# การสร้างโปรตีนสายผสมและการศึกษาหน้าที่ของบีตากลูโคซิเดส และเอกโซบีตากลูคาเนสจากข้าว

นางสาวบุษราคัม ป้อมทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี
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ปีการศึกษา 2550

# RECOMBINANT PROTEIN EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF β-GLUCOSIDASE AND EXO-β-GLUCANASE FROM RICE

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**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry**

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**RECOMBINANT PROTEIN EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF β-GLUCOSIDASE AND EXO-β-GLUCANASE FROM RICE**

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

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บุษราคัม ป้อมทอง : การสร้างโปรตีนสายผสมและการศึกษาหน้าที่ของบีตากลูโคซิเดสและเอกโซบีตากลูคาเนสจากข้าว (RECOMBINANT PROTEIN EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF β-GLUCOSIDASE AND EXO-β-GLUCANASE FROM RICE) อาจารย์ที่ปรึกษา : ผศ. ดร. รจนา โอภาสศิริ, 208 หน้า

สารพันธุกรรมที่บรรจุรหัสทางพันธุกรรม (cDNA) สำหรับยีนของบีตากลูโคซิเดสไอโซไซม์ Os4bglu12 และ GH5BG ซึ่งจัดไว้ในไกลโคซิลไฮโดรเลสกลุ่มที่1 และ 5 ตามลำดับ ได้ถูกเพิ่มปริมาณและวิเคราะห์ลำดับพันธุกรรม cDNA ของ Os4bglu12 และ GH5BG ต่างบรรจุรหัสสำหรับสังเคราะห์กรดอะมิโนจำนวน 510 ตัว การวิเคราะห์ลำดับกรดอะมิโนด้านปลายอะมิโนด้วยโปรแกรม PSORT คาดว่าโปรตีนทั้งสองนี้จะถูกส่งออกนอกเซลล์ โปรตีนสายผสมของเอนไซม์ Os4bglu12 และ GH5BG ซึ่งต่ออยู่กับโปรตีนไทโอรีดอกซินได้ถูกผลิตขึ้นใน *Escherichia coli* สายพันธุ์ Origami B(DE3) ในสภาพที่สามารถทำงานได้ เอนไซม์ Os4bglu12 สามารถย่อยสลายโอลิโกแซคคาไรด์ที่ประกอบด้วยกลูโคส 3-5 หน่วย เชื่อมต่อกันด้วยพันธะ β-(1,4) และไดแซคคาไรด์ที่ประกอบด้วยกลูโคส 2 หน่วย เชื่อมต่อกันด้วยพันธะ β-(1,3) ได้อย่างมีประสิทธิภาพ น้ำตาลไดแซคคาไรด์ที่ประกอบด้วยกลูโคส 2 หน่วย เชื่อมต่อกันด้วยพันธะ β-(1,4) สามารถยับยั้งการทำงานของเอนไซม์ Os4bglu12 บีตากลูโคซิเดสในการย่อยสับสเตรตดังกล่าว เมื่อพิจารณาจากลำดับกรดอะมิโนของเอนไซม์ GH5BG สามารถคาดคะเนได้ว่าเป็นเอนไซม์ที่มีสองโดเมนประกอบด้วย เอกโซบีตา-(1,3)กลูคาเนสโดเมนและโดเมนที่คล้ายกับฟาสซิน ซึ่งไม่ค่อยพบในเอนไซม์ของพืชทั่วไป ถึงแม้ GH5BG จะจัดเป็นเอนไซม์เอกโซบีตากลูคาเนสที่ย่อยพันธะ β-(1,3) ในบีตากลูโคซิเดส แต่การทำงานของเอนไซม์นี้ในการย่อยโอลิโกแซคคาไรด์กับคล้ายกับบีตากลูโคซิเดสที่อยู่ในไกลโคซิลไฮโดรเลสกลุ่มที่1 เอนไซม์ GH5BG สามารถย่อยสลายโอลิโกแซคคาไรด์ที่ประกอบด้วยกลูโคส 2-5 หน่วย เชื่อมต่อกันด้วยพันธะ β-(1,4) และไดแซคคาไรด์ที่ประกอบด้วยกลูโคส 2 หน่วย เชื่อมต่อกันด้วยพันธะ β-(1,3) ได้อย่างมีประสิทธิภาพ นอกจากนี้ทั้งเอนไซม์ Os4bglu12 และ GH5BG ยังสามารถย่อยพาราไนโตรฟีนอลไกลโคไซด์ได้หลายชนิด ซึ่งแสดงให้เห็นว่าบริเวณจับกับสับสเตรทตำแหน่งที่ -1 ของเอนไซม์ทั้งสองมีความจำเพาะต่อชนิดน้ำตาลที่เข้าจับต่ำ เอนไซม์ทั้งสองชนิดไม่สามารถย่อยกลูโคโอลิโกแซคคาไรด์และโพลิเมอร์ของกลูโคสที่มีกลูโคสต่อกันด้วยพันธะไกลโคซิดิกแบบ β-(1,3) และโพลิเมอร์ของกลูโคสที่มีพันธะแบบ β-(1,3),(1,4) ได้

