed germination and flower expansion, and release of the coenzyme pyridoxine from its glucose-conjugated storage form.

Forty β-glucosidase genes (GH1) have been identified in the rice genome, including 2 possible endophyte genes, 2 likely pseudogenes, 2 gene fragments, and 34 apparently competent rice glycosidase genes. The Phylogenetic analysis revealed that GH1 members with closely related sequences have similar gene structures and are often clustered together on the same chromosome (Figure 8) (Opassiri et al., 2006).

Why does rice need 34 β-glucosidase genes? is a very interesting question. The β-glucosidase in rice must have different specific functions. However, up to now scientists do not yet fully understand the exact functions. Therefore, the determination of the function of some β-glucosidase isozymes by RNAi is the aim of this research.

Figure 8 Relationship between rice and other plant GH1 protein sequences described by a phylogenetic tree rooted by *Os11bglu36* (Opassiri et al., 2006). The

sequences were aligned with ClustalX, then manually adjusted, followed by removal of N-terminal, C-terminal and large gap regions to build the data model. The tree was produced by the neighbor joining method and analyzed with 1000 bootstrap replicates. The internal branches supported by a maximum parsimony tree made from the same sequences are shown as bold lines. The sequences other than rice include: ME AAB71381, *Manihot esculenta* linamarase; RSMyr BAB17226, *Raphanus sativus* myrosinase; BJMyr AAG54074, *Brassica juncea* myrosinase; BN CAA57913, *Brassica napus* zeatin-O-glucoside-degrading β-glucosidase; HB AAO49267, *Hevea brasiliensis* rubber tree β-glucosidase; CS BAA11831, *Costus speciosus* furostanol glycoside 26- O-β-glucosidase (F26G); PS AAL39079*Prunus serotina* prunasin hydrolase isoform PH B precursor; PA AAA91166, *Prunus avium* ripening fruit β-glucosidase; TR CAA40057, *Trifolium repens* white clover linamarase; CA CAC08209, *Cicer arietinum* epicotyl β-glucosidase with expression modified by osmotic stress; DC AAF04007, *Dalbergia cochinchinensis* dalcochinin 8'-O-β-glucoside β-glucosidase; PT BAA78708, *Polygonum tinctorium* β-glucosidase; DL CAB38854, *Digitalis lanata* cardenolide 16-O-glucohydrolase; OE AAL93619, *Olea europaea* subsp. europaea β-glucosidase; CR AAF28800, *Catharanthus roseus* strictosidine β-glucosidase; RS AAF03675, *Rauvolfia serpentina* raucaffricine-O-β-D-glucosidase; CP AAG25897, *Cucurbita pepo* silverleaf whiteflyinduced protein 3; AS CAA55196, *Avena sativa* β-glucosidase; SC AAG00614, *Secale cereale* β-glucosidase; ZM AAB03266, *Zea mays* cytokinin β-

glucosidase; ZM AAD09850, *Zea mays* β glucosidase; SB AAC49177, *Sorghum bicolor* dhurrinase; LE AAL37714, *Lycopersicon esculentum* β-mannosidase; HV AAA87339, barley BGQ60 β glucosidase; HB AAP51059, *Hevea brasiliensis* latex cyanogenic β-glucosidase; PC AAC69619*Pinus contorta* coniferin β-glucosidase; GM AAL92115, *Glycine max* hydroxyisourate hydrolase; CS BAC78656, *Camellia sinensis* β-primeverosidase (Opassiri et al., 2006).

2.6 Research objectives

In this research, the pHELLSGATE8 (RNAi vector) derived vectors was designed to knock down β-glucosidase genes in rice. The construction of the vector was done by Gateway cloning technology. The coding region and 3'UTR were targets to knock down β-glucosidase genes. The recombinant RNAi vectors were transformed into the rice callus by *Agrobacterium*. The transformed calli were selected on selection medium and induced to plantlet on regeneration medium. The effect of knock down of β-glucosidase genes was studied. The summary of the objectives of this research is listed below.

1. To increase the understanding of rice β-glucosidase genes functions.
2. To develop and find suitable conditions for rice tissue culture and produce the knock down lines.
3. To determine the effects on RNA expression and small interfering molecule in knock down lines.
4. To determine the effect of loss of the gene function in callus and plantlet.

CHAPTER III

Materials and methods

3.1 Plant material

The cultivated rice, *Oryza sativa* cultivar KDML105 (indica) and Koshihikari (japonica) were used in this study. KDML105 seeds were obtained from Khonkaen University and Koshihikari seeds were obtained from Chiang-Mai Rice Research Center.

3.2 Accession numbers of β-glucosidase

Os03g0703000 (*Os3bglu7*) and 4 related genes (Os03g0703100 (*Os3bglu8*), Os12g0420100 (*Os12bglu38*), Os07g0656200 (*Os7bglu26*) and Os01g0508000 (*Os1bglu1*)) (Opassiri et al., 2006, http://ppdb.gene.nagoya-u.ac.jp/cgi-bin/go_slim_list.cgi?organism=Os&id=0016787) were used in this study.

3.3 Primers

The target sequence regions for the knock down of β-glucosidase in this research were divided into 2 parts. The first part was designed from the 3'UTR (three prime untranstated regions) to knock down each β-glucosidase gene individually. The other was designed from the coding region of *Os3bglu7* to knock down all 5 β-glucosidase genes in the group (5 Bglu group) in one construct (Figure 9 and 10).

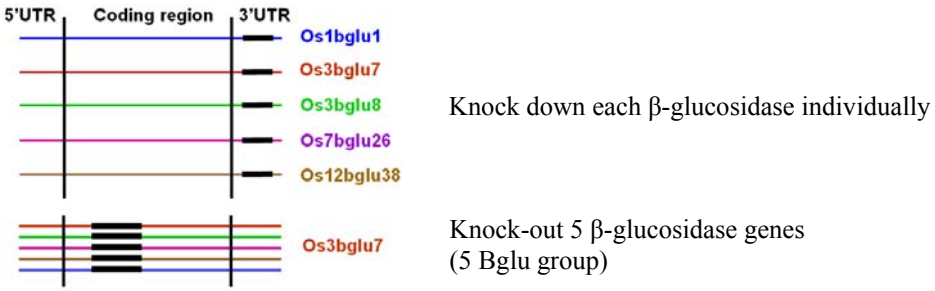


Figure 9 The knock down region of each β-glucosidase and the group of 5 β-glucosidases.

```
Os3bglu8        GCGGGCTGAGCCGGGCCGCGTTCCCCAAGGGGTTCGTGTTCGGGACGGCGACGTCGGCAT 275
Os3bglu7        GCGGGCTGAGCCGCGCCGCGTTCCCCAAGCGGTTCGTGTTCGGGACGGCCACGTCCGCGT 264
Os1bglu1        GCGGGCTGAGCCGGCGGAGCTTCCCGGCGGGGTTCGTGTTCGGGACGGCGGCGTCGGCGT 358
Os7bglu26       GCGGGCTGAGCCGGCGAGCCTTCCCGGAGGGGTTCGTGTTCGGCACGGCCGCGTCGGCGT 177
Os12bglu38      CCGACCTCACGCGGGAGACGTTCCCCGCGGGCTTCGTCTTCGGCACCGCGTCGTCGGCGT 160
                  **  ** *  **       *****   * * ***** ***** ** **  **** ** *

Os3bglu8        TCCAGGTCGAGGGCATGGCGGCGTCCGGCGGCCGCGGGCCGTCCATCTGGGACCCCTTCG 335
Os3bglu7        ACCAGGTCGAGGGCATGGCGGCGTCCGGCGGCCGCGGGCCGTCCATCTGGGACGCCTTCG 324
Os1bglu1        ACCAGGTGGAGGGCATGGCGCTCAAGGATGGCCGCGGCCCTAGCATTTGGGACGCCTTCG 418
Os7bglu26       ACCAGGTGGAGGGCATGGCGAAGCAGGGTGGCCGGGGCCCTAGCATCTGGGACGCTTTCA 237
Os12bglu38      ACCAGGTGGAGGGGAACGCCCTCCAGTATGGCCGAGGGCCCTGCATCTGGGACACCTTCC 220
                  ****** ***** *  **          ***** ** **   *** ****** * ***

Os3bglu8        TCCACACCCCAGGGAATATTGCGGGAAATGGGAATGCAGATGTTACTACAGATGAATATC 395
Os3bglu7        CGCACACCCCGGGAAATGTTGCAGGAAATCAGAATGGAGATGTTGCGACAGATCAATATC 384
Os1bglu1        TCAAGACTCCCGGTGAAATAGCAAATAATGCTACGGCAGATGTTACTGTTGATGAGTACC 478
Os7bglu26       TAGAGAAACCAGGGACGATCCCTAATAATGCCACAGCCGATGTGACGGTTGATGAGTATC 297
Os12bglu38      TGATGCAACCTGGTGTAACTCCTGATAATTCGACCGCGAATGTGACCGTCGACGAGTACC 280
                        ** **        *    ***   *  *   ****  *    **  * ** *

Os3bglu8        ATCGCTACAAGGAAGATGTTGATCTCTTGAAAAGCCTGAATTTCGATGCATATCGGTTTT 455
Os3bglu7        ATCGCTATAAGGAAGATGTTAATCTCATGAAAAGTTTGAATTTTGATGCCTACCGGTTTT 444
Os1bglu1        ATCGCTACAAGGAGGACGTAAACATCATGAAAAGTATGGGTTTCGATGCGTACCGCTTCT 538
Os7bglu26       ATAGGTACAAGGAAGATGTGAACATAATGAAGAACATGGGCTTTGATGCGTATAGATTTT 357
Os12bglu38      ACCGCTACATGGATGATGTGGACAACATGGTGAGAGTGGGCTTCGACGCGTATCGCTTCT 340
                *  * ** * *** ** **  *     **   *   **   ** ** ** **  * ** *

Os3bglu8        CAATCTCGTGGTCAAGGATTTTCCCTGATGGAGAGGGAAAAGTTAACACGGAAGGTGTGG 515
Os3bglu7        CAATCTCATGGTCCAGGATCTTCCCAGATGGTGAGGGACGAGTTAACCAAGAAGGCGTAG 504
Os1bglu1        CAATCTCATGGTCAAGAATATTCCCAACTGGAACTGGGAAAGTAAATTGGAAAGGTGTGG 598
Os7bglu26       CGATCTCTTGGTCAAGAATTTTACCAAATGGGACTGGGATGGTGAACCAGGAAGGAGTTG 417
Os12bglu38      CGATCTCCTGGTCTCGCATTTTCCCCAGTGGACTTGGGAAGATTAACAAAGACGGCGTGG 400
                * ***** *****  * ** ** **   ***    **     * **     * ** ** *

Os3bglu8        CATATTACAATAATCTAATAGATTATGTAATTAAGCAAGGGCTTATTCCTTACGTCAATC 575
Os3bglu7        CATATTACAACAATCTTATAAACTACCTTCTGCAGAAAGGTATCACTCCTTATGTCAATC 564
Os1bglu1        CATACTATAACAGATTGATAAACTATATGCTGAAGATAGGCATTACACCTTATGCCAATT 658
Os7bglu26       ATTATTACAACAGGTTAATAGATTACATGGTTAAGAAAGGCATCAAACCGTACGCAAACC 477
Os12bglu38      ATTATTACCACAGGCTCATTGATTACATGCTTGCTAACAACATTATTCCATATGTTGTGC 460
                  ** **  * *   * **  * **  *  *           * *  ** ** *

Os3bglu8        TGAACCACTACGATCTCCCACTTGCACTTCAGAAAAAGTATGAAGGCTGGTTAAGCCCAA 635
Os3bglu7        TTTACCACTACGATCTCCCTCTTGCGCTTGAGAAGAAGTACGGAGGCTGGTTGAATGCAA 624
Os1bglu1        TGTATCACTATGACTTACCAGAGGCACTAGAGGTGCAATATGGAGGACTGTTGAACAGAA 718
Os7bglu26       TCTACCACTATGACCTACCATTAGCACTCCATGAGCAGTACTTAGGCTGGCTAAGCCCAA 537
Os12bglu38      TCTACCACTACGACCTTCCACAGGTGCTCCATGATCAATACAAGGGATGGCTACACCCCA 520
                *  * ***** **  * **    *  **  *     * **    **   * *       *
```

Figure 10 The alignment of the nucleotide sequence of the 5 β-glucosidase genes (ClustalW). The two boxes show the location of forward and reverse primers to amplify target gene to knock down 5 β-glucosidase genes.

The primer pairs for amplification of the coding region of *Os3bglu7* to knock down the 5 genes (5 Bglu group (399 bp)) were designed in a highly similar sequence region of the 5 β-glucosidase genes from ClustalW sequence alignment program (Figure 10). The primer pairs to amplification in the 3'UTR region of *Os3bglu8* (260 bp), *Os12bglu38* (252 bp), *Os7bglu26* (256), *Os3bglu7* (283 bp) and *Os1bglu1* (271 bp) were designed from different sequence regions of the 5 β-glucosidase genes. The sequence of primer pairs showed in table 1.

Table 1 The sequence specific primer of β-glucosidase genes.

Specific primer		**Sequence***	**region**
Os3bglu8	Forward	**CACC**CTCGAGAAGTAGTGGATGCCAGCAG	3'UTR
	Reverse	GGGAATTCAGGCCAAAGTCCAGGATATC	
Os12bglu38	Forward	**CACC**CTCGAGCACGTTGGTTCAGGAAG	3'UTR
	Reverse	GGGAATTCCTGCCTCTCTTATCACC	
Os7bglu26	Forward	**CACC**CTCGAGTGCAGACAAAAGGATCAAGC	3'UTR
	Reverse	GGGAATTCTAGTCCCTTCTGTCAGCTC	
Os3bglu7	Forward	**CACC**CTCGAGGTCGACTTCAACACGCTC	3'UTR
	Reverse	GGGAATTCCACCAAGCCAAATCTCATC	
Os1bglu1	Forward	**CACC**CTCGAGGGCTACTTCGCCTGGTCC	3'UTR
	Reverse	GGGAATTCCAATCTTGAATGATG	
5 Bglu Group	Forward	**CACC**CTCGAGGTTCCCCAAGCGGTTCGTG	Coding
	Reverse	GGGAATTCGCATTCAACCAGCCTCCG	region

* CACC for directional cloning into pENTR/D TOPO vector, *Xho*I sites are underline, *EcoR*I sites are double underlined.

The primers were obtained from Proligo/Geneset (Singapore). The CACC sequence was added to the forward primer at the 5' end. The CACC sequence is the specific sequence for directional cloning into the pENTR™/D-TOPO cloning vector (Invitrogen) in the position of GGTG (Figure 11) to control the orientation of the target sequence.

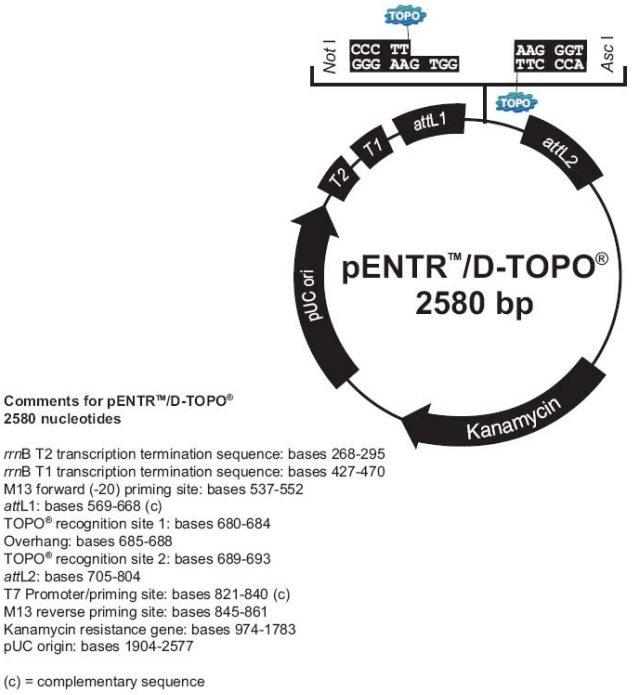


Figure 11 The map of pENTR™/D-TOPO cloning vector (Invitrogen)

After the CACC sequence, the *XhoI* site (CTCGAG) was added to the forward primers. The *EcoR*I site (GAATTC) was added to the reverse primer. These restriction enzyme sites were used to cut and move the fragment from the pENTR™/D-TOPO cloning vector to pHELLSGATE8 vector (CSIRO, the Commonwealth Scientific and Industrial Research Organisation, Australia's National Science Agency) (Figure 12).

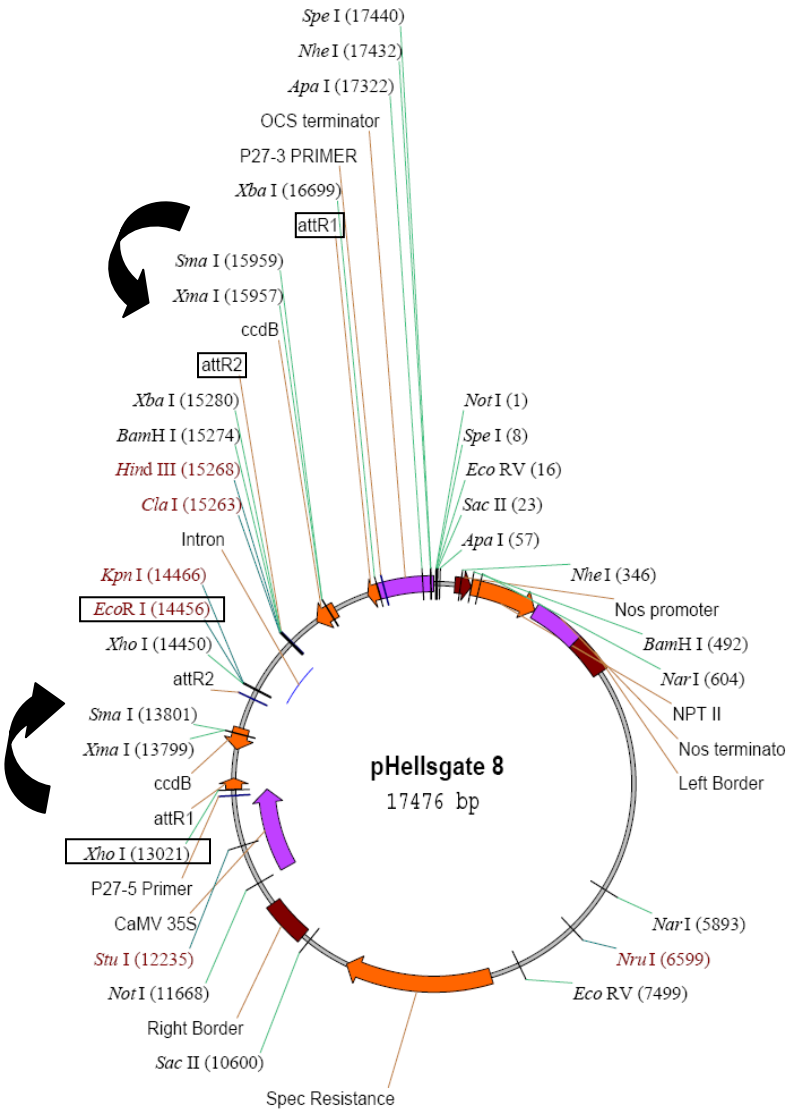


Figure 12 The map of pHELLSGATE8 vector (http://www.pi.csiro.au/tech_licensing_biol/MapsProtocol.htm). The arrows outside the plasmid indicate the regions that are replaced with β-glucosidase sequences.

3.4 RNAi vector (pHELLSGATE8) construction

RNA silencing constructs carrying fragments of endogenous genes were made using the pHELLSGATE8 vector. The vector pHELLSGATE8 has a special insertion

sequence containing an intron (pyruvate orthophosphate dikinase; pdk) flanked by 2 specific multiple cloning sites (MCSs) (Figure 12). This vector has attachment sites (attR site) that are important for the LR clonase enzyme to move the insert fragment from an entry vector (pENTR^{TM}/D-TOPO cloning vector; attL site) to the destination vector (pHELLSGATE8).

3.4.1 Amplification of rice 3'UTR and coding region of *Os3bglu7*

3.4.1.1 DNA extraction

Rice seeds were germinated on tissue paper under light/dark conditions at room temperature for 2 weeks. The DNA extraction was based on a potassium acetate method modified from Dellaporta et al. (1993). The extraction buffer was freshly prepared and preheated at 65^{o}C for 30 min. Zero point one eight gram of rice leaf was weighed and grinded in a mortar with liquid nitrogen until the sample became powder. The samples were then moved into a 1.5 mL micro centrifuge tube. Then 720 µL of extraction buffer was added and the tube was immediately vortexed. The tubes were incubated at 65^{o}C for 15 min in a heat box. The samples were vortexed every 5 min. After that, 225 µL of 5 M potassium acetate was added and mixed thoroughly by inverting the tube several times and the tubes were incubated on ice for 20 min on a shaker. The samples were then centrifuged for 15 min at 14,000 x g. The supernatant was decanted into a new tube. Ice-cold isopropanol (2/3 volume of supernatant) was added and the tubes were mixed gently by inverting slowly. Centrifugion was performed for 15 min (14,000 x g) to precipitate the DNA. The supernatant was removed from the tube and the DNA was washed with 30 µL of 70% cold ethanol gently and centrifuged for 7 min (3,000 x g) to precipitate DNA. The

supernatant was discarded by pipetting and the tubes were dried for 10 min at room temperature. Fifty microliter of TE was added in the tubes and were then stored at -20^{o}C.

3.4.1.2 PCR amplification

The PCR reaction mix includes DNA template (from 3.4.1.1), 2 µL of 2.5 mM dNTPs, 1.5 µL of 25 mM $MgCl_2$, 2.5 µL of 10X buffer (Promega), 0.3 µL of 10 mM forward primer, 0.3 µL of 10 mM reverse primer, 0.5 µL of *Taq* DNA polymerase (homemade) and 16.9 µL of sterile distilled water to make the final volume 25 µL. The PCR amplification was done at 94^{o}C for 5 min at the initial denaturation step, followed by 35 cycles of 94^{o}C for 30 seconds, annealed at a specific temperature (71^{o}C for 5 Bglu group, 55^{o}C for *Os3bglu8*, 56.6^{o}C for *Os7bglu26*, 52.6^{o}C for *Os12bglu38*, 51.4^{o}C for *Os3bglu7* and 59^{o}C for *Os1bglu1*) for 30 seconds, kept on 72^{o}C for 30 seconds and the final extension of 5 min at 72^{o}C was done after the last cycle.

3.4.1.3 DNA analysis by agarose gel electrophoresis

The amplified PCR products were analyzed on 1.5% agarose gel electrophoresis in 1X TAE buffer (0.04 M Tris-HCL pH 8.0, 0.04 M acetic acid, 0.001 M EDTA pH 8.0) as described by Sambrook et al. (1989). The 1.5% gel was prepared from low EEO, molecular biology grade agarose (Research Organics, Inc.) DNA samples were prepared by mixing 5:1 with 6X loading dye (Fermentas) and applied to the gel wells. Electrophoresis was performed at a constant 100V for 40 min. After electrophoresis, the gel was stained in ethidium bromide solution and destained with

distilled water. The band in the gel was visualized by UV light transillumination. The sizes of DNA fragments were estimated by comparing with GeneRuler 100bp DNA Ladders (Fermentas).

3.4.1.4 Purification of PCR products from agarose gels

The DNA bands in the agarose gel were extracted with the QIAquick gel extraction kit (QIAGEN) followed the handout recommended protocol. Briefly, the PCR products from 100 µL reaction mix were separated on 1% TAE agarose gels. The DNA bands were excised with a clean and sharp scalpel and placed in a 1.5 mL microcentrifuge tube. The small piece of excised gel was weighed. Three volumes of Buffer QG were added to one volume of gel and the tube was incubated at 65^{o}C for 10 min or until the gel was completely dissolved. To help to dissolve the gel, mixing was done by vortexing the tube every 2-3 min during the incubation. After the gel slice has dissolved completely, one gel volume of isopropanol was added to the sample and mixed again (this step was done to increase the yield of DNA fragments lower than 500 bp and higher than 4 bp). The sample was applied to the QIAquick spin column and centrifuged for 1 min at 14,000 x g. The flow through fraction was discarded and the column was washed with 0.75 µL of buffer PE and centrifuged for 1 min at 14,000 x g. The flow through was discarded again and the column was centrifuged for an additional 1 min at 14,000 x g. The column was placed in a clean 1.5 mL microcentrifuge tube and the DNA was eluted by adding 30 µL of buffer EB into the center of the column membrane, storing it at room temperature for 5 min and centrifuging the column at 14,000 x g for 1 min. The eluted DNA was stored at -20^{o}C.

3.4.2 Vector preparation for ligation reaction

3.4.2.1 Competent cell preparation

A single colony of *E. coli* DB3.1 from a fresh LB plate was used to start as a starter inoculum of 3 mL LB broth. It was grown overnight at 37°C with 200 rpm shaking. One milliliter of the starter was inoculated in a 250 mL flask that contained 50 mL LB broth and was shaken at 200 rpm at 37°C for 3 hr (measure OD_{600} equals to 0.6) and the flask was then placed on ice. The cell culture was placed into a 50 mL Falcon tube and centrifuged (Centrifuge 5810R, Eppendorf) at 4,000 x g 4°C for 7 min. The tube was placed on ice and the supernatant was immediately discarded leaving the cell pellet, then the cell pellet was resuspended in 30 mL ice-cold dH_2O and mixed gently until the cell pellet was completely dissolved in the dH_2O. The tube was centrifuged again at 4,000 x g at 4°C for 7 min and then placed on ice. The supernatant was discarded and the cell pellet was resuspended in 30 mL ice-cold dH_2O and mixed gentle, then centrifuged to keep the pellet (4,000 x g at 4°C for 7 min). Then the tube was placed on ice again and the cell pellet was resuspended in 30 mL 10% glycerol and centrifuged at 4,000 x g at 4°C for 7 min. The tube was placed on ice and the cell pellet was resuspended in 500 µL of GYT medium (10% (v/v) glycerol, 0.125% (w/v) yeast extract and 25% (w/v) tryptone), finally one hundred microliter per tube was aliquoted into 1.5 mL microcentrifuge tube and the tubes were immediately frozen in liquid N_2 and stored at -70°C.

3.4.2.2 pHELLSGATE8

The plasmid pHELLSGATE8 in TE, obtained from CSIRO, was transformed into DB3.1 cells by electroporation; 100 µL of DB3.1 competent cells and 1 µL of the

plasmid was mixed and placed into the Gene Pluser Cuvette (0.1 cm electrode gap, BIORAD). Then the cuvette was moved to an electroporator (2510 Eppendorf) and electrophorated at 1800 V. Three hundred microliters of SOC medium was added in the cuvette immediately and the cells were moved to a 1.5 mL microcentrifuge tube and shaken at 37°C for 30 min. Then, the DB3.1 competent cells were spread on an LB agar medium containing 100 mg/L antibiotic (spectinomycin, Sigma) and were incubated at 37°C for 16 hr to select the transformed cells.

3.4.2.3 Plasmid preparation

The colonies were selected from the LB plate and cultured in 3 mL LB broth containing 100 mg/L spectinomycin at 37°C for 16 hr. The QIAprep miniprep plasmid extraction kit (QIAGEN) was used to purify the plasmid. The cultured cells were pelleted by centrifugation at 14,000 x g for 1 min at room temperature. The cell pellets were resuspended completely by vortexing in a 250 μL P1 buffer containing RNase A. Then 250 μL of P2 buffer was added to resuspend the cells and mixed thoroughly gentle by inverting the tube 4-6 times to prevent the shearing of genomic DNA. After adding 350 μL N3 buffer to the viscous and slightly clear suspension cell solution and mixing immediately and thoroughly by inverting the tube 4-6 times, the solution became cloudy. The tube was centrifuged at 14,000 x g for 10 min to compact the pellet. The supernatant was applied to the QIAprep spin column by pipetting and was centrifuged at 14,000 x g for 1 min. The flow through was discarded and the QIAprep spin column was washed by adding 0.5 mL PB buffer, and then centrifuged for 1 min. The flow through was discarded and the column was washed by adding 0.75 mL PE buffer and centrifuged for 1 min. The flow through

was discarded and the column was centrifuged for an additional 1 min to remove the residual wash buffer. The QIAprep column was placed in a clean 1.5 mL microcentrifuge tube and the plasmid DNA was eluted by adding 50 µL EB buffer to the center of the column, keeping it at room temperature for 5 min and finally centrifuging the column at 14,000 x g for 1 min. The plasmid DNA was stored at -20^{o}C.

