STUDYING THE GENETIC FUNCTION OF RICE β-GLUCOSIDASE VIA RNA INTERFERENCE

Dang Thi Thanh Tam

A Thesis Submitted in Partial Fulfillment of the Requirements for

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การศึกษาบทบาทและหน้าที่ของยีนบีตากลูโคสิเดสในข้าวโดยเทคนิค RNAi

แดง ทิ แทน ตั้ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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β-GLUCOSIDASE VIA RNA INTERFERENCE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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แดง ทิ แทน ตั้ม : การศึกษาบทบาทและหน้าที่ของยีนบีตากลูโคสิเดสในข้าวโดยเทคนิค RNAi (STUDYING THE GENETIC FUNCTION OF RICE β-GLUCOSIDASE VIA RNA INTERFERENCE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 89 หน้า.

การแสดงออกของยีน *Os3bglu7* และยีนบีตากลูโคสิเดสอีกสี่ยีนในข้าว ถูกยับยั้งการ (knocked down) โดยใช้พลาสมิด pOpOff2 ที่สามารถยับยั้งการแสดงออกของยีนด้วยเทคนิค RNAi เมื่อถูกกระตุ้นด้วยสาร dexamethasone (DEX) พลาสมิด pOpOff2/Bglu7 ซึ่งมีลำดับยีนเป้าหมายจำเพาะกับ 3'UTR ของยีน *Os3bglu7* ถูกสร้างเพื่อใช้ในการยับยั้งแสดงออกของยีน *Os3bglu7* และพลาสมิด pOpOff2/Kn5 ถูกออกแบบเพื่อ ยับยั้งการแสดงออกของกลุ่มยีนบีตากลูโคสิเดสจำนวน 5 ยีน โดยใช้ลำดับนิวคลีโทด์ที่จำเพาะต่อยีน *Os3bglu7* โดยเชื้ออะโกรแบคทีเรีย สายพันธุ์ EHA105 ถูกนำมาใช้เพื่อถ่ายยีนเข้าสู่ข้าว อีกทั้งรีคอมบิแนนท์บีตากลูโคสิเดส (rOs3BGlu7) และ/หรือ DEX ถูกนำมาเติมในขบวนการถ่ายยีน เพื่อศึกษาบทบาทและหน้าที่ของยีนบีตากลูโคสิเดสในข้าว จากผลการทดลองพบว่า แคลลัสที่เลี้ยงบนอาหารที่ไม่เติม DEX และ rOs3BGlu7 (กลุ่มควบคุม) มีจำนวนแคลลัสที่ตายน้อยกว่าแคลลัสที่เลี้ยงบนอาหารคัดเลือกที่เติม DEX และ rBGlu7 นอกจากนี้ การเติม DEX เพื่อ ยับยั้งการแสดงออกของกลุ่มยีนบีตากลูโคสิเดสทั้ง 5 ยีน ส่งผลให้ปริมาณเชื้อ อะโกรแบคทีเรียที่ปนเปื้อนบนผิวของแคลลัสเพิ่มขึ้นเมื่อเทียบกับกลุ่มควบคุม สำหรับการยับยั้งการแสดงออกของยีน *Os3bglu7* พบว่าการเติม DEX และ rOs3BGlu7 ไม่มีผลต่อการเปลี่ยนแปลงปริมาณของเชื้ออะโกรแบคทีเรีย

นอกจากนี้ยังศึกษา การงอกและการพัฒนาของข้าวดัดแปลงพันธุกรรม โดยหลังจากเติม DEX เพื่อ ยับยั้งการแสดงออกของยีนบีตากลูโคสิเดสทั้ง 5 พบว่ามีผลต่อการงอกของเมล็ด และการยืดยาวของยอด และราก จากนั้นนำยอดของต้นข้าวดังกล่าวมาทำการตรวจวัดระดับการแสดงออกของยีนบีตากลูโคสิเดสทั้ง 5 ด้วย real-time PCR ซึ่งพบว่า ยีนดังกล่าวถูกยับยั้งการแสดงออกหลังจากเติม DEX จากผลการทดลองแสดงให้เห็นว่า การแสดงออกของกลุ่มยีนบีตากลูโคสิเดสอาจจะมีบทบาทสำคัญต่อการเจริญของต้นอ่อน อย่างไรก็ตามเมื่อเติม DEX ที่ความเข้มข้น 150 μM พบว่ามีผลในทางลบต่อการงอกของรากทั้งในข้าวดัดแปลงพันธุกรรม และข้าวปกติ โปรโมเตอร์

pOp6 ที่ใช้ในการทดลองนี้ไม่เหมาะสมกับการแสดงออกในข้าว เพราะการย้อม GUS พบว่า GUS ไม่ได้มีการแสดงออกในเนื้อเยื่อทุกส่วน

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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

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β-GLUCOSIDASE/ DEXAMETHASONE/ RNA INTERFERENCE/ *AGROBACTERIUM* TRANSFORMATION.

The highly expressed *Os3bglu7* and four other rice β-glucosidase genes were knocked down via a high-throughput dexamethasone (DEX) induced RNAi vector, pOpOff2. In the first construct (pOpOff2/Bglu7), the highly expressed *Os3bglu7* was knocked down with a construct that contains the target specific 3'UTR sequence. The second construct, pOpOff/Kn5, was designed to knock down a group of five closely related rice β-glucosidase genes. In this construct, the target sequence was amplified from the coding region of *Os3bglu7*. The rice transformation was done with *Agrobacterium* strain EHA105. Recombinant β-glucosidase protein (rOs3BGlu7) and/or DEX were added in the transformation processes. The results show that control treatment (no dexamethasone, no recombinant β-glucosidase protein added) had a lower number of dead calli indicating higher efficiency of transformation on selection medium than the treatments that applied DEX and supplemented with recombinant protein in both constructs. Moreover, adding DEX to knock down the five β-glucosidase genes increased the *Agrobacterium* population on the surface of the calli when compared to the control. No effects on the *Agrobacterium* population were observed for addition of DEX to knock down *Os3bglu7* and the supplementation with rOs3BGlu7.

Furthermore, when DEX was added to knock down the β-glucosidase genes' expression in germination and in the development of transgenic plantlets, the knocking down of these genes suppressed the germination of transgenic rice seed and the elongation of the shoot. The expression of these five β-glucosidase genes in shoots were measured by real-time PCR, which showed that they were partially knocked down after DEX treatment. These data indicated that these β-glucosidase genes may play important roles in the development of plantlet shoot. However, the effect of high concentration of DEX (150 μM) was observed on the development of root in both wild type and transgenic rice. The pOp6 promoter is not strong in rice, since GUS staining did not express in every tissue.

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LIST OF ABBREVIATIONS

^{0}C	=	Degrees Celsius
2,4 D	=	2,4 Dichlorophenoxyacetic acid
bp	=	Base pair
DEX	=	Dexamethasone
DNA	=	Deoxyribonucleic acid
DNaseI	=	Deoxyribonuclease I
et al.	=	Et alia (and other)
g	=	Gram
GUS	=	β-glucuronidase
kDa	=	Kilo Dalton
Kinetin	=	6-Furfurylaminopurine
l	=	Liter
LB	=	Luria-bertani
L-proline	=	(S)-2-Pyrrolidinecarboxylic acid
mRNA	=	Messenger ribonucleic acid
MS	=	Murashige and Skoog medium
NAA	=	α-Naphthaleneacetic acid
nm	=	Nano meter
(n/μ/m)g	=	(Nano/micro/mili) gram
OD	=	Optical density

LIST OF ABBREVIATIONS (Continued)

PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
rOs3BGlu7	=	Recombinant Os3BGlu7 protein
Timentin	=	Ticarcillin disodium salt
(μ/m)M	=	(Micro/mili) molar
UTR	=	Untranslated region

CHAPTER I

INTRODUCTION

1.1 Introduction

Rice (*Oryza. Sativa* L.) is the most important food crop in the world. It is also a good model plant for research. Nowadays, the developments of rice genome mapping, sequencing and molecular biology have provided useful tools for studying the gene functions. However, despite a lot of scientist's endeavor, the exact biological functions of many rice genes are still unknown. One example is the beta-glucosidase homologues. Most plant β-glucosidases are glycoside hydrolase family 1 (GH1) enzymes. In plant physiology, these proteins have many important functions, such as (1) cell wall lignification (Dharmawardhana et al., 1995; Trevino et al., 2006), (2) cell wall degradation in endosperm during germination (Leah et al., 1995), (3) bioactivation of defense compounds (Halkier & Gershenzon, 2006; Schroeder & Nambara, 2006) and (4) activation of phytohormones (Brzobohaty et al., 1993; Kristoffersen et al., 2000; Lee et al., 2006). However, the understanding about rice β-glycosidases is still not complete. In rice, 40 β-glucosidase genes have been found and 34 GH1 genes are expressed in a range of organs and stages of rice, based on the cDNA and EST sequences in public databases (Opassiri et al., 2006). Nevertheless, only a few rice β-glucosidases have been characterized for their expression, physiology, biochemical properties, and structure (Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006, 2010; Chuenchor et al., 2008, 2011; Seshadri et al., 2009;

Kuntothom et al., 2009, 2010; Wakuta et al., 2010). The physiological functions of specific β-glucosidases in defense mechanisms as well as in the development of rice are still controversial. To date, the largest number of studies about rice β-glucosidase has been conducted on *Os3bglu7* (*bglu1*). Opassiri et al. (2003) suggested that the highly expressed Os3BGlu7 protein may play a role in cell wall expansion, cell division, in activation of phytohormone and in defense of young tissue. In a sequence homology-based phylogenetic tree, Os3BGlu7 is grouped in the same phylogenetic cluster with four other rice β-D-glucosidase isoenzymes (Os1BGlu1, Os3BGlu8, Os7BGlu26 and Os12BGlu38) (Opassiri et al., 2006). The existence of these enzymes in rice may contribute to important functions in rice physiology. Our previous studies suggested that β-glucosidases may protect rice from the infection of *Agrobacterium*. In this research, our goal was to identify the functions of these β-glucosidase genes in *Agrobacterium* transformation process. To investigate the functions of these genes, the RNA interference method was used.

Nowadays, RNA interference (RNAi) has emerged as a powerful tool for studying gene function by knocking down targeted genes in many organisms. This mechanism is mediated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The dsRNA is processed into small interfering RNA (siRNA) by an enzyme called Dicer, and the siRNAs are then incorporated into a multi-component RNA-induced silencing complex (RISC), which finds and cleaves the target mRNA. In application, by introducing the double-stranded RNAs (dsRNAs) into cells, the mechanism will be activated automatically and leads to the knock down of target genes. For studies in plants, hairpin (hp) RNA expression cassettes are typically constructed on binary plasmids and delivered into cells by *Agrobacterium*-

mediated genetic transformation. In model plant species, such as *Arabidopsis* and rice, RNAi has been routinely used to characterize gene function and to engineer novel phenotypes (Fu et al., 2007). Therefore, we decided to knock down the *Os3bglu7* and the other four β-glucosidase genes in same cluster by RNAi to investigate the physiology functions of these genes in the rice callus transformation processes.

1.2 Research Objectives

The objectives of this research are as follows:

1. Clone the knock-out target sequence from rice and produce the recombinant RNAi vectors (pOpOff2).
2. Determine the effect of gene silencing during callus transformation with and without the supplementation of dexamethasone and recombinant β-glucosidase protein.
3. Detect the effect of DEX and the expression level of β-glucosidase genes in transgenic rice with and without dexamethasone treatment.

CHAPTER II

LITERATURE REVIEW

2.1 β-glucosidases

2.1.1 β-glucosidase functions in plant

Beta-glucosidases are enzymes, the plant representatives of which mainly belong to glycoside hydrolase family (GH1). They catalyze the hydrolysis of the β-glucosidic bond between two carbohydrate moieties or a carbohydrate and an aglycone moiety (http://www.cazy.org/fam/GH1.html; Cantarel et al., 2009). These enzymes have been found in all living organisms and play many functions. Plant β-glucosidases contribute to variety of physiology mechanisms, including lignification, cell wall degradation, chemical defenses, activation of phytohormones, biotic and abiotic stress respomses as well as plant secondary metabolism (Dharmawardhana et al., 1995; Forslund et al., 2004; Sue et al., 2000; Brzobohatý et al., 1993; Kristoffersen et al., 2000; Leah et al., 1995; Morant et al., 2008).

Plants are immobile and therefore need to defend themselves by synthesizing defense compound against herbivore or pathogen attack. The defense compounds of plant are classified into two classes: phytoalexin and phytoanticipin based on how they are produced. Phytoanticipins are preformed defense compounds that serve as the first chemical barrier, but phytoalexins are defense compounds synthesized in response to herbivore or pathogen attack (VanEtten et al., 1994). Some of the most well characterized classes of phytoanticipins are activated by β-

glucosidases, such as cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates. In general, many defense compounds are stored in a non-active glucosylated form and separated from the enzymes (β-glucosidases). Upon cell disruption, the defense compounds are bio-activated via hydrolysis of the glucosidic linkage catalyzed by the β-glucosidase (Morant et al., 2008). The defense compounds (glucosides) tend to be stored in the vacuole, while their corresponding β-glucosidases are often found in the apoplast or plastid (Ketudat-Cairns & Esen, 2010). Moreover, Ketudat-Cairns & Esen (2010) also reviewed that several GH1 hydrolases are found in other compartments such as peroxisome or ER-derived compartment called the ER body. The synthesis and storage of the two components of this defense system (glucosides and β-glucosidase) have highest amount in seeding and young parts in order to respond to the herbivore and/or pathogen attack (Forslund et al., 2004; Sue et al., 2000).

Plant cell wall is the highest repository of carbohydrates in nature; it has many β-linked glycosyl residues, so it is not surprising that β-glucosidases play importance role in cell wall development (Ketudat-Cairns & Esen, 2010). In barley, the seed specific β-glucosidase BGQ60 accumulates to high levels during late seed development and may plays a role in degrading several endosperm cell wall polysaccharides during germination (Leah et al., 1995). Several rice seedling β-glucosidases have also been shown to hydrolyze oligosaccharides. The rice cell wall-bound β-glucosidases's activity increase eight-fold within five days of germination, suggesting that the enzyme is likely to play an important role in germination process (Akiyama et al., 1998). Rice Os3BGlu7 β-glucosidase may play a role in the glycosylation or deglycosylation of pyridoxine in germinating rice seed in addition to

acting on oligosaccharides (Opassiri et al., 2004). The Os3BGlu7 and Os3BGlu8 isoenzymes are widely expressed in rice tissues and show increasing efficiency of hydrolyzing cellooligosaccharides as the degree of polymerization increases from 2 to 6, so they may be needed to release the glucose from oligosaccharides generated in cell remodeling at various stages of plant development (Opassiri et al., 2004; Kuntothom et al., 2009; Ketudat-Cairns & Esen, 2010).

By releasing the monolignols from their glycosides, β-glucosidases participate in the formation of intermediates in cell wall lignification. The coniferin β-glucosidase in the xylem of lodgepole pine tree was detected and was found to have high sequence similarity to known plant β-glucosidases. In situ localization also showed the exclusive presence of β-glucosidase activity in the differentiating xylem, consistent with a role in lignifications (Dharmawardhana et al., 1995). Recently, the two *Arabidopsis thaliana* β-glucosidases (BGLU45 and BGLU46) that cluster with lodgepole pine coniferin β-glucosidase were found to hydrolyze monolignol β-glucosidase, thus supporting the involvement of a monolignol glucoside/ β-glucosidase system in *Arabidopsis* lignification (Trevino et al., 2006).

In plants, many phytohormones have been recognized. The role of β-glucosidase in the release of biologically active phytohormons from apparently physiologically inactivate phytohormone glucosyl conjugates has been identified. The maize Zm-p60.1 gene encodes a β-glucosidase that cleaves the biologically inactive hormone conjugates cytokine-O-glucosides and kinetin-N3-glucosides to active cytokinin (Brzobohatý et al., 1993; Kristoffersen et al., 2000). In 2006, Lee et al. also elucidated that the cleavage of glucose-conjugated ABA by an ABA-specific β-glucosidase (AtBG1- an *Arabidopsis* β-glucosidase homolog localized to the ER) is a

new way to produce bioactive ABA in response to dehydration stress and also day/night conditions. This report may be the clearest evidence for the role of β-glucosidase in phytohormone activation and suggests that other phytohormone glucoconjugates also serve as β-glucosidase activated storage forms (Ketudat-Cairns & Esen, 2010).

2.1.2 Rice β-glucosidases

Although, research about other plant β-glucosidases, such as several from *Arabidopsis thaliana* has been well documented, only a few studies about rice β-glucosidase have been reported. In 2006, Opassiri et al. reported 40 rice β-glucosidase genes, including 34 full-length genes, 2 pseudogenes, 2 gene fragments and 2 intronless genes. Moreover, the relationships of rice GH1 member with other plant β-glucosidase enzymes were also discussed based on phylogenetic analysis (Figure 2.1). From this number of genes, some β-glucosidases have been characterized from rice seeding (Akiyama et al., 1998; Opassiri et al., 2003). A cell wall-associated β-glucosidase has been purified from germinated rice seeds and characterized; this enzyme preferred to hydrolyse glucose oligosaccharides (Akiyama et al., 1998). Some other β-glucosidase genes were characterized for their structure, enzymatic property and physiological function such as Os3BGlu6 (Seshadri et al., 2009); Os4BGlu12 (Opassiri et al., 2006, 2010); Os3BGlu8 and Os7BGlu26 (Kuntothom et al., 2009); and tuberonic acid glucoside (TAG)-hydrolyzing beta-glucosidase (OsTAGG1) (Wakuta et al., 2010). OsTAGG1 is specific β-glucosidase belonging to the rice GH family 1. It can hydrolyze TAG, which releases the physiologically active TA. OsTAGG1 was predicted play a role in cell wall metabolism since it had sewuence

similar to Os4BGlu12 (Opassiri et al., 2006) and had high exoglucanase activity as Os4BGlu12.

One of the β-glucosidase genes highly expressed in rice in many stages of development, such as germinating seed, shoot and flower is Os3BGlu7 (BGlu1). Several studies have been done on this gene and protein (Opassiri et al., 2003, 2004; Chuenchor et al., 2008, 2010; Kuntothom et al., 2009, 2010). Opassiri et al. (2003) reported that Os3BGlu7 protein can specifically hydrolyse several natural and synthetic substrates. Later enzyme kinetics of this enzyme also was reported by Opassiri et al., (2004); the crystal structure of Os3BGlu7 was determined by Chuenchor et al. (2008, 2011). Based on protein sequence relationship, Os3BGlu7 has been grouped in the same phylogenetic cluster with four other rice β-glucosidases isoenzymes (Os1BGlu1, Os3BGlu8, Os7BGlu26 and Os12BGlu38). These β-glucosidases isoenzymes cluster together with HvBII, and tomato and *Arabidopsis* β-D-mannisidases. The sequence analysis of these enzymes was discussed by Kuntothom et al (2009). The amino cacid sequences, substrate specificites, structure of these enzymes help us understand more about their functions in rice. However, until now, the exact physiology functions of the Os3BGlu7 protein and the other rice β-glucosidases protein in this same cluster of phylogenetic tree in rice (Opassiri et al., 2006) are still yet to be proven. Therefore, the effect of loss of function of these β-glucosidase isoenzymes by RNAi gene knockdown technique is the aim of this research.

Figrure 2.1 Relationship between rice and other plant GHI protein sequences described by a phylogenetic tree rooted by Os11bglu36 (adapted from 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 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 whitefly induced 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; LEAAL37714, *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).

2.2 *Agrobacterium* transformation

Agrobacterium tumefaciens is a soil phytopathogen that has been utilized routinely for plant transformation. In nature, *Agrobacterium tumefaciens* can transform plant cells and the transfer DNA (T-DNA) that carries a set of oncogenes and opine catabolism genes. The transferred DNA will be integrated into the plant genome with resultant of production of a crown gall, which is a plant tumor (Hiei et al., 1997). A general genetic mechanism of *Agrobacterium* transformation of plant cells is outlined in Figure 2.2. The transformation process comprises 10 major steps and begins with binding of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two component signal-transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2-DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10) (Tzfira & Citovsky, 2006). The processes of the *Agrobacterium*-mediated genetic transformation have been discussed in detail in recently reviews (Gelvin, 2000, 2010; McCullen & Binns, 2006).

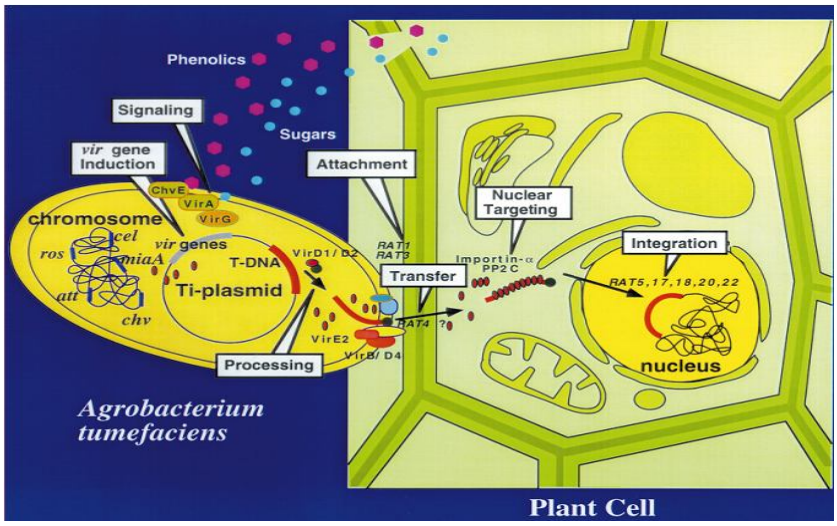

Figure 2.2 A model for the *Agrobacterium*-mediated genetic transformation (Gelvin, 2000).

In recombinant *Agrobacterium* Ti plasmids, the native T-DNA has been replaced with genes of interest. They represent the most efficient vehicles used today for the introduction of foreign genes into plants and for the production of transgenic plant species. *Agrobacterium*-mediated transformation has a lot of advantageous features, including the transfer of pieces of DNA (T-DNA) with defined ends and with minimal rearrangement, the transfer of relatively large segments of DNA, the integration of small numbers of copies of genes into plant chromosomes, and the high quality and fertility of resultant transgenic plants (Komari et al., 1998). Thus, *Agrobacterium*-mediated transformation has become a dominant technology used for the production of genetically modified transgenic plants.

In recent years, *Agrobacterium*-mediated transformation of rice has emerged as a main and highly applicable tool for transferring genes of interest into the rice genome (Giri & Lamix, 2000; Tyagi & Mohanty, 2000; Bajaj & Mohanty, 2005). Since the first report of rice transformation with *Agrobacterium*-mediated

transformation, many efficient systems of *Agrobacterium*-mediated transformation have been developed for *Japonica, Javanica* and *Indica* cultivars (Hiei et al., 1994; 2008; Toki, 1997; Yara et al., 2001; Rachwamati 2004; Zhang et al., 1997; Saika & Toki, 2010). Normally, success in plant genetic transformation depends on the efficiency of plant regeneration and transgene integration into the plant. Several factors influencing *Agrobacterium*-mediated transformation of monocotyledonous plants (including rice) have been elucidated, including the screening of the responsive genotype and explants, *Agrobacterium* strains, binary vectors, selectable marker genes and promoters, inoculation and co-culture conditions, and tissue culture and regeneration medium (Shrawat, 2006). Moreover, the use of actively growing tissue, acetosyringone and temperatures of 22-28^{o}C during co-cultivation are the necessary conditions for successful transformation in rice.

2.3 *Agrobacterium* and plant defense

In nature, *A. tumefaciens* is as an agent of disease for plants; it tries to deliver and expresses the T-DNA into the host cell genome. In response, the plants use their defense system to battle against this infection. The success of *Agrobacterium* transformation relies on both the activity of bacterial virulence proteins (utilized for host recognition, attachment and T-stand production) and on the activity of many host cellular proteins and other biological systems of the host cell (Citovsky et al., 2007). The plant defense system could interfere at any step of *Agrobacterium*-mediated transformation. Ditt et al. (2001) proved that the expression of a number of general defense related genes in plants change during *Agrobacterium* infection. The expression level of the defense genes correlates with the efficiency of transformation,

suggesting that *Agrobacterium* binding to host cell may weaken the plant defense response (Ditt et al., 2005). The activation of plant defense responses to *Agrobacterium* infection has been observed. In tobacco, the defense response was induced 6-12 h after *Agrobaterium* inoculation, and then suppressed later (24-36 h) in the infection (Veena et al., 2003). Whereas, the *Agrobacterium* induced the *Arabidopsis* defense response at 24-48 h post infection (Ditt et al., 2006).

The plant immune pathways in response to *Agrobacterium* have been discussed (Gaspar et al., 2004; Zipfel et al., 2006; Dunoyer et al., 2006; Anand et al., 2008). The first pathway mentioned is the perception of bacterial pathogen-associated molecular patterns (PAMPs). In this pathway, early PAMPs responses include ion fluxes across the plasma membrane, the production of reactive oxygen, late callose deposition to reinforce the cell wall and synthesis of the anti-microbial compounds (Ishikawa, 2009). The second pathway is systemic acquired resistance (SAR); this is a mechanism of induced defense which requires the accumulation of salicylic acid (SA) and the activation of pathogenesis related (PR) genes (reviewed in Durrant & Dong, 2004). The last pathway, reviewed by Bisaro (2006), is RNA silencing that directly cleaves and/or inhibits the translation of the target mRNA, which function as antiviral defense plants. PAMPs perception defense (Zipfel et al., 2006; Nurberger & Kemmerling, 2006; Ishikawa, 2009), SAR (Gaspar et al., 2004; Anand et al., 2008) and RNA silencing (Dunoyer et al., 2006) are all evolved in the plant defense response to the *Agrobacterium*. However, to date, the relationship between β-glucosidase genes and *Agrobacterium* is still unknown. Understanding the relationship between *Agrobacterium* and host defense mechanism and the adjustment of the biological systems of the host cell in *Agrobacterium* infection will help

improve the use of *Agrobacterium* as a unique and powerful experimental tool as well as increase the understanding of the plant defense systems.

2.4 RNA interference

2.4.1 The molecular mechanism of RNAi

RNAi is a post-transcriptional event that leads to specific gene silencing through degradation of the target mRNA. At first, RNAi was described as a mechanism that protects the genome from viruses and other insertable genetic elements. It also regulates gene expression during the development (Fire et al., 1998; Jorgensen, 1990). Recently, RNAi has been described as a mechanism for inhibiting gene expression.

In the RNAi pathway, the effector molecules that guides mRNA degradation are small dsRNAs (from 21 to 25 nucleotides), termed small interfering RNAs (siRNAs), that are produced by the cleavage of long dsRNAs. These short interfering RNAs are produced by the conserved cytoplasmic Dicer family of RNase III-like enzymes. Processing of dsRNAs by Dicer yields 21-25 nt dsRNA duplexes with 5' phosphates and 2 nucleotide 3' overhangs (Bernstein et al., 2001; Elbashir et al., 2001). These siRNAs then provided specificity to the endonuclease-containing RNA-induced silencing complex (RISC). The complex sits on the target mRNA and cleaves the mRNA.

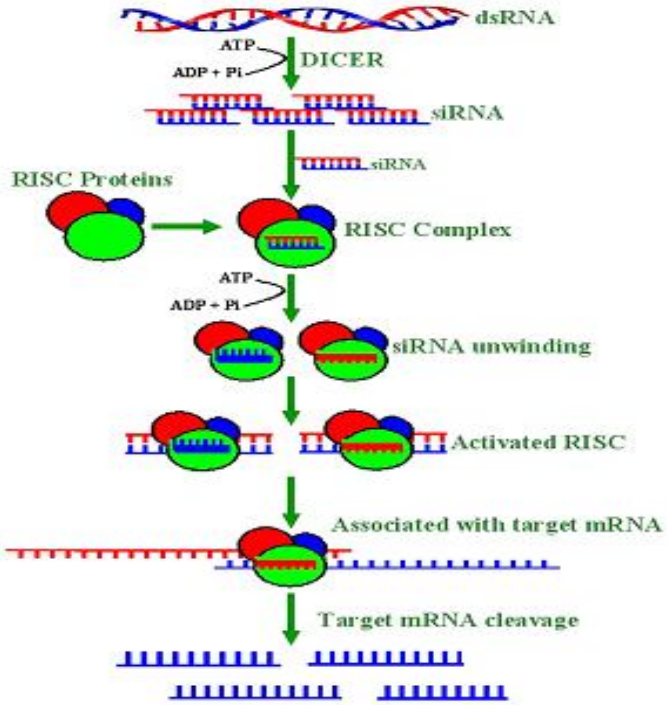

Figure 2.3 The RNA interference process. Double-stranded RNA is cut into short pieces (siRNA) by the endonuclease Dicer. The antisense strand is loaded into the RISC complex and links the complex to the mRNA strand by base-pairing. The RISC complex cuts the mRNA strand and the mRNA is subsequently degraded (Mahmood-ur-Rahman et al., 2008).

Normally, the traditional RNAi vector systems are generated by restriction enzyme digestion and ligation. However, the normal cloning method usually requires a lot of time and many steps with low efficiency to deliver the genes. Gateway cloning technology can be used to transfer the DNA fragments between cloning vector and expression vector without restriction enzyme digestion and ligation, while maintaining the reading frame (www.invitrogen.com). Gateway cloning plays an important role in producing the recombinant RNAi vectors and has become a popular

way to produce them. To produce the RNAi vectors, two cloning steps are needed. Firstly, the target gene's PCR product is cloned to entry vector to create an entry clone containing the *att*L1, *att*L2 sites. Secondly, the target gene fragment in the entry vector will be transferred to the Gateway destination vector (RNAi vector) to generate the expression vector by the Gateway LR reaction. The cells that contain the recombinant expression vectors with target gene insertion can grow on specific selection medium (www.invitrogen.com).

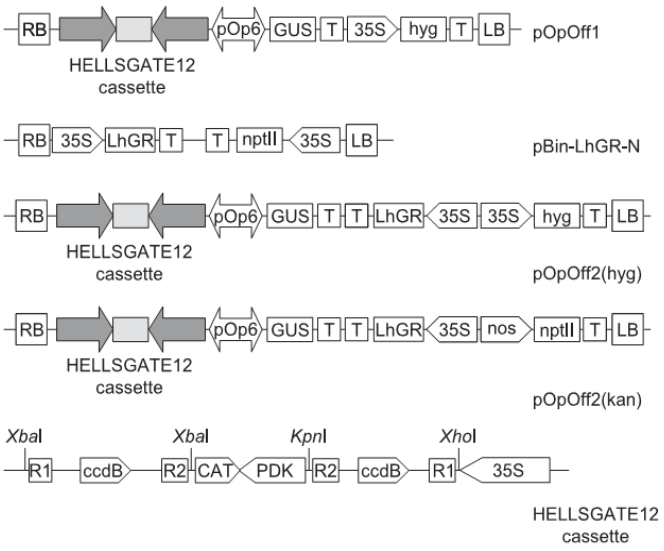

Figure 2.4 Schematic diagram of plasmid vectors and HELLSGATE12 cassette. (T, terminator; LB, left border; RB, right border; hyg, hygromycin resistance gene; nptII, kanamycine resistance gene; R1, Gateway™ *att*R1 site; R2, Gateway™ *att*R2 site (Wielopolska et al., 2005).

Recently, inducible RNAi vector systems have been used to knockout genes in plants because of their many advantages. Firstly, the silencing gene expression can be done at specific developmental stages or in specific tissues. To

silence in a specific tissue one can use a tissue specific promoter to produce the dsRNA. Secondly, the ability to manipulate gene expression in this way offers the prospect of dissecting the functions of genes that give lethal or complex pleiotropic phenotypes in knockout mutants or stable RNAi lines. One of the inducible RNAi systems that satisfies most features to generate plant lines with regulated RNAi is the pOpOff vectors (Wielopolska et al., 2005). These vectors were constructed by combining a inducible pOp6/LhGR promoter system (Craft et al., 2005) with the pHELLSGATE 12 Gateway^{TM} gene silencing vectors (Helliwell & Waterhouse, 2003). Dexamethasone ($C_{22}H_{29}FO_5$) (Figure 2.5), a potent synthetic member of the glucocorticoid class of steroid drugs (http://en.wikipedia.org/wiki/Dexamethasone), can be use as an inducer of the pOp6/LhGR promoter. The production of hairpin RNAi from this system can be turned on and off by the application and removal of dexamethasone. These vectors had also been constructed with GUS reporter gene and selectable marker genes, such as the kanamycin resistance gene (nptII) or hygromycin resistance gene (hyg). The pOpOff vectors are used to insert hairpin arms from a Gateway ^{TM} entry clone (Figure 2.4). Up to now, the pOpOff2 (hyg) vector has never been reported to have been applied to research in rice. However, the inducible vector pOpOff2 (hyg) was used to study function of genes in *Arabidopsis thaliana* (Wielopolska et al., 2005; Hradilova et al., 2007; Xua et al., 2008), and *Medicago truncatula* (Mantiri et al., 2008; Chen et al., 2009).

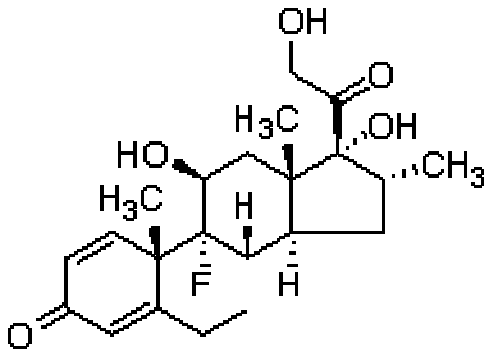

Figure 2.5 Structure of dexamethasone molecule (http://en.wikipedia.org/wiki/Dexamethasone).

2.4.2 Application of RNAi in studying rice genetic function

Nowadays, RNA interference has been shown to be a natural regulatory mechanism for silencing in eukaryotes as well as a powerful tool for investigating gene function. It is also a quick method for creating mutants, performing "gene knockout" and loss of "phenotype" studies. In rice, RNAi is often achieved by introducing dsRNA through a special vector that can be transformed into cells via *Agrobacterium* or other methods. The stable RNAi transformants are good models to investigate the gene function in different tissues, stages of development, and environment conditions. Up to now many gene functions of rice have been discovered and updated by use of RNAi to silence the genes. For example, in 2009, the contribution of salicylic acid glucosyltransferase, OsSGT1, to chemically induced disease resistance in rice plant was proven (Umemura et al., 2009). In another case, the OsSPX1 gene was demonstrated to act via a negative feed back loop to optimize growth under phosphate-limited conditions (Wang et al., 2009). The *PAIR3* (*HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS*) gene plays a crucial role in homologous chromosome pairing and synapsis in meiosis (Yuan et al., 2009).

RNAi was also used to show that the OsCPL1 gene (*Oryza sativa* CTD phosphatase-like 1), which encodes a protein containing a conserved carboxy-terminal domain (CTD), represses differentiation of the abscission layer during panicle development (Ji et al., 2009). Normally, the RNAi lines were produced and treated in test conditions to find out the function of specific genes through the phenotype in the development and in relation with other genes of rice when silencing target genes.

To date, rice RNAi has been used successfully to identify the functions of many genes. RNAi is a useful and flexible tool to study gene function in rice. The potential of using RNAi technique to knockout gene will have significant impacts on rice functional gene analysis in the upcoming years (Fu et al., 2007).

CHAPTER III

MATERIALS AND METHODS

3.1 RNAi vector construction

3.1.1 DNA extraction

Rice seeds (*Oryza sativa* L., Japonica cultivars) were germinated on tissue paper at room temperature for 2 weeks and then young leaves were collected. The DNA extraction followed the potassium acetate method modified from the Dellaporta method (1983). Briefly, small amounts of young leaves (0.18 g) were homogenized in liquid nitrogen until the sample become powder. After that, the sample was moved into a new tube and 720 µl of extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 1.25% SDS) was added and vortex immediately. The DNA extraction buffer was freshly prepared and preheated at 65 ^{o}C for 30 minute. The tube was incubated at 65 ^{o}C for 15 minute. The sample was vortexed every 5 minutes. Then 225 µl of 5 M potassium acetate was added and mixed several times. The tube was incubated on ice for 20 minutes on shaker and then centrifuged for 15 minutes at 14000 x g. The supernatant was decanted into a new tube, and then ice-cold iso-propanol was added. The tube was mixed gently by inverting slowly and then centrifuged for 10 minutes at 14000 x g to precipitate DNA. The DNA pellet was gently washed with 300 µl of 70% cold ethanol and centrifuged for 7 minutes at 3000 x g. The supernatant was removed and the DNA pellet was dried for 10 minutes at room temperature. Fifty microliters of TE was added and then the tube was stored at -

20 ^{o}C. Finally, the extracted DNA was checked by agarose gel electrophoresis method.