3.4.2.4 pENTR^{TM} TOPO cloning vector

The pENTR^{TM} directional TOPO cloning kits was purchased from Invitrogen. The pENTR^{TM} TOPO reagents were stored at -20^{o}C and the One Shot TOP10 chemically competent *E. coli* was stored at -70^{o}C.

3.4.3 Ligation and transformation

3.4.3.1 Construct pENTR^{TM} TOPO cloning vector

The DNA fragments from the 3'UTR region of each β-glucosidase genes and the 5 knock down genes obtained from step 3.4.1 were cloned into pENTR^{TM} TOPO followed the recommended protocol with minor modifications. Four microliters of fresh PCR product, 1 µL salt solution, and 1 µL pENTR^{TM} TOPO vector were mixed briefly. Then the reaction was mixed gently and incubated at room temperature for 1 hr or overnight. The reaction was placed on ice and then 2 µL of the pENTR^{TM} TOPO cloning reaction was added into a vial of the One Shot chemically competent *E. coli* (TOP10 competent cell, Invitrogen). They were mixed gently and incubated on ice for 30 min. The cells were heat-shocked at 42^{o}C without shaking for 30 seconds, immediately cooled, by transferring the tube to ice, and incubated for 2 min.

The SOC medium (250 µL) was added in the tube and the tube was shaken horizontal (200 rpm) at 37°C for 30 min, then 100 µl from the transformation was spread on a LB plate containing 100 mg/L of kanamycin and incubated overnight at 37°C.

3.4.3.2 DNA sequencing

The transformed colonies (obtained from step 4.3.1) were selected to confirm the correct sequence of the target genes. The colonies were selected from the LB plate and the cells were cultured in a 3 mL LB broth containing 100 mg/L of kanamycin at 37°C for 16 hr. The plasmid was prepared following the protocol in 3.2.1.1. The plasmid was then sent to Macrogen Company, Korea (http://dna.macrogen.com) for DNA sequencing. The primers for sequencing are shown in Table 2.

Table 2 The sequence specific primer of sequencing the target gene fragment in pENTR™ TOPO vector.

Specific primer	Sequence
M13F (-20)	GTAAAACGACGGCCAGT
M13R-pUC (-40)	CAGGAAACAGCTATGAC

3.4.3.3 The construction of the pHELLSGATE8

- Restriction enzyme digestion

The pENTR™ TOPO vector containing the correct target gene sequence obtained in step 3.4.3.2 and the pHELLSGATE8 vector were digested with *Eco*RI (Fermentas). The digestion reaction mix included 5 µg pENTR™ TOPO vector, 1.5 µL 10X buffer *Eco*RI, 10 units of *Eco*RI and sterile distilled water to bring the volume

up to 15 µL. The reaction was incubated at 37°C overnight. After the restriction enzyme digestion reaction of the pENTR™ TOPO vector and the pHELLSGATE8 vector, the plasmids were precipitated following the protocol 3.4.2.3, the two vectors were digested again with the second enzyme, *Xho*I (Fermentas). The digestion reaction mix included 5 µg vector, 1.5 µL 10X buffer R, 10 units of *Xho*I and sterile distilled water to bring the volume to 15 µL. The reactions were then incubated at 37°C overnight.

- Precipitation of the restriction enzyme digestion reaction

The plasmid was precipitated by the sodium acetate method to remove the buffer and the enzyme. The precipitation mix includes 15 µL of restriction enzyme digestion reaction, 4.5 µL of 3 M sodium acetate, 30 µL dH_2O and 112.5 µL absolute ethanol, was mixed by vortexing and was incubated on ice for 20 min. The reaction was centrifuged (centrifuge 5415C, Eppendorf) at 14,000 x g for 10 min. The supernatant was discarded and 250 µL of 70% ethanol was added, mixed gentle and centrifuged at 14,000 x g for 10 min. The supernatant was discarded and the pellet was dried until the ethanol was evaporated. Then the DNA pellet was resuspended in dH_2O.

- Agrose gel electrophoresis; plasmid and target gene fragment purification

The pENTR™ plus insert restriction enzyme digestion reaction was loaded into the 1.5% agarose gel in 1X TAE buffer (as described in 3.4.1.3). The inserted band was purified from the gel (as described in 3.4.1.4).

3.4.3.4 Ligation and transformation

The first piece of the target gene fragment was transferred to pHELLSGATE8 vector by T4 DNA ligase and the second piece in reverse orientation was transferred by LR clonase enzyme (Invitrogen) recombination reaction to insert the first piece, the sticky-end ligation of purified target gene fragment into pHELLSGATE8 vector was done by T4 DNA ligase (BioLabs) according to the supplier's directions. The reaction mix consisted of 100-200 ng of pHELLSGATE8 vector, 300-500 ng of target gene fragment, 1 μL of 10X ligase buffer, 1 unit of T4 DNA ligase and sterile distilled water to bring the volume up to 10 μL. The ligation reaction was then incubated at 16^{o}C overnight. The ligation reaction was transformed into DB3.1 cells by electroporation. To insert the second piece, the complete pHELLSGATE8 vector was produced by the LR clonase enzyme. The reaction mix included 300 ng of entry clone (pENTR^{TM} TOPO vector containing the target gene fragments from 4.3.1), 50 ng of destination vector (pHELLSGATE8 vectors containing the first piece of the target gene fragments), 2 μL 5x LR clonase reaction buffer, 2 μL of LR clonase enzyme (return it to the -70^{o}C storage immediately after use) and sterile distilled water to bring the volume to 10 μL. The reaction was incubated at 25^{o}C over night. The reaction was terminated by adding 2 μL of the proteinase K solution (invitrogen) and vortexing briefly, and then the sample was incubated at 37^{o}C for 10 min. Two microliter of LR reaction was transformed into 100 μL of DH5α competent cell by the electroporation method and the colonies grown on LB spectinomycin were collected for plasmid extraction. The correct constructs were transformed to *Agrobacterium* strain EHA105.

3.5 Plant tissue culture

3.5.1 Seed sterilization

The mature rice seeds were dehusked to remove the seed coat. The seeds were surface sterilized by soaking the seed in 100 mL of 70% ethanol for 1 min and washed with 100 mL of sterile water 3 times to remove the 70% ethanol. Two percent of sodium hypochlorite was used to sterilize the seeds with shaking for 30 min and the seeds were washed with 100 mL sterile water 6 times. The seeds were blotted and dried in a petri dish for 30 min and then the seeds were moved to the callus induction medium.

3.5.2 Callus induction

The sterile seeds of KDML105 were cultured on a MS and N6D medium (pH 5.8). The MS medium was composed of 30 g/L of sucrose, 4.43 g/L of Murashige&Skoog basal medium w/vitamins (Phytotechnology, USA), 2 mg/L of 2, 4-D and 4 g/L of gellengum (phytotechnology, USA). The cultures were incubated at 28^{o}C. The seeds of Koshihikari were cultured on only N6D medium (pH 5.8) containing with 30 g/L of sucrose or 30g/L of maltose, 3.98 g/L of CHU basal salt mixture (phytotechnology, USA), 300 mg/L of casamino acids, 2.878 g/L of L-proline, 5 mL/L of 100X N6 vitamin, 2 mg/L of 2, 4-D and 4 g/L of gellengum (phytotechnology, USA) for callus induction. The cultures were incubated at 28^{o}C.

3.5.3 Co-cultivation

The embryogenic calli (secondary calli) were separated and subcultured on fresh N6D medium for 3 days. *Agrobacterium* strain EHA105 harbouring recombinant

pHELLSGATE8 were streaked on solid AB medium composed of 100 mg/L spectinomycin, 5 g/L of Glucose, 50 mL/L of 20XAB buffer, 50 mL/L of 20XAB salt and 15 g/L of bactoagar. The bacterial cultures were incubated in the dark at 28^{o}C for 3 days.

The bacterial culture on the AB plate were resuspended in AAM liquid medium (pH 5.2) containing 1 mL/L of AA-1 (1000X), 1 mL/L of AA-2 (1000X), 1 mL/L of AA-3 (1000X), 1 mL/L of AA-4 (1000X), 1 mL/L of AA-5 (1000X), 5 mL/L of AA-6 (200X), 1 mL/L of AA-sol (100X), 0.5 g/L of casamino acid, 68.5 g/L of sucrose, 39 g/L of glucose, 0.90 g/L of L-glutamine, 0.30 g/L of L-aspatic acid, 3 g/L of KCl and containing a different concentration of 100 and 200 μM of acetosyringone. The AAM medium was shaken to disperse the bacterial clump and the density of the bacterial suspension was adjusted to an OD_{600} of 0.02 with AAM medium.

The 3-day old of pre-culture of secondary calli after subculturing on fresh media from 3.5.2 were moved to dry on a petri dish for 30 min. The secondary calli were then immersed in a bacterial suspension for 5 min and the excess bacterial suspension was removed by blotting dry the calli on sterile tissue paper. The calli were transferred to a co-cultivation medium (2N6-MS, pH 5.2) that contains 30 g/L of sucrose, 10 g/L of glucose, 3.98 g/L of CHU basal salt mixture, 300 mg/L casamino acid, 5 mL/L of 100X N6 vitamin, 2 mg/L of 2,4-D and 4 g/L of gellengum. The infected calli were incubated in the dark at 25^{o}C or 28^{o}C for 3 days.

3.5.4 Callus selection

After 3 days, the infected calli were washed to remove *Agrobacterium*. The calli were washed 4 times with sterile distilled water until the water turns clear,

followed by 3 times washing with sterile distilled water that contains 300 mg/L of timentin or 300 mg/L of carbenicilin or cefotaxime. The calli were blotted on sterile tissue paper and transferred to N6D medium (8 g/L of agar A, Biobasic science Inc.) containing 100 mg/L of paromomycin and 300 mg/L of timentin. The calli were then incubated in the dark at 28^{o}C for 2 weeks and then the healthy portions of calli were moved onto a fresh selection medium. The calli were moved onto a fresh selection medium every 2 weeks (4 times) and then some 8-week old calli were moved onto regeneration medium. The rest of the calli were moved on to a new selection medium for about 2-4 weeks to increase the amount of calli for DNA and RNA extraction.

3.5.5 Plant regeneration

After 4 rounds of selection, actively growing pieces of calli were transferred to MS regeneration medium (pH 5.8) containing 30 g/L of sucrose, 30 g/L of sorbitol, 4.33 g/L MS basal salts mixture vitamin (phytotechnology, USA), 2 g/L casamino acid, 0.5 mg/L of NAA, 2 mg/L of kinetin, 30 mg/L of paromomycin and 8 g/L of agar A (Biobasic science Inc.). The calli were incubated under a 16 hr light and 8 hr dark photoperiod. Shoot and root regeneration was observed after 6 weeks and the calli were transferred to a rooting medium (MS medium, pH 5.8, containing 4.33 g/L of MS basal salt mixture vitamin, 30 g/L of sucrose and 4 g/L of gellangum). The 2 week old plantlets were then removed from a bottle by washing the root to eliminate the gellangum and transferred to soil. The plantlets were covered with plastic bags to maintain moisture for 7 days and then the plastic bag was removed and the plantlets were transferred to a greenhouse.

3.6 Checking of transgenic callus and plantlet by PCR

3.6.1 DNA extraction and PCR amplification

The resistant calli on selection medium and the transgenic plants were collected for DNA extraction according to section 3.4.1.1. The *nptII* gene primers (forward primer: GCTATTCGGCTATGACTG and reverse primer: CGGCCATTTTCCACCATG, specific product size 730 bp) were used to confirm the integration of the pHELLSGATE8 T-DNA into the rice genome.

The PCR reaction mix includes DNA template (from 3.4.1.1), 2 µL of 2.5 mM dNTP, 1.5 µL of 25 mM $MgCl_2$, 2.5 µL of 10X buffer, 0.3 µL of 10 mM forward primer, 0.3µL of 10 mM reverse primer, 0.5 µL of Taq DNA polymerase (homemade) and 16.9 µL of sterile distilled water to make the final volume 25 µL. The PCR amplification was done at 94^{o}C for 5 min for the initial denaturation step, followed by 35 cycles of 94^{o}C for 30 seconds, annealed at 55^{o}C for 30 seconds, and at 72^{o}C for 35 seconds and then the final extension of 5 min at 72^{o}C was done after the last cycle. The amplified PCR products were analyzed on 1.5% agarose gel electrophoresis in 1X TAE buffer. Electrophoresis was performed at a constant 100 V for 40 min. After electrophoresis, the gel was stained in an ethidium bromide solution and destained with distilled water. The band in the gel was visualized by UV light transillumination (Chemidoc, Biorad). The sizes of DNA fragments were estimated by comparing them with GeneRuler 100bp DNA Ladders (Fermentas).

3.7 Checking the effect of knock down of the β-glucosidase gene

3.7.1 RNA extraction

Total RNA was extracted from the calli with TRizol reagent (Invitrogen). The 100 mg calli were homogenized with liquid nitrogen in a mortar. One milliliter of TRizol was added to the sample and mixed well. Then 0.2 mL of chloroform was added to the tube with the sample and was shaken vigorously by hand for 15 seconds and the sample was incubated at room temperature for 3 min. Then the samples were centrifuged at 12,000 x g for 10 min at 4^{o}C. The aqueous phase was transfrerred to a fresh tube and the RNA was precipitated by mixing with 0.5 mL of isopropyl alcohol. The samples were incubated at -20^{o}C overnight and then centrifuged at 12,000 x g for 10 min at 4^{o}C. The supernatant was removed and the RNA pellet was washed once with 1 mL of 75% ethanol. The sample was mixed by vortex and centrifuged at 8,000 x g for 5 min at 4^{o}C. The supernatant was removed and the RNA pellet was dried. After adding 50 μL RNase free dH_2O the sample was stored at -70^{o}C.

3.7.2 RT-PCR (Reverse-transcription PCR)

The RNA sample was treated with DNaseI (Promega) to destroy the DNA. Total RNA from 3.7.1 was treated with 1 μL of DNase buffer (10X) and 1 μL of DNaseI and incubated at 37^{o}C for 45 min. Then 1 μL of DNase stop solution (Promega) was added and the reaction was incubated at 70^{o}C for 20 min.

SuperScript^{TM}III Reverse Transcriptase (Invitrogen) was used to synthesize the first-strand cDNA. It provides an increased specificity and a higher yield of cDNA. One microliter of 50 μM Oligo (dT) primer, 1 μL of 10 mM dNTPs and the 3 μg of treated total RNA were mixed and heated at 65^{o}C for 15 min. The samples were

moved on ice immediately for 1 min to denature the secondary structure and centrifuged briefly for 2-3 seconds. After that, 4 µL of 5X first-strand buffer, 1 µL of 0.1 M DTT, 1 µL of RNaseOUT and 1 µL of SuperScript III RT were added. The reactions were mixed and incubated at 55°C for 60 min in a PCR Sprint Thermal Cycler (HBSP05220). The reactions were inactivated by heating the tube at 70°C for 15 min and then placed on ice. The first strand cDNAs were kept at -20°C and is ready for use in a PCR reaction.

One microliter of first-strand cDNA synthesis was used as a template to check the rice β-glucosidase mRNA expression. The PCR reaction was made by adding 2 µL of 2.5 mM dNTP, 2.5 µL of 10X buffers, 1.5 µL of 2.5 mM $MgCl_2$, 0.3 µL of 10 µM of each primer for β-glucosidase and β-actin, 0.5 µL *Taq* DNA polymerase and H_2O to have a final volume of 25 µL. The PCR of each β-glucosidase gene was done under the following PCR conditions: keep at 94°C for 5 min at the initial denaturation step, followed by 35 cycles of 94°C for 30 seconds, annealed at 55°C for *Os3bglu8*, 56.6°C for *Os7bglu26*, 52.6°C for *Os12bglu38*, 51.4°C for *Os3bglu7*, 59°C for *Os1bglu1* and 53°C for β-actin for 30 second, and at 72°C for 35 second and then the final extension of 5 min at 72°C was done after the last cycle

3.7.3 siRNA extraction

One hundred milligrams of calli were homogenized with liquid nitrogen and moved to a 1.5 mL microcentrifuge tube. One milliliter of TRizol reagent was immediately added into the powder sample and mixed until the sample clearly dissolved in the reagent. The sample was stored at room temperature for 5 min. The phase separation was done by adding 0.2 mL of chloroform and mixing by vortexing

and incubating the sample at room temperature for 3 min. The sample was centrifuged at 11,000 x g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The RNA remained exclusively in the aqueous phase. The aqueous phase (about 60% of the volume TRizol reagent) was moved carefully, without disturbing the interphase, into a fresh 1.5 mL microcentrifuge tube. The RNA was precipitated from the aqueous phase by adding 0.5 mL of isopropanol and incubating at -20°C overnight. The incubated sample was centrifuged at 11,000 x g for 15 min at 4°C. The supernatant was removed and the pellet was washed with 1 mL of 75% ethanol. The sample was mixed and centrifuged at 11,000 x g for 5 min at 4°C. The supernatant was removed by pipetting and the pellet was air-dry for 10 min. The pellet was resuspended in 35 µL of DEPC-treated water by passing the solution a few times through the pipette tip.

3.7.4 Northern blot analysis

The siRNA was detected by DIG Northern Starter kit (Roche).

3.7.4.1 DNA template for RNA probe

The plasmid DNA of each constructs (pENTR™/D-TOPO) containing the target knock down gene fragment was digested with *Not*I. Five microgram of Plasmid DNA, 1.5 µL of buffer (10X), 0.5 µL of *Not*I and 8 µL of dH_2O, was mixed and was incubated at 37°C overnight. The reaction was purified by QIAquick PCR Purification Kit (QIAGEN). The digested plasmid was stored at -20°C. The digested plasmid was then use as template for making RNA probe (3.7.4.2).

3.7.4.2 Probe preparation

The RNA probe is labeled in an in vitro transcription reaction with digoxigenin -11-UTP using a labeling mixture and an optimized transcription buffer. The probe was synthesized by adding 0.5 µg linearized plasmid DNA (3.7.4.1), 2 µL of labeling mix (5X), 2 µL of Transcription buffer (5X) and 1 µL of T7 RNA polymerase. The reaction was mixed, centrifuged and incubated for 1 hour at 42^{o}C. Two microliters of DNaseI (RNase free) were added and incubated for 15 min at 37^{o}C to remove DNA. The reaction was stopped by adding 2 µL of 0.2 M EDTA (pH 8.0). The RNA probe was immediately used for hybridization or stored at -70^{o}C under RNase free conditions.

3.7.4.3 Polyacrylamide gel preparation and separation of siRNA

The gel system used was the Bio-Rad mini-PROTEIN system. The electrophoresis equipment was cleaned with sterile distilled water and the glass plates were wiped with 70% ethanol. A 15% denaturing polyacrylamide gel (7 M urea) was prepared with 4.2 g of urea, 0.5 mL of 10X TBE, 3.75 mL of 40% (w/v) 19:1 acrylamide:bis-acrylamide and 2.5 mL of water. The solution was stirred at room temperature to dissolve the urea and 70 µL of 10% (w/v) ammonium persulphate (APS) and 3.5 µL of TEMED were added. The solution was immediately poured on to the glass plates and kept at room temperature for 30 min to polymerize.

The electrophoresis apparatus was assembled and the wells were rinsed with running buffer (0.5X TBE) using a pipet and the gel was pre-runned at 100V for 30 min. The RNA samples (15-20 µg) were mixed with 6X loading buffer (Fermentas) and denatured at 65^{o}C for 10 min and then the tubes were placed on ice immediately.

The well was washed and loaded with the RNA samples. The gel was run at 50V until the dyes enter the gel (30 min). The small RNAs run between the bromophenol blue and xylene cyanol in a 15% denaturing polyacrylamide gel. The well was then washed with micropipette to remove the high molecular weight RNA and then the electrophoresis was continued at 100V until the bromophenol blue reached the bottom of the gel. The section of the gel for blotting on membrane was chosen from the area under the xylene cyanol to the bottom of the gel for detects the siRNA.

3.7.4.4 Small RNA transfer by capillary blotting

The gel was soaked in 50 mL of 20X SSC for 10 min and a membrane (Nylon membrane positively charged, Roche) was cut to the size of the gel. The membrane was equilibrated in dH_2O and soaked in 20X SSC for 5 min. The capillary blot (Figure 13) of RNA was done overnight. The capillary blot system was dismantled and the membrane was placed on a Whatman 3MM-paper soaked with 2X SSC. The RNA was crosslinked to the membrane with UV (365 nM) for 2 min and the membrane was rinsed briefly in dH_2O and then air dried. The membrane was stored dry at 4^oC.

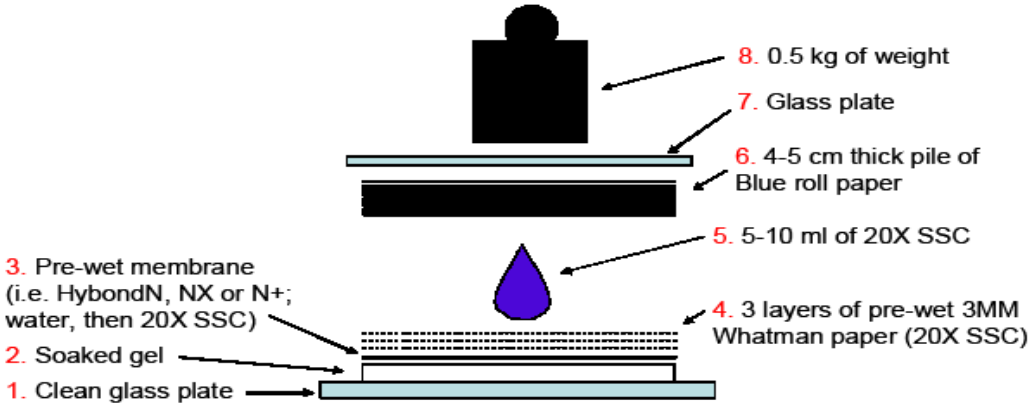


Figure 13 Capillary blotting without reservoir followed the protocol of Molnár et al. (2007). The system was setup following the numbers and the transfer of RNA was done overnight.

3.7.4.5 Hybridization

An appropriate volume of the DIG Easy Hyb Granules (Roche) was pre-warmed at 68°C for 30 min. The membranes were placed in a conical tube of 50 mL and 2 mL of the pre-warmed DIG Easy Hyb was added with gentle agitation at 68°C for 30 min. The DIG-labeled RNA probes (3.7.4.2) were denatured by boiling for 5 min and rapidly cooled on ice for 5 min. The denatured probes were added into 2 mL of pre-warmed DIG Easy Hyb and mixed well (avoiding foaming bobbles). Then, the prehybridization solution was poured off and the probe/hybridization mixture was added to the membrane and incubated at 42°C overnight with gentle agitation.

3.7.4.6 Immunological detection

The membranes were removed from the conical tube and placed in a glass petri dish. The 2X SSC and 0.1% SDS was added to wash the membrane two times

under constant agitation for 5 min and followed by washing with 0.1X SSC and 0.1% SDS two times at 50^{o}C under constant agitation for 15 min. After hybridization and stringency washing, the membranes were rinsed briefly for 3 min in 1X washing buffer and incubated for 30 min in 100 mL of blocking solution. Then the membranes were incubated for 30 min in 50 mL of antibody solution. The antibody solution was prepared by centrifuging anti-digoxigenin-AP for 5 min at 10,000 x g in the original vial and pipetting the necessary amount carefully from the surface with diluting anti-digoxigenin-AP 1:10,000 in blocking solution. The membranes were washed twice for 15 min in 100 mL of washing buffer and equilibrated for 5 min in 100 mL of detection buffer. Then the membrane was placed, with RNA side facing up, in a plastic bag and quickly 2-3 drops of CDP-Star ready-to-use solution were applied until the membrane was evenly soaked and then it was incubated under dark condition in chemi DOC (BIORAD) for 5 min. The excess liquid was squeezed out and the membrane was exposed by the imaging device for 5-20 min.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Primer design for knock down β-glucosidases gene

The 3'UTR is a particular section of messenger RNA (mRNA) (Figure 14), which follows the coding region but is not translated into protein. It is believed to contain binding sites for proteins that affect the mRNA stability, translation efficiency and location in the cell (polyadenylation signals). The 3'UTR generally shows diverse sequence in different organisms and in gene families (Chen et al., 2006, Friedberg et al., 2003, Jan et al., 1997, Greener et al., 2002). In rice, the 3'UTR of GH1 β-glucosidase genes show sequence specific for each gene. Many researches have used the 3'UTR as a specific region to knock down of specific genes (Miki et al., 2003, Sundaram et al., 2006).

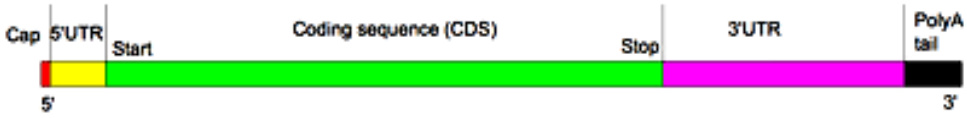


Figure 14 The structure of mRNA including the untranslated regions (UTRs).

Therefore, the primers for specific knock down of each β-glucosidase gene were designed from the 3'UTR. The 3'UTR region primers for *Os3bglu7* (Figure 15), *Os3bglu8* (Figure16), *Os12bglu38* (Figure 17), *Os7bglu26* (Figure 18) and *Os1bglu1* (Figure 19) were designed.

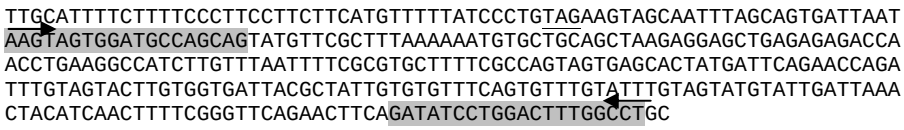


Figure 15 The position of forward and reverse primer pair in the 3'UTR of partial sequence of *Os3bglu8*. The underlined sequence is the stop codon and the gray boxes are the locations of the forward and reverse primers.

Figure 16 The position of forward and reverse primer pair in the 3'UTR of partial sequence of AK071058 (*Os12bglu38*). The underlined sequence is the stop codon and the gray boxes are the locations of the forward and reverse primers.

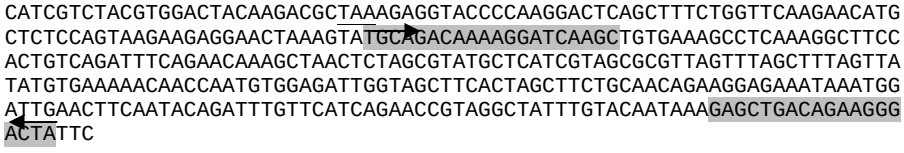


Figure 17 The position of forward and reverse primer pair in the 3'UTR of partial sequence of *Os7bglu26*. The underlined sequence is the stop codon and the gray boxes are the locations of the forward and reverse primers.

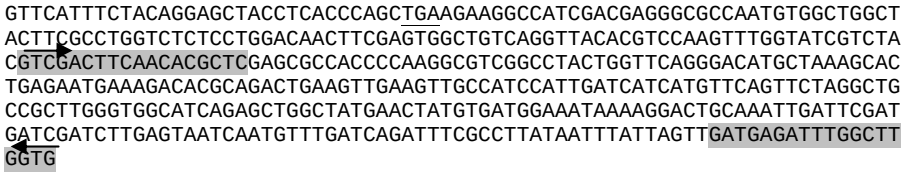


Figure 18 The position of forward and reverse primer pair in the 3'UTR of partial sequence of *Os3bglu7*. The underlined sequence is the stop codon and the gray boxes are the locations of the forward and reverse primers.