3.1.2 Total RNA extraction

Total RNA was extracted from the leaves of transgenic plants with TRIzol (Invitrogen). One hundred milligrams of leaves was homogenized with liquid nitrogen in a mortar. The sample was mixed with 1 ml of TRIzol in an Eppendorf tube. To the tube, 0.2 ml of chloroform was added and shaken vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. Then the sample was centrifuged at 12,000 x g, 4 ^{o}C for 10 minutes. The supernatant was transferred to a new tube. The RNA was precipitated by mixing with 0.5 ml of cold isopropyl alcohol. The sample was incubated at 15-30 ^{o}C for 10 minutes and then centrifuged at 12,000 x g, 4 ^{o}C for 10 minutes. The solution was removed and the RNA pellet was washed with 1 ml of 75% ethanol and mixed by vortex, then centrifuged at 8000 x g, 4 ^{o}C for 5 minutes. The ethanol was removed and the RNA pellet was dried. Finally, 50 µl of RNase free diH_2O was added and the sample was stored at -70 ^{o}C.

3.1.3 Amplification of target sequences

A group of five rice β-glucosidase genes (*Os1bglu1, Os3bglu7, Os3bglu8, Os7bglu26* and *Os12blgu38*) was chosen to be knocked down. To knock down these genes, a conserved region in the coding region of *Os3bglu7* was used as the target sequence. The target sequence to knock down only the *Os3bglu7* (the highly expressed gene) is the sequence located at the 3'UTR region which is specific to only this gene. Primers for the amplification of these fragments are shown in the Table 3.1. These primers are the same as those that were used in Wanthanaleart (2009).

Table 3.1 The sequences of specific primers for β-glucosidase genes.

Specific primer	Type	Sequence	Region
Os3bglu7 (Bglu7: 350 bp)	Forward	**CACC**CTCGAGGTCGACTTCAACACGCTC	3'UTR
	Reverse	GGGAATTCCACCAAGCCAAATCTCATC	
5 Bglu group (Kn5: 399 bp)	Forward	**CACC**CTCGAGGTTCCCCAAGCGGTTCGTG	Coding region
	Reverse	GGGAATTCGCATTCAACCAGCCTCCG	

CAAC for directional cloning into pENTR/D TOPO vector.

For the first construct to knock down only the highly expressed *Os3bglu7* gene, the target sequence from the 3'UTR region of this gene was amplified from genomic DNA. The expected size is about 350 base pairs. For the second construct to knock down 5 genes, the target sequence was amplified from a cDNA clone of *Os3bglu7*. The cDNA was synthesized from total RNA of 7 day old rice leaves (3.1.2). The expected size of this sequence is 399 base pairs. This sequence was named knock-down Kn5. The PCR amplifications were done at 95 ^{o}C for 2 minutes as the initial denaturation step, followed by 35 cycles of 95 ^{o}C for 30 seconds, annealed at a specific temperature (65 ^{o}C for primers to amplify the Kn5 sequence, 52 ^{o}C for primers to amplify the Bglu7 sequence) for 30 seconds, kept at 72 ^{o}C for 30 seconds and the final extension of 5 minute at 72 ^{o}C was done after the last cycle. The PCR

products were purified with a QIAGEN PCR purification kit and detected by agarose gel electrophoresis.

3.1.4 Construct pENTR/D-TOPO® cloning vector

The pENTR/D-TOPO® cloning vector is a Gateway® destination vector from Invitrogen Company. In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) (Figure 3.1) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation.

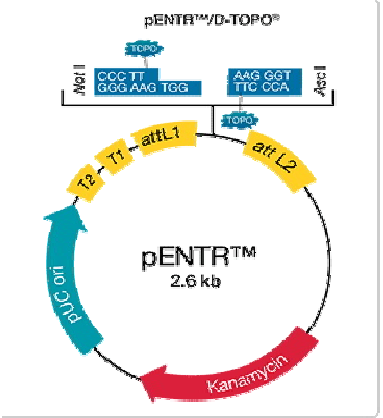

Figure 3.1 The map of pENTR/D-TOPO® cloning vector (Invitrogen) (*att*L sites flanking the PCR production; pUC origin for high plasmid yields; kanamycin resistance gene for selection in *E. coli*).

The target sequences (PCR products) with CACC overhang were cloned into pENTR/D-TOPO® (Invitrogen) following the recommended protocol. Then, the reaction was transformed into One Shot Top 10 chemically competent cells by the heat-shock method and selected on LB plates containing 100 mg/L of kanamycin. The positive colonies were checked by colony PCR using specific primers. The

recombinant plasmids was extracted with a QIAGEN plasmid prep kit and send to sequence at Macrogen Company (Korea) with M13F and M13R primers (Table 3.2).

Table 3.2 Primers using for sequencing inserted genes.

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

3.1.5 pOpOff2 construction

The target gene fragments in pENTR/D-TOPO® were then transferred into pOpOff2 by LR clonase enzyme (Invitrogen) reaction. The pre-reaction (total volume 8 µl) includes 40 ng of entry vector (recombinant pENTR/D-TOPO®), 300 ng of pOpOff2 vector and TE buffer, pH 8.0. Two microliters of the LR Clonase™ II enzyme were added for each reaction and then samples were incubated at 25 °C overnight (16 hours). Then 1 µl of proteinase K solution was added to terminate the reaction and samples were incubated at 37 °C for 10 minutes. Two microliters of the LR reaction were transformed into 100 µl of DH5α competent cells by electroporation and then spread on LB plates containing 100 mg/L spectinomycin. The positive colonies were checked by colony PCR with specific primers. Then, the plasmids were extracted from positive colonies and digested with the restriction enzyme Fast Digest™ *Xba*I (Fermentas). Two microliters of recombinant plasmid (1 µg/ 2 µl) were mixed with 2 µl of FastDigest buffer, 1 µl of FastDigest enzyme and 15 µl of nuclease-free water. The reaction was incubated at 37 °C in a heating block for 10 minutes; then the enzyme was inactivated by heating for 20 minutes at 60 °C. The

product was checked by agarose gel electrophoresis. After that, the pOpOff2 plasmids that contained the inverted repeats of the target genes, named pOpOff2/Bglu7 and pOpOff2/Kn5, were transformed into *Agrobacterium* strain EHA105 by electroporation.

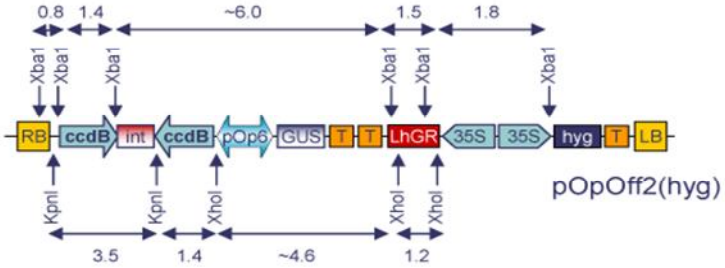

Figure 3.2 The partial map of pOpOff2 vector. The Bglu7 and Kn5 sequences were inserted into the pOpOff2 plasmids by LR reaction, replacing the two ccdB genes.

3.2 Expression and purification of recombinant β-glucosidase enzyme

The recombinant BGlu7 protein (rOs3BGlu7) was expressed following the method of Opassiri et al. 2003. The recombinant expression vector pET32a(+) Os3BGlu7 was expressed in E. *coli* (Origami (DE3)). For expression, the newly transformed selected clones were grown in LB broth containing 100 mg/L ampicillin, 12.5 mg/L tetracyclin, 15 mg/L kanamycin at 37 ^{0}C until reaching OD_{600} of 0.5-0.6. Then, IPTG was added to the final concentration of 0.3 mM and the cultures were incubated at 20 ^{o}C for 8 h. The cell pellets were collected by centrifugation and keep at -80 ^{o}C before extraction.

Soluble proteins were extracted from cell pellets by using extraction buffer including 20 mM Tris-HCl, pH 8.0, 200 µg/ml lysozyme, 1 mM

phenylmethylsulfonylfluoride (PMSF) by vortex and sonication. Purification of soluble protein was done with an immobilized metal affinity chromatography (IMAC) column (equilibrated with Co^{2+}). The concentration of protein was measured by Bradford assay (1976) and albumin protein was used as standard.

The activity of the purified rice β-glucosidase was assayed using the method of Evans (1985). This method used a spectrophotometric assay to measure the release of p-nitrophenol (pNP) from p-nitrophenol-β-D-glucopyranoside (pNP-Glu) by β-glucosidase reactions after alkalization with sodium carbonate.

3.3 Plant tissue culture

3.3.1 Plant material

The *Japonica* rice cultivar, Koshihikari was used in this study. Rice seeds were stored at 4 ^{o}C and changed every 6 months.

3.3.2 Callus induction

Mature rice seeds were dehusked, sterilized in 70% ethanol for 1 minute and 3% hypochlorite for 30 minutes. After six rinses with sterile water, rice seeds were placed on solid N6D medium (pH 5.8) that contained 30 g/L sucrose, 3.98 g/L CHU basal salt mixture (Phytotechnology), 300 mg/L casamino acid, 2.878 g/L L-proline, 5 ml/L 100x N6 vitamin, 2 mg/L 2,4D and 4 g/L gellengum (Phytotechnology). The plates were incubated at 28 ^{o}C in the dark for 5-6 weeks to produce primary and secondary calli.

3.3.3 Co-cultivation

After 6 weeks, the secondary calli (embryogenic calli) were separated and subcultured on a fresh N6D medium for 3-4 days to activate the tissue before transformation. *Agrobacterium* strain EHA105 containing recombinant pOpOff2 was streaked on AB medium composed of 100 mg/l spectinomycin, 5 g/l glucose, 50 ml/l 20xAB buffer, 50 ml/l 20xAB salt and 15 g/l bactoagar. The bacterial plates were incubated in the dark at 28 ^{o}C for 3 days.

Before co-culture, *Agrobacterium* was resuspended in AAM liquid medium (pH 5.2) containing 200 µM acetosyringone. The density of the *Agrobacterium* suspension was adjusted to give an OD_{600} of 0.02 with AAM liquid medium. After that, the secondary calli were immersed in the bacteria suspension for 5 minutes then moved to sterile tissue paper and blotted dry for 15 min. Then, the calli were transferred to co-cultivation medium (2N6-MS, pH 5.2) that contained 30 g/l sucrose, 10 g/l glucose, 3.98 g/l CHU basal salt mixture (Phytotechnology), 300 mg/l casamino acid, 5 ml/l 100x N6 vitamin, 2 mg/l 2,4D and 4 g/l gellengum (Phytotechnology). The plates were sealed with micropore tape to control moisture inside and incubated in the dark at 25 ^{o}C for 3 days.

3.3.4 Callus selection

After 3 days, the infected calli were washed 4 times with sterile water and 3 times with water including 300 mg/l timentin. The calli were then transferred to selection medium (N6D callus induction medium containing 300 mg/l timentin and 50 mg/l hygromycin) for 4 weeks; the plates were incubated at 28 ^{o}C. The calli were changed to new plates every 2 weeks.

3.3.5 Plant regeneration

After 2 rounds of selection (4 weeks), the fresh healthy calli were transferred to MS regeneration medium (pH 5.8) containing 30 g/l sucrose, 30 g/l sorbitol, 4.33 g/l MS basal salts mixture vitamin (Phytotechnology), 2 g/l casamino acid, 0.5 mg/l of NAA, 2 mg/l of kinetin, 50 mg/l hygromycin and 8 g/l agar A (Biobasic Science Inc.). After 1 month on the regeneration medium, the green spots appeared and grew up to small plants. Small plants were transferred to hormones free MS medium for 2 weeks to generate roots. Then, transgenic plants were transferred to soil.

3.4 Experimental design

The *Agrobacterium* transformations were treated as outlined in Table 3.3 with pOpOff2/Bglu7 and pOpOff2/Kn5 RNAi constructs (Kn5: knock-down 5 β-glucosidase genes; Bglu7: knock-down *Os3bglu7*).

Table 3.3 Treatments of transformation experiment.

Treatments	**Constructs**	**DEX**[1/]	**rOs3BGlu7**[2/]	**ddiH_2O (200 µl)**[3/]
CT	pOpOff2/Kn5 Or pOpOff2/Bglu7	-	-	+
T1	pOpOff2/Bglu7	+	-	+
T2	pOpOff2/Bglu7	-	+	-
T3	pOpOff2/Bglu7	+	+	-
T4	pOpOff2/Kn5	+	-	+
T5	pOpOff2/Kn5	-	+	-
T6	pOpOff2/Kn5	+	+	-

[1/] DEX: 128 µg of dexamethasone per plate (an inducer for RNAi production). One hundred microliters of DEX stock (3.33 mM) were spread on the surface of co-cultivation medium.

[2/] rOs3BGlu7: 0.3 mg of purified active recombinant Os3BGlu7 β-glucosidase protein (BGlu7) per plate.

[3/] ddiH_2O was used to balance the moisture inside the plate during the transformation protocol.

3.5 Quantification of the *Agrobacterium* population on the calli after co-cultivation

Follow the transformation experiments, the population of *Agrobacterium* was also determined. After 3 days of co-cultivation, 30 pieces of calli (2-3 mm) in each treatment were selected and rinsed in 10 ml of sterile water, and then 1 ml was used to measure OD_{600nm} to quantify the number of *Agrobacterium*. The experiments were done in triplicates for each treatment and 3 transformation experiments were done.

3.6 Transgenic calli and plants confirmation

The transgenic calli (after 4 week of selection step) and the leaves of transgenic plants (RNAi lines) were checked by PCR. The DNA of calli and plants were extracted following the protocol mention above (3.1.1). The Hyg (hygromycin) primers (forward primer: CTTGACATTGGGGAATTCAG and reverse primer GCCCAATGCGCAGCATATAC) were used to confirm the integration of pOpOff2 T-DNA into the rice genome. The PCR amplifications were done at 95 ^{o}C for 2 minutes as the initial denaturation step, followed by 35 cycles of 95 ^{o}C for 30 second, annealed at 58 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds and the final extension of 5 minute at 72 ^{0}C was done after the last cycle.

3.7 Supplement of dexamethasone (DEX) and GUS staining

3.7.1 Supplement of DEX

Supplement of DEX in transformation experiment

The stock of DEX (10 mM) was dissolved in 95% ethanol and stored at –20 ^{o}C. For transformation experiments, DEX was diluted 3 times in 95% ethanol and 100 µl of diluted DEX (3.33 mM) or 128 µg was spread on the surface of solid medium.

Checking the effect of DEX on rice seed germination

Transgenic rice seeds (T1 of pOpOff2/Kn5 (RNAi lines)) and normal rice (wild type) were dehusked, sterile and then put on agar solid MS medium containing DEX (final concentrations: 0, 50, 100, 150 µM at 28 ^{o}C. Hygromycin (50 mg/l) was added to the MS medium for transgenic rice seeds to select the transgenic plantlets. DEX and Hygromycine were added in the agar MS medium prior to plate pouring. The data were collected at day 3 and day 10 of germination. After 10 days, the plantlets were collected to check for the expression of β-glucosidase genes.

Checking the effect of DEX on plantlet growth

Transgenic rice seeds (T1 of pOpOff2/Kn5 (RNAi lines)) and normal rice were dehusked, sterile and then geminated on sterile tissue paper that soak in liquid MS medium. For transgenic rice seeds, hygromycin (50 mg/l) was added to the MS medium to select the transgenic plantlets. After 3 days of germination, rice plantlets were transferred to liquid MS medium containing 150 µM DEX. The height of the plants and the lengths of roots were collected at day 7 after DEX treatments. The RNA samples were also collected to check for the rice β-glucosidase genes expression.

3.7.2 GUS staining

The samples (from 3.7.1) after treating with DEX were collected to do GUS staining. The X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidecyclohexy lammonium) (Fermentas) was dissolved in dimethylformamide with the concentration of 40 mg/ml as stock solution, then wrapped in foil and keep at -20 ^{o}C. Staining buffer was prepared as listed in Table 3.4. One hundred milliliters of X-Gluc was diluted in 8 ml of staining buffer. Samples were put in small plates then staining solution was poured to cover the samples. The plates were sealed and then coved with foil to protect them from light. The plates were incubated at 37 ^{o}C up to 2 days.

Table 3.4 Components of GUS staining buffer

No.	Component	Weight	Final concentration
1	NaH_2PO4	14 g	100 mM
2	Na_2EDTA	3.7 g	10 mM
3	Potassium ferrocyanide	210 mg	0.5 mM
4	Potassium ferricyanide	170 mg	0.5 mM
5	Triton X-100	1 ml	0.1%
Adjusted the pH to 7.0 with NaOH and brought volume to 1 liter			

3.8 Expression level of β-glucosidase genes in transgenic rice

3.8.1 cDNA synthesis by reverse transcription

The total RNA sample (step 3.1.2) was treated with DNase to destroy the DNA before cDNA synthesis. The reaction includes 8 µl of total RNA, 1 µl of Dnase buffer (10x) and 1 µl of DNase I (Promega). The reaction was incubated at 37 ^{o}C for 30 minutes. Then, 1 µl of DNase stop solution (Promega) was added and the reaction was incubated at 65 ^{o}C for 10 minutes.

The first-strand cDNA was synthesized using SuperScript^{TM}III Reverse Transcriptase (Invitrogen). The pre-reaction (total volume 13 µl) includes 1 µl of 50 µM Oligo (dT) primer, 1 µl of 10 mM dNTPs, 3 µg of DNase-treated total RNA and RNase free diH_2O. The samples were mixed and heated at 65 ^{o}C for 5 minutes to denature the secondary structure. Then, the samples were moved onto ice immediately for 2-3 minutes 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 ^{o}C for 60 minutes in the heating block of the PCR machine. Then the reactions were inactivated by heating at 70 ^{o}C for 15 minutes. The first-strand cDNAs were diluted and kept at -20 ^{o}C before using in PCR reactions.

3.8.2 Real-time PCR analysis of gene expression

The quantification of all gene transcripts was done by real-time PCR. One microliter of 10 times diluted first-strand cDNA synthesis reaction (3.8.1) was used as template to check the rice β-glucosidase gene's expression. The sequences of β-actin primers and other β-glucosidase primers are shown in Table 3.5. Expression of the β-

actin gene was used as the control to compare with expression of β-glucosidase genes. These β-glucosidase primers are specific to the 3' UTR region of each gene. It has been shown that these primers amplify only their genes (Wanthanalert, 2009).

Table 3.5 The specific primer sequences for β-actin and β-glucosidase genes.

Primers	Sequence	Length of amplified sequence
β-actin	F*: ACTCTGGTGATGGTGTCAGCC R*: GTCAGCAATGCCAGGGAACATA	451 bp
Os1bglu1	F: GGCTACTTCGCCTGGTCC R: CAATCTTGAATGATG	270 bp
Os3bglu7	F: GTCGACTTCAACACGCTC R: CACCAAGCCAAATCTCATC	350 bp
Os3bglu8	F: AAGTAGTGGATGCCAGCAG R: AGGCCAAAGTCCAGGATATC	259 bp
Os7bglu26	F: TGCAGACAAAAGGATCAAGC R: TAGTCCCTTCTGTCAGCTC	255 bp
Os12bglu38	F: CACGTTGGTTCAGGAAG R: CTGCCTCTCTTATCACC	251 bp

F*: Forward; R*: Reverse

DyNAmo^{TM} capillary SYBR^{R} Green 2-steps qRT-PCR Kit (Finnzymes) was used for the PCR reaction. The reaction included 10 µl of SYBR Green, 1 µl of 5 mM forward specific primer, 1 µl of 5 mM reverse specific primer, 7 µl of DNase-

RNase free water and 1 µl of diluted cDNA template. The conditions for real time PCR reaction are shown in Table 3.6. The data were analyzed according to the Pfaffl (2001) method (Appendix B).

Table 3.6 Protocol setup for real-time PCR reaction

	Protocol Setup
1	Temperature Control: Sample Calculation
2	Lid Mode: Constant 100 ^{o}C; Shutoff < 30 ^{o}C
3	Incubate at 95 ^{o}C for 00:15:00
4	Incubate at 94 ^{o}C for 00:00:30
5	Incubate at 58 ^{o}C for 00:00:30 (annealing temperature for all primers)
6	Incubate at 72 ^{o}C for 00:00:30
7	Plate Read
8	Go to line 2 for 34 more times
9	Incubate at 72 ^{o}C for 00:01:00
10	Melting Curve from 60 ^{o}C to 95 ^{o}C with the increment 1 ^{o}C/min
11	Incubate at 25 ^{o}C forever → END

CHAPTER IV

RESULT AND DISCUSSION

4.1 Target sequence for RNAi vectors

RNAi-based techniques have been employed as an effective experimental tool for the elucidation of gene function by influencing the gene expression (Hirai & Kodama, 2008). For the application, the plant RNAi vector consists of an inverted repeat harboring target sequences under the control of a strong promoter. The inverted repeat sequences are separated by a spacer fragment (an intron).

Here, the pOpOff2 (RNAi vector) system was used to analyze the function of β-glucosidase genes. Normally, RNAi inactivates gene expression in a sequence-specific manner. There are several factors that affect the effectiveness of RNAi-based gene-silencing. Double-stranded RNA (dsRNA) is one of an effective trigger of RNAi. In many studies, the target sequences to generate dsRNAs were amplified from the 3'UTR region of the gene of interest (Miki et al, 2005). The 3'UTR is a particular section of the messenger RNA (mRNA) that follows the coding region but is not translated into protein. It is believed to be a binding site for proteins that affect mRNA stability and translational regulation (Mazumder et al, 2003). The 3'UTRs show both diversity and conservation in different organisms and in gene families (Friedberg et al, 2003; Chen & Rajewsky, 2006). The 3'UTR region of the rice GH1 β-glucosidase gene family show high diversity. Therefore the sequences are specific for each gene. To knock down the expression of *Os3bglu7*, the target sequence was amplified from

the 3'UTR region of this gene. The first construct of pOpOff2 has the target length (bglu7) of 350 bp.

For the second construct, to knock down five β-glucosidase genes (*Os1bglu1, Os3bglu7, Os3bglu8, Os7bglu26* and *Os12blgu38*), a conserved sequence in the coding region of *Os3bglu7* was amplified to use as the target sequence (Kn5) in the RNAi vector. The sequence of target sequence (Figure 4.1) is 399 base pairs (bp) and is conserved among the 5 rice β-glucosidase genes.

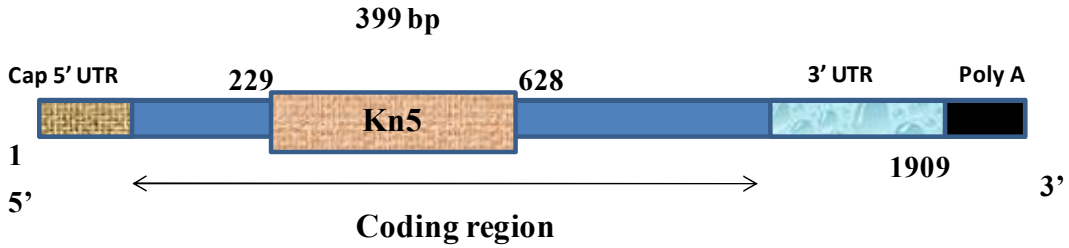

Figure 4.1 The mRNA structure of *Os3bglu7* including target sequence Kn5 (399 bp) to knock down five β-glucosidase genes (*Os1bglu1, Os3bglu7, Os3bglu8, Os7bglu26* and *Os12blgu38*)**.**

This Kn5 target sequence not only can knock down the *Os3bglu7* gene expression, but it can also interfere with gene expression of the four other β-glucosidase genes. It showed high identity of 69% to 84% with mRNA sequence of other β-glucosidase genes (Table 4.1) and high coverage (85-98%) to three of the β-glucosidase genes (*Os1bglu1*, *Os3bglu8* and *Os7bglu26*).

Table 4.1 Identity between the Kn5 target sequence in the RNAi vector and mRNA sequence of the five β-glucosidase genes.

Accession	Description	Max identity	Query coverage
OSU28047	*Os3bglu7* (1909 bp)	399/399 (100%)	100%
AK120790	*Os3bglu8* (2082 bp)	330/393 (84%)	98%
AK068499	*Os7bglu26* (1897 bp)	270/392 (69%)	97%
AK069177	*Os1bglu1* (1833 bp)	249/345 (72%)	85%
AK071058	*Os12bglu38* (1796 bp)	76/102 (74%)	25%

Although, the coverage of Kn5 sequence on *Os12blgu38* mRNA sequence is not high (about 25%), the coverage is concentrated in one region of about 100 base pairs (Figure 4.2). It should be enough to generate the small interfering RNA (21-25 nucleotides) when double strand RNA is present in the cell. Therefore, the Kn5 target gene of the RNAi vector has the potential to knock down the expression of these five β-glucosidase genes in rice.

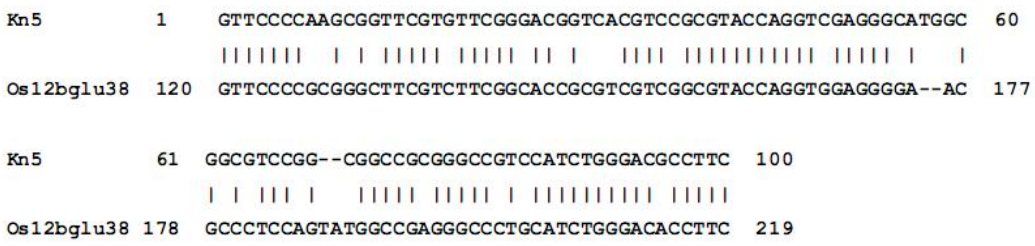

Figure 4.2 Alignment of a partial Kn5 sequence with partial a mRNA sequence of *Os12blgu38*.

4.2 RNAi vectors construction

The genomic DNA (Kosihikari) was used as a template to amplify the target sequences to knock down the *Os3bglu7*. The first construct to knock down the highest expressed gene, *Os3bglu7*, was done by amplifying 350 bp of the 3'UTR region of this gene from genomic DNA. The second construct to knock down 5 genes, the target sequence was amplified from a cDNA clone of *Os3bglu7*. The expected size of this sequence is 399 base pairs. The PCR products were purified and verified by agarose gel electrophoresis.

After amplification, the DNA products were ligated into pENTR^{TM}/D TOPO cloning vector and transformed using the heat shock method to One shot $^{®}$ TOP 10 competent *E. coli* cell (Invitrogen). The transformed colonies were screened on LB-kanamycin plates. Checking for the recombinat colonies was done by PCR amplification with the M13 forward and M13 reverse primers. The plasmids from positive colonies were extracted and sequenced. The sequence showed the same nucleotide sequence as expected.

The recombinant plasmids were extracted using the QIA plasmid extraction kit (QIAGEN). The LR clonase reaction was done to transfer the target gene from the pENTR^{TM}/D TOPO cloning vector to the pOpOff2 vector. Colonies were screened on LB-spectinomycin plates and the inserted gene was checked with specific primers by colony PCR. The results of the colony PCR are shown in Figures 4.3 and 4.4.

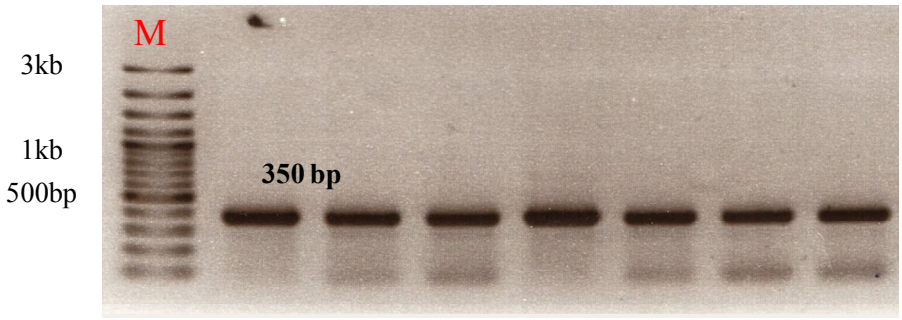

Figure 4.3 Agarose gel electrophoresis of colony PCR products of recombinant pOpOff2/Bglu7 plasmid (M: 100bp marker).

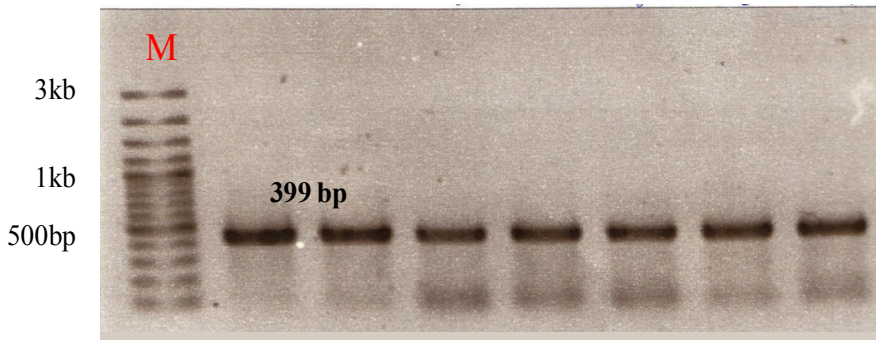

Figure 4.4 Agarose gel electrophoresis of colony PCR products of recombinant pOpOff2/Kn5 plasmid (M: 100bp marker).

All selected colonies of pOpOff2/Bglu7 and pOpOff2/Kn5 had the expected bands. It can be concluded that these colonies contain recombinant plasmids and the plasmids have at least one insert. All of the colonies were used for plasmid extraction and the plasmids were digested by restriction enzymes. In this experiment, *Xba*I was used to cut the plasmids to find the plasmids that contain 2 pieces of the target genes. A diagram of fragments of pOpOff2 cut with *Xba*I, *Xho*I, *Kpn*I is shown in Figure 4.5. The agarose gel electrophoresis of the plasmids checked by restriction digestion is shown in Figure 4.6.

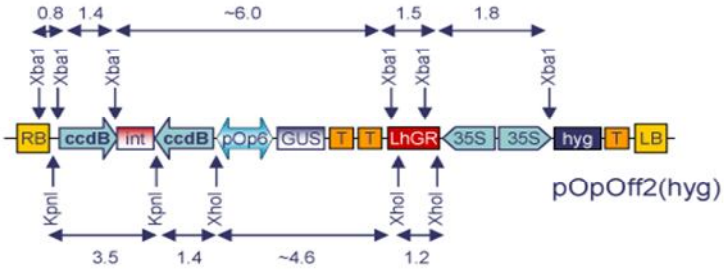

Figure 4.5 The restriction sites of *Xba*I, *Xho*I and *Kpn*I enzymes on the pOpOff2 vector (http://www.pi.csiro.au/RNAi/vectors.htm).

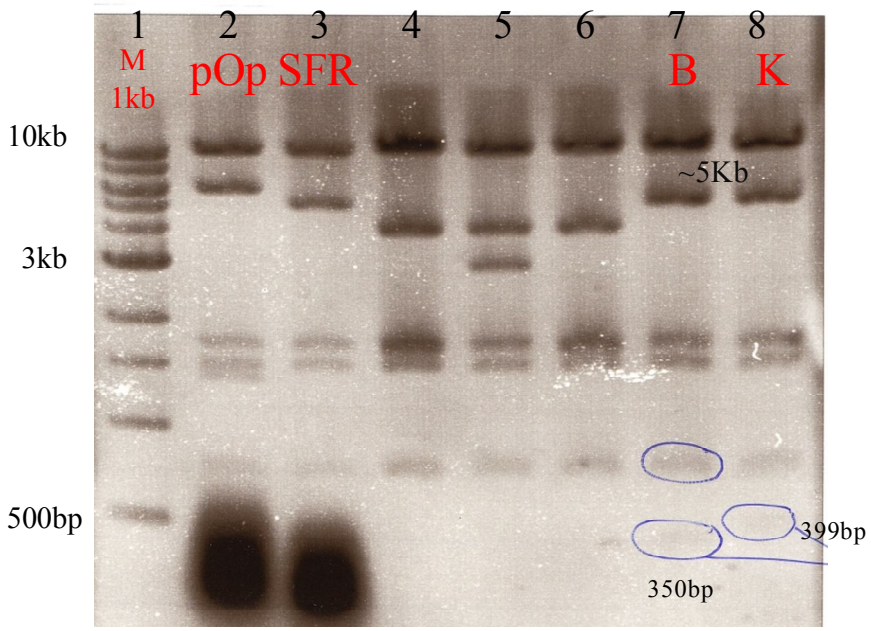

Figure 4.6 The agarose gel electrophoresis of plasmids cut with *Xba*I restriction enzyme. M (100 bp marker), B (pOpOff2/Bglu7), K (pOpOff2/Kn5), pOp (pOpOff2 control), SFR (pOpOff2/SFR control), 4-6 (incorrectly inserted plasmids).

The results showed that the pOpOff2 had 6 fragments after cutting and the smallest was about 800 base pairs. Lane 2 is the result when the pOpOff2/ SFR plasmid was cut. The pOpOff2/SFR was used as a positive control. The

pOpOff2/SFR has the insert of about 350 base pairs. In comparison with the pOpOff2 and pOpOff2/SFR, the important fragments to confirm the right plasmid are ~5kb and <500bp. Thus, the result in lanes 4, 5 and 6 show that they are the not correct double inserts plasmids. Lanes 7 and 8 indicate that the vectors have two pieces of target gene fragments in the correct position.

The correct plasmid was transformed into *Agrobacterium* (EHA105) for rice transformation and producing the transgenic knock down rice plants.

4.3 Recombinant β-glucosidase protein production

The β-glucosidase protein (Os3BGlu7) was expressed from the pET32a(+) vector (Opassiri et al., 2003). The pET32a(+) vector expressed protein contains thioredoxin and a six His-tag sequence in the N-terminal region. Soluble proteins were extracted from cell pellets and purified by IMAC (Co^{2+}) following the protocol of Opassiri (2003). The SDS-PAGE of each step of the protein purification is shown in Figure 4.7. The 66 kDa expected recombinant Os3BGlu7 (rBGlu7) can be seen. Most of the BGlu7 protein was eluted out in the 100 mM imidazone elution fraction (E100). The buffer of the E100 fraction was changed to 20 mM Tris-Cl, pH 8.0, by centrifugal ultrafiltration.

After changing the buffer, the protein concentration was measured by the Bradford assay (1976) and protein activity was assayed by the method of Evans (1985). Purified active recombinant β-glucosidase protein was kept at 4 ^{o}C. The active recombinant rBGlu7 β-glucosidase protein 0.3 mg per plate was added in the transformation experiments.

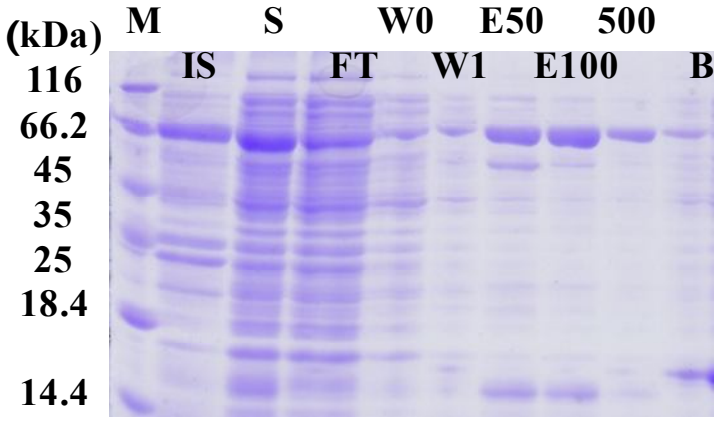

Figure 4.7 SDS-PAGE of Recombinant BGlu7 protein expressed in Origami (DE3) E. *coli*. Bio-RAD Marker (M), insoluble (IS), soluble (S), W0-W1 (washing buffer included different concentration of imidazole from 0, 10) E50-E100 (elution with buffers containing 50 and 100 mM imidazole), 500 (500 mM imidazole elution), B (Bead IMAC, after 500 mM imidazole elution).

4.4 Effect of treatments on the *Agrobacterium* population

The transformations were done with *Agrobacterium* (EHA105) carrying RNAi constructs to knock down *Os3bglu7* alone (pOpOff2/Bglu7) and the 5 β-glucosidase genes (pOpOff2/Kn5) in Koshihikari rice calli with different treatments. The first treatment was performed by adding DEX (induce chemical) in the co-cultivation step of *Agrobacterium* transformation to induce the production of double strand RNAi. The second treatment was done by supplementing active recombinant BGlu7 β-glucosidase protein in the co-cultivation step of *Agrobacterium* transformation. The third treatment was performed by adding both DEX and active recombinant β-

glucosidase protein (rBGlu7) in the co-cultivation step of *Agrobacterium* transformation. After *Agrobacterium* infection, all treatments were incubated at 25 ^{o}C for 3 days in the dark. The plates of each treatment were shaken two times per day in order to rotate the surface of the calli to touch the surface of the medium. In the same replication, all treatments of each construct were infected with *Agrobacterium* at the same times and the same concentration of *Agrobacterium* suspension.