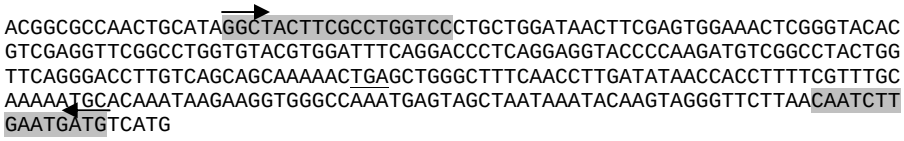


Figure 19 The position of forward and reverse primer pair of the coding region/3'UTR of partial sequence of *Os1bglu1*. The underlined sequence is the stop codon and the gray boxes are the locations of the forward and reverse primers.

Knocking down single β-glucosidases might not be enough to show the effect of lose of functions in calli or plantlet because rice has 34 β-glucosidases genes. Therefore, the coding region of *Os3bglu7* was used to design primers and used as template to amplify conserved region to knock down *Os3bglu8*, *Os12bglu38*, *Os7bglu26* and *Os1bglu1*. The highly conserved nucleotide and protein sequence of this region of the 5 genes were observed from the alignment, showed in Figures 20 and 21, respectively.

```
Os3bglu7        CGCTGTTGGCGGCGC--GGGATGCTG-GTGCCGCGGCGGTGCCCAAGCCCAACTGGCTGG 204
Os3bglu8        CGCTGGTCGTGCTCG--ACCGTGCCG-GGGCCCGGGTCCGCGCCGCGGACGACGACACGG 215
Os1bglu1        CGAAGACGGCATTCGCGGCGGCGCCG-GCGCCGATCATCAGGAGGCGGCCGGGATCACGG 298
Os7bglu26       -GCTGCTGACGCTGCCACCGGCACAATGCTACTGGCTCAACCCGGAGATCTACGACGCCG 117
Os12bglu38      TACTCCT--CATCGCCATCGTCGTCGTCTCCCTCTCCCATGGCAACGGGGAGCA----GA 100
                                                *                 *

Os3bglu7        GCGGGCTGAGCCGCGCCGCGTTCCCCAAGCGGTTCGTGTTCGGGACGGCCACGTCCGCGT 264
Os3bglu8        GCGGGCTGAGCCGGGCCGCGTTCCCCAAGGGGTTCGTGTTCGGGACGGCGACGTCGGCAT 275
Os1bglu1        GCGGGCTGAGCCGGCGGAGCTTCCCGGCGGGGTTCGTGTTCGGGACGGCGGCGTCGGCGT 358
Os7bglu26       GCGGGCTGAGCCGGCGAGCCTTCCCGGAGGGGTTCGTGTTCGGCACGGCCGCGTCGGCGT 177
Os12bglu38      CCGACCTCACGCGGGAGACGTTCCCCGCGGGCTTCGTCTTCGGCACCGCGTCGTCGGCGT 160
                 **  ** *  **        *****   * * ***** ***** ** **  **** ** *

Os3bglu7        ACCAGGTCGAGGGCATGGCGGCGTCCGGCGGCCGCGGGCCGTCCATCTGGGACGCCTTCG 324
Os3bglu8        TCCAGGTCGAGGGCATGGCGGCGTCCGGCGGCCGCGGGCCGTCCATCTGGGACCCCTTCG 335
Os1bglu1        ACCAGGTGGAGGGCATGGCGCTCAAGGATGGCCGCGGCCCTAGCATTTGGGACGCCTTCG 418
Os7bglu26       ACCAGGTGGAGGGCATGGCGAAGCAGGGTGGCCGGGGCCCTAGCATCTGGGACGCTTTCA 237
Os12bglu38      ACCAGGTGGAGGGGAACGCCCTCCAGTATGGCCGAGGGCCCTGCATCTGGGACACCTTCC 220
                 ****** ***** *  **          ***** ** **   *** ****** * ***

Os3bglu7        CGCACACCCCGGGAAATGTTGCAGGAAATCAGAATGGAGATGTTGCGACAGATCAATATC 384
Os3bglu8        TCCACACCCCAGGGAATATTGCGGGAAATGGGAATGCAGATGTTACTACAGATGAATATC 395
Os1bglu1        TCAAGACTCCCGGTGAAATAGCAAATAATGCTACGGCAGATGTTACTGTTGATGAGTACC 478
Os7bglu26       TAGAGAAACCAGGGACGATCCCTAATAATGCCACAGCCGATGTGACGGTTGATGAGTATC 297
Os12bglu38      TGATGCAACCTGGTGTAACTCCTGATAATTCGACCGCGAATGTGACCGTCGACGAGTACC 280
                        ** **        *    ***   *  *   ****  *    **  * ** *

Os3bglu7        ATCGCTATAAGGAAGATGTTAATCTCATGAAAAGTTTGAATTTTGATGCCTACCGGTTTT 444
Os3bglu8        ATCGCTACAAGGAAGATGTTGATCTCTTGAAAAGCCTGAATTTCGATGCATATCGGTTTT 455
Os1bglu1        ATCGCTACAAGGAGGACGTAAACATCATGAAAAGTATGGGTTTCGATGCGTACCGCTTCT 538
Os7bglu26       ATAGGTACAAGGAAGATGTGAACATAATGAAGAACATGGGCTTTGATGCGTATAGATTTT 357
Os12bglu38      ACCGCTACATGGATGATGTGGACAACATGGTGAGAGTGGGCTTCGACGCGTATCGCTTCT 340
                *  * ** * *** ** **  *     **   *   **   ** ** ** **  * ** *

Os3bglu7        CAATCTCATGGTCCAGGATCTTCCCAGATGGTGAGGGACGAGTTAACCAAGAAGGCGTAG 504
Os3bglu8        CAATCTCGTGGTCAAGGATTTTCCCTGATGGAGAGGGAAAAGTTAACACGGAAGGTGTGG 515
Os1bglu1        CAATCTCATGGTCAAGAATATTCCCAACTGGAACTGGGAAAGTAAATTGGAAAGGTGTGG 598
Os7bglu26       CGATCTCTTGGTCAAGAATTTTACCAAATGGGACTGGGATGGTGAACCAGGAAGGAGTTG 417
Os12bglu38      CGATCTCCTGGTCTCGCATTTTCCCCAGTGGACTTGGGAAGATTAACAAAGACGGCGTGG 400
                * ***** *****  * ** ** **   ***    **     * **     * ** ** *

Os3bglu7        CATATTACAACAATCTTATAAACTACCTTCTGCAGAAAGGTATCACTCCTTATGTCAATC 564
Os3bglu8        CATATTACAATAATCTAATAGATTATGTAATTAAGCAAGGGCTTATTCCTTACGTCAATC 575
Os1bglu1        CATACTATAACAGATTGATAAACTATATGCTGAAGATAGGCATTACACCTTATGCCAATT 658
Os7bglu26       ATTATTACAACAGGTTAATAGATTACATGGTTAAGAAAGGCATCAAACCGTACGCAAACC 477
Os12bglu38      ATTATTACCACAGGCTCATTGATTACATGCTTGCTAACAACATTATTCCATATGTTGTGC 460
                  ** **  * *   * **  * **  *  *           * *  ** ** *

Os3bglu7        TTTACCACTACGATCTCCCTCTTGCGCTTGAGAAGAAGTACGGAGGCTGGTTGAATGCAA 624
Os3bglu8        TGAACCACTACGATCTCCCACTTGCACTTCAGAAAAAGTATGAAGGCTGGTTAAGCCCAA 635
Os1bglu1        TGTATCACTATGACTTACCAGAGGCACTAGAGGTGCAATATGGAGGACTGTTGAACAGAA 718
Os7bglu26       TCTACCACTATGACCTACCATTAGCACTCCATGAGCAGTACTTAGGCTGGCTAAGCCCAA 537
Os12bglu38      TCTACCACTACGACCTTCCACAGGTGCTCCATGATCAATACAAGGGATGGCTACACCCCA 520
                *  * ***** **  * **    *  **  *     * **    **   * *       *
```

Figure 20 Partial result of ClustalW alignment of nucleotide of five β-glucosidase genes. The gray boxes showed the location of te forward and reverse primers used to amplified the coding region to knock down five β-glucosidase genes.

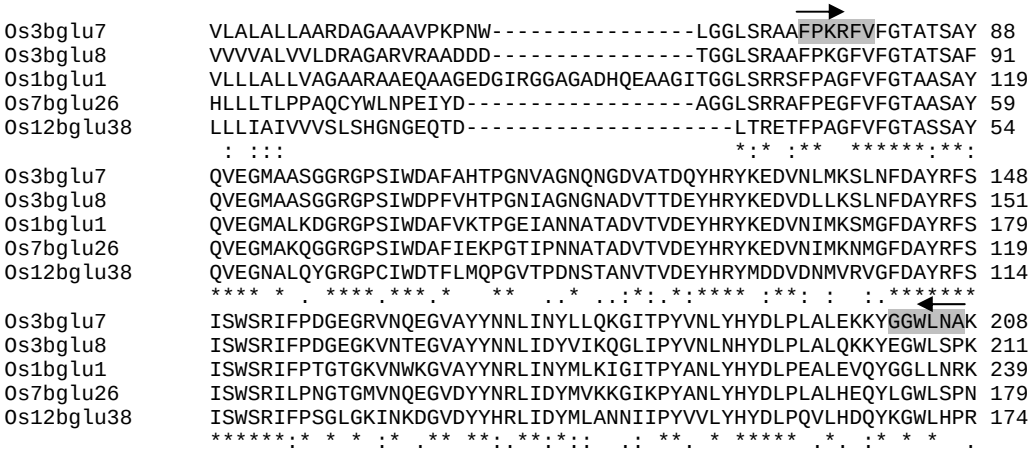


Figure 21 Partial alignment of the protein sequence of the five β-glucosidase genes. The gray boxes show the location of forward and reverse primers used to amplify the coding region to knock down the five β-glucosidase genes.

The score of whole sequence homology of nucleotide (table 3) between *Os3bglu7* and other 4 genes were calculated by clustalW. The coding regions of *Os3bglu7* and other genes indicate the nucleotide and amino acid sequence identities among the member are 62-83% and 58-81%, respectively.

Table 3 The 399 nucleotide sequence length identity at coding region of each β-glucosidases.

Sequence A name		Sequence B name		Score % identity
1	*Os3bglu7*	2	*Os3bglu8*	83
1	*Os3bglu7*	3	*Os1bglu1*	69
1	*Os3bglu7*	4	*Os7bglu26*	67
1	*Os3bglu7*	5	*Os12bglu38*	62
2	*Os3bglu8*	3	*Os1bglu1*	70
2	*Os3bglu8*	4	*Os7bglu26*	70
2	*Os3bglu8*	5	*Os12bglu38*	64
3	*Os1bglu1*	4	*Os7bglu26*	73
3	*Os1bglu1*	5	*Os12bglu38*	65
4	*Os7bglu26*	5	*Os12bglu38*	70

The suppression efficiency was generally correlated with the score of sequence identity between the target sequence and trigger sequence. The high identity between target and trigger sequence will lead to the high knock down level. Miki and group (2005) suggested that a conserved region of one gene could be used to suppress the expression of a set of genes that share conserved sequences in different knock down level. In table 4, the sequence of 3'UTR region used to knock down each β-glucosidase genes were compared for the percent identity. The 3'UTR of *Os3bglu7* and other genes indicate the nucleotide sequence identities among the member are only 5-16%. So, the target 3' UTR regions of each gene should be highly specific to knock down individual β-glucosidase genes.

Table 4 The 3'UTR nucleotide target sequence identity of each β-glucosidases.

Sequence A name		Sequence B name		Score % identity
1	*Os3bglu8*	2	*Os12bglu38*	11
1	*Os3bglu8*	3	*Os7bglu26*	10
1	*Os3bglu8*	4	*Os3bglu7*	12
1	*Os3bglu8*	5	*Os1bglu1*	8
2	*Os12bglu38*	3	*Os7bglu26*	6
2	*Os12bglu38*	4	*Os3bglu7*	6
2	*Os12bglu38*	5	*Os1bglu1*	6
3	*Os7bglu26*	4	*Os3bglu7*	5
3	*Os7bglu26*	5	*Os1bglu1*	8
4	*Os3bglu7*	5	*Os1bglu1*	16

4.2 β-glucosidase genes amplification and pHELLSGATE8 construction

The genomic DNA of rice (Koshihikari) was used as a template to amplify the β-glucosidase genes. The first construct for knock down 5 genes with one RNAi vector was done by amplified the coding region of *Os3bglu7*. The PCR product was

determined by agarose gel electrophoresis. The expected size of 399 bp was seen on the gel (Figure 22)

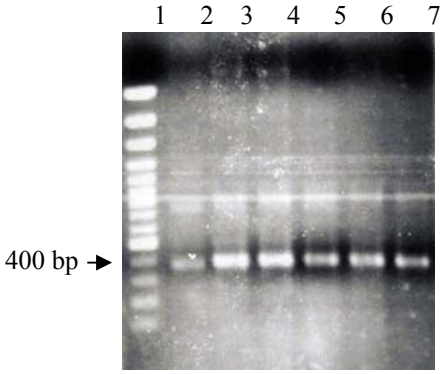


Figure 22 Agarose gel electrophoresis of amplified fragment from PCR reactions of *Os3bglu7* with 5 β-glucosidase group forward and reverse primers. Lane 1, 100 bp marker; lanes 2-7, PCR products generated with a gradient annealing temperatures of 70.0°C, 70.8°C, 71.7°C, 72.9°C, 74.2°C and 75.6°C.

The DNA size about 400 bp (major product) after amplified with 70.8°C were excised and eluted from agarose gel then cloned into pENTR™/D TOPO cloning vector. Eight of the transformant colonies were selected from the LB-kanamycin plate to examine for the insertion of target gene sequence by colony-PCR method. The result is shown in Figure 23.

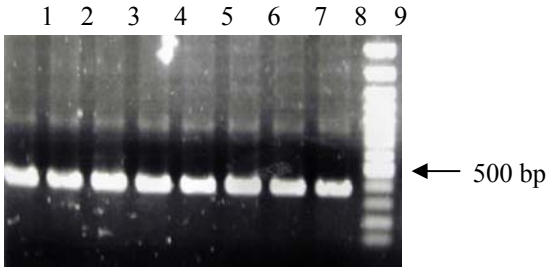


Figure 23 Agarose gel electrophoresis of colony-PCR method of recombinant plasmid in One Shot® TOP10 *E. coli*. Lane 1 to 8 are PCR product using recombinant plasmid from LB-kanamycin plate as template, Lane 9: 100 bp markers.

From the colony-PCR method, the results showed that all colonies selected have the expected band of about 400 bp. It can be concluded that the 8 colonies contained recombinant plasmid that had a DNA sequence of coding region of *Os3bglu7*. One from 8 colonies was sequenced. The sequence showed the same nucleotide sequence as expected. The recombinant plasmid was extracted using QIAprep miniprep plasmid extraction kit (QIAGEN) and the LR reaction was done to transfer the target gene from pENTR™/D TOPO cloning vector to pHELLSGATE8 vector. The LR reaction was transformed into *E.coli* strain DH5α. The transformant colonies were screened with LB-spectinomycin plate. Ten colonies which grew on the plate were used for plasmid extraction and screen for the correct recombinant pHELLSTAGE8 by restriction enzyme digestion to confirm the 2 target gene sequence insertion instead of the ccdB genes. The plasmid was cut with *Xho*I and *Xba*I in separate reaction. The result of gel electrophoresis found that no insertion of the target genes size 400 bp were obtain when compare with the control

pHELLSTAGE8, which showed the ccdB band size at about 1500 bp after cut with *Xba*I and *Xho*I. After several LR reactions were repeated, the screenings of the recombinant pHELLSTAGE8 that contain 2 pieces of the target genes were still unsuccessful. So the plasmid was investigated by DNA sequencing to see what was wrong with the recombinant plasmid. The sequencing result of the recombinant plasmid that did not give the correct size insert when cut with *Xho*I and *Xba*I is shown in Figure 24.

Figure 24 The sequence of the wrong pHELLSGATE8 with one gene fragment insertion.

The result showed that the recombinant pHELLSGATE8 contained only 1 piece of the target gene fragment and the intron was not found. So, new primers pairs were designed by adding the restriction sequences of *Xho*I and *Eco*RI at the forward and reverse primer, respectively. The new primers for knock down 5 β-glucosidase genes with one RNAi plasmid and each β-glucosidase genes are shown in table 1.

The PCR amplification of *Os3bglu7* for knock down of 5 genes was done with the new primer pair with an annealing temperature of 71^{o}C. The 400 bp PCR product (Figure 26) was excised and eluted from the gel and cloned into pENTR^{TM}/D TOPO.

The transformed colonies that grew on an LB-kanamycin plate were selected for plasmid extraction and sequencing to confirm the correct nucleotide sequence. After that, the correct 5 genes knock down insertion fragment in pENTR™/D TOPO was transferred to pHELLSGATE8 by digest of the pENTR™/D TOPO (containing the insert fragment gene) and pHELLSGATE8 with *EcoR*I and *Xho*I. The agarose gel electrophoresis of pENTR™/D TOPO and pHELLSGATE8 digestion are shown in Figure 25.

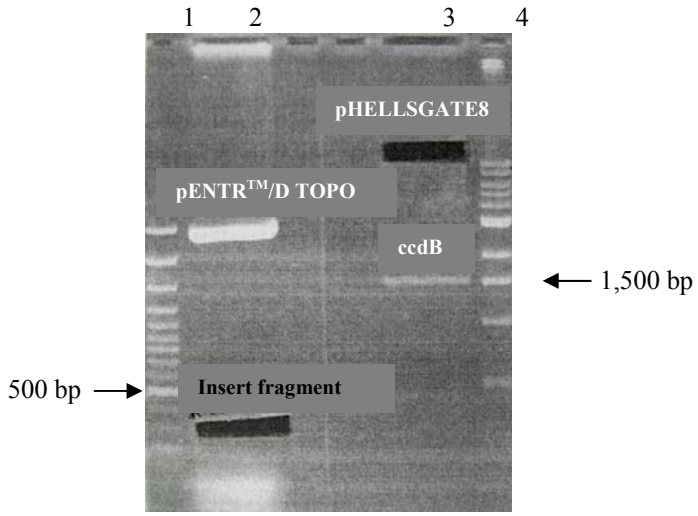


Figure 25 Agarose gel electrophoresis of pHELLSGATE8 and pENTR™/D TOPO. Lane 1: 100 bp marker, lane 4: 1 kb marker, lane 2 and 3: recombinant pENTR™/D TOPO and pHELLSGATE8 vectors after digests with *EcoR*I and *Xho*I.

The result showed that the ccdB fragment of 1435 bp was separated from the pHELLSGATE8. This long fragment is ccdB gene, attR1, attR2 and restriction site (*Xma*I, *Sma*I) was replaced with the target gene fragment for knock down 5 genes

from pENTR^{TM}/D TOPO vector at the next step. The pHELLSGATE8 (upper band) and the insert gene fragment were gel purified and ligation together.

The plasmids from 3 of the transformant colonies were examined for the insertion of target gene sequence in pHELLSGATE8 by digestion with *EcoR*I and *Xho*I, respectively, as shown in Figure 26.

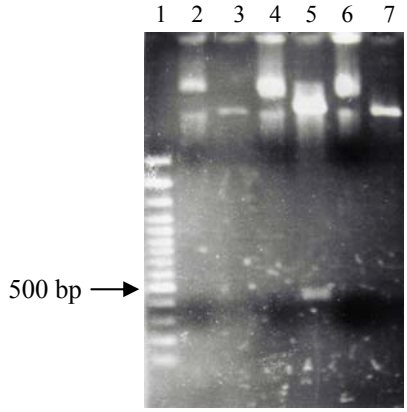


Figure 26 Agarose gel electrophoresis of pHELLSGATE8. Lane 1: 100 bp marker, lane 2, 4 and 6: uncut recombinant pHELLSGATE8, lanes 3, 5 and 7: recombinant pHELLSGATE8 were digested with *EcoR*I and *Xho*I.

The result showed that only one sample from three samples contained the target gene fragment of about 400 bp. So this plasmid was used to transfer the second piece of a target gene from pENTR^{TM}/D TOPO vector (containing the target gene) by LR clonase reaction. In this experiment 2 colonies were found on the antibiotic plate. Then, the cells were grown in LB-spectinomycin broth for plasmid extraction and examine the second insertion of the target gene by digestion with *Xba*I. Figure 27 shows that both colonies contained the second piece of target gene insertion. The size of the target gene showed about 450 bp that was longer than a target gene size about

400 bp because the *Xba*I cut the the plasmid in region included the attR1 and attR2 sites, which were 25 and 24 bp, respectively

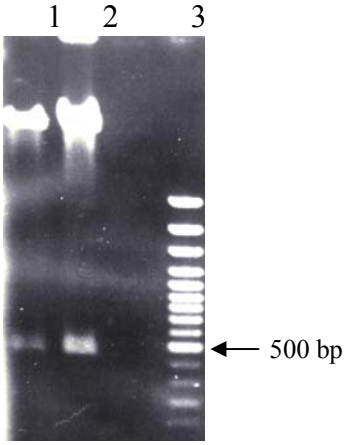


Figure 27 Agarose gel electrophoresis of digested recombinant pHELLSGATE8 with 2 inserts. Lane 1-2: recombinant pHELLSGATE8 with 2 inserts, lane 3: 100 bp marker.

After the digestion of the second fragment from the recombinant pHELLSGATE8 with 2 inserts with *Xba*I, the insertion of the second target gene was found. The sequence of the recombinant pHELLSGATE8 with 2 inserts confirmed that the two target gene insertions were in the right positions and in opposite orientations to each other, as shown in Figure 28. The result indicated that the 2 pieces of the target gene fragments to knock down 5 β-glucosidase genes were put in the correct position and orientation. The intron between the 2 pieces of the target gene to form the loop structure is also in the correct position. After that, the recombinant pHELLSGATE8 with 2 inserts was transformed into *Agrobacterium* for rice transformation and produced the transgenic knock down rice lines.

Figure 28 The sequence of two target gene fragment insertions in pHELLSGATE8.

The construct for knock down using the 3'UTR of each β-glucosidase genes (*Os3bglu7*, *Os3bglu8*, *Os12bglu38*, *Os7bglu26* and *Os1bglu1*) were done with the same method as the knock down 5 group β-glucosidases genes. The 3'UTR of each β-glucosidases gene were amplified and cloned into the pENTR^{TM}/D TOPO vector. The plasmids were sequenced to confirm the correct nucleotide sequence. The first piece of the 3'UTR fragment were cloned into pHELLSGATE8 from the pENTR^{TM}/D TOPO vector by digestion with *EcoR*I and *Xho*I and litation, while the second piece was done by LR reactions. The correct plasmids were transformed into *Agrobacterium* for rice transformation and produced the transgenic knock down rice line.

4.3 Rice transformation

4.3.1 Callus induction in Koshihikari and KDML105

Embryogenic callus induction is the first step in rice transformation. It is the most critical step for the success for rice transformation. The frequencies of callus induction and plant regeneration in rice tissue culture are influenced by many factors

such as culture medium composition, explant source, genotype and environment (Torbert et al., 1998). Among these the genotype and nutrient composition are regarded to be the major sources of variation in *in vitro* culture (Khanna and Raina, 1998).

In this research, 2 rice varieties, Koshihikary and KDML105 were studied. The transformation of target genes into rice was done by *Agrobacterium* EHA105. The methods started with callus induction followed by co-cultivation, transformant callus selection and finally regeneration to plantlet. The first step, the seed coat of Koshihikari and KDML105 were removed by hand or gentle grinding in a mortar to avoid the embryo damage and then sterile with sodium hypochlorite before transfer the seeds to callus induction medium.

The seeds of Koshihikari and KDML105 showed the same yellow color when sterilized in 2% of sodium hypochlorite for 30 min. Sodium hypochlorite is a strong oxidizer solution that burns tissues and induces the brown color. The seeds that show yellow color normally will died, which leads to low efficiency callus induction (Figure 29). Therefore, the seeds were washed with sterile water many times to remove excess sodium hypochlorite until the seeds were white in color.

Figure 29 The seeds on callus induction medium turn brown due to the effect of sodium hypochlorite.

Primary calli developed from the scutellum region of the embryo (http://www.goldenrice.org/image/why_grain.jpg) were visible within 7-10 days. The calli of KDML105 (Figure 30) were induced on MS and N6D medium. The calli of Koshihikari (Figure 31) were induced only on N6D medium. The Koshihikari and KDML105 secondary calli started to separate from the primary calli after about 4-6 weeks (Figure 32). The secondary calli of Koshihikari showed different growth rate, shape and size when compare with KDML105. The Koshihikari calli grew faster with global, smooth appearance and big size than KDML105. The suitable time for induction of the secondary calli for *Agrobacterium* transformation of Koshihikari was about 4-6 weeks, but KDML105 needed a longer time (about 6-7 weeks). Koshihikari gave higher amounts of secondary calli than KDML105 (Figure 33). The pre-culture of Koshihikari secondary calli on N6D medium showed good health and were of high quality for transformation when compared to KDML105 (Figure 34). The different number, color, size and shape of calli between Koshihikari and KDML105 may derive from the variation between the rice genotypes. It's also depend on the type of basal medium and environmental factors but the genotype appears to be an important factor influencing the high-quality rice calli in culture (Lee et al., 2002).

Hartke and Lorz (1989) tested 15 *indica* rice lines and found that seven of them produced embryogenic calli. Abe and Fustsuhara (1986) tested 66 *indica* and *japonica* cultivars and reported that japonica varieties displayed a higher rate of callus induction than those of indica. Their result showed that many agronomically important rice genotypes are poor callus induction.

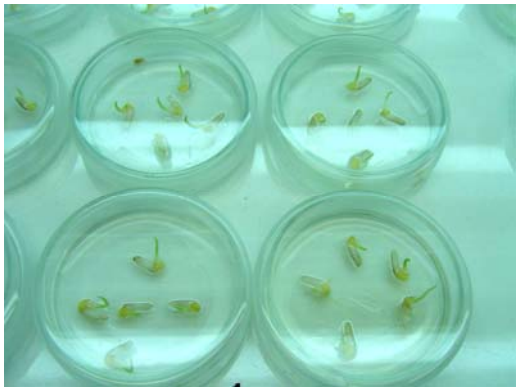


Figure 30 The primary calli of KDML after 2 week on N6D medium.

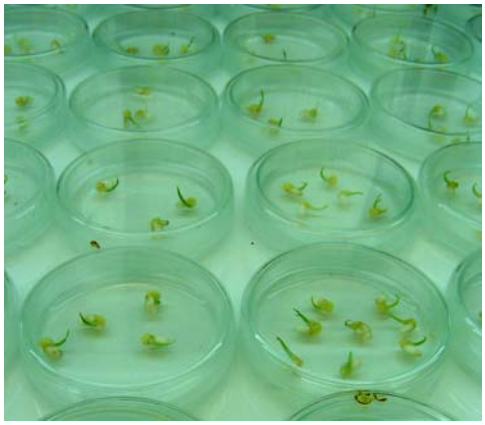


Figure 31 The primary calli of Koshihikari after 2 week on N6D medium.

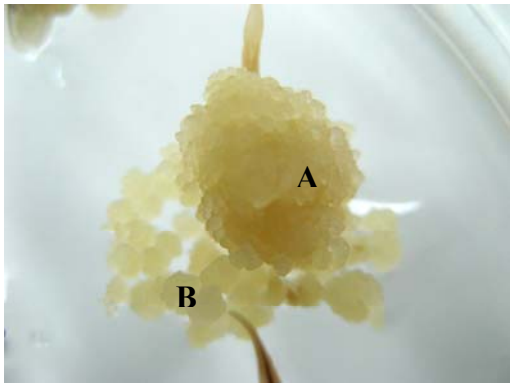


Figure 32 Primary calli (A) and secondary calli (B).