After 3 days of co-cultivation, 30 pieces of calli (2-3 mm) of each treatment were selected and rinsed in 10 ml of sterile water. The *Agrobacterium* populations on the calli after co-cultivation were determined by measuring the OD_{600} value of *Agrobacterium* suspension. The results of *Agrobacterium* population carrying construct pOpOff2/Bglu7 and pOpOff2/Kn5 are shown in Table 4.2 and Table 4.3, respectively.

Table 4.2 The relative levels of *Agrobacterium* population of treatments with pOpOff2/Bglu7 construct.

Treatments	***Agrobacterium* population (OD_{600})**	
	Replication 1	**Replication 2**
CT 1 (pOpOff2/Bglu7)	0.027 (+)	0.022 (+)
T1 (pOpOff2/ Bglu7 + DEX)	0.024 (+)	0.028 (+)
T2 (pOpOff2/ Bglu7 + rBGlu7)	0.025 (+)	0.025 (+)
T3 (pOpOff2/ Bglu7 + DEX + rBGlu7)	0.027 (+)	0.030 (+)

Note: DEX: 128 µg/plate; rBGlu7: 0.3 mg/plate; (+) means the same *Agrobacterium* population as control.

In the experiment of the pOpOff2/Bglu7 construct, all treatments (T1, T2, T3) showed similar *Agrobacterium* populations on the surface of calli after co-cultivation. Treatments with DEX and/or recombinant β-glucosidase protein did not affect the growth of *Agrobacterium* on the surface of calli in comparison with control treatment (CT 1). These data proved that adding the induction chemical (DEX) to induce RNAi production to repress the expression of *Os3bglu7* did not influence the development of *Agrobacterium* in the co-cultivation medium. Moreover, active recombinant β-glucosidase protein (rBGlu7) or the combination of DEX and rBGlu7 did not limit or affect the *Agrobacterium* population in the *Agrobacterium* transformation process.

Table 4.3 The *Agrobacterium* population of treatments with the pOpOff2/Kn5 construct.

Treatments	***Agrobacterium* population (OD_{600})**		
	Replication 1	**Replication 2**	**Replication 3**
CT 2 (pOpOff2/Kn5)	0.025 (+)	0.010 (+)	0.012 (+)
T4 (pOpOff2/Kn5 + DEX)	0.037 (++)	0.014 (++)	0.022 (++)
T5 (pOpOff2/Kn5 + rBGlu7)	0.023 (+)	0.010 (+)	0.012 (+)
T6 (pOpOff2/Kn5 + DEX + rBGlu7)	0.034 (++)	0.017 (++)	0.036 (++)

Note: DEX: 128 µg/plate; rBGlu7: 0.3 mg/plate; (+) means the same *Agrobacterium* population as control; (++) means the *Agrobacterium* population higher than control.

In the experiment of pOpOff2/Kn5 construct, the results showed that the treatment supplemented with DEX (T4 and T6) to activate the production of double

strand RNA (Kn5) had a higher *Agrobacterium* population than the control treatment (CT 2). However, when recombinant β-glucosidase protein was added (T5), number of *Agrobacterium* population when compared to the control treatment was not different (Table 4.3). These data proved that when adding DEX to induce RNAi production to knock down the 5 target genes; it promoted the growth of *Agrobacterium* on the surface of the calli. Conversely, recombinant β-glucosidase protein did not seem to affect the population of *Agrobacterium* after 3 days co-cultivation of calli. The combination of DEX and recombinant β-glucosidase protein (T6) in co-cultivation also led to an increase in the *Agrobacterium* population on the surface of calli when compared with the control treatment (CT 2).

In conclusion, adding DEX in the co-cultivation medium induced the RNAi production to knock down the expression of β-glucosidase genes. When all five β-glucosidase genes (pOpOff2/Kn5) were knocked down, the *Agrobacterium* population increased (Table 4.3). However, when knocking down only one β-glucosidase gene (*Os3bglu7*) was not enough to affect the growth of *Agrobacterium* on the surface of calli. No effects of recombinant β-glucosidase protein added to the co-cultivation on the *Agrobacterium* population were detected with either construct (pOpOff2/Bglu7 or pOpOff2/Kn5). The combination of DEX and rBGlu7 (T3, T6) treatment had the same effect on the number of *Agrobacterium* on the surface of the calli as when adding only DEX (T2, T5).

4.5 Effect of knocking down β-glucosidase genes on the transformation efficiency

In this experiment, DEX was added to the surface of the co-culture medium (T1, T4) and the efficiency of transformation was compared with the control treatment (no DEX, no rBGlu7 protein added). After infecting with *Agrobacterium*, the calli were co-cultured with *Agrobacterium* for 3 days in the dark at 25 ^{o}C on co-culture medium containing DEX. After extensive washing, the calli were transferred to selection medium. The results of these treatments are shown in Table 4.4 and Figure 4. 8.

Table 4.4 The percentages of brown calli in treatments adding DEX.

Treatments	Total number of calli	Number of replications	Percent of brown calli	
			Week 1	Week 2
CT (pOpOff2/Bglu7)	730	4	11^{a}	26.7^{c}
T1 (pOpOff2/Bglu7 + DEX)	290	2	12.1^{a}	39.2^{d}
T4 (pOpOff2/Kn5 + DEX)	515	4	17.3^{b}	43.7^{e}

Note: 0.3 mg of rBGlu7 was added per plate. Values with different superscripts (a,b,c,d,e) are significantly different ($P< 0.05$)

Theoretically, on selection medium, non-transformed calli will turned brown with time. The percentage of brown calli on selection medium was evaluated to reflect

the efficiency of transformation of different treatments. When DEX was added on co-culture medium for 10 days, no effect of DEX on un-infected calli was observed. After 10 days of DEX treatment of uninfected calli on co-culture medium, no effect was observed, indicating that DEX treatment does not harm uninfected calli. DEX also did not show any effect on the growth of *Agrobacterium*.

The control treatment (no DEX) had a lower number of brown calli (26.7%) after 2 weeks on selection medium than the other 2 treatments (39.2% and 43.7%) (Figure 4.8). It showed that when adding DEX into the co-culture medium, control treatment had higher transformation efficiency after 2 weeks of selection (73%) than the other two treatments of pOpOff2/Bglu7 (60%) and pOpOff2/Kn5 (56%). This indicated that adding DEX to knock down expression of β-glucosidase genes affected transformation efficiency.

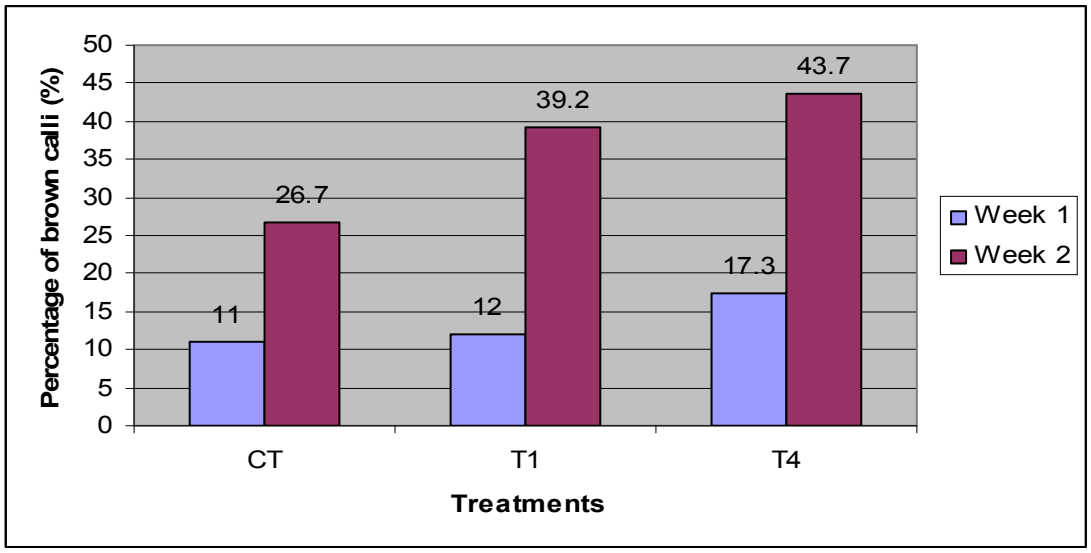

Figure 4.8 The percentages of brown calli in treatments adding DEX.
(CT: pOpOff2/Bglu7; T1: pOpOff2/Bglu7 + DEX; T4: pOpOff2/Kn5 + DEX)

When adding DEX in order to induce the RNAi to knock down 5 β-glucosidase genes (T4), the results showed higher population of *Agrobacterium* (Table 4.3) and also a higher percent of brown calli (Table 4.4). Numerous studies prove that the suppression of the expression of target gene by double-stranded RNA can happen within 24 hours in many organisms (Akashi et al., 2001; Makimura et al., 2002; Wang et al., 2005; Rothermel et al., 2006). In this research, the calli were co-cultured with *Agrobacterium* for 3 days. The T-DNA to knock down the 5 β-glucosidase genes could be integrated into the rice genome and dsRNA expressed and induce the knocking down of β-glucosidase genes via the RNAi mechanism. It indicates that when five β-glucosidase genes were knocked down, the *Agrobacterium* developed better and caused over growth of *Agrobacterium*.

The structures of calli in treatment T1 and T4 were broken. Although the calli after 3 days of co-culture were washed extensively to get rid of the *Agrobacterium*, the calli were not healthy on the selection medium. After 2 weeks, these calli became weak, turned brown and died. The brown and dead calli can be both the non-transformed and the transformed but damaged calli.

In the treatment that added DEX inducer in order to induce the RNAi to knock down *Os3bglu7* expression (T1), a higher percentage of brown calli was observed than the control treatment, but it was lower than the treatment of knocking down 5 β-glucosidase genes (T4). However, the knock-down *Os3bglu7* (T1 treatment) did not show a difference in the *Agrobacterium* population with the control treatment. Therefore, these data indicated that the effect of knocking down *Os3bglu7* on *Agrobacterium* transformation of rice calli may not be related to the growth of *Agrobacterium* on the surface of calli but related to the ability of *Agrobacterium* to

infect calli in the transformation process. When the expression of 5 β-glucosidase genes as knocked down, this may have caused the defense system of calli to become inactive, so that *Agrobacterium* could easily break down the calli defense mechanism. Consequently, the calli became more sensitive and damaged.

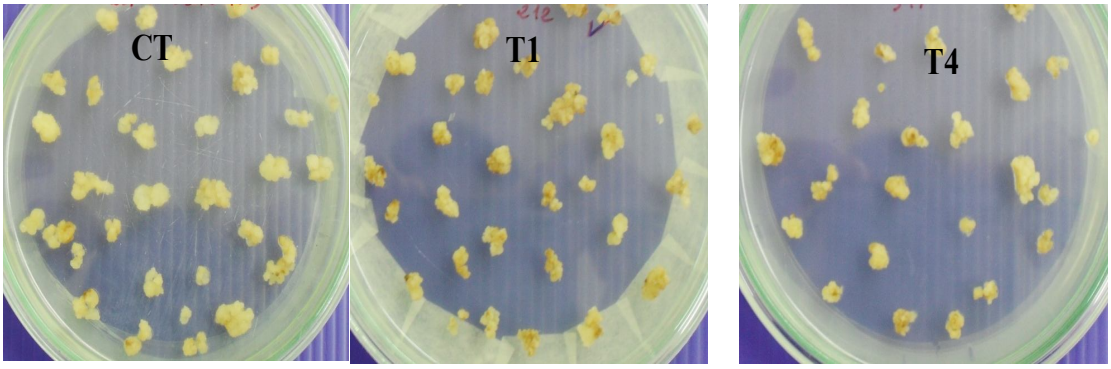

Figure 4.9 The calli after 2 weeks on the selection medium.
(CT: pOpOff2/Bglu7; T1: pOpOff2/Bglu7 + DEX; T4: pOpOff2/Kn5 + DEX).

4.6 Effect of recombinant β-glucosidase protein on transformation efficiency

In this experiment, transformations were done by adding active recombinant Os3BGlu7 β-glucosidase protein on the surface of the co-culture medium (T2 and T5 treatments). The amount of active protein used for each plate was 0.3 mg.

Table 4.5 The percentage of brown calli in treatments that add recombinant β-glucosidase protein.

Treatments	Total number of calli	Number of replications	Percent brown calli	
			Week 1	Week 2
CT (pOpOff2/Bglu7)	730	4	11[a]	26.7[b]
T2 (pOpOff2/Bglu7 + rBGlu7)	365	2	29[b]	45.5[c]
T5 (pOpOff2/Kn5 + rBGlu7)	360	2	28.9[b]	46.7[c]

Values with different superscripts (a,b,c) are significantly different ($P < 0.05$).

The result showed that higher percentage of brown calli was observed when recombinant β-glucosidase protein was added comparing to the control treatment. After 2 weeks, the control treatment had less than 30 percent of brown calli while treatments T2 and T5 had more than 45 percent of the calli turned brown and died on

the selection medium. Interestingly, there is no significant difference in the data of percents of brown calli between the two constructs of pOpOff2/Bglu7 and pOpOff2/Kn5. These data indicated that the recombinant β-glucosidase protein added in the treatment strongly reduced the efficiency of transformation. Opassiri et al. (2003) reported that rice recombinant β-glucosidase protein (BGlu1/BGlu7) can hydrolyzed various pNP-derivaties, the cyanogenic glycosides prunasin, dhurrin and amygdalin. Moreover, BGlu1 (Os3BGlu7) was also inhibited by IAA and GA3, which might indicate that BGlu1 plays a role in phytohormone activation (Opassiri et al., 2003). In this case, it can be suggested that β-glucosidase protein may be involved in the secondary metabolism that serves as plant protection from *Agrobacterium*. The recombinant β-glucosidase protein may also activate other proteins and these proteins release toxic molecules to protect calli against T-DNA transfer by *Agrobacterium*. So, in the *Agrobacterium* transformation process, the appearance of recombinant β-glucosidase protein interfered with the infection ability of *Agrobacterium*. Therefore, the supplementation of β-glucosidase protein reduced the efficiency of transformation in the *Agrobacterium* transformation process.

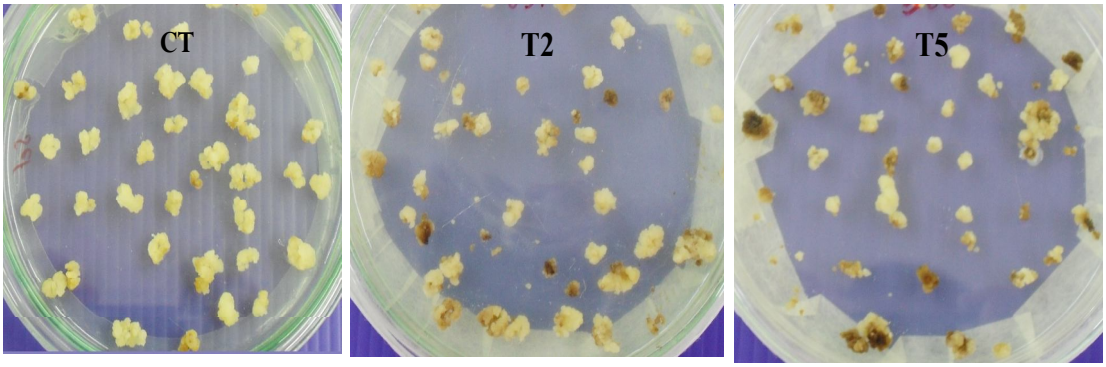

Figure 4.10 The calli after 1 week on the selection medium of treatment adding recombinant β-glucosidase protein. (CT: pOpOff2/Bglu7; T2: pOpOff2/Bglu7 + rBGlu7; T5: pOpOff2/Kn5 + rBGlu7).

4.7 Transgenic calli and plants confirmation

To confirm the integration of T-DNA fragments in hygromycin-resistant calli and transgenic plants (Figures 4.11 and 4.12), polymerase chain reaction (PCR) analysis was carried out using Hyg specific primers (Al-Forkan et al., 2004 and Methods section 3.6). Figures 4.13 and 4.14 show the analysis of PCR amplifications of genomic DNA with Hyg primers of hygromycin-resistant calli and transgenic plants (pOpOff2/Bglu7 and pOpOff2/Kn5). The specific bands (>500 bp) were found in all resistant calli after transformation with constructs for knock down *Os3bglu7* and knock down 5 β-glucosidase genes and transgenic plants.

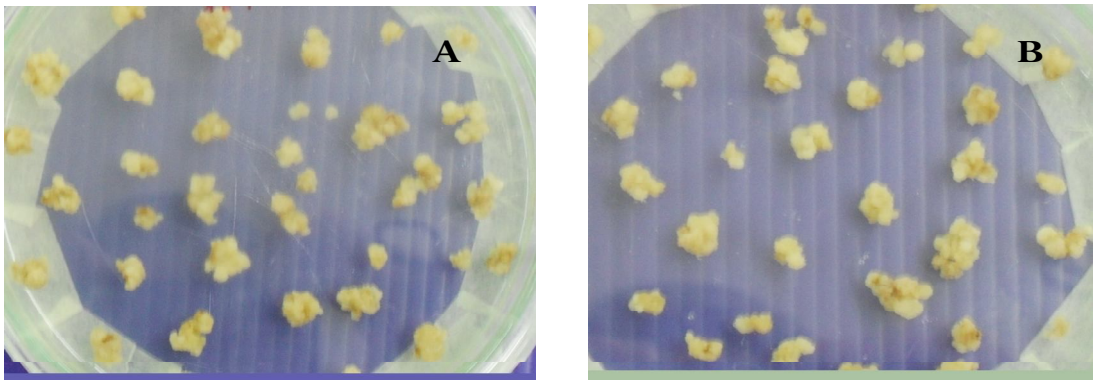

Figure 4.11 The hygromycin-resistant calli (A: pOpOff2/Bglu7 construct; B: pOpOff2/Kn5 construct).