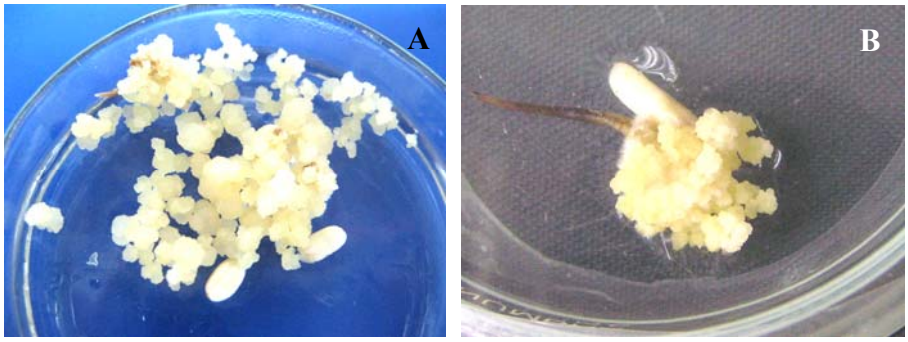


Figure 33 The calli induction on N6D medium. Koshihikari (A), KDML105 (B).

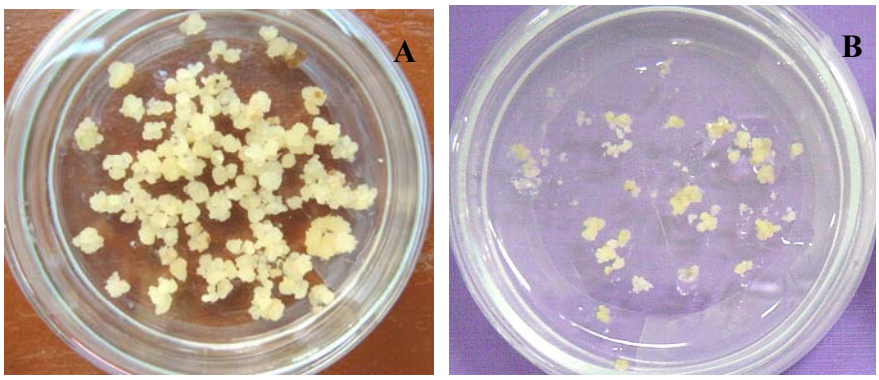


Figure 34 The calli pre-culture on N6D medium at 3 days. Koshihikari (A), KDML105 (B).

Light is a very important physical factor for callus induction, cell growth and production of plant secondary metabolites (Vom Endt et al., 2002). However the level of responsiveness to light depends on cell type and plant species and cultivar. The effect of light to induce secondary callus of KDML105 and Koshihikari were observed. The secondary callus of KDML105 and Koshihikari were induced in light/dark (16/8h) and dark condition under the constant temperature of 28^{o}C in a growth chamber. From my observation, I found that light was not an important factor,

it does not effect the induction of the secondary callus neither for KDML105 nor Koshihikari but the medium for callus induction has more effect. Qian et al. (2004) reported that light influences the rate of callus induction and browning in indica rice variety Pei'ai64s. Their research suggested that light increased the induction frequency, but it is no different when compared with cultured in the dark. Moreover, light makes the callus grow faster, but also increases the browning rate. Several investigators suggested that the dark condition is preferred for shoot induction and somatic embryogenesis from some species. Thadavong et al. (2002) reported the effect of light on callus formation in rice cultivar TDK1. The seeds cultured under a light condition gave higher average callus formation frequency (92%) and larger average size of callus (3.88 mm) than those cultured under the dark condition (87% and 3.44 mm, respectively). Similar results were also observed in the indica rice cultivar RD6 (Pipatpanukul et al., 2004). In japonica rice (Nipponbare and Kitake), higher growth of calli was observed under the light condition rather than under the dark condition. Pipatpanukul et al. (2004) reported that the effect of light and dark conditions mainly depended on the concentration of 2,4-D in the medium. Although the light condition generated a high percent of callus induction at 4.5, 9.0, 18.0 and 22.5 μM concentrations of 2,4-D but at 13.5 μM of 2,4-D higher callus induction was seen in the dark condition. The differences between this researches with those previously reported, seemed to depend on the varieties of rice and the intensity of light and environment.

The effect of temperature on the induction of secondary calli of KDML105 and Koshihikari were observed. The effect of incubation temperature on calli growth showed no difference from 25-30^{o}C. However, temperatures higher or lower than

25-30^{o}C affected the secondary calli induction. The calli at temperatures higher than 30^{o}C were soft, light brown in color and friable, due to the evaporation of water from the medium onto the cover of the glass plate. The water droplet also dropped onto the calli. Most of the secondary calli died, and those that lived gave low transformation efficiency. The callus morphology of calli incubated at temperatures lower than 25^{o}C was dry and yellowish in color with compact appearance. The highest amounts of secondary calli were obtained at 28^{o}C in a constant temperature chamber in constant darkness. This condition gave good quality secondary calli for transformation.

The effect of culture medium on calli growth was observed and is shown in table 5. The primary calli induction of KDML105 was not different between both medium (MS and N6D), but the influence of different culture media on secondary callus growth of KDML105 was observed. Calli on N6D medium gave higher amount in number and weight of secondary calli than MS medium (Figure 35). The calli on MS and N6D medium were compact and yellowish. These results were similar to the research of Niroula et al. (2005). They reported that the composition of the culture medium influenced growth of plant cell culture and that N6D medium was the most efficient for callus induction, cell proliferation and plant regeneration from seeds of various rice genotypes.

High quality and high number of calli induction were obtained from the seeds that had been harvested within 3 months. The efficiency of calli induction of KDML105 and Koshihikari seeds stored at room temperature or cold room (4^{o}C) after 6 months decreased. Seeds stored longer than 6 months also gave low efficiency of secondary callus induction. So the seeds in this experiment were changed every 6 months (if possible) to maintain the quality and callus induction efficiency. Very

small amounts of KDML105 (Figure 36) and Koshihikari secondary calli were obtained from the seeds after 6 months storage even grown on N6D medium. The breaking off of primary calli to induce the secondary calli leads to browning and poor callus formation. Therefore, the secondary calli should be grown from the primary calli until the secondary callus separated itself from the primary calli onto the medium. Agar with its solidifying effect on the medium is likely to limit water uptake by tissues. A high percentage of gelling agents reduces the water potential of the medium resulting in the formation of hard, compact, drier and friable calli that are more amenable for transformation. In this research 4% gellen gum was used for callus induction.

Table 5 The percent of callus induction of KDML105 on MS and N6D medium and Koshihikari on N6D medium.

Replication	**KDML105**		**Koshihikari**
	MS	**N6D**	**N6D**
	% callus induction*	**% callus induction***	**% callus induction***
1	92.40	94.80	94.00
2	96.80	90.40	97.20
3	91.60	96.00	93.60
4		95.00	91.33
5		91.33	95.33
6		94.00	92.33
7		94.50	99.00
8		96.12	98.25
9		97.25	97.62
10		96.00	95.75
11			88.20
12			89.00
13			87.60
14			88.00
Average	**94 ± 3 %**	**95 ± 2 %**	**93 ± 4 %**

* callus induction frequency (%) = $\frac{\text{No. of seeds produced calli}}{\text{No. of seeds cultured}} \times 100$

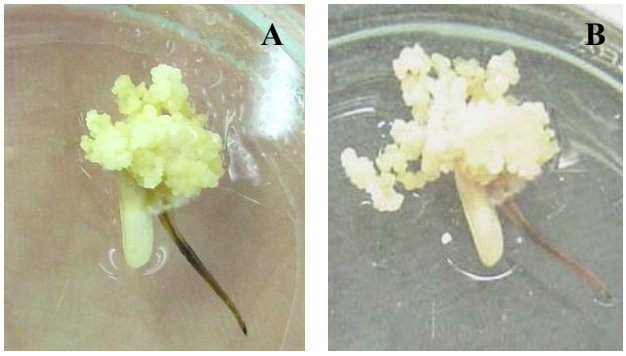


Figure 35 The callus induction of KDML105 on MS (A) and N6D (B) medium.

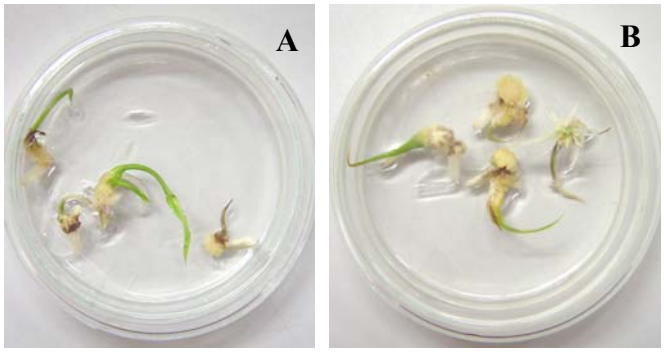


Figure 36 The low efficiency of calli induction of KDML105 seeds older than 6 months old on MS medium (A) and N6D medium (B) at 4 weeks.

4.3.2 Transformation efficiency

It has been almost sixteen years since the initiation of experiments on rice transformation using *Agrobacterium*. Chen et al. (1993) successfully transformed rice using this method. However the transformation efficiency was low. After Hiei et al. (1994) reported high efficiency *Agrobacterium*-mediated transformation of fertile and heritable transgenic rice plants (japonica cultivars Tsukinohikari, Asanohikari and Koshihikari), this technology was adopted by many laboratories. *Agrobacterium*-

mediated transformation has several advantages, including higher transformation efficiency, ability to transfer large pieces of DNA with minimal rearrangement, integration of a relatively lower number of transgene copies, and low experimental costs.

The efficiency of *Agrobacterium*-mediated T-DNA transfer to plant cells depends not only on the successful recognition and colonization of plant cells by the *Agrobacterium*, but also on the responses of the plant cells to the *Agrobacterium* infection process (Zambryski, 1988, Binns, 1990). Cultivar variability in susceptibility to *Agrobacterium* in rice has been reported although these authors used only three or four rice cultivars (Hiei et al., 1994). The essential causes of genotypic differences are not known, but they are probable related to the reduction efficiency in one or more steps in T-DNA transfer, integration into host cells, expression of T-DNA genes, or cell response to T-DNA produced phytohormones (Aldemita and Hodges, 1996).

The various factors affecting gene delivery have been evaluated by studying the expression of *gus* or *gfp* genes after co-cultivation between callus and *Agrobacterium*. The optimization of these factors was considered for the establishment of successful transformation systems in rice.

It was earlier reported that *Agrobacterium* does not induce the hypersensitive response in target plants, but the bacterium introduces several proteins into the host cell (Robinette and Matthysse, 1990). Now days, several reports of high necrosis and poor survival rates of target plant tissues during the process of *Agrobacterium*-mediated T-DNA transfer have been reported (Pu and Goodman, 1992, Deng et al., 1995, Perl et al., 1996, Mercuri et al., 2000, Chakrabarty et al., 2002, Das et al., 2002).

Inoculation of plant tissue with *Agrobacterium* is a disruptive process and triggers a hypersensitive response in the tissue. The plant tissues recognized the invading of pathogens and activate defense signal transduction leading to necrotic responses. These factors may be the result of hypersensitive defense reaction in plants to *Agrobacterium* infection, which may involve the recognition of specific signals from the *Agrobacterium* that triggers the burst of reactive oxygen species at the infection site.

Most studies of *Agrobacterium* transformation of rice use actively growing embryogenic cells such as those in immature embryos and callus induced from scutellum. The transformation of Koshihikari and KDML105 in this research were done by pre-culturing the secondary callus on N6D medium for 3-6 days (Figure 37 A) and then co-culturing for 3 days with *Agrobacterium* that had been pre-cultured on AB medium (Figure 37 B). Hiei et al. (1994) reported the pre-culture of calli in fresh medium for four days prior to infection was important for the transformation of japonica rice. Figure 38 showed results of KDML105 and Koshihikari calli after *Agrobacterium* infection. More than 70% of KDML105 calli died after co-cultivation. In contrast, higher numbers of Koshihikari calli survived after co-cultivation.

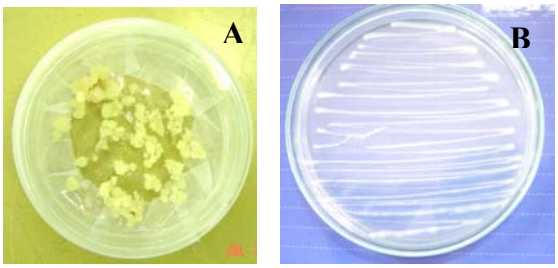


Figure 37 Callus pre-culture on N6D medium (A) and *Agrobacterium* preculture on AB medium (B) at 3 days.

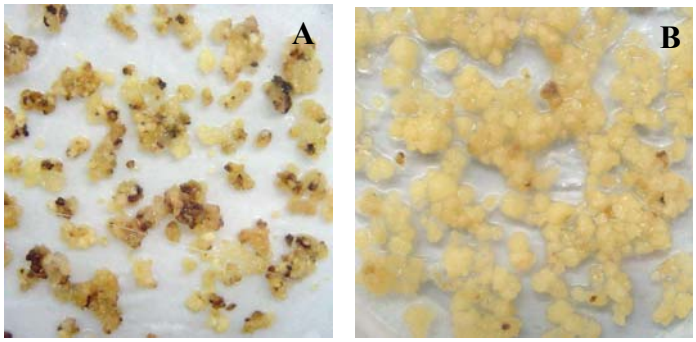


Figure 38 The KDML105 calli (A) and Koshihikari calli (B) after *Agrobacterium* infection for 3 days.

Hansen (2000) worked with maize tissues and observed that co-cultivation with *Agrobacterium* leads to rapid tissue necrosis and cell death. In this case, the degree of necrotic reaction appears to depend on several transformation parameters, including co-cultivation duration, temperature, pH, co-culture medium, inoculation time and the concentration of *Agrobacterium*. These factors are also important factors for *Agrobacterium*-mediated gene transfer efficiency in rice.

4.3.2.1 Co-cultivation duration

The co-cultivation of callus and *Agrobacterium* for 2-3 days is generally considered suitable for *Agrobacterium* transformation, as reported for many plant species. In this research, the callus of KDML105 and Koshihikari were co-cultivate with *Agrobacterium* for 3 days because co-cultivation for more than 3 days led to decrease in transformation frequency and more than 80% calli died, especially KDML105 (data not shown), because small and rough surface of KDML105 calli makes it difficult to wash away the *Agrobacterium*. If the *Agrobacterium* overgrow, it is hard to wash out and get rid of the bacteria that are in the deep rough surface of the

calli. It will induce necrosis and kill the entire callus after 3 days of co-cultivation (Figure 39). This result is similar to the results of Samiphak and Siwarungson (2006). They reported that 3 days co-cultivated calli gave approximately two fold higher green fluorescent proteim (GFP) positive calli than 2 days co-cultivation. Tyagi et al. (2007) showed that the co-cultivation of calli with *Agrobacterium* for more than two and a half days resulted in the tendency to repeat *Agrobacterium* infection, with a loss of regeneration efficiency. Thus, co-cultivation for two and a half days was found to be the most suitable for optimum transformation. Hoque et al. (2005) reported the effect of co-cultivation period in rice variety Moulata at 1, 2, 3, 4 and 5 days. They found that GUS activity was only observed from the calluses co-cultivated from 2 to 5 days. Although calli co-cultivated for 4 and 5 days showed GUS activity but the tissue were harmfully affected by prolonged cultivation with *Agrobacterium*. The highest GUS activity was observed after 3 days co-cultivation.

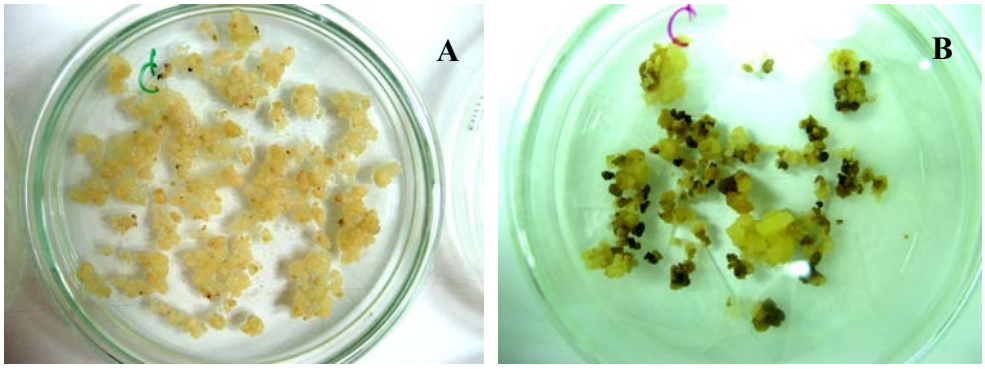


Figure 39 The Koshihikari on selection medium after co-cultivation with *Agrobacterium* at 3 days (A) and 4 days (B).

4.3.2.2 The temperature and pH for co-cultivation

Co-cultivation of Khosihikari with *Agrobacterium* was incubated 3 days in the dark condition at 25 and 28^{o}C. The result indicated that 25^{o}C led to higher transformation efficiency (21%) than the callus co-cultivated at 28^{o}C (10%) (Table 6). This result was similar to Ozawa (2009), who reported the best conditions for co-cultivation were found to be 25°C. Co-cultivation at 25^{o}C appears to be a suitable temperature for plant cell infection and T-DNA insertion into the rice callus in many reports. This might be the optimal temperature of the rice cell for *Agrobacterium* infection. Saharan et al. (2004) reported that the co-cultivation of embryogenic rice calli with *Agrobacterium* at 28±1°C in indica rice cultivars HKR-46 and HKR-126 was the good condition. However, in this research show a different result. KDML105 (indica) showed weak callus and overgrowth of *Agrobacterium* when co-cultivation at 28^{o}C and most calli died on selection medium after the *Agrobacterium* was removed.

Table 6 The transformation efficiency of Koshihikari callus at 25 and 28 ^{o}C.

Replication	25 ^{o}C			28 ^{o}C		
	No. callus*	**Resistant callus**	**Transformation efficiency (%)***	**No. callus***	**Resistant callus**	**Transformation efficiency (%)***
1	150	24	16.00	180	14	7.78
2	150	31	20.67	180	11	6.11
3	150	28	18.67	180	8	4.44
4	240	51	21.25	180	15	8.33
5	240	46	19.17	300	39	13.00
6	240	53	22.08	300	41	13.67
7	300	69	23.00	300	27	9.00
8	300	71	23.67	300	35	11.67
9	300	56	18.67	300	46	15.33
10	300	68	22.67	300	29	9.67
Average	**237**	**50**	**21 ± 2^{a}**	**252**	**27**	**10 ± 3^{b}**

*The callus on selection medium at week 4.

Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

Low pH during co-cultivation is an important factor influencing *Agrobacterium* transformation. Although the gene transfer was observed over the entire range of pH 5.0 - 5.8, but pH 5.2 co-cultivation medium enhanced the transformation efficiency (Saharan et al., 2004, Zhu and Wu, 2008). The callus of Koshihikari and KDML105 after co-cultivation with *Agrobacterium* on co-cultivation medium with pH 5.8 showed severe necrosis after 2 days of co-cultivation but a higher number of Koshihikari callus could grow and survive when compared to KDML105 on the same selection medium. The stimulatory effect of low pH on transformation frequency may be due to the fact that an acidic pH induces the virulence genes of *Agrobacterium*. A set of *Agrobacterium* operons required for pathogenesis is coordinately induced during plant infection by the *VirA* and *VirG* proteins. The intracellular concentration of *VirG* increases in response to acidic media, and this response was proposed to be regulated at the level of transcription at a promoter P2 that strongly induced by low pH (Mantis and Winans, 1992).

4.3.2.3 The concentration of *Agrobacterium*

The concentration of *Agrobacterium* is one of the important factors that control the optimum growth rate of bacteria on co-cultivation medium. In this experiment, the *Agrobacterium* concentration at OD_{600} = 0.02 in AAM medium was used for co-cultivation for 5 min and the excess *Agrobacterium* was removed by blotting the infected calli on sterile tissue paper and moving the calli onto the co-cultivation medium for 3 days. Higher concentrations of *Agrobacterium* caused overgrown and calli damage. Other reports showed that the best conditions for co-cultivation were *Agrobacterium* concentration of OD_{600} = 0.04 to 0.2. The

transformation efficiency using *Agrobacterium* at a concentration of OD_{600} = 0.04 was significantly higher than that obtained at the higher *Agrobacterium* concentration of OD_{600} = 0.2, and this was probably because excess *Agrobacterium* damaged the calli (Ozawa, K. 2009). This confirms the earlier observations of Kumria et al. (2001) that high bacterial density (OD_{600} = 0.7-1.0 with 10 min infection) or prolonged infection time (15-30 min with the optimal OD_{600} = 0.3-0.6) are harmful and affect the growth and regeneration of callus of indica rice during *Agrobacterium*-mediated transformation. Similarly, Chakrabarty et al. (2002) reported that exposure of cauliflower hypocotyl explants to undiluted culture of *Agrobacterium* (OD_{600} = 0.5) resulted in severe necrosis of the explants whereas diluted culture (1:10 and 1:20 dilution) reduced necrosis.

The concentration OD_{600} = 0.5 of *Agrobacterium* culture suspended in AA-AS medium and cocultivation on media overlaid with Whatman no. 1 filter paper reduced the browning of calli after cocultivation, possibly because of reduced damage to explants during *Agrobacterium* infection, which resulted in less phenolic production and better recovery of calli during selection (Kumar et al., 2005). Although many researches used a Whatman no.1 paper to put on the cocultivation medium and added 1-5 mL of infection medium on Whatman paper, in this research, I found that there was no different between used or not used Whatman paper. The best condition to decrease the calli damage from *Agrobacterium* were to remove the excess bacterial cells by moving the infected calli from infection liquid medium onto sterile tissue paper and allowing them to air dry for about 5 min before putting the calli on co-cultivation medium. The calli on co-cultivation should be separated from each other because the colony of calli causes high humidity and that is the cause for

Agrobacterium growth. Even though, the necrotic response of the rice calli in this research was not observed during growth in cocultivation medium, it was observed at 2 days after transfer of the calli to *Agrobacteria*-free medium.

4.3.2.4 The effect of *Agrobacterium* on callus

This research indicated that calli were still damaged by the inoculation of *Agrobacterium*. The brown and black calli were still observed in both Koshihikari and KDML105 calli (Figure 40). This damage decreased the transformation efficiency. The better characteristics of Koshihikari callus led to higher tolerance to *Agrobacterium* infection. *Agrobacterium* do not grow well on dry plates that contain high amounts of a gelling agent. The dryness of the surface of the solid medium could decrease *Agrobacterium* growth. However, it is difficult to control the level of dryness on the surface of the solid medium. So the amount of *Agrobacterium* should be controlled by immersed the callus in a low concentration of bacterial suspension for a short time and blotting the calli dry on sterile paper before putting the calli on cocultivation medium. Then the plates were wrapped with surgical tape to let the air flow and decrease the humidity in the plates. *Agrobacterium* over growth happened in high humidity and led to necrosis of calli. Calli coated with a high amount of *Agrobacterium* after 3 days could not be cleared of the overgrowth of *Agrobacterium* and died.

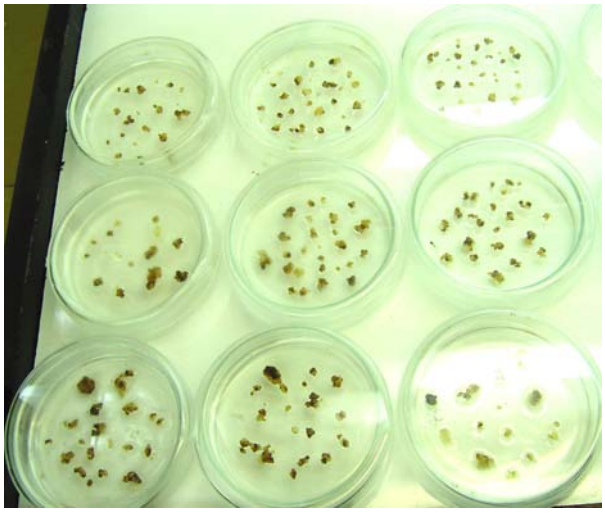


Figure 40 The KDML105 after co-cultivation with *Agrobacterium* for 3 days.

Necrosis and cell death may occur in the cell layer when the T-DNA is transferred. Transgenic cells that show necrotic tissues may inhibit the regeneration and reduce the recovery of transgenic cells (Potrykus, 1990). Necrotic tissues are also known to accumulate antimicrobial substances (Goodman and Novacky, 1994) that may inhibit the potential of *Agrobacterium* to colonize plant cells and transfer T-DNA. Generally, the cells that release the chemical signal, which induces the *vir* genes in *Agrobacterium*, include only in living cell but not in dead necrotic cells (Shaw et al., 1991). Moreover, dead necrotic cells may also attract opportunistic microorganisms under *in vitro* conditions, which leads to contamination that subsequently inhibits plant growth and regeneration. It has earlier been shown that the first step in the transfer of T-DNA molecule from *Agrobacterium* to plant is the recognition of a susceptible plant cell (Zambryski, 1988). Plant cells can be highly susceptible or nonsusceptible to *Agrobacterium* infection, depending on the genotype of the host plant and the strain of the *Agrobacterium* (Jordan and Hobbs, 1994).

4.3.2.5 The effect of acetosyringone

Acetosyringone has been reported as a key component to successful transformation in rice. It induces the expression of *Agrobacterium vir* genes and activates transfer of the T-DNA into the rice genome. The addition of acetosyringone during co-cultivation has been reported to induce *vir* genes, extend the host range of some *Agrobacterium* strains, and to be essential for rice transformation. It also increases the number of transformed calli (Saharan et al., 2004). In monocots, phenolic compounds like acetosyringone for plant/bacterial interaction are not synthesized to support the gene transfer. In this research, the 200 μM concentration of acetosyringone resulted in higher numbers of transformant calli when compared to 100 μM upon co-cultivation at 25 ^{o}C (Table 7).

Table 7 The transformation efficiency of Koshihikari callus with 100 and 200 μM acetosyringone.

Replication	100 μM			200 μM		
	No. callus*	**Resistant callus**	**Transformation efficiency (%)**	**No. callus***	**Resistant callus**	**Transformation efficiency (%)**
1	240	21	8.75	210	34	16.19
2	240	32	13.33	210	43	20.48
3	240	27	11.25	240	39	16.25
4	270	35	12.96	240	45	18.75
5	270	41	15.19	240	42	17.50
6	300	47	15.67	300	57	19.00
7	300	53	17.67	300	61	20.33
8	300	64	21.33	300	72	24.00
Average	**270**	**40.00**	**15 ± 4[b]**	**255**	**49**	**19 ± 3[a]**

* The callus on selection medium at week 4.

Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

Terada et al. (2004) reported that 200 μM acetosyringone appeared to be the best concentration for rice transformation. Hiei et al. (1994) reported that transformation efficiency was extremely low when acetosyringone was omitted. The optimum concentration of acetosyringone in cocultivation medium may vary between different cultivars of rice (Hiei et al., 1997, Vijayachandra et al., 1995). It was interesting that Rao and Rao (2007) reported the ability to transform target genes into indica (Pusa Basmati1) rice callus under acetosyringone free conditions. They added acetosyringone to both pre-induction medium (PIM) and cocultivation medium (CCM). They added only sucrose to both pre-induction and cocultivation media and found that the transient expression levels were similar to those obtained by adding acetosyringone. Thus, phenolics compound like acetosyringone may not be essential for induction of *vir* genes. So the development of transgenic *indica* rice may possible under acetosyringone free conditions.

4.3.3 The effect of antibiotic to callus selection and regeneration

To confirm the transformed calli on selection and regeneration medium, two types of antibiotics were added. The first type of antibiotic was used to kill *Agrobacterium* and the other group was used to select the transformed calli.

After cocultivation with *Agrobacterium* for 3 days, the calli were washed with sterile water containing antibiotic. In this research 3 antibiotics (carbenicilin, cefotaxime and timentin) were used to kill *Agrobacterium*. The concentrations of these antibiotics were between at 200-500 mg/L. The carbenicilin showed the highest toxic effect on callus when compared to cefotaxime and timentin at the same concentration (data not shown). This result was similar to the experiment of

Pipatpanukul et al. (2004) that cefotaxime and carbenicillin concentrations up to 250 mg/L had the highest phytotoxicity to the plant at the regeneration step. In this experiment, 300 mg/L of timentin was found to be a suitable concentration for high efficiency to eliminate *Agrobacterium* with less damage to the callus in the washing and selection step.