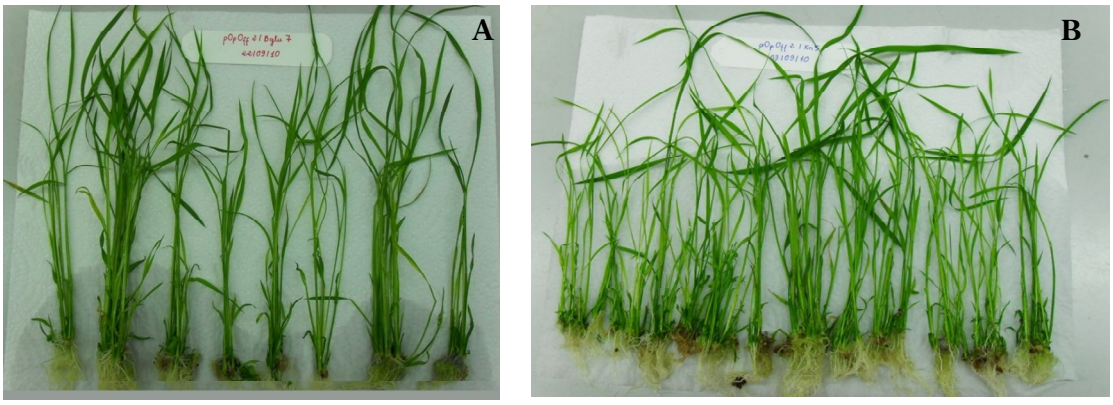

Figure 4.12 The transgenic plants (T0). (A: plants transformed with the pOpOff2/Bglu7 construct; B: plants transformed with the pOpOff2/Kn5 construct).

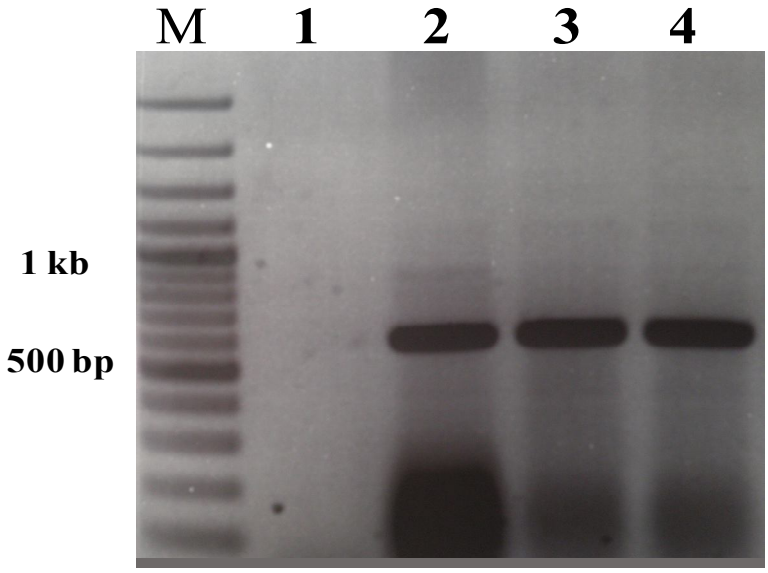

Figure 4.13 PCR products of *Hyg* genes from resistant calli trasformed with the pOpOff2/Bglu7 and pOpOff2/Kn5 constructs. (M: 100 bp marker, lane 1, non transgenic calli as template; lane 2, plasmid as positive control; lane 3 and 4 PCR products from hyg resistant calli of the pOpOff2/Bglu7 and pOpOff2/Kn5 constructs, respectively).

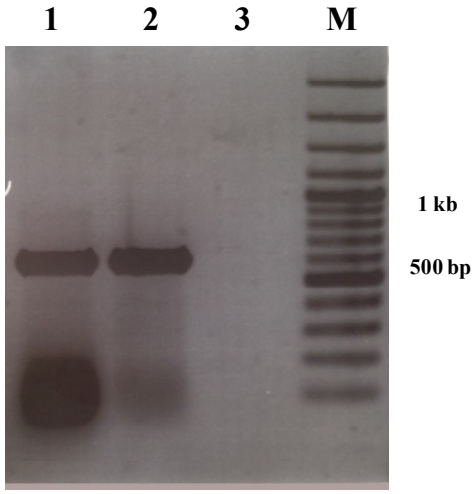

Figure 4.14 PCR products of *Hyg* genes from transgenic plants carring the pOpOff2/Bglu7 and pOpOff2/Kn5 construct. (M: 100 bp marker; lane 1 and 2, PCR product from transgenic plants of the pOpOff2/Bglu7 and pOpOff2/Kn5 constructs, respectively; lane 3, non transgenic plant as a control).

4.8 Effect of DEX on germination of rice seed and plantlet

The inducible RNAi vector pOpOff2 combines the dexamethasone-inducible pOp6/LhGR promoter system (Craft et al., 2005) with the pHellsGATE Gateway™ gene silencing vectors (Wielopolska et al., 2005). In this vector system, the dexamethasone-inducible pOp6 promoter is bidirectional and able to drive the expression of both the gene of interest and GUS reporter gene (Wielopolska et al., 2005).

Opassiri et al. (2003) showed that the *Os3bglu7* has high expression in shoot and young tissues. This suggested that it may play a role in growth, cell wall expansion and/or cell division (Opassiri et al., 2003). And this gene clusters together in same group with four other β-glucosidase genes (Opassiri et al., 2006). Therefore,

the experiments to test the function of these five β-glucosidase genes in germination of rice seeds and in the growth of plantlets of T1 RNAi lines of pOpOff2/Kn5 were carried out.

In the first experiment, the rice seed (T1) of transgenic plants (pOpOff2/Kn5-T0) and wild type line were harvested and dehusked before germination on MS medium with or without DEX. Thirty rice seeds were used for each treatment. The final concentrations of DEX were 0, 50, 100 and 150 μM. Hygromycin was also added to the media of transgenic rice.

The results in Table 4.6 showed that after 3 days on MS medium, most of the transgenic rice seeds germinated. However, the wild type rice seeds of control treatment (WT) had about only 60% of the seeds germinated, while other treatments adding DEX (W1, W2 and W3) had percentages of germination around 57% - 67%. However, the changes of percent germination were not correlated with the increasing DEX concentration. The low percent of germination of wild type appeared to be due to the immature and aged seeds obtained from the Chiang Mai Rice Research Center. It indicated that when the DEX concentration increased in the treatments (W1, W2, and W3), the percentages of germination were not affected.

In the experiments of transgenic rice seeds (T), the percent of germination was reduced incorrelation with the DEX concentration in the medium. The control treatment T (CT) had 93% germination while the treatment with 150 μM DEX had only 87% germination. Although the difference in values between control treatment and other treatments were not large, they may reflect the effect of knocking down the five β-glucosidase genes. The T1 transgenic rice seeds were germinated on MS

medium containing hygromycin (50 mg/L) to select only transgenic rice. However, we need to keep in mind that in T1 seedings only ¾ of the plants are transgenic (hyg resistant plants), assuming the inserted DNA followed the Mendelian segregation patterns when the T0 plants self-pollinated. However, germination on hygromycin plates after 3 days might not be able to select for transgenic or non transgenic plantlets yet.

Table 4.6 Effect of DEX on germination of rice seeds.

Treatments	DEX (µM)	Day 3 of germination	
		Shoots	**Roots**
		# (%)	**# (root length)**
W (CT)	0	18 (60%)	15 (1-2 cm)
W1	50	17 (57%)	13 (< 1 cm)
W2	100	20 (67%)	12 (< 1 cm)
W3	150	18 (60%)	10 (< 0.5 cm)
T (CT)	0	28 (93%)	15 (> 2 cm) and 8 (1-2 cm)
T1	50	27 (90%)	12 (< 1 cm)
T2	100	27 (90%)	10 (< 0.5 cm)
T3	150	26 (87%)	9 (< 0.5 cm)

Note: - W: wild type rice seeds; T: T1 rice seeds of pOpOff2/Kn5 line

- At day 10 of germination, the percents of germinated seeds did not change, but the lengthes of shoots and roots were longer. This data correlated with the data of day 3.

Besides percentage of germination, we also considered about the effect of DEX on the root length during germination of rice seeds. In this experiment, the lengthes of roots, the growth of roots seems to be limited by DEX on treatments of both wild type and transgenic plants. However, the DEX treatments on the transgenic rice showed stronger effects than the DEX treatments of wild type rice. With transgenic rice seeds, the control treatment showed 77% (15+8/30) of seeds had roots. In the treatments that supplemented with DEX, the length of roots decreased with increasing DEX concentration. In this case, we cannot conclude that the effect of DEX on the germination of the transgenic seeds was related to gene knock-down since DEX also affected the germination of normal rice seeds. However, this data indicated that DEX added in the medium to knock-down the five β-glucosidase genes' expression may have affected the lengthes of roots in germination of rice seedlings (Figure 4.15). To confirm our judgment, a second experiment was performed. The effect of DEX on the growth of plantlets was tested. In this experiment, ten plantlets were used for each treatment with the concentration of 150 μM DEX. After 7 days on the medium containing DEX, the lengthes of roots and shoots of the plantlets were measured (Table 4.7).

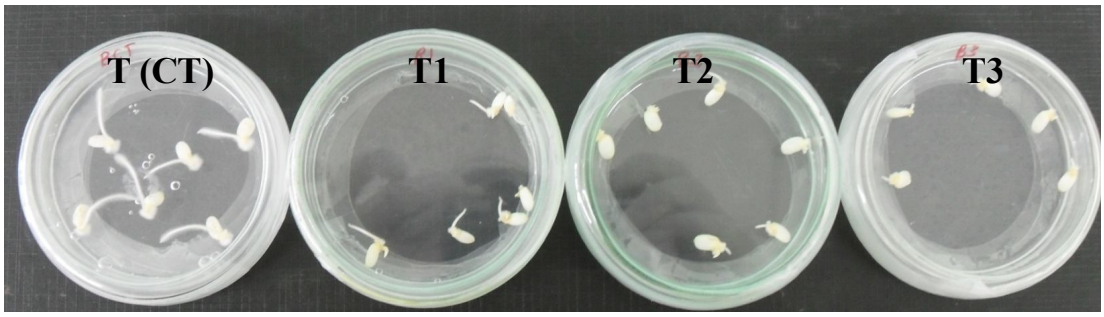

Figure 4.15 Germination of transgenic rice seeds (pOpOff2/Kn5) on MS medium with different treatments at day 3. (T (CT): 0 µM DEX, T1: 50 µM DEX, T2: 100 µM DEX, T3: 150 µM DEX).

Table 4.7 Effect of DEX on the growth of OpOff2/Kn5 plantlets at day 7.

Treatments	Average of shoot length (cm)	Length of root (cm)
W (CT)	2.44 [a]	> 2 cm (more than 3 roots per plant)
W3 (150 µM DEX)	2.28 [a]	< 0.5 cm (1-2 roots per plant)
T (CT)	5.15 [c]	> 3 cm (more than 3 roots per plant)
T3 (150 µM DEX)	3.36 [b]	< 3 cm (1-2 roots per plant)

W: wild type plantlets; T: T1 plantlets of pOpOff2/Kn5 lines

Values with different superscripts (a,b,c) are significantly different ($P < 0.01$)

In this experiment (Table 4.7), the DEX affected the number of roots and the length of root similar to the results seen in Table 4.6. The results showed different effects of DEX on the length of shoot on wild type rice and transgenic plantlets. In the treatments of wild type, the average shoot length of the control treatment W (CT) was 2.44 cm, while the average shoot length on DEX treatment (W3) was 2.88 cm, the

difference of two treatments is not significant (P<0.01) (Figure 4.16). In contrast, in the DEX treatment of transgenic plants (T3), the average of shoot length (3.36 cm) was strongly reduced when comparing with control treatment W (CT) (5.15 cm) (Figure 4.17). This data proved that when adding DEX to knock down β-glucosidase genes expression, the knocking down of these genes suppressed the elongation of shoot. The functions of β-glucosidases in rice and other plants were shown to be related to cell wall development in germinating seed and seedlings (Leah et al., 1995; Opassiri et al., 2004; Kuntothom et al., 2009; Cairns & Esen, 2010), lignification (Dharmawardhana et al., 1995; Trevino et al., 2006), and activation of phytohormones (Brzobohatý et al., 1993; Kristoffersen et al., 2000; Lee et al., 2006). These evidences support our data about the function of β-glucosidases in the growth of rice plantlets. It indicated that these highly expressed β-glucosidase genes may play importance roles in the development of shoots in the plantlet stage.

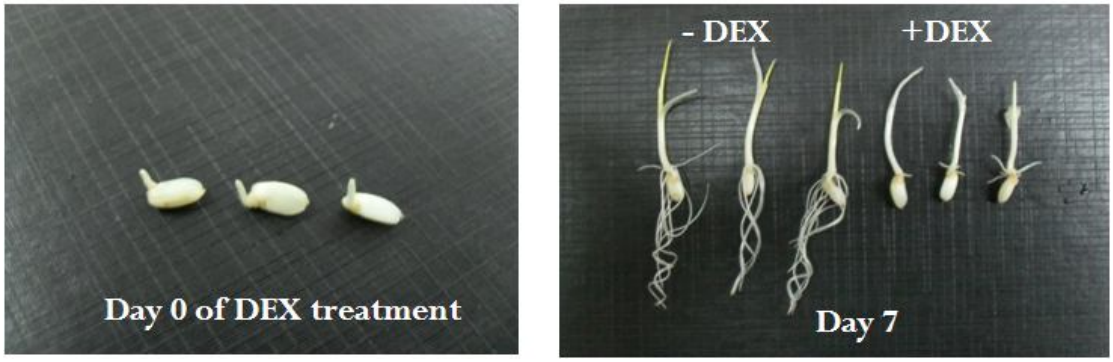

Figure 4.16 The effect of DEX on growth of wild type plantlets (Koshihikari)

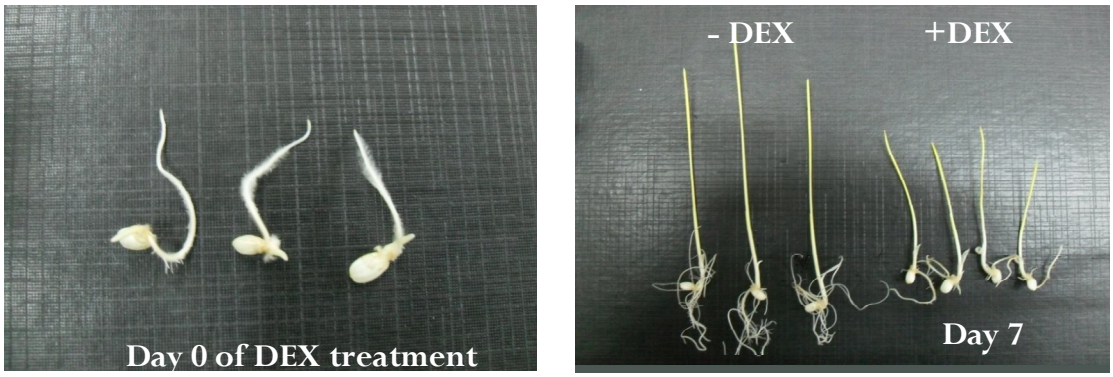

Figure 4.17 The effect of DEX on growth of OpOff2/Kn5 plantlets (T1)

4.9 Expression level of β-glucosidase genes in transgenic rice

To confirm that DEX can induce RNAi production, three samples of plantlets after treatment with high DEX concentration (150 μM) for 7 days were collected to check the knocking down of the β-glucosidase genes' expression by real-time RT-PCR. The control samples were transgenic plants without DEX treatment. The Real-Time RT-PCR data were analyzed following method of Pfaffl (2001). The Pfaffl mathematical model of relative expression ratio of Real-Time PCR was used. In this method, the ratio of target gene is expressed in a sample versus a control in comparison to reference gene expression. In this study, β-actin gene was used as the reference gene. The relative gene expression of β-glucosidase genes in DEX treated samples and controls are shown in Figure 4. 17.

The results (Figure 4.18) showed that all four β-glucosidase genes of pOpOff2/Kn5 plantlet were knocked down (76% for *Os1blgu1*, 73% for *Os3bglu7*, 61% for *Os3bglu8* and 23% for *Os7bglu26*) when treated with DEX. In the figure, the expression of *Os12bglu38* was not shown since it showed very low expression in control samples and the expression of this gene in the DEX treated samples was not

detected. The data indicated that DEX really reduced the expression of all five β-glucosidase genes. This also proved that the RNAi construct to knock down five β-glucosidase genes worked and can affect the expression of target genes in transgenic plants. However, the level of knocking down of each gene was different. And the RNAi construct only knocked down, but did not knocked out these β-glucosidase genes. In the control sample, the *Os3bglu7* is the most highly expressed gene, followed by the *Os1bglu1, Os7bglu26, Os3bglu8 and Os12bglu38*. The *Os12bglu38* has very low expression level. Other DEX treated transgenic calli and plantlets were used to do GUS staining (Figure 4.19; 4.20) to confirm the expression of the pOp6 promoter under DEX treatment control.

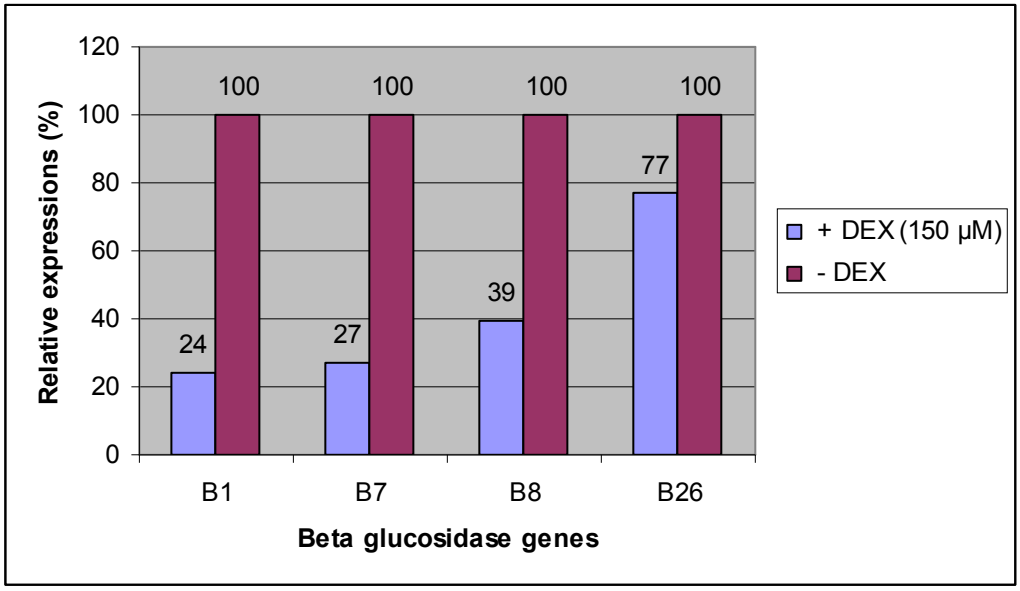

Figure 4.18 Relative expressions of β-glucosidase genes of pOpOff2/Kn5 plantlets (T1). (B1, *Os1bglu1*; B7, *Os3bglu7*; B8, *Os3bglu8*; B26, *Os7bglu26*).

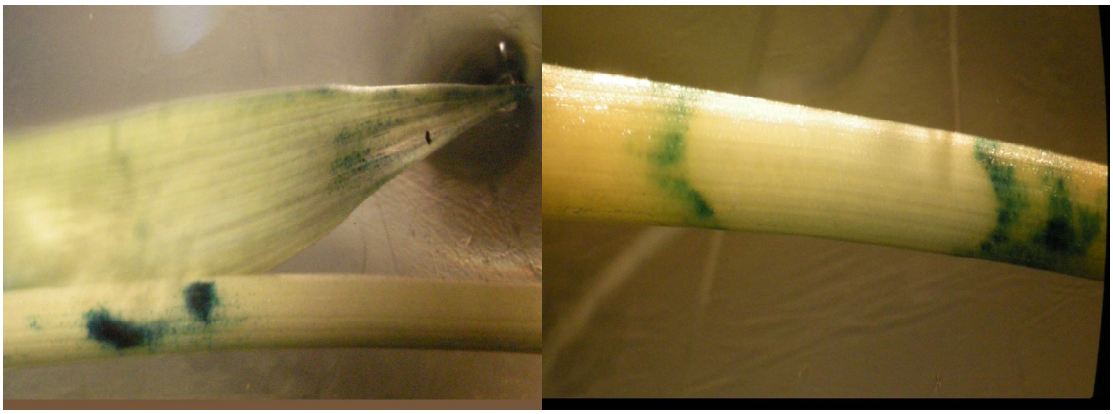

Figure 4.19 GUS staining on leaf and shoot of pOpOff2/Kn5 plantlets (T1).

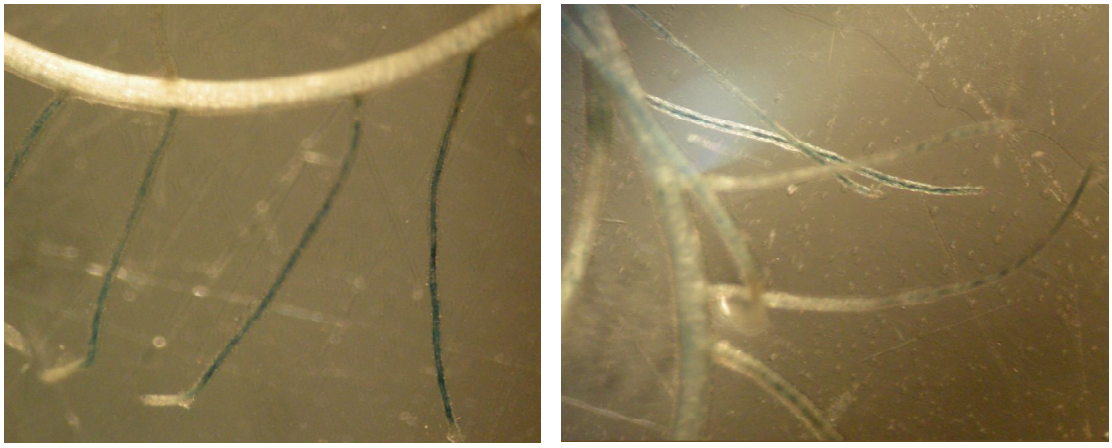

Figure 4.20 GUS staining on root of pOpOff2/Kn5 plantlets (T1).

Figure 4.19 and 4.20 showed that the GUS staining can be observed mainly in very young dividing tissues for example root hair and meristematic tissue, but not in the main root. This may be the answer to why the pOpOff2/Kn5 only knocked down, but did not knock out the five β-glucosidase genes' expression. It is because the pOp6 promoter is not a strong promoter for expression in every tissue to use for gene knock out in rice plant.

CHAPTER V

CONCLUSION

In this research, our goal was to identify the function of five rice β-glucosidase genes (*Os1bglu1, Os3bglu7, Os3bglu8, Os7bglu26 and Os12bglu38*) in the *Agrobacterium* transformation process by the RNAi method. Two constructs of inducible RNAi vectors to knock down these genes (pOpOff2/Bglu7 and pOpOff2/Kn5) were produced. In the first construct, to knock down the highly expressed *Os3bglu7* gene, the target sequence (Bglu7) was amplified from the 3'UTR of this gene and cloned into pOpOff2. The second construct contained a target gene (Kn5) amplifying from the coding region of *Os3bglu7*, which was meant to knock-down all five genes. The recombinant constructs were cloned into *Agrobacterium* (EHA105) before rice transformation. Different treatments of two RNAi constructs were tested during the co-cultivation step of *Agrobacterium* transformation by supplementing inducer (DEX) and/or recombinant Os3BGlu7 (rBGlu7).

The *Agrobacterium* population on the surface of the calli increased when DEX was added to knock down the five β-glucosidase genes compared with the control. However, the supplementation of DEX in transformations with the pOpOff2/Bglu7 construct did not increase the *Agrobacterium* population. The effect of exogenously added rBGlu7 on the *Agrobacterium* population was not observed. The combination of DEX and rBGlu7 had the same effect on the number of *Agrobacterium* on the surface of the calli as did adding only DEX.

In the *Agrobacterium* transformation process, adding DEX to knock down the expression of β-glucosidase genes affected the transformation efficiency of both constructs. After two weeks on selection medium, the control treatment showed 26.7% percent of the calli were brown which was lower than the pOpOff2/Kn5 construct (43.7%) and pOpOff2/Bglu7 construct (39.2%). When adding DEX, the expression of the 5 β-glucosidase genes was knocked down, which may inactivate the defense systems of the calli, allowing *Agrobacterium* to easily break down the calli defense mechanism. Consequently, calli became more sensitive and damaged. Therefore, the transformation efficiencies were reduced. Moreover, the supplementation of β-glucosidase protein in the co-cultivation step also reduced the *Agrobacterium* transformation efficiency. The β-glucosidase may activate other compounds and these compounds can release toxic molecules to protect the calli against the T-DNA transfer by *Agrobacterium*. So, in the *Agrobacterium* transformation process, the appearance of recombinant β-glucosidase protein interfered with the infection ability of *Agrobacterium*. These data support the hypothesis that β-glucosidase plays a role in defense of callus to *Agrobacterium* infection.

An effect of DEX on seed germination was observed. The percent of germinated rice seeds was reduced, which correlated with the increase of DEX concentration for transgenic rice seeds but not for wild type rice seeds. However, high DEX concentration affected the growth of roots in both transgenic and wild type rice. In addition, in the development of transgenic plantlets, adding DEX to knock down β-glucosidase genes expression suppressed the elongation of shoots. This proved that

these high expression β-glucosidase genes may play an important role in the development of shoot and root in the plantlet stage. The data of real-time RT-PCR confirmed that the expression of these five β-glucosidase genes were knocked down after DEX treatment. All of these data indicated that the five β-glucosidase genes may play an important role in the development of young rice seedlings.

REFERENCES

REFERENCES

Akashi H, Miyagishi M and Taira K (2001) Suppression of gene expression by RNA interference in cultured plant cells. **Antisense and Nucleic Acid Drug Development** 11:359-367.

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

Al-Forkan M, Power JB, Paul Anthony, Lowe KC and Davey MR (2004) *Agrobacterium*-mediated transformation of bangladeshi indica rices. **Cellular & Molecular Biology Letters** 9: 287-300.

Anand A, Uppalapati SR, Ryu C-M, Allen SN, Kang L, Tang Y and Mysore KS (2008) Salicylic acid and systemic acquired resistance play a role in attenuating crown gall disease caused by *Agrobacterium tumefaciens*. **Plant Physiology** 2008:703-715.

Bajaj S and Mohanty A (2005) Recent advances in rice biotechnology toward genetically superior transgenic rice. **Plant Biotechnology Journal** 3:275-307.

Bernstein E, Caudy AA, Hammond SM and Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. **Nature** 409:363-366.

Bisaro DM (2006) Silencing suppression by geminivirus proteins. **Virology** 344:158-168.

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J and Palmet K (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. **Science** 262.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009). The Carbohydrate-Active enZymes database (CAZy): an expert resource for Glycogenomics. **Nucleic Acids Research** 37:D233-D238.

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.

Chen S-K, Kurdyukov S, Kereszt A, Wang X-D, GresshoV PM and Rose RJ (2009) The association of homeobox gene expression with stem cell formation and morphogenesis in cultured *Medicago truncatula*. **Planta** 230:827-840.

Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR, Esen A, Chen C-J, Opassiri R, Svasti J and Ketudat-Cairns JR (2008) Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysisand transglycosylation. **Journal of Molecular Biology** 377:1200-1215.

Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Svasti J and Ketudat-Cairns JR (2011) The structural basis of oligosaccharide binding by rice BGlu1 beta-glucosidase. **Journal of Structural Biology** 173:169-179.

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafny-Yelin M, Vyas S, Tovkach A and Tzfira T (2007) Biological systems of the host cell involved in *Agrobacterium* infection. **Cellular Microbiology** 9:9-20.

Craft J, Samalova M, Baroux C, Townley H, Martinez A, Jepson I, Tsiantis M and Moore1 I (2005) New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in *Arabidopsis*. **The Plant Journal** 41:899-918.

Dharmawardhana DP, Ellis BE and CarIson JE (1995) A beta-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. **Plant Physiol** 107:331-339.

Ditt RF, Kerr KF, Figueiredo Pd, Delrow J, Comai L and Nester EW (2006) The *Arabidopsis thaliana* transcriptome in response to *Agrobacterium tumefaciens* **Mol Plant Microbe Interact** 19:665-681.

Ditt RF, Nester E and Comai L (2005) The plant cell defense and *Agrobacterium tumefaciens*. **FEMS Microbiology Letters** 247:207-213.

Ditt RF, Nester EW and Comai L (2001) Plant gene expression response to *Agrobacterium* tumefaciens. **Proceeding of the National Academy of Sciences** 98:10954-10959.

Dunoyer P, Himber C and Voinnet O (2006) Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. **Nature Genetics** 38:258-263.

Durrant WE and Dong X (2004) Systemic acquired resistance. **Annual Review of Phytopathology** 42:185-209.

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

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. **Nature** 391:806-811.

Forslund K, Morant M, Jørgensen B, Olsen CE, Asamizu E, Sat 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 Physiology** 135:71-84.

Friedberg F, Saunders AA and Rhoads AR (2003) Conservation of the 3'-untranslated regions of calmodulin mRNAs in cetaceans. **Molecular Biology Reports** 30:193-198.

Fu D, Uauy C, Blechl A and Dubcovsky J (2007) RNA interference for wheat functional gene analysis. **Transgenic Research** 16.

Gaspar YM, Nam J, Schultz CJ, Lee L-Y, Gilson PR, Gelvin SB and Bacic A (2004) Characterization of the arabidopsis lysine-rich arabinogalactan protein *AtAGP17* mutant (*rat1*) that results in a decreased efficiency of *Agrobacterium* transformation. **Plant Physiology** 135.

Gelvin SB (2000) *Agrobacterium* and plant gene involved in T-DNA transfer and integration. **Annual Review of Plant Physioloy and Plant Molecular Biology** 51:223-256.

Gelvin SB (2010) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. **Annual Review Phytopathology** 48:45-68.

Giri CC and Laxmi GV (2000) Production of transgenic rice with agronomically useful genes: an assessment. **Biotechnology Advances** 18:653-683.

Halkier BA and Gershenzon J (2006) Biology and biochemistry of glucosinolates. **The Annual Review of Plant Biology** 57:303-333.

Helliwell C and Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. **Methods** 30:289-295.

Hiei Y and Komari T (2008) *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. **Nature Protocols** 3:824-834.

Hiei Y, Komari T and Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. **Plant Molecular Biology** 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. **The Plant Journal** 6:271-282.

Hradilova J, Malbeck J and Brzobohaty B (2007) Cytokinin regulation of gene expression in the AHP gene family in *Arabidopsis thaliana*. **Journal of Plant Growth Regulation** 26:229-244.

http://en.wikipedia.org/wiki/Dexamethasone

http://www.cazy.org/fam/GH1.html

http://www.pi.csiro.au/RNAi/vectors.htm

Ishikawa A (2009) The Arabidopsis G-protein beta-subunit is required for defense response against *Agrobacterium tumefaciens*. **Bioscience, Biotechnology and Biochemistry** 73:47-52.

Ji H, Kim S-R, Kim Y-H, Kim H, Eun M-Y, Jin I-D, Cha Y-S and Ahn B-O (2010) Inactivation of the CTD phosphatase-like gene *OsCPL1* enhances the development of the abscission layer and seed shattering in rice. **The Plant Journal** 61:96-106.

Jorgensen R (1990) Altered gene expression in plants due to trans interactions between homologous genes. **Trends in Biotechnology 8**:340-344.

Ketudat-Cairns JR and Esen A (2010) β-Glucosidase **Cellular and Molecular life sciences** 67:3389-3405.

Komari T, Hiei Y, Ishida Y, Kumashirot T and Kubo T (1998) Advances in cereal gene transfer. **Current Opinion in Plant Biology** 1:161-165.

Kristoffersen P, Brzobohaty B, Hohfeld I, Bako L, Melkonian M and Palme K (2000) Developmental regulation of the maize Zm-p60.1 gene encoding a beta glucosidase located to plastids. **Planta** 210:407-415.

Kuntothom T, Luang S, Harvey AJ, Fincher GB, Opassiri R, Hrmova M and Ketudat-Cairns JR (2009) Rice family GH1 glycoside hydrolases with β-D-glucosidase and β-D-mannosidase activities. **Archives of Biochemistry and Biophysics** 491:85-85.

Kuntothom T, Raab M, Tvaroska I, Fort S, Pengthaisong S, Canada J, Calle L, Jimenez-Barbero J, Ketudat-Cairns JR and Hrmova M (2010) Binding of β-D-Glucosides and β-D-Mannosides by rice and barley β-D-Glycosidases with distinct substrate specificities. **Biochemistry** 49:8779–8793.

Leah R, Kigel J, Svendsen I and Mundy J (1995) Biochemical and molecular characterization of a barley seed beta-glucosidase. **The Journal of Biological Chemistry 270**:15789-15797.

Lee KH, Piao HL, Kim H-Y, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee I-J and Hwang I (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. **Cell** 126:1109-1120.

Mahmood-ur-Rahman, Ali I, Husnain T and Riazuddin S (2008) RNA interference: The story of gene silencing in plants and humans. **Biotechnology Advances** 26:202-209.

Makimura H, Mizuno TM, Mastaitis JW, Agami R and Mobbs CV (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. **BMC Neuroscience** 3:18.

Mantiri FR, Kurdyukov S, Lohar DP, Sharopova N, Saeed NA, Wang X-D, VandenBosch KA and Rose RJ (2008) The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in *Medicago truncatula*. **Plant Physiology** 146:1622-1636.

Mazumder B, Seshadri V and Fox PL (2003) Translational control by the 3′-UTR: the ends specify the means **Trends in Biochemical Sciences** 28:91-98.

McCullen CA and Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. **Annual Reviews of Cell and Developmental Biology** 22:101-127.

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

Morant AV, Jørgensen K and Jørgensen C (2008) Beta Glucosidases as detonators of plant chemical defense. **Phytochemistry** 69:1795-1813.

Nurnberger T and Kemerling B (2006) Receptor protein kinases-pattern recognition receptors in plant immunity. **Trends in Plant Science** 11:519-522.

Opassiri R, Hua Y, Wara-Aswapati O, Svasti J, Akiyama T, Esen A and Ketudat-Cairns JR (2004) β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. **Biochemical Journal** 379:125-131.

Opassiri R, Ketudat-Cairns JR, Akiyama T, Aswapati OW, Svasti J and Esen A (2003) Characterization of a rice beta-glucosidase highly expressed in flower and geminating shoot. **Plant Science** 165:627-638.

Opassiri R, Maneesana J, Akiyama T, Pomthong B, Jind S, Kimurae A and Ketudat-Cairns JR (2010) Rice Os4BGlu12 is a wound-induced beta-glucosidase that hydrolyzes cell wall-β-glucan-derived oligosaccharides and glycosides. **Plant Science** 179:273-280.

Opassiri R, Pomthong B, Onkoksoong T, Akiyama T, Esen A and Ketudat-Cairns JR (2006) Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 beta-glucosidase. **BMC Plant Biology** 29:33.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. **Nucleic Acids Research** 29:2002-2007.

Rachmawati D, Hosaka T, Inoque E and Anzai H (2004) *Agrobacterium*-mediated transformation of javanica rice cv. rojolele. **Bioscience, Biotechnolology and Biochemistry** 68:1193-1200.

Rothermel A, Volpert K, Burghardt M, Lantzsch C, Robitzki AA and Layer PG (2006) Knock-down of GFR_4 expression by RNA interference affects the development of retinal cell types in three-dimensional histiotypic retinal spheres. **Investigative Ophthalmology Visual Science** 47:2716-2725.

Saika H and Toki S (2010) Mature seed-derived callus of the model indica rice variety Kasalath is highly competent in *Agrobacterium*-mediated transformation. **Plant Cell Reports** 29:1351-1364.

Schroeder JI and Nambara E (2006) A quick release mechanism for abscisic acid. **Cell** 126:1023-1025.

Seshadri S, Akiyama T, Opassiri R, Kuaprasert B and Ketudat-Cairns JR (2009) Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosidesand (1/3)- and (1/2)- linked disaccharides. **Plant Physiology** 151:47-58.

Shrawat AK and Lörz H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. **Plant Biotechnology Journal** 4:575-603.

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

Toki S (1997) Rapid and efficient *Agrobacterium*-mediated transformation in rice. **Plant Molecular Biology Reporter** 15:16-21.

Trevino LE, Chen W, Card ML, Shih MC, Cheng CL and Poulton JE (2006) *Arabidopsis thaliana* beta-glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. **Phytochemistry** 67:1651-1660.

Tyagi AK and Mohanty A (2000) Rice transformation for crop improvement and functional genomics. **Plant Science** 158:1-18.

Tzfira T and Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. **Current Opinion in Biotechnology** 17:147-154.

Umemura K, Satou J, Iwata M, Uozumi N, Koga J, Kawano T, Koshiba T, Anzai H and Mitomi M (2009) Contribution of salicylic acid glucosyltransferase, OsSGT1, to chemically induced disease resistance in rice plants. **The Plant Journal** 57:463-472.

VanEtten HD, Mansfield JW, Bailey JA and Farmer EE (1994) Two classes of plant antibiotics: phytoalexins versus phytoanticipins. **Plant Cell** 6:1191-1192.