After the calli were washed and blotted dry on sterile tissue paper, then were moved onto the selection medium. The recombinant pHELLSGATE8 vector contains a selectable marker gene (*nptII*) that caused resistance to kanamycin, paromomycin, geneticin (G418) and neomycin. Only paromomycin and kanamycin were used for selection of transformed calli in this experiment.

In plants, kanamycin is the most commonly used as a selective agent, normally in concentrations ranging from 50 to 500 mg/l. It is very effective in inhibiting the growth of dicoteledonous untransformed cells. However, kanamycin is ineffective as a selection marker for several legumes and gramineae. For KDML105 and Koshihikari in this experiment, kanamycin seems to interfere with the regeneration of transformed cells to green plantlets. It was also not good for transformed calli selection. This result is in agreement with with Caplan et al. (1992) that demonstrated that kanamycin is an effective selective agent for transformed rice protoplasts, but normal rice callus shows a natural resistance to this antibiotic. It has also been found that for protoplast-derived calli selection on kanamycin is very inefficient in terms of regeneration, and that a large number of albino plants occured on some experiments (Toriyama et al., 1988). Raineri et al. (1990) used 200 mg/L of kanamycin to select rice calli derived from mature embryos, but no transgenic plants were recovered. The presence of kanamycin decreased the number of shoots and percent of regeneration.

Complete inhibition of plant regeneration from rice calli occurred at 150 mg/L kanamycin as reported by Pipatpanukul et al. (2004).

Selection of calli on G418 is more effective than kanamycin, maybe because G418 is more toxic than kanamycin to untransformed rice cells. G418 selection also induces the recovery of a higher proportion of fertile, green and transgenic plants (Ayres and Park, 1994). Even so, the overall efficiency of the regeneration was still low. Chan et al. (1992) used G418 antibiotic in *Agrobacterium*-mediated transformation of rice roots and immature embryos. In both cases, it was possible to generate G418-resistant calli, but after selection with selectable and screenable markers for rice transformation, only three transgenic plants could be recovered when using embryos as the target tissue. However, a total of just four transgenic plants were produced following selection on 40 mg/L of G418. In *Agrobacterium*-mediated transformation, stably transformed calli can be produced efficiently with G418, but the absence of regenerants suggests that exposure of cells to G418 for a long period of time inhibits regeneration (Aldemita and Hodges, 1996). An alternative selectable antibiotic is paromomycin. It can be used for selecting *nptII*-transformed rice cells. This research found that the concentration of 100 mg/L of paromomycin is enough to screen for transformed rice calli.

4.3.4 The callus screening on selection medium

The cocultivation of calli with *Agrobacterium* carrying recombinant pHELLSGATE8 was washed with sterile water that contained 300 mg/L of timentin. The calli were then transferred to the selection medium and incubated in darkness at 28^{o}C. The selection medium contained 300 mg/L of timentin to kill *Agrobacterium*

and 100 mg/L paromomycin for selection of the paromomycin resistant calli. These growing calli were transferred to fresh selection medium every 2 weeks for 8 weeks. Continuous selection on paromomycin-containing medium resulted in the appearance of proliferating and apparently resistant calli.

Three days after transfer the washed-calli onto selection medium, they showed yellow color (Figure 41 A). After 1 week, 50% of the calli changed to brown color. At week 3, the non-paromomycin-resistant calli could not grow on the selection medium and more than 70% of the callus died (Figure 41 B). The surviving calli after 2 weeks (from Figure 41 B) were separated on a new selection medium with about 30 calli/plate (Figure 41 C). After two weeks of selection, only the paromomycin resistant calli were obtained and new calli were generated. The non-paromomycin-resistant calli died (Figure 41 D). The paromomycin resistant calli of each construct were observed. After 2 weeks, the calli were transferred into the new selection medium to increase the amount of paromomycin resistant calli to be enough for subculture on regeneration medium, DNA, RNA and siRNA extraction. Two weeks later, the calli were bigger in size. The amount of calli also increased about 3-5 times (Figure 41 E).

However, the calli could not generate to the new generation calli after subculture on the selection medium several times or more than 3 months because the calli turned brown, soft texture and died. Therefore, the high quality of calli for subculture and move on regeneration medium were done within 2 months.

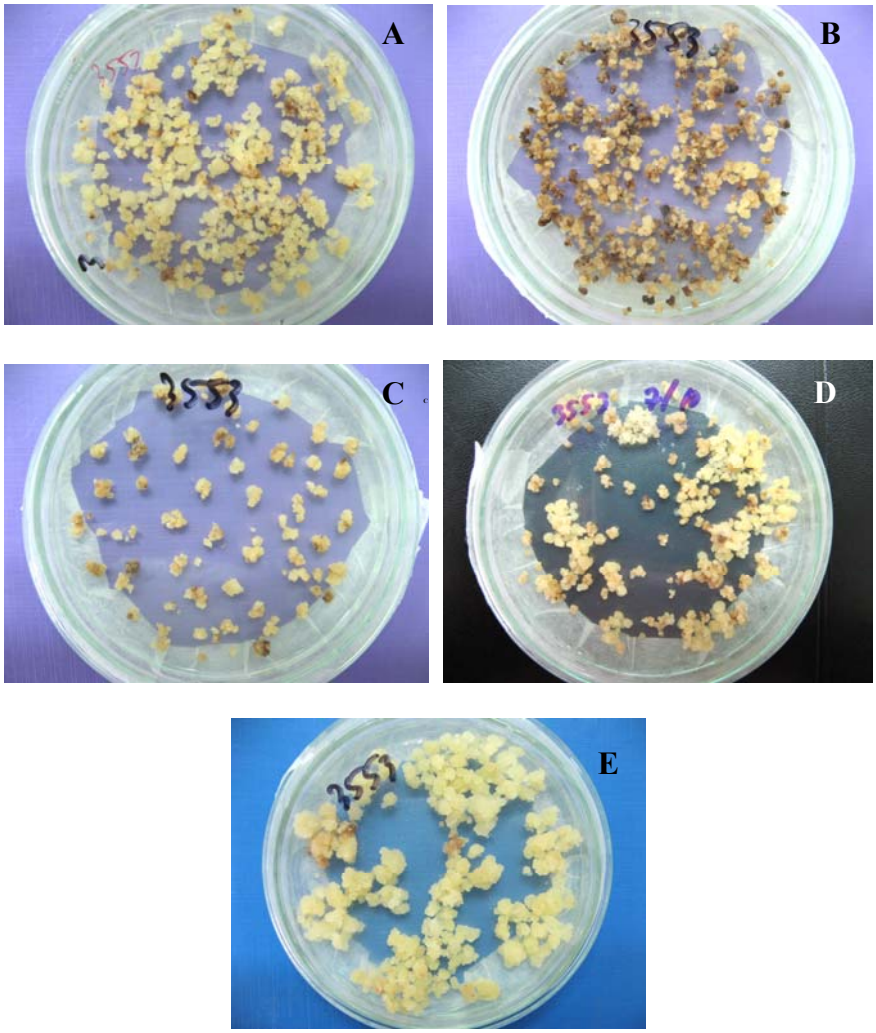


Figure 41 Koshihikari calli on selection medium. Three days after transfer the washed-calli on to selection medium (A), at week 2 on new selection medium (B), the surviving calli after 2 weeks separated on new selection medium (C), the paromomycin resistant calli at week 4 (D) and the paromomycin resistant calli at week 8 (E).

The percent transformations of 6 constructs were observed. The result indicated that the paromomycin resistant calli transformed with the control construct

constructs. The transformation efficiency was not different among the 5 genes constructs (Table 8).

Table 8 The transformation efficiency of paromomycin resistant calli.

Construct	Replication	No.callus	No.resistant callus	Transformation efficiency (%)*
Control	1	150	24	16.00
	2	150	31	20.67
	3	150	28	18.67
	4	240	51	21.25
	5	240	46	19.17
	6	240	53	22.08
	7	300	69	23.00
	8	300	71	23.67
	9	300	56	18.67
	10	300	68	22.67
	11	210	34	16.19
	12	210	43	20.48
	13	240	39	16.25
	14	240	45	18.75
	15	240	42	17.50
	16	300	57	19.00
	17	300	61	20.33
Average		**242**	**48.12**	**20 ± 2[a]**
Os3bglu7	1	150	21	14.00
	2	150	19	12.67
	3	150	27	18.00
	4	150	28	18.67
	5	240	36	15.00
	6	240	31	12.92
	7	240	43	17.92
	8	240	46	19.17
	9	300	36	12.00
	10	300	44	14.67
	11	300	51	17.00
	12	300	48	16.00
	13	300	39	13.00
	14	300	47	15.67
Average		**240**	**36.86**	**15 ± 2[b]**

Table 8 The transformation efficiency of paromomycin resistant calli (Continued).

Construct	Replication	No.callus	No.resistant callus	Transformation efficiency (%)*
Os3bglu8	1	150	22	14.67
	2	150	16	10.67
	3	240	32	13.33
	4	240	35	14.58
	5	240	47	19.58
	6	240	34	14.17
	7	300	53	17.67
	8	300	49	16.33
	9	300	41	13.67
	10	300	60	20.00
Average		**246**	**38.90**	**15 ± 3[b]**
Os12bglu38	1	150	25	16.67
	2	240	34	14.17
	3	240	28	11.67
	4	240	23	9.58
	5	240	35	14.58
	6	300	47	15.67
	7	300	41	13.67
	8	300	57	19.00
	9	300	64	21.33
	10	300	59	19.67
Average		**261**	**41.30**	**16 ± 4[b]**
Os7bglu26	1	150	26	17.33
	2	150	21	14.00
	3	240	33	13.75
	4	240	25	10.42
	5	300	49	16.33
	6	300	55	18.33
	7	300	51	17.00
	8	300	65	21.67
	9	300	50	16.67
	10	300	41	13.67
Average		**258**	**41.60**	**16 ± 3[b]**
Os1bglu1	1	150	17	11.33
	2	150	20	13.33
	3	240	36	15.00
	4	240	27	11.25
	5	240	29	12.08
	6	240	38	15.83
	7	300	54	18.00
	8	300	63	21.00
	9	300	47	15.67
	10	300	59	19.67
Average		**246**	**39.00**	**15 ± 3[b]**

* Transformation efficiency = $\frac{\text{No. resistant callus}}{\text{No. callus}} \times 100$

Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

4.3.5 Confirmation of paromomycin resistant calli by PCR

To confirm the integration of T-DNA fragments in paromomycin-resistant calli, polymerase chain reaction (PCR) analysis was carried out with *nptII* primers.

Figures 42 to 47 show the analysis of the PCR amplification of genomic DNA of paromomycin-resistant calli amplified with *nptII* primers. The expected 730 bp fragment band was found in all calli after transformation with empty pHELLSGATE8 and constructs for knock down *Os1bglu1*, *Os7bglu26*, *Os3bglu8*, *Os12bglu38* and *Os3bglu7*. No *nptII* band was found in the non-transformed calli (lane 2 in Figures 43-45 and lane 1 in figures 46-47).

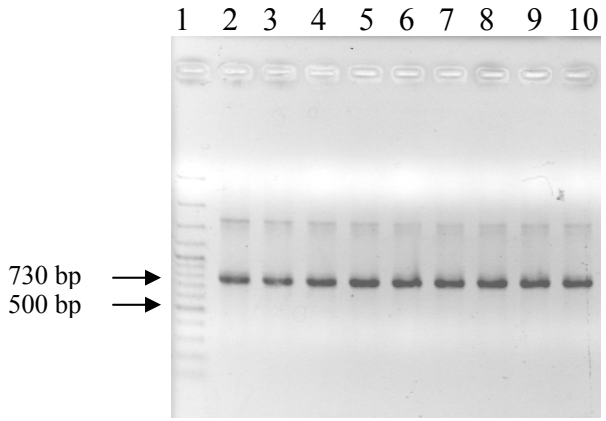


Figure 42 The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with control (empty pHELLSGATE8). Lane 1, 100 bp marker; lanes 2-10, PCR product of the *nptII* gene from different transgenic calli.

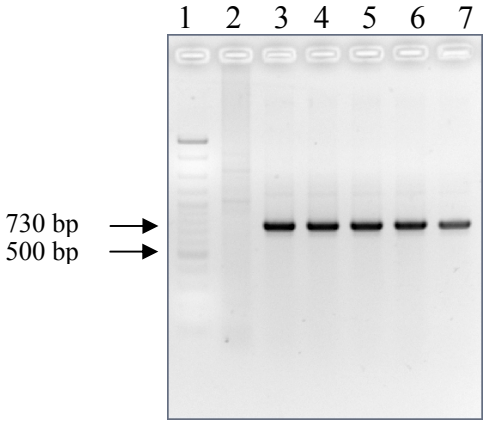


Figure 43 The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with construct to knock down *Os1bglu1*. Lane 1, 100 bp marker; lane 2, nontransgenic calli genomic DNA template as a control; lanes 3-7, PCR products of *nptII* gene from different transgenic calli.

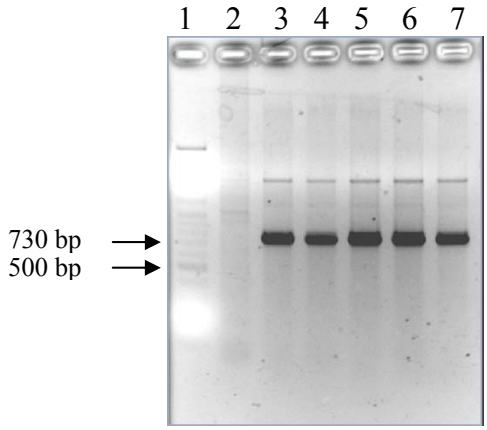


Figure 44 The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with construct to knock down *Os7bglu26*. Lane 1, 100 bp marker; lane 2, nontransgenic calli genomic DNA template as a control; lanes 3-7, PCR products of *nptII* gene from different transgenic calli.

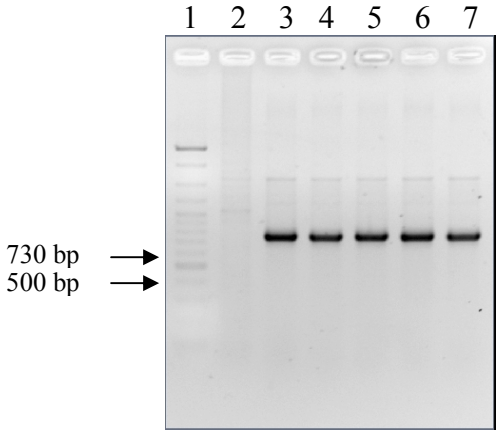


Figure 45 The The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with construct to knock down *Os3bglu8*. Lane 1, 100 bp marker; lane 2, nontransgenic calli genomic DNA template as a control; lanes 3-7, PCR products of *nptII* gene from different transgenic calli.

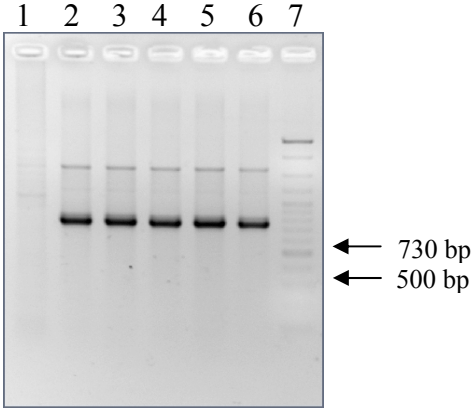


Figure 46 The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with construct to knock down *Os12bglu38*. Lane 1, nontransgenic calli genomic DNA template as a control; lanes 2-6, PCR product of *nptII* gene from different transgenic calli; lane 7, 100 bp marker.

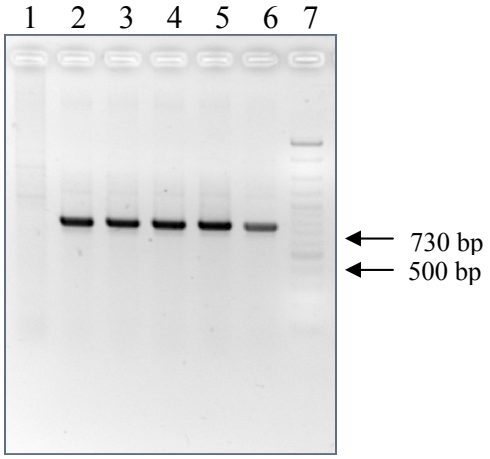


Figure 47 The *nptII* gene PCR products from genomic DNA of paromomycin resistant calli after transformation with construct to knock down *Os3bglu7*. Lane 1, nontransgenic calli genomic DNA template as a control; lanes 2-6, PCR product of *nptII* gene from different transgenic calli; lane 7, 100 bp marker.

4.3.6 Effect of transformation of the construct knocking down 5 genes

The transformation of the knock down 5 genes construct into the calli of KDML105 was done at least 15 times, but all calli were contaminated with *Agrobacterium* (Figure 48 A, B). The major problem is the shape and surface of KDML105 calli. The rough surface and very small size of calli lead to *Agrobacterium* contamination on selection medium. Although the calli were washed with water containing antibiotic several times, the *Agrobacterium* still survived in the folds of the calli surface and could not easily be washed out. The calli washed with sterile water containing antibiotic more than 6 times with continuous stirring were able to survive on the medium without *Agrobacterium* contamination. However, the breaking of calli from continuous stirring led to weak, brown calli and all calli died after 1 week

(Figure 48 C). This research could not transformed KDML105 calli with the knock down 5 genes construct.

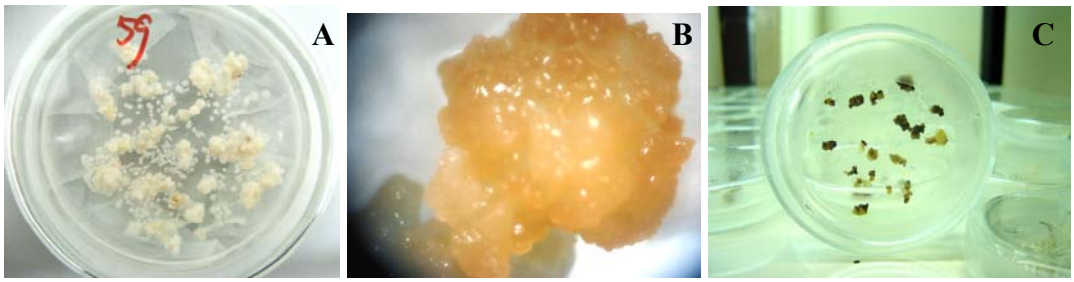


Figure 48 *Agrobacterium* contamination (A, B) and the calli on selection medium (C).

After the lack of success with KDML105 transformation, koshihikari were chosen for transformation of the knock down 5 β-glucosidase genes construct (5 Bglu group). The result showed that the calli of Koshihikari still have the same contamination problem of *Agrobacterium* as in KDML105 calli after tried at least 10 times. It is interesting that both KDML105 and Koshihikari show the same effect. The problem of *Agrobacterium* contamination was not a technical problem, since all the technique used was able to transform other constructs into rice calli (see 4.3.8). The surface of contaminated calli under microscope did not show different with non-transformed calli. Akash et al. (2001) reported that the suppression by double-stranded RNA (dsRNA) of the expression of a target gene was observed within 24 hours after the introduction of dsRNA expression plasmids into tobacco BY-2 cells. Other reports also showed that the RNAi mechanism can happen within 24 hr in many organisms after the dsRNA or siRNA occured in the cell (Murakami et al., 2005, Rothermel et al., 2006, Makimura et al., 2002, Lu et al., 2008). The calli after co-cultivation with *Agrobacterium* for 3 days could possible produce the dsRNA of target

gene from the integration of pHELLSGATE8 in the rice genome within 24 hr and induce the knock down β-glucosidase genes via the RNAi mechanism in the cytoplasm.

In this work the calli with knock down 5 genes construct show susceptible to *Agrobacterium* infection maybe due to the involvement of the β-glucosidases in hydrolysised of the secondary metabolites that serve as plant antibiotics, known as phytoalexins. The major roles of plant secondary metabolites are to protect plants from attack by insect, herbivores and pathogens, or to survive other biotic and abiotic stresses. Secondary metabolite accumulation has been shown in rice cell or rice suspension-cultured cells (Nojiri et al., 1996). Salicylic acid (SA) has been proposed to be an endogenous signal for systemic acquired resistance to infection by pathogens in the plant. In general, most SA is found in an inactive form as inert glucose conjugate (salicylic acid 2-O-β-D-glucoside or SAG) (Defraia et al., 2008). SAG seems to be a storage form of SA from which bioactive SA can be generated (Seo et al., 1995). Recent reports indicate that β-glucosidase might be involved in regulating the signaling activity of phytohormones. Therefore, it seems likely that β-glucosidase might also play an important role by regulating the level of free SA. The SA can directly shut down the expression of the *vir* regulon and specifically inhibit the expression of the *Agrobacterium virA*/*G*, two component regulatory systems that tightly control the expression of the *vir* regulon, including the *repABC* operon on the Ti plasmid (Yuan et al., 2007). Therefore, plants defective in SA accumulation were more susceptible to *Agrobacterium* infection.

Blanchard et al. (2001) reported β-glucosidase aggregated with an other proteins, the β-glucosidase aggregating factor (BGAF), and could not be extracted

efficiently but it did show activity. From this discovery, the scientists knew the enzyme was there, but something was keeping it in a large aggregate. BGAF was a hybrid protein with two distinct regions or domains, a disease-response region and a carbohydrate-binding region (lectin) (Kittur et al., 2009, Subramanyam et al., 2008). In nature, the two occur as separate proteins, but in all the grass species studied so far, they were fused, probably millions of years ago in the ancestors of the grasses. Lectins are sugar-binding proteins which are highly specific for their sugar moieties. They typically play a role in biological recognition phenomena involving cells and proteins. The function of lectins in plants is still uncertain. Surfaces of cells have glycoproteins that lectins recognize by their carbohydrate portion to which they bind. The BGAF's lectin region is similar to lectins that recognize mannose sugar (Molina et al., 2004). The binding of glycoproteins on the surface of parasitic cells is also believed to be a function. Once thought to be necessary for rhizobia binding and defense when foreign cells, such as bacteria, fungus or viruses, try to enter the cell, BGAF probably binds foreign cells, marks them, and recruits other components of the defense system to eventually arrest the development of the foreign elements and kill them. Therefore, the β-glucosidase in BGAF aggregate form is involved in defense and helps the plant cell to against foreign cell infection. So β-glucosidases genes possible to knock down and it could not form BGAF and help the plant cell to against *Agrobacterium*.

4.3.7 Expression of β-glucosidase in transformed calli

To confirm that the β-glucosidases are expressed in the callus and the *Agrobacterium* contamination maybe involved in some effect after the β-glucosidases

knock down, RT-PCR was done. RNA samples extracted from nontransformed calli. Then, the total RNAs were used as a template for cDNA synthesis in two steps of RT-PCR. The primer in the region of 3'UTR of each β-glucosidase genes were used to estimate the expression of each gene. The results indicated that *Os12bglu38* transcripts cound not be detected in calli, but the other 4 genes are expressed at different level (Figure 49). The Expressed Sequence Tags database (dbEST) (National Center for Biotechnology Information) also indicated that *Os12bglu38* has not been found in callus.

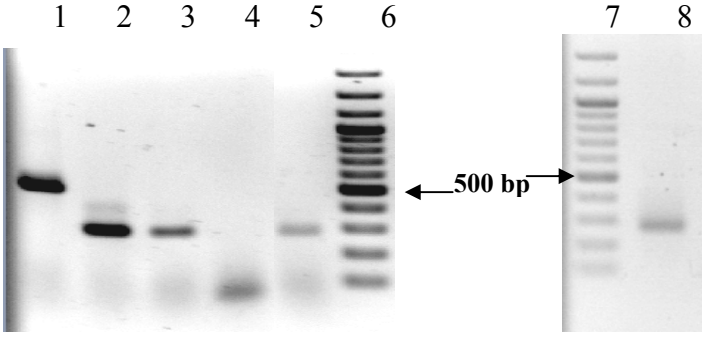


Figure 49 The RNA expressions of each β-glucosidase genes in non transformed calli. Lane 1, β-actin; lane 2, *Os3bglu7*; lane 3, *Os3bglu8*; lane 4, *Os12bglu38*; lane 5, *Os1bglu1*; lane 8, *Os7bglu26* and lanes 6 and 7, 100 bp marker; samples were load on 1.5% agarose gel.

4.3.8 The effect of knock down individual β-glucosidase genes on selection medium

The effect of knock down of individual 5 genes in the calli did not show any distinct phenotype on calli (Figure 50). This phenomenon was supported by Nishimura and Maeda (1984). They tested an effect of nojirimycin, which is a β-

glucosidase inhibitor in cell wall growth, on morphogenesis of root segments. They reported that no effect was observed in the initiation of lateral root primordial or callus, initials except for an inhibition of lateral root growth. A few contaminations from *Agrobacterium* were found after the washing step in all constructs but not as much as the knock down 5 β-glucosidase genes (5 Bglu group), see section 4.3.6.

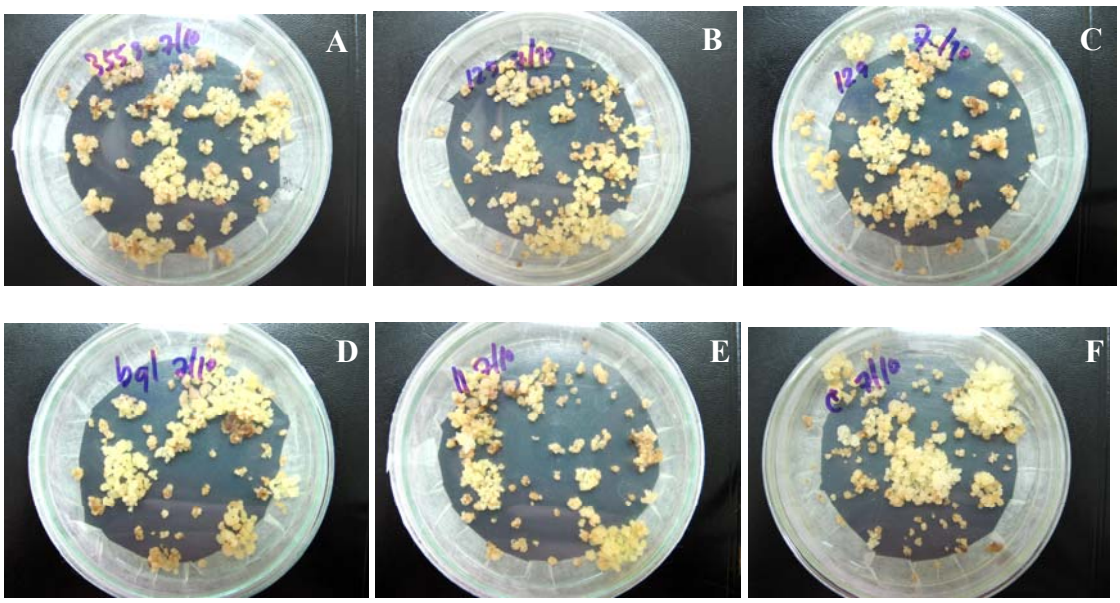


Figure 50 Paromomycin resistant calli after transformed with 6 constructs on the selection medium at 1 month. (A) *Os3bglu8*, (B) *Os1bglu1*, (C) *Os7bglu26*, (D) *Os3bglu7*, (E) *Os12bglu38* and (F) Control.

4.3.9 The regeneration of paromomycin resistant calli

Regeneration ability of plant is very important for producing transgenic plants by genetic engineering. The paromomycin resistant calli were transferred to regeneration medium containing basic MS salts and vitamins with 0.5 mg/L of NAA and 2 mg/L of kinetin. Tsukahara and Hirosawa (1992) indicated that high regeneration frequency was obtained with combinations of NAA (0.05–0.5 g/L) and

kinetin (0.5–2 mg/L). Brar et al. (1985) also reported that the presence of a cytokinin analog either BAP or kinetin is essential in promoting plant regeneration from cultured cells.

The combination of sucrose and sorbitol have been the most acceptable and able to stimulate the growth rate and improved the regeneration of Koshihikari calli. Sorbitol is a major factor for embrogenic callus formation in monocot plants (Swedlund and Locy, 1993). Cho et al. (2004) reported that the growth of calli is stimulated and multiple shoots of regenerated plants could be obtained in higher frequency when sorbitol in combination with sucrose or maltose and 5 mg/L of kinetin were used. The supplementation of sorbitol and proline in the medium was attributed to be more effective to obtain regenerated plants of rice rather than the medium containing sucrose or maltose alone. Kishor and Reddy (1986) also used sorbitol for restoring and enhancing the plant regeneration ability of rice calli. The addition of sorbitol (15-75 g/L) in the regeneration medium promotes plantlet regeneration (Tsukahara and Hirosawa, 1992). However, Yang et al. (1999) reported that addition of sorbitol suppressed the proliferation and regeneration of rice callus.

Light is usually an important factor affecting growth, organogenesis and the formation of plant products, including primary and secondary metabolites. The effect of light intensity and photoperiod on the formation plantlet from callus was investigated by Liu et al. (2001). Although they found that light irradiation during subculture was not a requirement for rice calli regeneration on solid medium, it evidently improved plantlet regeneration in liquid system. The growth rate was a little higher in a light cycle when compared to in darkness. The paromomycin resistant

calli of each construct in this study were subcultured onto regeneration medium and incubated at 28^{o}C under a 18/6h light/dark, cycle.

This study has found that 50-100 mg/L of kanamycin blocked the regeneration of KDML105 and Koshihikary. Moreover, four cycles of selection of transformed calli on selection medium are enough to screen for the real transformed calli and to kill all *Agrobacterium* and nontransformed calli. To avoid the effect of the antibiotic in regeneration step, the paromomycin resistant calli were grown on the free-antibiotic regeneration medium.

Plantlet can be regenerated from the paromomycin resistant calli after transformation with the control and the *Os12bglu38* constructs. The calli transformed with the other constructs could not regenerate to plantlet. The calli having high regeneration potential formed green spots on the surface of callus with fast growth for the first 2 weeks of cultivation (Figure 51 A). The size of growing of healthy paromomycin resistant calli increased from 3-4 mm (black arrow in Figure 51 A) to 1-1.5 cm (blue arrow in Figure 51 A) in diameter. The suitable number of calli on regeneration medium were about 10-15 calli per plate, which is good for calli to maintain enough space for growth.

However, only some green spots on the calli could form shoot after 3-6 weeks (Figure 51 B). Some green calli spots could not form shoot under the same condition. Shoot or root formation were not correlated with the size of calli. Some small calli could generate shoot and root similar to the big calli. The shoot and root induction of the calli emerged at different times and size of calli, which were dependent on the regeneration potential of each calli. Lai and Liu (1982) reported that shoot

regeneration frequency was dramatically different among rice calli induced from different varieties.

The regeneration medium in this research could stimulate shoot and root regeneration from the vigorous callus within one month (Figure 51 C). After the plantlet had obtained leaves of about 3 cm, the root emerged. They were then transferred into the MS hormone free medium (Figure 51 D). About 2 weeks later, the plantlets were moved to soil and covered with a plastic bag to maintain high humidity. The plants were grown under 28^{o}C in 18h/d light condition (Figure 51 E). One week later, the plastic bags were removed and transferred the plantlet in a net cage (Figure 51 F).

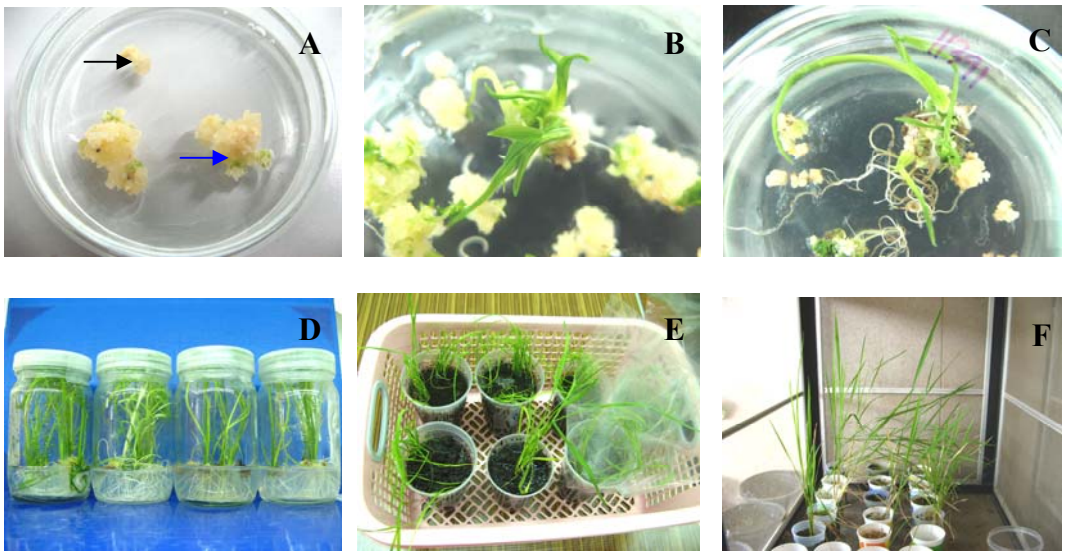


Figure 51 The rice regeneration from callus. The green spot callus after 2-3 weeks old calli (A), calli become green leaf on regeneration medium (B), shoot and root induction from calli (C), plantlet on MS medium-free hormone (D), plantlet cover with plastic bag before transfer to outdoors (E), the plantlets in a net cage (F).

The regeneration efficiency on the regeneration medium was calculated and shown in table 9.

Table 9 The regeneration efficiency of paromomycin resistant calli.

Construct	Replication	No.callus	No.plantlet	Regeneration efficiency (%)*
Control	1	150	8	5.33
	2	150	7	4.67
	3	150	12	8.00
	4	150	9	6.00
	5	150	4	2.67
	6	150	11	7.33
	7	150	7	4.67
Average		**150**	**8.29**	**6 ± 2[a]**
Os3bglu7	1	150	0	0
	2	150	0	0
	3	165	0	0
	4	180	0	0
	5	180	0	0
	6	195	0	0
	7	225	0	0
	8	225	0	0
	9	225	0	0
	10	225	0	0
Average		**192**	**0**	**0**
Os3bglu8	1	165	0	0
	2	165	0	0
	3	165	0	0
	4	180	0	0
	5	180	0	0
	6	180	0	0
	7	195	0	0
	8	195	0	0
	9	195	0	0
Average		**180**	**0**	**0**
Os12bglu38	1	150	5	3.33
	2	150	3	2.00
	3	165	6	3.64
	4	195	8	4.10
	5	195	2	1.03
	6	225	10	4.44
Average		**180**	**5.67**	**3 ± 1[b]**

Table 9 The regeneration efficiency of paromomycin resistant calli (Continue).

Construct	Replication	No.callus	No.plantlet	Regeneration efficiency (%)*
Os7bglu26	1	165	0	0
	2	180	0	0
	3	180	0	0
	4	180	0	0
	5	180	0	0
	6	195	0	0
	7	225	0	0
	8	225	0	0
	9	225	0	0
	10	225	0	0
Average		**198**	**0**	**0**
Os1bglu1	1	150	0	0
	2	150	0	0
	3	150	0	0
	4	150	0	0
	5	180	0	0
	6	180	0	0
	7	195	0	0
	8	225	0	0
	9	225	0	0
Average		**178**	**0**	**0**

* Regeneration efficiency = $\frac{\text{No. resistant callus}}{\text{No. Plantlet}} \times 100$

Within a column, values with the different superscripts (a, b) are significantly different ($P \leq 0.05$).

The result showed that only the calli transformed with control and *Os12bglu38* constructs could regenerate to plantlets while the other transferred construct could not regenerate to plantlet. Although in the beginning of this research some nontransformed calli in each of the individual gene knock down experiments escape the antibiotic selection medium and could grow and regenerate to plantlets, later on they were tested and found to be nontransformed plantlets. The low concentration of paromomycin (50 mg/L) and selection on selection medium for only 1-2 rounds led to the low efficiency of the paromomycin resistant calli screening. The regeneration

medium was not the main factor that prevented the regeneration because the control and *Os12bglu38* transformed calli still could regenerate to plantlet. However, the *Os12bglu38* transformed plantlet did not show any different phenotype when compared with the control plantet (empty pHELLSGATE8 transformed) or nontransformed rice. This might be due to the fact that *Os12bglu38* does not express in calli, although it is expressed at shoot, spikelet before heading, panicle and flower. This research may concluded that the effect of knock down four β-glucosidase genes expressed in callus may decreased the regeneration of calli to plantlet on regeneration medium.

Many publications reported the β-glucosidases were involed in differentiation and regeneration of plants, such as, Chena et al. (2009) studied the cell wall proteins (CWPs) in rice callus. A total of 292 proteins were identified, which included numerous classical CWPs and antioxidant proteins. Bioinformatics analysis showed that 72.6% of these proteins possessed a signal peptide, and a total of 198 proteins were determined to be CWPs in rice. Functional classification divided the extracellular proteins into different groups, including glycosyl hydrolases (23%), antioxidant proteins (12%), cell wall structure-related proteins (6%), metabolic pathways (9%), protein modifications (4%), defense (4%) and protease inhibitors (3%). The specific β-glucosidase activity varies in different tissues with the highest one in roots, and always higher in cell walls than in protoplast. The cell wall location of the enzyme was confirmed biochemically by its activity in intercellular washing fluids (IWF). Both aglycones and glycosides were present in these fluids (Pislewska et al., 2002). The plant hormones that are important for callus induction and regeneration have been studied by Fazelienasab et al. (2004). ABA is one of the five

classical plant hormones that act as inducer of somatic embryogenesis and maturation of these embryos in somatic embryogenesis in plants (Nadina et al., 2001). Free ABA was released by the incubation of ABA-glucose ester with IWF. IWF contains β-glucosidase activity which releases abscisic acid (ABA) from the physiologically inactive ABA-glucose conjugate pool in the barley leaf apoplast. The following data support this conclusion and give the first biochemical and physiological characterization of the extracellular glucosidase activity in barley (Dietz et al., 2000). Shin et al. (1991) reported the highest survival of dried calli and the highest regeneration rate of plantlets were observed in calli which had been pretreated with 10^{-5} M abscisic acid (ABA) in the presence of 90 g/L of sucrose and were regrown on an R-2 medium. In tissue culture of coconut, it has also been reported the concentration of ABA and its interaction with an appropriate auxin were critical in increasing callus induction and regeneration (Fernando et al., 2000).

The composition of cell wall may play an important role in the differentiation of plant tissues. Plant cells have rigid cell walls that define the size, form and stability of the plant cell. It is considerate for cell surface including cell wall would play an intercellular communication in tissue development. These cell walls are comprised of polymers of simple sugar monomers linked in a variety of linear or branched polymers known as polysaccharides. The most abundant simple sugar monomer is glucose, and the most abundant polymer is cellulose. Cellulose is a linear polymer comprised of β-1,4 linked glucose monomers. Other polysaccharides found in plant cell walls include hemicelluloses, which comprise a group of polysaccharides composed of β-1, 4 linked glucose monomers having side chains which may include sugars other than glucose, including xylose, fucose, arabinose, and galactose. β-glucosidase is a glucosidase

enzyme that acts upon β 1-4 bonds linking two glucose, as well as other linkages or glucose-substituted molecules and exocellulase with specificity for a variety of β-D-glycoside substrates. It catalyzes the hydrolysis of terminal non-reducing residues in β-D-glucosides with release of glucose. Therefore, β-glucosidase is involved in differentiation of plant tissues.

Plant cells possess totipotency that is whole plants can be regenerated from single cells by modulating culture conditions (Reinert, 1959). The mechanisms of totipotency are little understood so far, and are mainly discussed in relation to the concentration and ratio of phytohormones (Toonen and De Vries, 1996). The shoot regeneration was determined by the changes of sucrose, glucose, and starch contents (Huang and Liu, 1998). The result showed that sucrose, glucose and starch contents were higher at the initial stage of culture after they transferred the callus to regeneration medium. After being transferred to regeneration medium, glucose content increased prominently during the first day and decreased quickly after three days. The glucose content at the early regeneration stage may be an indicator for shoot regeneration in rice callus which is a regeneration-related factor in rice callus. (Huang and Liu, 2002)

4.3.10 Check the transformed plantlet by PCR

The analysis of PCR amplification of genomic DNA of plantlets transformed with empty pHELLSGATE8 (control) and *Os12bglu38* construct, which amplified with *nptII* primer are shown in Figures 52 and 53, respectively. The expected 730 bp fragment band of *nptII* was found in all plantlet samples transformed with empty pHELLSGATE8 and *Os12bglu38* construct. Suprisingly a band of higher molecular

weight was observed in the non transformed calli. However, No *nptII* size band were found. So the entire paromomycin resistant calli after 4 rounds on selection medium and moved to regeneration medium showed that they are actual transformed plantlet.

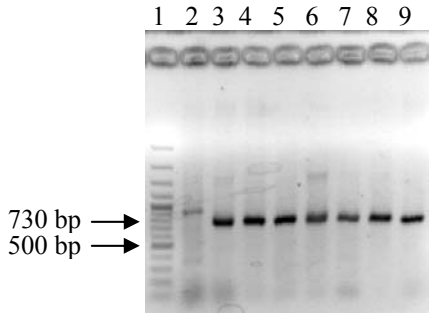


Figure 52 The *nptII* gene PCR products from genomic DNA of plantlets transformed with empty pHELLSGATE8. Lane 1, 100 bp marker; lane 2, nontransgenic calli; lanes 3-9, PCR products of *nptII* gene from different transformed plantlets.

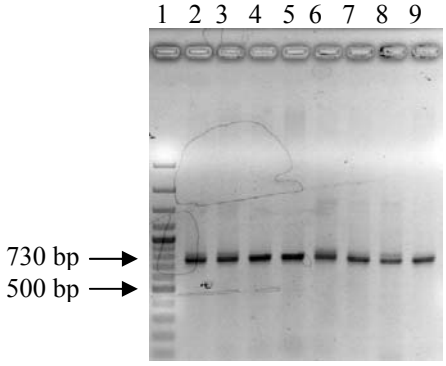


Figure 53 The *nptII* gene PCR products from genomic DNA of plantlets transformed with construct to knock down *Os12bglu38*. Lane 1, 100 bp marker; lanes 2-9, PCR products of *nptII* gene from different transformed plantlets.

4.4 The RNA expression in transformed calli

4.4.1 The detection of β-glucosidase gene expression by RT-PCR

The knock down effect on RNA level of each β-glucosidase gene in calli were checked by RT-PCR. The mechanisms of RNAi in cell induced by dsRNA or siRNA to silence the expression of target genes in cytoplasm. The dsRNA fragment of *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os1bglu1* 3'UTR, the product transcription of pHELLSGATE8 in part of T-DNA region, were processed into small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer in the initiation step. Then, the siRNAs assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), which are unwind the dsRNAs. The siRNA strands subsequently guide the RISCs to complementary mRNA target molecules, where they cleave and destroy the β-glucosidase mRNA at the 3'UTR region. It is a post-transcriptional process initiated by dsRNA molecules that induce degradation of a complementary target RNA.

Total RNA from paromomycin resistant calli of each construct after 4 round of selection were extracted. The *Os12bglu38* paromomycin resistant calli were not used for RNA extraction to detect the RNA expression because this gene does not normally expressed in callus. Therefore, only four β-glucosidase genes, which are *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os1bglu1*, were evaluated for RNA expression and RNA suppression from RNAi mechanism in calli. The paromomycin resistant calli after transformation with empty pHELLSGATE8 vector were used as a control to compare the RNA expression with the other paromomycin resistant calli. Total RNA was precipitated overnight to increase the yield and total RNA sample of each construct was evaluated in 1.5% agarose gel electrophoresis (Figure 54).

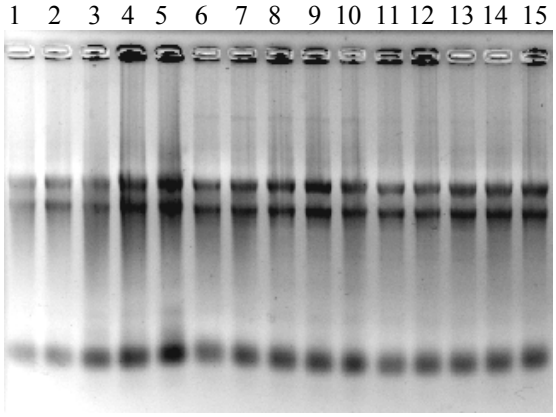


Figure 54 Total RNA of paromomycin resistant calli. Lanes 1-3, *Os3bglu7*; lanes 4-6, *Os1bglu1*; lanes 7-9, *Os7bglu26*; lanes 10-12, *Os3bglu8*; lanes 13-15, empty pHELLSGATE8. The samples were loaded on 1.5% agarose gel.

Total RNA was treated with DNaseI and used as a template for two step RT-PCR reaction. The intensities of *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os1bglu1* 3'UTR PCR products were compared with the β-actin gene. The results in Figure 55 showed that in paromomycin resistant calli of *Os3bglu8*, no *Os3bglu8* mRNA was detected in all calli samples. However, the calli transformed with empty pHELLSGATE8 vector (control) could detect the *Os3bglu8* mRNA expression. The suppression of non- specific genes was detected with the other primer for amplified *Os7bglu26*, *Os3bglu7* and *Os1bglu1*. The result showed the expression of mRNA of *Os7bglu26*, *Os3bglu7* and *Os1bglu1*. Therefore, no effect to knock down the other genes in paromomycin resistant calli of *Os3bglu8*.

The paromomycin resistant calli of *Os7bglu26* (Figure 56) and *Os1bglu1* (Figure 57) showed similar results of complete knock down of mRNA expression

level when compared with the control paromomycin resistant calli and no knock down of the other genes. The interesting result was found in the parmomycin resistant calli of *Os3bglu7*. The mRNA level of *Os3bglu7* was observed in all samples of paromomycin resistant calli when compare with the control paromomycin resistant calli (Figure 58). Although some sample showed almost complete knock down of *Os3bglu7* mRNA level (lane 2) or half level of mRNA expression in lane 3 when compared with control paromomycin resistant calli but the expression was not completely knock down as in the other promomycin resistant calli constructs.

Opassiri et al. (2006) demonstrated that *Os3bglu7* is the highest express β-glucosidase gene in rice. Maybe the dsRNA of *Os3bglu7* from pHELLSGATE8 are not sufficient enough to knock down all the highly expressed *Os3bglu7* or maybe the knock down of *Os3bglu7* activate some molecules to activate higher level of *Os3bglu7* expression to maintain the biological activity for the calli to survive. Suppression of RNAi by *Os3bglu7* dsRNA might be caused by loss of *Os3bglu7* mRNA level in the cell. Alternatively, the *Os3bglu7* dsRNA might suppress RNAi, because it contains a specific dsRNA sequence that is a potent competitor for the RNAi machinery or it might need long time to knock down all highly express of *Os3bglu7* by dsRNA in callus cell because the other genes do not show the high express as same as *Os3bglu7*. If the complete knock down of *Os3bglu7* happen after move the paromomycin resistant calli on regeneration medium. Therefore, it may cause the effect in some biological pathway that is involved in rice regeneration to plantlets, because in this experiment no plantlets were obtained to study the phenotype effect.

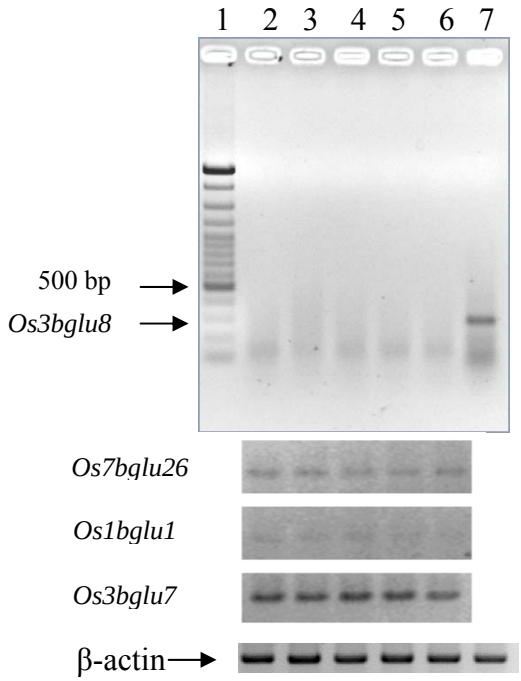


Figure 55 RT-PCR products from mRNA of β-glucosidase in transformed rice calli with construct *Os3bglu8*. Gene specific 3'UTR primers were used to generate *Os3bglu8*, *Os7bglu26*, *Os1bglu1* and *Os3bglu7* PCR fragment, β-actin was used as a control. Lane 1, 100 bp marker; lanes 2-6, paromomycin resistant calli transformed with *Os3bglu8*; lane 7, paromomycin resistant calli transformed with empty pHELLSGATE8.

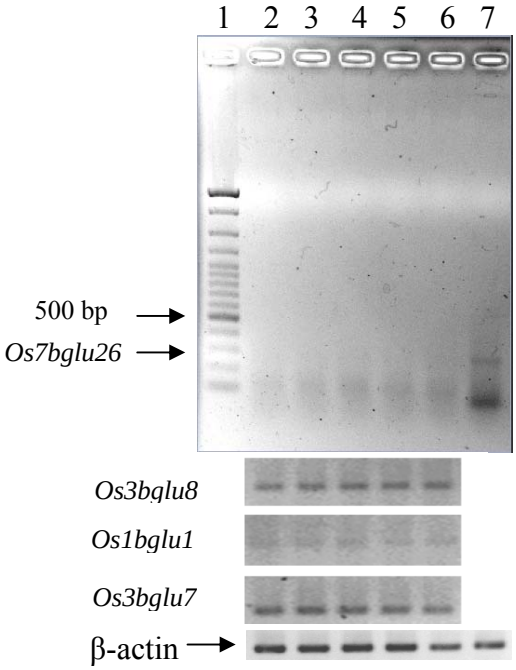


Figure 56 RT-PCR products from mRNA of β-glucosidase in transformed rice calli with construct *Os7bglu26*. Gene specific 3'UTR primers were used to generate *Os7bglu26*, *Os3bglu8*, *Os1bglu1* and *Os3bglu7* PCR fragment, β-actin was used as a control. Lane 1, 100 bp marker; lanes 2-6, paromomycin resistant calli transformed with *Os7bglu26*; lane 7, paromomycin resistant calli transformed with empty pHELLSGATE8.

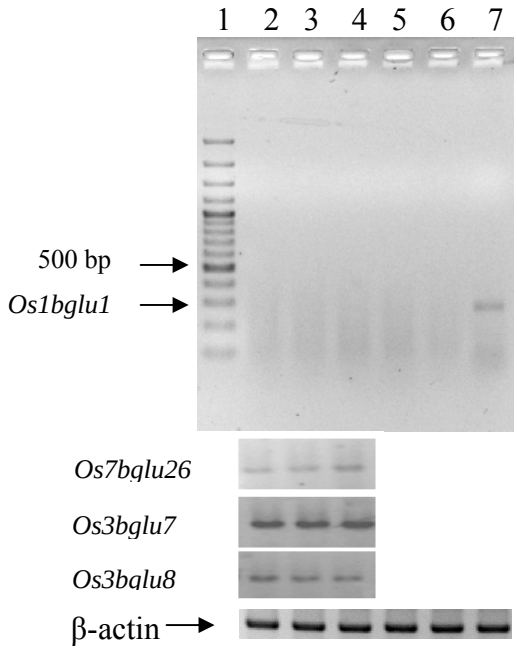


Figure 57 RT-PCR products from mRNA of β-glucosidase in transformed rice calli with construct *Os1bglu1*. Gene specific 3'UTR primers were used to generate *Os1bglu1*, *Os7bglu26*, *Os3bglu7* and *Os3bglu8* PCR fragment, β-actin was used as a control. Lane 1, 100 bp marker; lanes 2-6, paromomycin resistant calli transformed with *Os1bglu1*; lane 7, paromomycin resistant calli transformed with empty pHELLSGATE8.

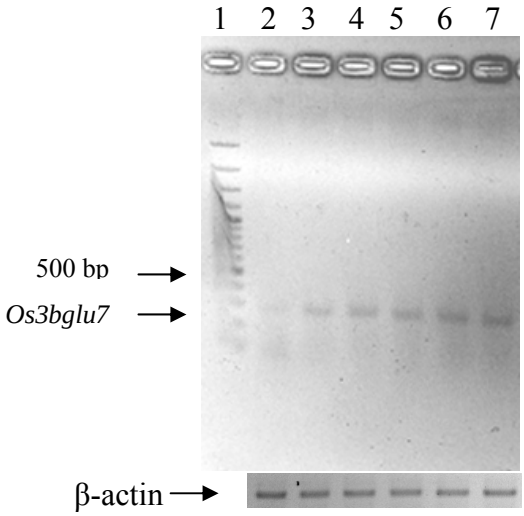


Figure 58 RT-PCR products from mRNA of β-glucosidase in transformed rice calli with construct *Os3bglu7*. Gene specific 3'UTR primers were used to generate *Os3bglu7* PCR fragment, β-actin was used as a control. Lane 1, 100 bp marker; lanes 2-6, paromomycin resistant calli transformed with *Os3bglu7*; lane 7, paromomycin resistant calli transformed with empty pHELLSGATE8.

4.4.2 The detection of siRNA by northern blot analysis

siRNA is a product of RNAi after dsRNA cleaved by dicer or Dicer-like (DCL) proteins. The rice genome has six putative DCL proteins (Liu et al., 2007). The mRNA target is directly cleaved into siRNA by sequence complementarity. Therefore, the presence of siRNAs indicates the occurrence of RNA silencing in cells. siRNAs can be detected by northern blot analysis. The method of detecting siRNAs involved isolating total RNA and separating the small RNA by 15% denaturing polyacrylamide gel electrophoresis (PAGE). Then, the RNA was blotted to a

membrane and hybridizing the RNA on the membrane with a labeled probe. In the final step, the hybridization signal could be detected. siRNA is the trigger molecule for RNAi. The accumulated of *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os1bglu1* siRNA in the paromomycin resistant calli should initiate degradation of the *Os3bglu7*, *Os3bglu8*, *Os7bglu26* and *Os1bglu1* transcripts with sequence complementarity. The levels of siRNA accumulation in paromomycin resistant calli of each construct are shown in Figure 59. The high accumulation of siRNA was found in *Os3bglu8* followed by *Os7bglu36*, *Os1bglu1* and *Os3bglu7*, respectively.

The result of siRNA accumulation showed the effect of mRNA expression in the previous result (3.4.4.1). The high expression of mRNA of *Os3bglu7* in most calli sample were in agreement with the low accumulation of siRNA. The low amounts of siRNA are not enough to knock down the high level of *Os3bglu7* mRNA expression in rice callus. The high amounts of siRNA give more chance to combine with the RISC complex for search and destroy the mRNA target. However, the mRNA expression of *Os3bglu8*, *Os7bglu36* and *Os1bglu1* show low level if expression in normal condition, so enough siRNAs were generated to completely knock down and the mRNA could not be detected.