Veena, Jiang H, Doerge RW and Gelvin SB (2003) Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and supresses host defense gene expression. **The Plant Journal** 35:219-236.

Wakuta S, Hamada S, Ito H, Matsuura H, Nabeta K and Matsui H (2010) Identification of a β-glucosidase hydrolyzing tuberonic acid glucoside in rice (*Oryza sativa* L.). **Phytochemistry** 71:1280-1288.

Wang C, Ying S, Huang H, Li K, Wu P and Shou H (2009) Involvement of OsSPX1 in phosphate homeostasis in rice. **The Plant Journal** 57:895-904.

Wang Y-h, Liu S, Zhang G, Zhou C-q, Zhu H-x, Zhou X-b, Quan L-p, Bai J-f and Xu N-z (2005) Knockdown of c-Myc expression by RNAi inhibits MCF-7 breast tumor cells growth in vitro and in vivo. **Breast Cancer Research** 7:R220-R228.

Wanthanalert W (2009) Functional genomic of rice beta-glucosidase, in *Biotechnology*: Suranaree University of Technology.

Wielopolska A, Townley H, Moore I, Waterhouse P and Helliwell C (2005) A high-throughput inducible RNAi vector for plants. **Plant Biotechnology Journal** 3:583-590.

www.invitrogen.com

Xua XM, Zhaoa Q, Rodrigo-Peirisa T, Brkljacica J, Hea CS, Müllerb S and Meiera I (2008) RanGAP1 is a continuous marker of the *Arabidopsis* cell division plane **Proceedings of the National Academy of Sciences, USA** 105:18637-18642.

Yara A, Otani M, Kusumi K, Matsuda O, Shimada T and Iba K (2001) Production of transgenic japonica rice (*Oryza sativa*) cultivar, Taichung 65, by *Agrobacterium*- mediated method. **Plant Biotechnology** 18:305-310.

Yuan W, Li X, Chang Y, Wen R, Chen G, Zhang Q and Wu C (2009) Mutation of the rice gene PAIR3 results in lack of bivalent formation in meiosis. **The Plant Journal** 59:303-315.

Zhang J, Xu R-j, Elfiott MC and Chen D-F (1997) *Agrobacterium*-mediated transformation of elite indica and japonica rice cultivars *Molecular* **Biotechnology** 8:223-231.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T and Felix G (2006) Perception of the bacterial PAMP EFTu by the receptor EFR restricts *Agrobacterium*-mediated transformation. **Cell** 125:749-760.

APPENDICES

APPENDIX A

A.1 AB medium

Table 1 AB medium component

	Components (g/L)
1	Glucose: 5g/L
2	AB buffer (20X): 50 ml/L (Autoclave separately) - K_2HPO_4 : 60g/L - $NaH_2PO_4.2H_2O$: 26g/L
3	AB salt (20X): 50 ml/L ((pH to 7 before autoclaving, autoclave separately) - NH_4Cl: 20g/L - $MgSO_4.7H_2O$: 6g/L - KCl: 3g/L - $CaCl_2.2H_2O$: 0.268g/L - $FeSO_4.7H_2O$: 0.05g/L

A.2 AAM Liquid Medium (Hiei, et al, 1994)

1. Sucrose	68.5	g/l
2. Glucose	36	g/l
3. Casamino acids	500	mg/l
4. AA Macro 10x	100	ml/l
5. AA Micro 1000x	1	ml/l
6. Fe_2EDTA (Iron) 100x	10	ml/l
7. AA Amino Acids 100x	10	ml/l
8. MS Vitamins 100x	10	ml/l

9. Adjust pH 5.2 before autoclave

AA Macro 10x Store at 4°C

KCl	29.50	g/l
$MgS0_4.7H_2O$	2.50	g/l
$CaCl_2.2H_2O$	1.50	g/l
$NaH_2PO_4.H_2O$	1.5	g/l
Substituting chemicals:		
$Ca\ Cl_2$	1.13	g/l
$MgS0_4$	1.2	g/l

AA Micro 1000x Store at 4°C

$MnS0_4.4H_20$	1000	mg/100mL
H_3BO_3	300	mg/100mL
$ZnS0_4.7H_20$	200	mg/100mL
KI	75	mg/100mL
$Na_2MoO_4.2H_2O$	25	mg/100mL
$CuSO_4.5H_2O$	2.5	mg/100mL
$CoCl_2.6H_2O$	2.5	mg/100mL

AA Amino Acids 100x

L-glutamine	8.76	g/100mL
L-aspartic acid	2.66	g/100mL
L-arginine	1.74	g/100mL
Glycine	0.075	g/100mL

MS Vitamins 100x (Murashige and Skoog, 1962) Store at 4°C

Myo-inositol	10	g/l
Nicotinic acid	50	mg/l
Pyridoxine-HCl	50	mg/l
Thiamine-HCl (10mg/mL)	1	ml/l
Glycine (100mg/mL)	20	µl/l
Substituting chemicals:		
Pyridoxime	41	mg/l

APPENDIX B

B.1 Mathematical model for relative quantification in real-time PCR (Pfaffl, 2001)

This method shows a mathematical model of relative expression quantification of a target gene in comparison to a reference gene. The relative expression ratio (R) of target gene is calculated based on the E and C(t) values of a sample versus a control, and expressed in comparison to a reference gene (equation). This is method has high accuracy and reproducibility (< 2.5% variation).

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{ref})^{\Delta Ct\ ref\ (control-sample)}}$$

$(E_{target})^{\Delta Ct\ target\ (control-treated)}$

E_{target} is the real-time PCR efficiency of the target gene transcript

E_{ref} is the real-time PCR efficiency of the reference gene transcript

$E= 10^{(-1/slope)}$ (Rasmussen, R., 2001)

Δ Ct target = C(t) of control – C(t) sample of the target gene transcript

Δ Ct ref = C(t) of control – C(t) sample of the reference gene transcript

In this research, the Actin gene was used as the reference gene and the target genes are *Os1bglu1, Os3bglu7, Os3bglu8, Os7bglu26* and *Os12blgu38*. The control samples were transgenic rice without DEX treatment and the samples were DEX-treated transgenic rice.

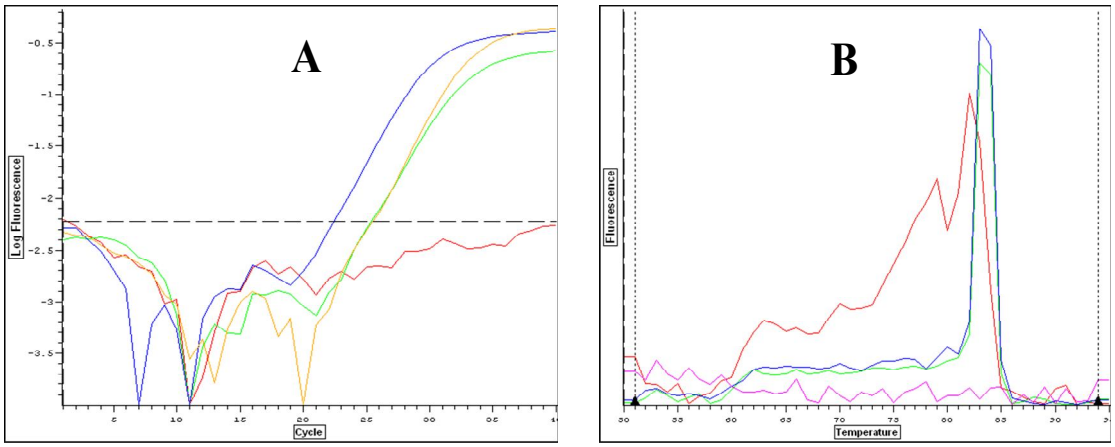

Figure 1. The amplification curve for *Actin* gene with different cDNA concentrations (A) and the melting curve of the PCR product of *Actin* gene (B) with different cDNA concentration.

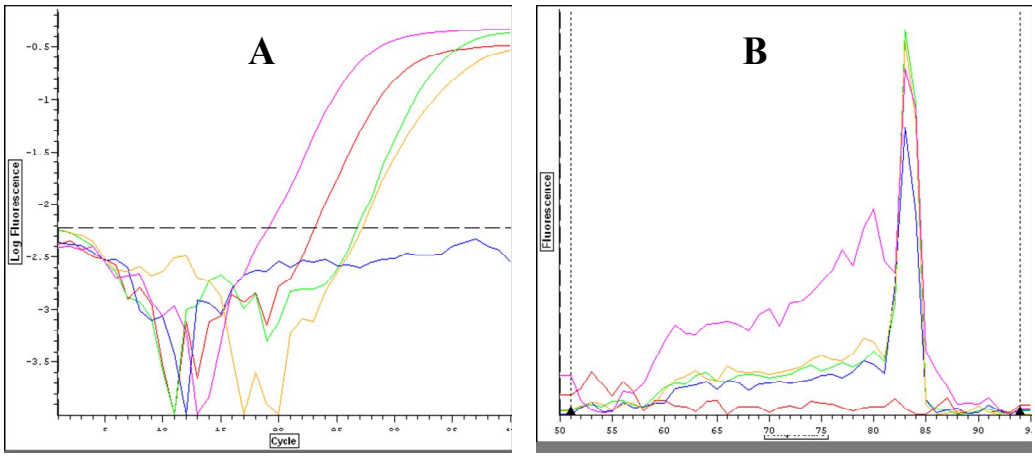

Figure 2. The amplification curve for *Os1bglu1* with different cDNA concentrations and the melting curve of the PCR product of *Os1bglu1* (B).

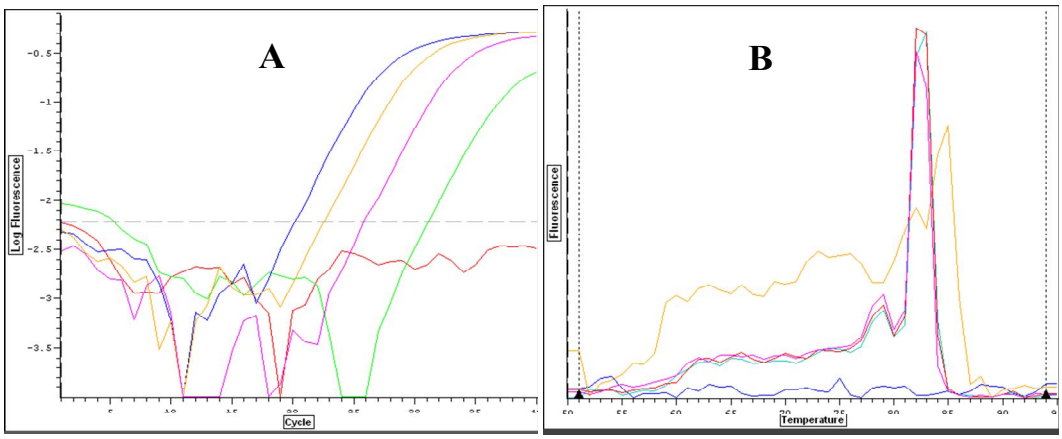

Figure 3. The amplification curve for *Os3bglu7* with different cDNA concentrations and the melting curve of PCR product of *Os3bglu7* (B).

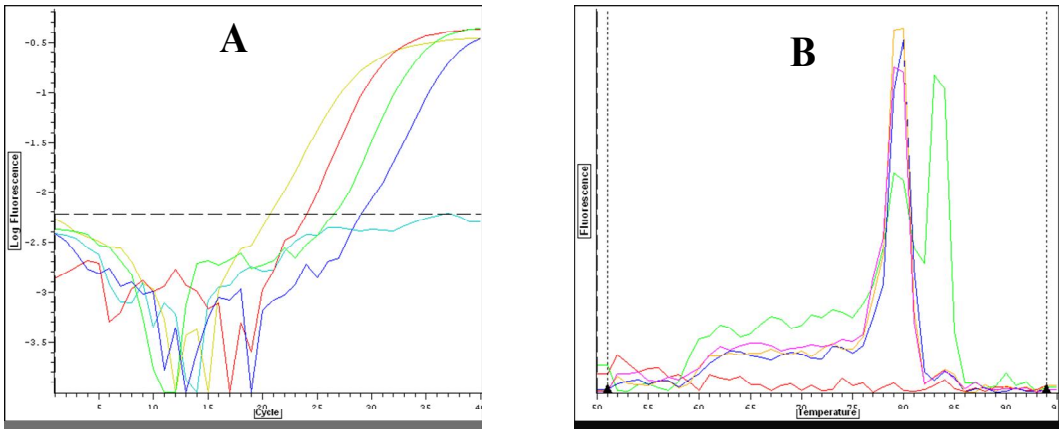

Figure 4. The amplification curve for *Os3bglu8* with different cDNA concentrations and the melting curve of the PCR product of *Os3bglu8* (B).

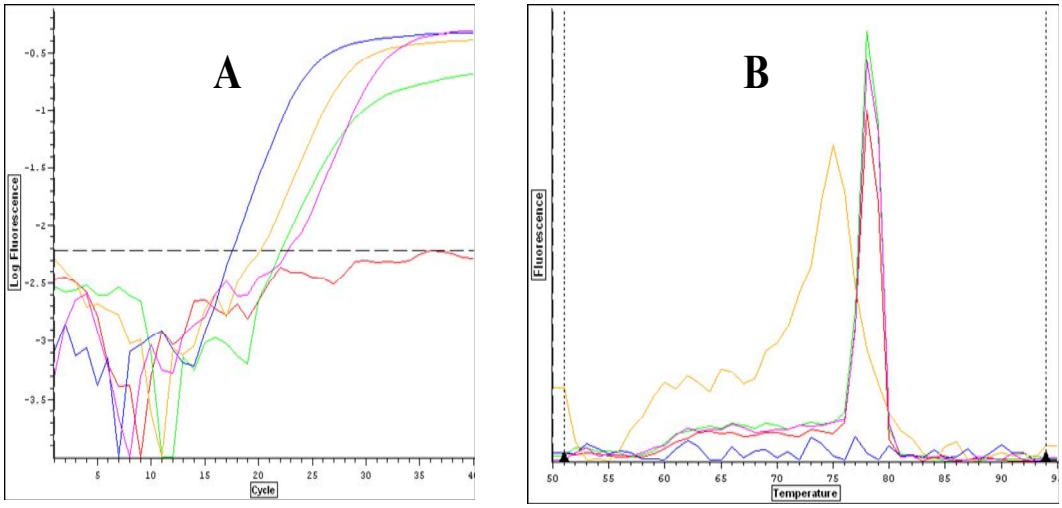

Figure 5. The amplification curve for *Os7bglu26* with different cDNA concentrations and the melting curve of the PCR product of *Os7bglu26* (B).

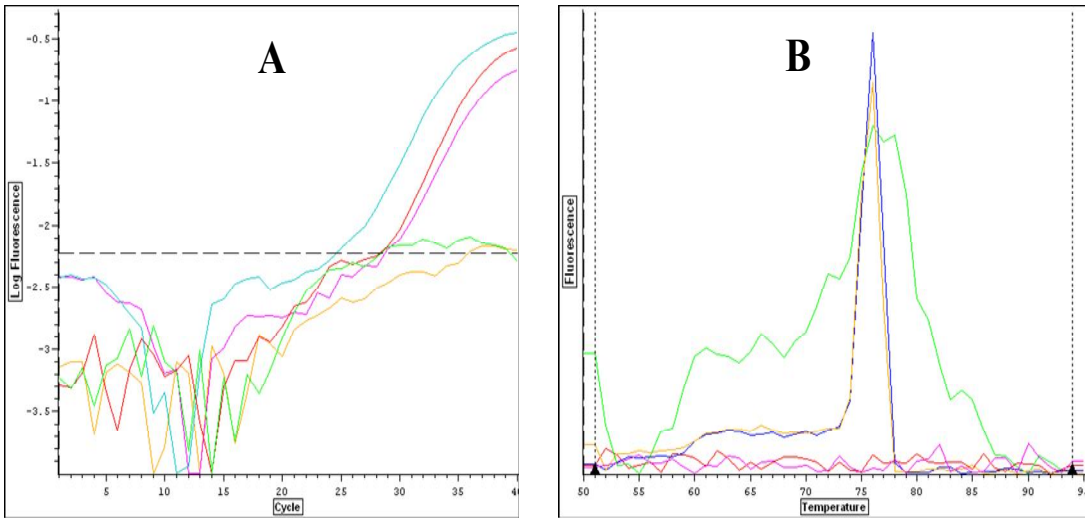

Figure 6. The amplification curve for *Os12bglu38* with different cDNA concentrations and the melting curve of the PCR product of *Os12bglu38* (B).

Table 1. The efficiency of amplification of target gene and reference gene.

Genes	Slop	E value (=$10^{(-1/slope)}$)
Actin (A)	-3.64	1.882
Os1bglu1 (B1)	-3.81	1.832
Os3bglu7 (B7)	-2.84	2.253
Os3bglu8 (B8)	-2.51	2.507
Os7bglu26 (B26)	-2.58	2.445

Table 2. Real-time PCR results of control plants.

C(t)	Control 1	Control 2	Average Control
A	22.33	30.16	26.25
B1	21.7	28.87	25.29
B7	21.31	28.09	24.70
B8	23.4	29.82	26.61
B26	23.67	28.15	25.91
B38	33.42	NA	NA

Table 3. Real-time PCR results of treated plants (samples).

C(t)	Sample 1	Sample 2	Sample 3	Average sample
A	27.73	21.6	21.20	23.51
B1	28.39	23.13	22.84	24.79
B7	29.42	22.11	21.04	24.19
B8	29.89	24.22	23.11	25.74
B26	27.75	23.16	21.89	24.27
B38	NA	NA	NA	NA

BIOGRAPHY

Miss Dang Thi Thanh Tam was born on January 17th, 1985 in Vinh City, Nghe An province, Viet Nam. She obtained her Bachelor degree in Biotechnology from Ha Noi University of Agriculture in 2007. After graduation, she worked as a young lecturer in the Plant Biotechnology Department, Biotechnology Faculty, Ha Noi University of Agriculture. During this time, she had the opportunity to practice skills in the field of molecular biology and plant biotechnology. In 2009, she received a scholarship to attend the Master Degree program from the SUT-Thai-European Union-French (SUT-TECF) project. Her research topic was "Studying the Genetic Function of Rice β-glucosidase via RNA Interference". The results from parts of this study have been presented at (1) the International Conference on Agriculture and Agro-Industry (ICAAI-2010; Food, Health and Trade), Mae Fah Luang University, Chiang Rai, 19-20 November, 2010; and (2) the 3rd SUT Graduate Conference 2010, Suranaree University of Technology, Nakhon Rachasima, 21-23 November, 2010.