Variation in siRNA levels maybe due to several reasons. First, T-DNA location in the genome could affect expression. In *Agrobacterium*-mediated plant transformation, T-DNA integrated into the host genome randomly. If the transgene inserted in the genome where active transcription occurs, the transgene would be active. Otherwise, it would be less active or even silent and could not regenerate the dsRNA of target genes. Second, the copy number of the transgene may be a contributing factor in expression levels. However, transgene activity may not be

directly proportional to its copy number due to co-suppression. Third, methylation of transgene may occur, especially at or near promoter if it is considered foreign. As a safeguard, the host generally has a mechanism to methylate and inactivate the transgene. This has been reported with foreign DNA expressing dsRNA in PTGS and is supported by a requirement of DNA methylase in initiating RNA-dependent DNA methylation (Vaistij et al., 2002; Mathieu and Bender, 2004)

RNAi interference (RNAi) induces sequence-specific degradation of mRNA and posttranscriptionally down-regulates gene expression. Target mRNAs are found on the basis of homology with short double stranded RNA, called small interfering RNA (siRNA). RNA interference is a widespread experimental tool for gene silencing. Successful silencing highly depends on the selection of siRNA sequence, because not all siRNAs are efficient. siRNA sequences were chosen at random. However, only some of them significantly decreased target gene expression suggesting that the rate of silencing may depend on siRNA sequence. siRNAs with UU 3′overhangs are more efficient. The symmetric 3′overhangs help to form RISCs with antisense and sense strands in equal ratio. The target mRNA should contain AA (N19) UU motif, where N indicate the target sequence (Elbashir et al., 2001). siRNAs have phosphorylated 5′ends because the phosphate group is essential for its function (Biao-Ma et al., 2005). dsRNA adopts a right-handed A-form helix conformation that is more tightly packaged than a B-from helix typical for DNA. Helical geometry could influence the RISC formation and loading of the guide strand. The antisense strand-target mRNA duplex also must be in an A-form helix for successful mRNA cleavage (Chiu and Rana 2002). It is possible that the sequence of *Os3bglu7* siRNA

has some problem to combine with the RISC complex, which led to the naked siRNA duplex in cell and can be degraded.

The effect of mRNA expression and siRNA accumulation in paromomycin resistant calli of *Os3bglu7* may involved in the secondary structures of the *Os3bglu7* for dicer binding protein and mRNA-binding protein may influence the accessibility to dsRNA and siRNA. Martin et al. (2006) indicated that mismatched siRNAs, designed to mimic miRNAs, would exhibit activity as a function of target site location within endogenous mRNAs and appear to reduce protein levels in a manner consistent with their effects on mRNA levels with those targeting the 3'UTR exhibiting greater activity.

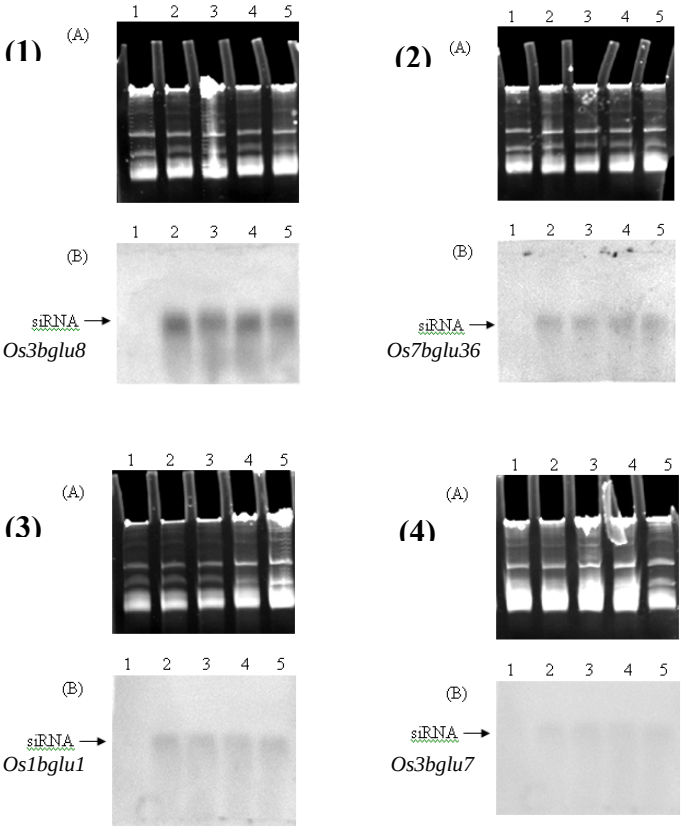


Figure 59 Northern blot analysis of siRNA using 3' UTR probes of *Os3bglu8* (1), *Os7bglu36* (2), *Os1bglu1* (3) and *Os3bglu7* (4). (A) Total RNA sample

load in 15% polyacrylamide gel (upper part of gel). (B) siRNA detection on membrane (lower part of gel was cut and moved to blot on the membrane). Lane 1 (A), (B), paromomycin resistant calli transformed with empty pHELLSGATE8; lanes 2-5 (A), (B), paromomycin resistant calli transformed with the construct to knock down each β-glucosidase genes.

The mechanism of β-glucosidase genes in rice is still unclear but in this research the effect after knock down of β-glucosidase genes in calli was observed. *Agrobacterium* contamination and inability to regenerate the transgenic rice may reflect the loss of function of β-glucosidase in rice calli.

CHAPTER V

CONCLUSIONS

One set of proteins that is involved in rice growth and responses to stress is β-glucosidase. The presence of many β-glucosidases in rice suggests that they may also have other functions that are yet unknown. In rice, *Os3bglu7* has the highest expression when compared with the other genes. The phylogenetic tree analysis showes that *Os3bglu7* is closely related with 4 other genes. Therefore, in this study, 5 β-glucosidase genes, *Os1bglu1*, *Os3bglu7*, *Os3bglu8*, *Os12glu38* and *Os7Bglu26* were chosen for functional analysis by RNAi. Five constructs using the 3'UTR of each of the 5 genes were used for RNAi knock down in the pHELLSGATE8. And one construct, using the conserved region of *Os3bglu7*, was used to try to knock down all 5 genes. The last construct, which is used as control, is the empty pHELLSGATE8. The pHELLSGATE8 were constructed by Gateway cloning technology with LR clonase emzyme and transferred to rice calli via the *Agrobacterium* strain EHA105.

The calli of KDML105 were induced from seeds on a callus induction medium (MS and N6D medium) and Koshihikari were induced on a N6D medium. The secondary callus was obtained after 4-6 weeks. Light was not an important factor for callus induction, but the culture medium has more effect on growing of secondary callus. The highest amount of secondary callus was obtained at 28 ^{o}C on a N6D medium in both rice varities. When the secondary callus properties of Koshihikari are compared to KDML105 the Koshihikari have a smoother surface, a bigger size and

grow faster. The small size and rough surface of KDML105 led to contamination of *Agrobacterium* easier than Koshihikari. Four to six weeks old calli were precultured on a new callus induction medium and co-cultivated with *Agrobacterium*. Transformation efficiency depended on many factors. (1) Acetosyringone is one factor to activate the expression of the *vir* gene and the transfer of T-DNA into the rice genome. In this research, 200 μM of acetosyringone was the best concentration for rice transformation. The lower concentration led to decreased transformation efficiency. (2) Co-cultivation of 3 days obtained higher transformation efficiency than in 4 days. The longer time led to a high percentage of calli with necrosis because of *Agrobacterium* infection. (3) Temperature is an important factor for controlling the *Agrobacterium* growth, the plant cell infection and the T-DNA insertion into the rice callus. 25 ^{o}C was a suitable temperature for co-cultivation conditions. (4) A pH of the co-cultivation medium of 5.2 induces the virulence genes of *Agrobacterium* and enhances the transformation efficiency. (5) *Agrobacterium* concentration is a critical factor to indicate the transformation efficiency. The high concentration of *Agrobacterium* led to necrosis of callus. *Agrobacterium* concentration at OD_{600} = 0.02 in infection medium reduced the necrosis of calli and resulted in a high transformation efficiency. The excess bacteria were removed by blotting dry the calli on sterile tissue paper before it was moved in co-cultivation medium. After the co-cultivation step, the calli were washed with antibiotics to eliminate the *Agrobacterium*. Timentin had a high efficiency to kill *Agrobacterium* and has less effect on calli, when compared with cefotaxime and carbenicilin.

The calli were then screened and selected. The callus induction medium was supplemented with 2 antibiotics for killing the *Agrobacterium* (timentin) and the

transformed calli (paromomycin) were selected. Only the construct for the knock down of 5 genes showed the contamination of *Agrobacterium* in both KDML105 and Koshihikari, while the other constructs for the knock down of individual β-glucosidase genes were not contaminated. Therefore, the knock down of 5 β-glucosidase genes may have been enough to prevent the chemical release of toxic molecules against *Agrobacterium*. For the individual knock down β-glucosidase genes there was no different character observed when compared with the control. The control construct showed the highest transformation efficiency (at 19%) when compared with the other constructs (15%). The calli were screened on a selection medium for 2 months and the T-DNA insertion was confirmed by PCR with *nptII* primers. This research indicated that the paromomycin resistant calli were transgenic calli. RT-PCR indicated a complete knock down of mRNA expression of *Os1bglu1*, *Os3bglu8* and *Os7Bglu26* in paromomycin resistant calli, but the *Os3bglu7* showed a different knock down mRNA level. siRNA confirmed the RNAi mechanism to occur in the cell, this research indicated that the siRNA were found in the transgenic calli. The highest amount of siRNAs was found in *Os3bglu8* followed by *Os1bglu1*, *Os7bglu36* and the lowest was found in *Os3bglu7*. The paromomycin resistant calli were regenerated to plantlets on a regeneration medium. Only the control and the *Os12bglu38* knock down construct were able to regenerate and the obtained regeneration efficiency of 5% and 3%, respectively. The plantlet of the control and the *Os12bglu38* plantlets showed the integration of *nptII* genes. However, the *Os12bglu38* transgenic plantlets did not show any different phenotype when compared with the control plantlet.

REFERENCES

Abe, T. and Futsuhara, Y. (1986). Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). **Theor Appl Genet**. 72: 3-10.

Aharoni, A., Giri, A. P., Deuerlein, S., Griepink, F., Kogel, W., Verstappen, W. A., Verhoeven, H. A., Jongsma, M. A., Schwab, W., and Bouwmeester, H. J. (2003). Terpenoid metabolism in wild-type and transgenic arabidopsis Plants. **Plant Cell**. 15(12): 2866–2884.

Akashi, H., Miyagishi, M. and Taira, K. (2001). Suppression of gene expression by RNA interference in cultured plant cells. **Anti nucle acid drug develop**. 11(6): 359-67.

Akiyama, T., Kaku, H. and Shibuya, N. (1998). A cell wall-bound β-glucosidase from germinated rice: purification and properties. **Phytochemistry**. 48: 49-54.

Aldemita, R. R. and Hodges, T. K. (1996). *Agrobacterium*-mediated transformation of *japonica* and *indica* rice varieties. **Planta**. 199: 612–617.

Amin M. A., Uddin, M. A. and Hossain M. A. (2004). Regeneration study of some indica rice cultivars followed by Agrobacterium-mediated transformation of highly regenerable cultivar, BR-8. **J Biol Sci**. 4(2): 207-211.

Ayres, N. M. and Park, D. (1994). Genetic transformation of rice. **Plant Sci**. 13(3): 219-239.

Bahassi, E. M., O'Dea, M. H., Allali, N., Messens, J., Gellert, M. and Couturier, M. (1999). Interactions of CcdB with DNA gyrase. Inactivation of GyrA, poisoning

of the gyrase-DNA complex, and the antidote action of CcdA. **J Biol Chem**. 274: 10936-10944.

Bak, S., Paquette, S. M., Morant, M., Rasmussen, A. V., Saito, S., Bjarnholt, N., Zagrobelny, M., Jørgensen, K., Hamann, T. and Osmani, S. (2006). Cyanogenic glycosides; a case study for evolution and application of cytochromes. **Phytochem Rev**. 5: 309–329.

Bellincampi, D., Camardella, L., Delcour, J. A., Desseaux, V., D'Ovidio, R., Durand, A., Elliot, G., Gebruers, K., Giovane, A., Juge, N., Sorensen, J. F., Svensson B. and Vairo, D. (2004). Potential physiological role of plant glycosidase inhibitors. **Biochim Biophys Acta**. 12(2): 265–74.

Biao Ma, J., Yuan, Y. R., Meister, G., Pei, Y., Tuschl, T. and Patel, D. J. (2005). Structural basis for 5'-end-specific recognition of guide RNA by the *A. fulgidus* Piwi protein. **Nature**. 434: 666-670.

Binns, A. N. (1990). *Agrobacterium*-mediated gene delivery and the biology of host range limitations. **Physiol Plant**. 79: 135-139.

Blanchard, D. J., Cicek, M., Chen, J. and Esen, A. (2001). Identification of β-glucosidase aggregating factor (BGAF) and mapping of BGAF binding regions on maize β-glucosidase. **J Biol Chem**. 276(15): 11895-11901.

Brar, D. S., Ling, D. H. and Yoshida, S. (1985). Plant regeneration from somatic cell cultures of some IR varieties of rice. In M. S. Swaminathan (ed.), Biotechnology in International Agricultural Research. IRRI Manila, Philipines. 169-177.

Bücker, C. and Grambow, H. J. (1990). Alterations in 1, 4-benzoxazinone levels following inoculation with stem rust in wheat leaves carrying various alleles for resistance and their possible role as phytoalexins in moderately resistant

leaves. **Z Naturforsch**. 45: 1151–1155.

Busto, M. D. and Perez-Mateos, M. (1995). Extraction of humic-β-glucosidase fractions from soil. **Biol Fer Soi**. 20: 77–82.

Cao, X., Liu, Q., Rowland, L. J. and Hammerschlag, F. A. (1998). GUS expression in blueberry (*Vaccinium* spp.): factors influencing Agrobacterium-mediated gene transfer efficiency. **Plant Cell Rep**. 18: 266–270.

Caplan, A., Dekeyser, R. and Van Montagu, M. (1992). Selectable markers for rice transformation. **Methods in Enzymol**. 216: 426-441.

Chakrabarty, R., Viswakarma, N., Bhat, S. R., Kirti, P. B., Singh, B. D. and Chopra, V. L. (2002). *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. **J Biosci**. 27: 495-502.

Chan, M. T. Tse-Min Lee. T. M. and Chang, H. H. (1992). Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. **Plant Cell Physiol**. 33(5): 577-583.

Chen, M. T., Chang, H. H., Ho, S. L., Tong, W. F., and Yu, S. M. (1993). *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/S-glucuronidase gene. **Plant Mol Biol**. 22: 491-506.

Chen, K. and Rajewsky, N. (2006). Deep conservation of microRNA-target relationships and 3'UTR motifs in vertebrates, flies, and nematodes. **Cold Spring Harb Symp Quant Biol**. 71: 149-156.

Chena, X. Y., Kimb, S. T., Chob, W., Rima, Y., Kimd, S., Kima, S. W., Kang, K., Parkd, Z. Y. and Kim, J. Y. (2009). Proteomics of weakly bound cell wall

proteins in rice calli. **Plant Physiol**. 166: 675-685.

Cheng, X., Sardana, R., Kaplan, H. and Altosaar, I. (1998). *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. **Appli Bio Sci**. 95: 2767-2772.

Cheng, M., Lowe, B., Spencer, M., Ye, X. and Armstrong, C. L. (2004). Invited review: factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. **In vitro cell Dev Biol Plant**. 40: 31-45.

Chiu, Y. L. and Rana, T. M. (2002). RNAi in human cells: basic structural and functional features of small interfering RNA. **Mol Cell**. 10(3): 549-561.

Cho, J. H., Lee, J. Y. and Kim, Y. W. (2004). Improvement of shoot regeneration from scutellum-derived callus in rice. **Korean J Crop Sci**. 49(1): 52-60.

Cicek, M. and Esen, A. (1998). Structure and expression of a Dhurrinase (β-glucosidase) from sorghum. **Plant Physiol**. 116: 1469-1478.

Cicek, M., Blanchard, D., Bevan, D. R. and Esen, A. (2000). The Aglycone Specificity-determining Sites Are Different in 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (Maize β-Glucosidase) and Dhurrinase (Sorghum β-Glucosidase). **J Biol Chem**. 275: 20002-20011.

Conn, E. E. (1980). Cyanogenic compounds. **Ann Rev Plant Physiol**. 31: 433-451.

Cottrell, T. R. and Doering, T. L. (2003). Silence of the strands: RNA interference in eukaryotic pathogens. **Trends Microbio**. 11(1): 37-43.

Coutinho, P, M and Henrissat, B. (1999). Carbohydrate-active enzymes. AFMB-CNRS. http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html.

Crawford, G. W. and Shen, C. (1998). The origins of rice agriculture: recent progress

in east asia. **Antiquity**. 72: 858-866.

Davies**,** G. and Henrissat, B. (1995). Structures and mechanisms of glycosyl hydrolases. **Structure**. 3: 853-859.

Datta, K. and Datta, S. K. (2006). Indica rice (*Oryza sativa*, BR29 and IR64). **Plant Sci**. 343: 201-212.

Das, D. K., Reddy, M. K., Upadhyaya, K. C. and Sopory, S. K. (2002). An efficient leaf-disk culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). **Plant cell Rep**. 20: 999-1005.

Defraia, C. T, Schmelz, E, A. and Mou, Z. (2008). A rapid biosensor-based method for quantification of free and glucose-conjugated salicylic acid. **Plant Methods**. 31(4): 2-8.

Deng, W., Pu, X. A., Goodman, R. N., Gordon, M. P. and Nester, E. W. (1995). TDNA genes responsible for inducing a necrotic response on grape vines. **Mol Plant Microbe Interact**. 8: 538-548.

Dharmawardhana, D. P., Ellis, B. E. and Carlson, J. E. (1995). A [β]-Glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. **Plant Physiol**. 2: 331-339.

Dietz, K. J., Sauter, A., Wichert, K., Messdaghi, D. and Hartung, W. (2000). Extracellular ß-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. **J Experimental Botan**y. 51(346): 937-944.

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988). Plant Genetic Transformation and Gene Expression, A Laboratory Manual, Blackwell Scientific Publications Ltd, London.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003). Killing the messenger:

short RNAs that silence gene expression. **Nat Rev Mol Cell Biol**. 4: 457-467.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. **EMBO J**. 20(23): 6877-6888.

Esen, A. and Bandaranayake, H. (1998). Insertional polymorphism in introns 4 and 10 of the maize β-glucosidase gene *glu1*. **Genome**. 41(4): 597-604.

Fan, T. W. M. and Conn, E. E. (1985). Isolation and characterization of two cyanogenic β-glucosidases from flax seeds. **Arch Biochem Biophys**. 243: 361-373.

Faure, D., Henrissat, B., Ptacek, D., Bekri, A.M. and Vanderleyden, J. (2001). The *celA* gene, encoding a glycosyl hydrolase family 3 β-glucosidase in *Azospirillum irakense,* is required for optimal growth on cellobiosides. **Applied Envir on Micro**. 67(5): 2380-2383.

Fazelienasab, B., Omidi, M. and Amiritokaldani, M., (2004). **Crop Sci**. http:// www.cropscience.org.au/icss2004/poster/3/4/2/207 fazelienasab.htm? print=1.

Feng, Q., Zhang, Y., Hao, P., Wang, S., Fu, G., Huang, Y., Li, Y., Zhu, J., Liu, Y., Hu, X., Jia, P., Zhang, Y., Zhao, Q., Ying, K., Yu, S., Tang, Y., Weng, Q., Zhang, L., Lu, Y., Mu, J., Lu, Y., Zhang, L. S., Yu, Z., Fan, D., Liu, X., Lu, T., Li, C., Wu, Y., Sun, T., Lei, H., Li, T., Hu, H., Guan, J., Wu, M., Zhang, R., Zhou, B., Chen, Z., Chen, L., Jin, Z., Wang, R., Yin, H., Cai, Z., Ren, S., Lv, G., Gu, W., Zhu, G., Tu, Y., Jia, J., Zhang, Y., Chen, J., Kang, H., Chen, X., Shao, C., Sun, Y., Hu, Q., Zhang, X., Zhang, W., Wang, L., Ding, C., Sheng, H., Gu, J., Chen, S., Ni, L., Zhu, F., Chen, W., Lan, L., Lai, Y., Cheng, Z., Gu, M., Jiang, J., Li, J., Hong, G., Xue, Y. and Han, B. (2002). Sequence and analysis of rice chromosome 4. **Nature**. 420(6913): 316-20.

Fernando, S. C. and Gamage, C. K. A. (2000). Abscisic acid induced somatic in immature embryo explant of coconut (*Cocos nucifera* L.). **Plant Sci**. 151: 193-198.

Fia, G., Giovani, G. and Rosi, I. (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. **J Appl Microbiol**. 99: 509-517.

Fire, W. W. and Sun, L. (1998). Functional genomics double-stranded RNA posses puzzle. **Nature**. 391: 744-745.

Forslund, K., Morant, M., Jørgensen, B., Olsen, C. E., Asamizu E., Sato, S., Tabata, S. and Bak, S. (2004). Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in *Lotus japonicus*. **Plant Physiol**. 135: 71-84.

Friedberg, F., Saunders, A. A. and Rhoads, A. R. (2003). Conservation of the 3′-untranslated regions of calmodulin mRNAs in cetaceans. **Mol Bio Rep**. 30: 193-198.

Garris, A., Tai, T., Coburn, J., Kresovich, S. and McCouch, S. R. (2005). Genetic structure and diversity in *Oryza sativa* L. **Genetics**. 169: 1631-1638.

Gaudin, V., Vrain, T. and Jouanin, L. (1994). Bacterial genes modifying hormonal balances in plants. **Plant Physiol Biochem**. 32: 11-29.

Gelvin, S. B. (2003). *Agrobacterium*-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. **Micro Mol Biol Rev**. 67: 16-37.

Goodman, R. N. and Novacky, A. J. (1994). The hypersensitive reaction in plants to pathogens. St Paul, APS Press.

Greener, M. J., Sewry, C. A., Muntoni, F. and Roberts, R. G. (2002). The 3'UTR

region untranslated region of the dystrophin gene conservation and consequences of loss. **European J Human Gen**. 10: 413-420.

Guo, J., Cho, J, H., Jo, H. J., Seong, E. S. and Wang, M. H. (2007). *Agrobacterium*-mediated transformation of rice 'Ilmibyeo' using *HPT* selection marker gene. **Korean J Plant Res**. 20(3): 242-246.

Hannon, G.J. (2002). RNA interference. **Nature**. 418: 244-251.

Hansen, G. (2000). Evidence for *Agrobacterium*-induced apoptosis in maize cells. **Mol Plant Microbe Interact**. 13: 649-657.

Hartke, S. and Lorz, H. (1989). Somatic embryogenesis and plant regeneration from various indica rice (*Oryza sativa* L.) genotypes. **J Gene Breeding**. 43: 205-214.

Hartung, W., Sauter, A. and Hose, E. (2002). Abscisic acid in the xylem: where does it come from, where does it go to?. **J Exp Bot**. 53: 27-32.

He, S. and Withers, S. G. (1997). Assignment of sweet almond β-glucosidase as a family 1 glycosidase and identification of its active site nucleophile. **J Biol Chem**. 272(40): 24864-24867.

He, X., Miyasaka, S., Fitch, M., Moore, P. and Zhu, Y. (2008). Agrobacterium tumefaciens-mediated transformation of taro (Colocasia esculenta (L.) Schott) with a rice chitinase gene for improved tolerance to a fungal pathogen Sclerotium rolfsii. **Plant Cell Rep**. 27(5): 903-909.

Henrissat, B. and Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. **Biochem J**. 316: 695-696.

Hiei, Y., Komari, T. and Kubo, T. (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. **Plant Mol Biol**. 35: 205-218.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. **Plant J**. 6: 271-282.

Hiei. Y. and Komari, T. (2008). Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. **Nat Protoc**. 3(5): 824-34.

Hoque, M. E., Mansfield, J. W. and Bennett, M. H. (2005). *Agrobacterium*-mediated transformation of indica rice genotypes: an assessment of factors affecting the transformation efficiency. **Plant Cell Tiss Organ Cult**. 82: 45-55.

Hood, E. E., Gelvin, S. B., Melchers, L. S. and Hoekema, A. (1993). New *Agrobacterium* helper plasmids for gene transfer to plants. **Transgenic Res**. 2: 208-218.

Hösel, W., Surholt, E. and Borgmann, E. (1978). Characterization of β-glucosidase isoenzymes possibly involved in lignification from chick pea (*Cicer arietinum* L.) cell suspension cultures. **Eur J Biochem**. 84(2): 487-92.

Hösel, W., Tober, I., Eklund, S. H. and Conn, E. E. (1987). Characterization of β-glucosidases with high specificity for the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L) moench seedlings. **Arch Biochem Biophys**. 252: 152-162.

Hu, H., Xiong, L.and Yang, Y. (2005). Rice SERK1gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. **Planta**. 222: 107-117.

Huang, W. L. and Liu, L. F. (1998). Promotion of shoot regeneration from rice (*Oryza sativa* L.) callus induce on the medium containing high concentration of sucrose.

Chinese Agron. J. 8: 91-100.

Huang, W. L. and Liu, L. F. (2002). Carbohydrate metabolism in rice during callus induction and shoot regeneration induced by osmotic stress. **Bot Bull Acad Sin**. 43: 107-113.

Ignacimuthu, S. and Arockiasamy, S. (2006) *Agrobacterium*-mediated transformation of an elite indica rice for insect resistance. **Current sci**. 90(6): 256-264.

Ignacimuthu, S., Arockiasamy, S. and Terada, R. (2000). Genetic transformation of rice: current status and future prospects. **Current sci**. 79(2): 186-195.

Islam, S. M., Miyazaki, T., Tanno, F. and Itoh, K. (2005). Dissection of gene function by RNA silencing. **Plant Biotech**. 22: 443-446.

Jahn, G. C, Litsinger, J. A., Chen, Y. and Barrion, A. (2007). Integrated pest management of rice: ecological concepts. In ecologically based integrated pest management (eds. O. Koul and G.W. Cuperus). **CAB International**. 315-366.

Jan, E., Yoon, J. W., Waterhouse, D., Iannaccone, P. and Goodwin, E. B. (1997). Conservation of the *C.elegans* TRA-2 3'UTR translational control. **EMBO J**. 16: 6301-6313.

Jiang, C. J. and Li, Y. Y. (1999). Studies on testing conditions of β-*D*-glucosidase activity in tea. **J Anhui Agric Univ**. 22: 212-215.

Jones, P. R. and Vogt, T. (2001). Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. **Planta**. 213: 164-174.

Jordan, M. C. and Hobbs, L. A. (1994). The transformation of legumes using *Agrobacterium tumefaciens*. In Biotechnological Applications of Plant Cultures (Shargool, PD, Ngo TT, eds.), CRC press, Boca Raton. 61-76.

Kaisa, M., Mikko, L., Taina, L., Merja, T., Pekka, S. and Kurt, V. F. (2003).

Developmental lignification and seasonal variation in β-glucosidase and peroxidase activities in xylem of Scots pine, Norway spruce and silver birch**.** **Tree physiol**. 23(14): 977-86.

Keri, E. S., Josephine, A. and MariaElena, A. (2004). Expression of β-glucosidase and its role in activating zeatin-O-glycoside during cold-stress recovery in *Zea mays* root tip. Plant Biology 2004: 24-28, Lake Buena Vista, FL USA.

Khanna, H. K. and Raina, S. K. (1998). Genotype culture media interaction effects on regeneration response of three indica rice cultivars. **Plant Cell Tiss Organ Cult**. 52: 145-153.

Kishor, P. B. and Reddy, G. M. (1986). Osmoregulation and organogenesis in callus tissues of rice. **Indian J Plant Physiol**. 24: 700-702.

Kittur, F. S, Yu, H. Y., Bevan, D. R and Esen, A. (2009). Homolog of the maize {β}-glucosidase aggregating factor from sorghum is a jacalin-related GalNAc-specific lectin but lacks protein aggregating activity. **Glycobiology**. 19(3): 277-287.

Klose, S. and Tabatabai, M. A. (2002). Response of glycosidases in soils to chloroform fumigation. **Bio Fer Soi.** 35: 262-269.

Konno, H., Yamasaki, Y. and Katoh, K. (1996). A β-glucosidase associated with cell walls from cell suspension cultures of carrot. **Phytochemistry**. 43: 1157-1161.

Kumar, K. K., Maruthasalam, S., Loganathan, M., Sudhakar, D. and Balasubramanian, P. (2005). An improved *Agrobacterium*-Mediated transformation protocol for recalcitrant elite indica rice cultivars. **Plant Mol Bio Rep**. 23: 67-73.

Kumria, R., Waie, B. and Rajam, M. V. (2001). Plant regeneration from transformed embryogenic callus of an elite indica rice via *Agrobacterium*. **Plant Cell Tiss**

Organ Cult. 67: 63-71.

Kupkanchanakul, T. (1998). Khao Dawk Mali 105 yield improvement through agronomic practices. **Thai agri res J**. 16(2): 144-150.

Kuroki, G. W. and Poulton, J. E. (1987). Isolation and characterization of multiple forms of *prunasin hydrolase* from black cherry (*Prunus serotina* Ehrh.) seeds. **Arch Biochem Biophys**. 255: 19-26.

Lai, K. L. and Liu, L. F. (1982). Induction and plant regeneration of callus from immature embryos of rice plants (*Oryza sativa* L.). **Jpn J Crop Sci**. 51: 70-74.

Le. L., Lorenz, Y., Scheurer, S., Fötisch, K., Enrique, E., Bartra, J., Biemelt, S., Vieths, S. and Sonnewald, U. (2006). Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. **Plant Biotechnol J**. 4(2): 231-242.

Lee, K. H., Piao, H. L., Kim, H., Choi, S. M., Jiang, F., Hartung, W., Hwang, I., Kwak, J. M., Lee, I. J. and Hwang, I. (2006). Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic Acid. **J cell**. 126: 1109-1120.

Li, D., Liu, H., Yang, Y., Zhen, P. and Liang, J. (2009). Down-regulated expression of *RACK*1 gene by RNA Interference enhances drought tolerance in rice. **Rice sci**. 16(1): 14-20.

Lichner, Z., Silhavy, D. and Burgyán, J. (2003). Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences. **J. Gen Virol**. 84: 975-980.

Lin, T., He, X.W., Yang, L., Shou, H.X. and Wu, P. (2005). Identification and characterization of a novel water-deficit-suppressed gene OsARD encoding

an acid-reductone-dioxygenase-like protein in rice. **Gene**. 360: 27-34.

Liu, L. H., Wan, X. C., Wen, Y. and Zhang, Z, Z. (2003). Variations of β-glucosidase activities during the processing of Qimen black tea. **J Anhui Agric Univ**. 30: 386-389.

Liu, M., Zhu, J., Sun, Z. X. and Xu, T. (2007). Possible suppression of exogenous β-1,3-glucanase gene gluc78 on rice transformation and growth. **Plant Sci**. 172: 888-896.

Liu, J., Xie, C. H., Yu, Z. S. and Liu, Y. (2001). Research on the propagation of *Amorphophallus* in vitro. **J Huazhong Agric Univ**. 20: 283-285.

Londo, J. P. and Chiang, Y. (2006). Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, *Oryza sativa*. **PNAS**. 103(25): 9578-9583.

Lu, J., Yue, B., Wang, C., Bai, S. and Sheng, G. (2008). Efficacy of RNAi-induced down-regulation of wild-type FLT3 on NF-κB pathway in THP-1 cell line. **Life Science J**. 5(2): 15-20.

Luo, A.D., Liu, L., Tang, Z.S., Bai, X.Q., Cao, S.Y. and Chu, C.C. (2005). Down-regulation of OsGRF1 gene in rice rhd1 mutant results in reduced heading date. **J integrative plant biology**. 47(6): 745-752.

Ma, Q., Dai, X., Xu, Y.,Guo, J., Liu, Y., Chen, N., Xiao, J., Zhang, D., Xu, Z., Zhang, X. and Chong, K. (2009). Enhanced tolerance to chilling stress in *OsMYB3R-2* transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. **Plant Physiol**. 150(1): 244-256.

Macino, G., and Cogoni, C. and Romano, N. (1994). Suppression of gene expression by homologous transgenes. **J Gen M**. 65: 205-209.

Macías, F. A., Chinchilla, N., Varela, R. M., Marín, D. and Molinillo, J. M. G. (2005). Structure-activity relationships of benzoxazinoids on *Echinochloa crus galli* (L.)P./Beauv. 4th Allelopathy Congress.

Maicas, S. and Mateo, J. J. (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. **Appl Microbiol Biotechnol**. 67: 322-335.

Makimura, H., Mizuno, T. M., Mastaitis, J. W, Agami, R., Mobbs, C. V. (2002). Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. **BMC Neuroscience**. 3(18): 1-6.

Mantis, N. J. and Winans, S. C. (1992). The Agrobacterium tumefaciens vir gene transcriptional activator *virG* is transcriptionally induced by acid pH and other stress stimuli. **J Bacteriol**. 174(4): 1189-1196.

Martin, S. E. and Matasha, J. (2006). Mismatched siRNAs downregulate mRNAs as a function of target site location. **FEBS Letters**. 580: 3694-3698.

Masciandaro, G., Macci, C., Doni, S., Maserti, B. E., Leo, A. C., Ceccanti, B. and Wellington, E. (2008). Comparison of extraction methods for recovery of extracellular β-glucosidase in two different forest soils. **Soil Biochem**. 40: 2156-2161.

Mattiacci, L., Dicke, M. and Posthumus, M. A. (1995). β-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. **PNAS**. 92: 2036-2040.

Mathieu, O. and Bender, J. (2004). RNA-directed DNA methylation. **J Cell Sci**. 117: 4881-4888.

Mazzuca, S., Spadafora, A. and Innocenti, A. M. (2006). Cell and tissue localization of

β-glucosidase during the ripening of olive fruit (*Olea europaea*) by *in situ* activity assay. **Plant Sci**. 171: 726-733.

Mercuri, A., Benedetti, L. D., Burchi, G. and Schiva, T. (2000). *Agrobacterium*-mediated transformation of African violet. **Plant Cell Tiss Organ Cult**. 60: 39-46.

Miki, D., Morutoh, S. and Shimamoto, K. (2003). Gene-specific RNAi using 3'UTR-dsRNA in rice. **Plant Cell Physiol**. 44: 234-241.

Miki, D., Itoh, R. and Shimamoto, K. (2005). RNA silencing of single and multiple members in a gene family of rice. **Plant Physiol**. 138: 1903-1913.

Mizutani, M., Nakanishi, H., Ema, J., Ma, S. J., Noguchi, E., Inohara-Ochiai, M., Fukuchi-Mizutani, M., Nakao, M. and Sakata, K. (2002). Cloning of β-primeverosidase from tea leaves, a key enzyme in tea aroma formation. **Plant Physiol**. 130: 2164-2176.

Mkpong, E., Yan, H., Chism, G. and Sayre, R.T. (1990). Purification, characterization, and localization of linamarase in cassava. **Plant Physiol**. 93: 176-181.

Mok, D.W. and Mok, M.C. (2001). Cytokinin metabolism and action. **Plant Mol Biol.** 52: 89-118.

Molina, J., Landa, A., Bautista, G., Martínez, M. and Córdoba, F. (2004). Molecular association of lectin and β-glucosidase in corn coleoptile. **Biochim Biophys Acta**. 1674(3): 299-304.

Molnar, A., F., Schwach, D. J., Studholme, E. C., Thuenemann and Baulcombe, D. C. (2007). miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. **Nature**. 447: 1126-1129.

Montoya, A.L., Chilton, M.D., Gordon, M.P. Sciaky, D. and Nester, E.W. (1977).

Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumor cells: role of plasmid genes. **J Bacteriol**. 129(1): 101-107.

Morant, M., Bak, S., Møller, B. L. and Werck-Reichhart, D. (2003). Plant cytochromes P450: tools for pharmacology, plant protection and phytomediation. **Curr Op Biotechnology**. 14: 151-162.

Morant, A. V., Jorgensen, K., Jorgensen, C. Paquette, S.M., Sánchez-Pérez, R., Møller, B. L. and Bak, S. (2008). Glucosidases as detonators of plant chemical defense. **Phytochemistry**. 69(9): 1795-1813.

Murakami, M., Ota, T., Nukuzuma, S. and Takegami, T. (2005). Inhibitory effect of RNAi on japanese Encephalitis virus replication *in vitro* and *in vivo*. **Microbio Immuno**. 49 (12): 1047-1056

Napoli, C., Lemieux, C. and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. **Plant Cell**. 2(4): 279-289.

Nadina, N., Martinez, M. E., Castillo, R. and Gonzalez, O. (2001). Effect of abscisic acid and jasmonic acid on partial desiccation of encapsulated somatic embryos of sugarcane. **Plant Cell Tiss Organ Cult**. 65(1): 15-21.

Nematollahi, W. P. and Roux, S. J. (1999). A novel β-glucosidase from the cell wall of maize (*Zea mays* L.): rapid purification and partial characterization. **J. Plant Physiol**. 155: 462-469.

Niemeyer, H. M. (1988). Hydroxamic acid (4-hydroxy-1, 4-benzoxazin-3-ones), defence chemicals in the Graminece. **Phytochemistry**. 27: 3349-3358.

Nisius, A. (1988). The stromacentre in *Avena* plastids: an aggregation of β-glucosidase responsible for the activation of oat-leaf saponins. **Planta**. 173: 474-481.

Nishimura, S. and Maeda, E. (1984). Enhancement of callus initation and growth with 2, 6-dicholorobenzonitrile, a cellulose inhibitor, in rice roots. **Japan J Crop Sci.** 53(1): 109-112.

Nikus, J. and Lisbeth M. V. (1999). Tissue localization of β-glucosidase in rye, maize and wheat seedlings. **Physiologia Plantarum**. 107(4): 373-381.

Nojiri, H., Sugimori, M. Yamane, H., Nishimura, Y., Yamada, A., Shibuya, N., Kodama, O., Murofushi, N. and Omori, T. (1996). Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. **Plant Physiol**. 110: 387-392.

Ogawa, K., Moon, J. H., Guo, W. F., Yagi, A., Watanabe, N. and Sakata, K. (1995). A study on tea aroma formation mechanism: Alcoholic aroma precursor amounts and glycosidase activity in part of the tea plant. **Z Naturforsch.** 50: 493-498.

Opassiri, R. (2003). Isolation and characterization of rice β-glucosidase. Ph.D. thesis, Suranaree University of technology, Thailand.

Opassiri, R., Pomthong, B., Onkoksoong, T., Akiyama, T., Esen, A., and Ketudat Cairns, J. R. (2006). Analysis of rice glycosyl hydrolase family 1 and expression of *Os4bglu12* β-glucosidase. **BMC Plant Biology**. 6: 33-39.

Osbourn, A. E. (2003). Saponins in cereals. **Phytochemistry**. 62: 1-4.

Osbourn, A. E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. **Plant Cell**. 8: 1821-1831.

Ozawa, K. and Kawahigashi, H. (2006). Positional cloning of the nitrite reductase gene associated with good growth and regeneration ability of calli and establishment of a new selection system for *Agrobacterium*-mediated transformation in rice (*Oryza sativa* L.). **Plant sci**. 170: 384-393.

Ozawa, K. (2009). Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). **Plant sci**. 176(4); 522-527.

Penga, L. T., Shia, Z. Y., Lia, L., Shenc, G. Z. and Zhanga, J. L. (2007). Overexpression of transcription factor OsLFL1 delays flowering time in *Oryza sativa*. **J plant physiology**. 165: 876-885.

Perl, A., Lotan, O., Abu-Abied, M. and Holland, D. (1996). Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): The role of antioxidants during grape *Agrobacterium* interactions. **Natur Biotechnol**. 14: 624-628.

Pipatpanukul, T., Bunnag, S., Theerakulpisut, P. and Kosittrakul, M. (2004). Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by *Agrobacterium tumefaciens*. **Songklanakarin J Sci Technol.** 26(1): 1-13.

Pisithkul, K., Jongkaewwattana, S., Wongpornchai, S., Tulyathun, W. and Meechui, S. (2006). Modifying cooking quality of khao dawk mali 105 rice. **Agricultural Sci J**. 37(5): 187-190.

Pitson, S. M., Seviour, R. J. and Mcdougall, R. M. (1999). Intercellular and cell wall associated [β]-glucanases and [β]-glucosidases of *Acremonium persicinum*. **Mycological Research**. 103: 1217-1224.

Pislewska, M., Bednarek, P., Stobiecki, M., Zielinska, M. and Wojtaszek, P. (2002). Cell wall-associated isoflavonoids and β-glucosidase activity in *Lupinus albus* plants responding to environmental stimuli. **Plant cell environ**. 25(1): 29-40.

Pocsi, I., Kiss, L., Hughes, M. A. and Nanasi, P. (1989). Kinetic investigation of the substrate-specificity of the cyanogenic β-D-glucosidase (linamarase) of white clover. **Arch Biochem Biophys**. 272: 496-506.

Pongtongkam P., S. Peyachoknagul**,** P. Sripichit, A. Thongpan, K. Klakhaeng, S. Ketsagul and K. Lertsirirungson. (2004). Effects of L-lysine on callus formation, plant regeneration and flowering of Thai rice cv. KDML105. **Kasetsart J (Nat. Sci.).** 38: 190-195.

Potrykus, I. (1990). Gene transfer to cereals. **Bio/Technology**. 8: 535-542.

Poulton, J. E. (1990). Cyanogenesis in plants. **Plant Physiol**. 94: 401-405.

Prasad**,** K. and Vijayraghavan**,** U. (2003). Double-Stranded RNA Interference of a rice *PI*/*GLO* paralog, *OsMADS2*, uncovers its second-whorl-specific function in floral organ patterning. **Genetics**. 165: 2301-2305.

Pu, X. A. and Goodman, R. N. (1992). Induction of necrosis by *Agrobacterium tumefaciens* on grape explants. **Physiol Mol Plant Pathol**. 41: 245-254.

Pulawskaa, J., Willems, A. and Sobiczewski, P. (2006). Rapid and specific identification of four Agrobacterium species and biovars using multiplex PCR. **Syst Appli Micro**. 29: 470-479.

Qian, H., Zhang, X., Xue, Q. (2004). Factors affecting the callus induction and GUS transient expression in indica rice Pei'ai64s. **Pakistan J Bio Sci**. 7(4): 615-619.

Quintela, J. C., Caparrhs, M. and Pedro, M. A. (1995). Variability of peptidoglycan structural parameters in gram-negative bacteria. **FEMS Micro Letters**. 125: 95-100.

Raineri, D. M., Bottino, P., Gordon, M. P. and Nester, E. W. (1990). *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). **Bio Technology**. 8: 33-38.

Rao, M. V. R. and Rao, G. J. N. (2007). *Agrobacterium*-mediated transformation of *indica* rice under acetosyringone-free conditions. **Plant Biotech**. 24: 507-511.

Reinert, J. (1959). Uber die kontrolle der morphogenes und die induction von

adventuvembryonen an gewebekulturen aus karotten. **Planta**. 53: 318-333.

Richard, S., Saffman, E. E., Styhler, S., Rother, K., Li, W. and Lasko, P. (1999). Premature translation of *oskar* in oocytes lacking the RNA-binding protein Bicaudal-C. **Mol Cell Biol**. 18: 4855-4862.

Robinette, D. and Matthysse, A. G. (1990). Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas* syringae pv. phaseolicola. **J Bacteriol**. 172: 5742-5749.

Rodo, A. P., Brugiere, N., Vankova, R., Malbeck, J., Olson, J. M., Haines, S. C., Martin, R. C., Habben, J. E., Mok, D. W .A. and Mok, M. C. (2008). Over-expression of a zeatin O-glucosylation gene in maize leads to growth retardation and tasselseed formation. **J Exp Bot**. 59: 2673-2686.

Rongtian, L., Zhongming, Z. and Qifa, Z. (2002). Transformation of *japonica* rice with *RHL* gene and salt tolerance of the transgenic rice plant. **Chinese Sci Bul**. 47(12): 998-1002.

Rothermel, A., Volpert, K., Burghardt, M., Lantzsch, C., Robitzki, A. A. and Layer, P. G. (2006). Knock-down of GFRα4 expression by RNA interference affects the development of retinal cell types in three-dimensional histiotypic retinal spheres. **Invest Opht Vis Sci**. 47: 2716-2725.

Saharan, V., Yadav, R. C., Yadav, N. R. and Ram, K. (2004). Studies on improved *Agrobacterium*-mediated transformation in two *indica* rice (*Oryza sativa* L.) **African J Biotech**. 3(11): 572-575.

Sahi, S. V., Chilton, M. D. and Chilton, W. S. (1990). Corn metabolites affect growth and virulence of *Agrobacterium tomefaciens*. Proceddings of the National

Academy of Sciences USA. 87: 3879-3883.

Samiphak, K. and Siwarungson, N. (2006). Transformation and expression of green fluorescent protein and barley stress-induced embryogenic *hva1* gene in indica rice KDML 105 by *Agrobacterium tumefaciens* EHA 105. **J Sci Res Chula Univ.** 31(1): 46-54.

Sakata, K., Antonio, B.A., Mukai, Y., Nagasaki, H., Sakai, Y., Makino, K., and Sasaki, T. (2000). INE: A rice genome database with an integrated map view. **Nucleic Acids Res**. 28: 97-101.

Sasaki, T. and Burr, B. (2000). International Rice Genome Sequencing Project: the effort to completely sequence the rice genome. **Curr Opi Plant. Bio**. 3: 138-141.

Schell, J. and Van, M. M. (1977). The Ti-plasmid of *Agrobacterium tumefaciens*, a natural vector for the introduction of nif genes in plants?. **Basic Life Sci**. 9: 159-79.

Selmar, D., Lieberei, R. and Biehl, B. (1988). Mobilization and utilization of cyanogenic glycosides – the linustatin pathway. **Plant Physiol**. 86: 711-716.

Seo, S., Ishizuka, K. and Ohashi, Y. (1995). Induction of salicylic acid β-glucosidase in tobacco leaves by exogenous salicylic acid. **Plant Cell Physiol**. 36(3): 447-453.

Shaw, J. A., Mol, P. C., Bowers, B., Silverman, A. J., Valdivieso, M. H., Duran, A. and Cabib, E. (1991). The function of chitin synthase 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. **J Cell Bio**. 114-111.

Shin, D. H., Virigool, S., Shinozaki, K. Y. and Oono, K. (1991). Survival mechanism of dried calli and regeneration of plants in rice. **Japanese J Genetics**. 66(1):

13-25.

Shrawat, A. K and Lörz, H. (2006). *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. **Plant Bio J**. 4: 575-603.

Siritunga, D. and Sayre, R. (2003). Generation of cyanogen-free transgenic cassava. **Planta**. 217(3): 367-373.

Somerville, C. and Somerville, S. (1999). Plant functional genomics. **Science**. 4: 98-109.

Spadafora, A., Mazzuca, S., Chiappetta, F. F., Parise, A., Perri, E. and Innocenti, A. M. Oleuropein-specific-β-glucosidase activity marks the early response of olive fruits (*Olea europaea*) to mimed insect attack. **Agricultural Sci China**. 7(6): 703-712.

Subramanyam, S., Smith, D. F., Clemens, J. C., Webb, M. A., Sardesai, N and Williams, C. E. (2008). Functional characterization of *HFR1*, a high-mannose N-glycan-specific wheat lectin induced by Hessian fly larvae. **Plant Physio**. 147(3): 1412-1426.

Sue, M., Ishihara, A. and Iwamura, H. (2000). Purification and characterization of a β-glucosidase from rye (*Secale cereale* L.) seedlings. **Plant Sci**. 155: 67-74.

Sundaram, P., Echalier, B., Han, W., Hull, D. and Timmons, L. (2006). ATP-binding cassette transporters are required for efficient RNA interference in *Caenorhabditis elegans*. **MBC**. 17: 3678-3688.

Sunilkumar, G., Campbell, L., Puckhaber, L., Stipanovic, R. and Rathore, K. (2006). Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. **Proc Natl Acad Sci USA**. 103(48): 18054-18059.

Swedlund, B. and Locy, R. D. (1993). Sorbitol as the primary carbon source for the

growth of embryogenic callus of maize. **Plant Physiol**. 103: 1339-1346.

Terada, R., Asao, H. and Iida, S. (2004). A large-scale *Agrobacterium*-mediated transformation procedure with a strong positive–negative selection for gene targeting in rice (*Oryza sativa* L.). **Plant Cell Rep**. 22: 653-659.

Thadavong, S., Sripichitt, P., Wongyai, W. and Jompuk, P. (2002). Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. **Kasetsart J (Nat. Sci.)**. 36: 334-344.

Tian, L., Qi-hua, L., Ohsugi, R., Yamagishi, T. and Sasaki, H. (2006). Effect of high temperature on sucrose content and sucrose cleaving enzyme activity in rice grain during the filling Stage. **Rice Sci**. 13(3): 205-210.

Toonen, M. A. J and de Vries, S. C. (1996). Initiation of somatic embryos from single cells. In: Wang TL, Cuming A (eds) Embryogenesis, the Generation of a Plant. BIOS Scientific Publishers, Oxford, UK, pp 173-190.

Torbert, K. A., Rines, H. W. and Somers, D. A. (1998). Transformation of oat using mature embryo-derived tissue cultues. **Crop Sci**. 38: 226-231.

Toriyama, K., Arimoto, Y., Uchimiya, H. and Hinata, K. (1988). Transgenic rice plants after direct gene transfer into protoplasts. **Bio Technol**. 6: 1072- 1074.

Torregrosa, L., Iocco, P and Thomas, M. R. (2002). Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. **Am J Enol Vitic**. 53(3): 183-190.

Tsukahara, M. and Hirosawa, T. (1992). Characterization of factors affecting plantlet regeneration from rice (*Oryza sativa* L.) callus. **J Plant Research**. 105(2): 227-233.

Tulyathan, V. and Leeharatanaluk, B. (2007). Changes in quality of rice (*Oryza sativ*a

L.) cv. KDML 105 during storage. **J food biochem**. 31: 415-425.

Tyagi, A. K. (2004). Enhanced tolerance to abiotic stress in transgenic tobacco by overexpression of a zinc-finger protein gene from rice. **PNAS**. 101: 6309-6314.

Vasella, A., Davies, G. J. and Böhm, M. (2002). Glycosidase mechanisms. **Chemical Biol**. 6: 619-629.

Vaistij, F. E., Jones, L. and Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. **Plant cell**. 14(4): 857-67.

Vijayachandra, K., Palanichelvam, K. and Veluthambi, K. (1995). Rice scutellum induces *Agrobacterium tumefaciens* vir genes and T-strand generation. **Plant Mol Biol**. 29: 125-133.

Vom Endt, D., Kijne, J. W. and Memelink, J. (2002). Transcription factors controlling plant secondary metabolism: what regulates the regulators. **Phytochem**. 61(2): 107-114.

Wakasa, Y., Ozawa, K. and Takaiwa, F. (2007). *Agrobacterium*-mediated transformation of a low glutelin mutant of 'Koshihikari' rice variety using the mutated-acetolactate synthase gene derived from rice genome as a selectable marker. **Plant Cell Rep**. 26: 1567-1573.

Wang, L., Wang, Z., Xu, Y., Joo, S., Kim, S., Xue, Z., Xu, Z., Wang, Z. and Chong, K. (2009). OsGSR1 is involved in crosstalk between gibberellins and brassinosteroids in rice. **Plant J**. 57(3): 498-510.

Wang, Z., Chen, C., Xu, Y., Jiang, R., Han, Y., Xu, Z. and Chong, K. (2004). A Practical vector for efficient knockdown of gene expression in rice (*Oryza*

sativa L.). **Plant Mol Bio Rep**. 22: 409-417.

Waterhouse, P., Graham, M. and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. **Proc Natl Acad Sci USA**. 95(13): 959-964.

Waterhouse, P. and Wang, M. B. (2001). Gene silencing as an adaptive defence against viruses. **Nature**. 411: 834-842.

Xiao, H., Wang, Y., Liu, D., Wang, W., Li, X., Zhao, X., Xu, J., Zhai, W. and Zhu, L. (2003). Functional analysis of the rice -P3 homologue OsMADS16 by RNA interference. **Plant Mol Biol**. 52(5): 957–966.

Xu, F. Q., Li, X. R. and Ruan, Y. L. (2008). RNAi-mediated suppression of hexokinase gene OsHXK10 in rice leads to non-dehiscent anther and reduction of pollen germination. **Plant sci**. 674-684.

Yang, X. W., Wynder, C., Doughty, M. L. and Heintz, N. (1999). BAC-mediated gene-dosage analysis reveals a role for Zipro1 (Ru49/Zfp38) in progenitor cell proliferation in cerebellum and skin. **Nat Genet**. 22: 327-335.

Yang, W., Kong, Z., Ikerodah, E. O., Xu, W. and Li, Q. (2008). Calcineurin B-like interacting protein kinase OsCIPK23 functions in pollination and drought stress responses in rice (*Oryza sativa* L.). **J Genet Genomics**. 35: 531-543.

Yuan, Z. C., Edlind, M. P., Liu, P., Saenkham, P., Banta, L. M., Wise, A. A., Ronzone, E., Binns, A. N., Kerr, K. and Nester, E. W. (2007). The plant signal salicylic acid shuts down expression of the *vir* regulon and activates quormone-quenching genes in *Agrobacterium*. **Microbiology**. 104(28): 11790-11795

Zambryski, P. (1988). Basic processes underlying *Agrobacterium*mediated DNA transfer to plant cells. **Annu Rev Genet**. 22: 1-30.

Zhang, S., Wei, Y. and Pan, H. (2007). Transgenic rice plants expressing a novel antifreeze glycopeptide possess resistance to cold and disease. **Z. Naturforsch**. 62: 583-591.

Zhong, J., Wang, H., Zhang, D., Liu, B. and Wang, J. (2007). Rice repetitive DNA sequence RRD3: a plant promoter and its application to RNA interference. **J. Genetic and Genomics**. 34(3): 258-266.

Zhu, Z and and Wu, R. (2008). Regeneration of transgenic rice plants using high salt for selection without the need for antibiotics or herbicides. **Plant sci**. 174(5): 519-523.

APPENDIX

N6 Vitamin (200X)	Glycine	40 mg
	Nicotinic acid	10 mg
	Pyridoxine HCl	10 mg
	Thyamine HCl	20 mg
	Myo-inositol	2 g
AA-1 (1000X)	$MnSo_4.4\text{-}6H_2O$	1 g
	H_3BO_4	300 mg
	$ZnSo_4.7H_2O$	200 mg
	$Na_2MoO_4.2H_2O$	25 mg
	$CuSO4.5H_2O$	2.5 mg
	$CoCl_2.6H_2O$	2.5 mg
	KI	75 mg
AA-2 (1000X)	$CaCl_2.2H_2O$	15 g
AA-3 (1000X)	$MgSO_4.7H_2O$	25 g
AA-4 (1000X)	Fe-EDTA	4 g
AA-5 (1000X)	$NaH_2PO_4.2H_2O$	15 g
AA-6 (200X)	Nicotinic acid	20 mg
	Thaiamine HCl	200 mg
	Pyridoxine HCl	20 mg
	Myo-inosital	2 g
AA-sol (100X)	L-arginine	5.3 g
	Glycine	225 g
AB butter (20X)	K_2HOP_4	6 g
	NaH_2PO_4	2.6 g
AB salt (20X)	NH_4Cl	2 g
	$MgSO_4.7H_2O$	0.6 g
	KCl	0.3 g
	$CaCl_2.2H_2O$	0.0268 g
	$FeSO_4.7H_2O$	0.005 g

BIOGRAPHY

Miss Wipaporn Wanthanalert was born on July 17, 1978 in Bangkok, Thailand. She graduated the bachelor's degree of science in Crop Production Technology from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima in 1999. She used to attend 4 months in Genetic Conservation by Tissue culture project at The Royal Chitralada Projects in her bachelor's degree program. In 2000, she continued her Master degree in Crop Production Technology (Entomology) from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and graduated in 2003. She has opportunity to studied Doctoral degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima under the National Center for Genetic Enginnering and Biotechnology (BIOTEC) scholarship. In 2007, she got Honorable Mention Award, For Outstanding Poster Presentation in the TSB 19th Annual Meeting: Biotechnology for Gross National Happiness, Thailand in the topic of "β-glucosidase genes knock down via RNAi"