ในการศึกษาครั้งนี้ประสบความสำเร็จในการถ่ายฝากยีนด้วยพาหะสี่ชนิด ได้แก่ pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12* และ pMDC139-*GH5BG* เข้าสู่ข้าวสายพันธุ์ Yukihikari ด้วยการใช้ *Agrobacterium* สายพันธุ์ EHA 105 คาดว่ามีต้นข้าวที่ได้รับการส่งถ่ายยีน

ด้วยพาหะ pMDC139-*Os4bglu12* จำนวน 4 ต้น และ pMDC139-*GH5BG* จำนวน 3 ต้น ซึ่งสามารถตรวจสอบได้จากการตรวจสอบด้วยการเพิ่มปริมาณของ 35 S โปรโมเตอร์ ด้วยวิธี PCR

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ลายมือชื่อนักศึกษา_____
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BUSARAKUM POMTHONG : RECOMBINANT PROTEIN EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF β-GLUCOSIDASE AND EXO-β-GLUCANASE FROM RICE. THESIS ADVISOR : ASST.PROF. RODJANA OPASSIRI, Ph.D. 208 PP.

β-GLUCOSIDASE/EXO-β-GLUCANASE/OLIGOSACCHARIDE/ RICE/ *AGROBACTERIUM*/TRANSFORMATION

The full-length cDNAs of a glycosyl hydrolase family (GH) 1 gene, *Os4bglu12* β-glucosidase, and a GH5 gene, *GH5BG* β-glucosidase, were cloned and characterized. The isolated *Os4bglu12* and *GH5BG* cDNAs each encoded a 510-amino-acid-long precursor protein that was predicted to be secreted outside the cells, as judged by the PSORT program. The recombinant thioredoxin-Os4bglu12 and -GH5BG fusion proteins were functionally expressed in *E. coli* strain Origami B(DE3). The recombinant Os4bglu12 enzyme efficiently hydrolyzed β-(1,4)-linked oligosaccharides of 3-6 glucose residues and β-(1,3)-linked disaccharide. Cellobiose could inhibit Os4bglu12 enzyme in the hydrolysis of cellooligosaccharides and laminaribiose. The mature protein of GH5BG contains two major domains, a β-1,3-exoglucanase-like domain and a fascin-like domain, which is not commonly found in plant enzymes. GH5BG was designated a putative glucan exo-β-(1,3)-glucosidase based on sequence homology, however, its catalytic activity is somewhat like GH1 β-glucosidases, which show similar oligosaccharide preferences. The GH5BG could hydrolyzed β-(1,4)-linked oligosaccharides of 2-6 glucose residues and β-(1,3)-linked disaccharide. Both Os4bglu12 and GH5BG also hydrolyzed many kinds

of *p*NP-β-glycosides which indicates the low stringency at the -1 subsite of the enzyme. Hydrolysis of β-(1,3)-linked oligosaccharides with DP more than 2, laminarin and 1,3, 1,4-β-glucans by both enzymes could not be detected.

Successful *Agrobacterium*-mediated transformation was obtained in Yukihikari calli with 4 different plasmids, pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. Putative transgenic rice plants could be regenerated and specific PCR amplification of the 35S promoter indicated that 4 transgenic pMDC139-*Os4blu12* and 3 transgenic pMDC139-*GH5BG* plants were true transgenic rice plants.

School of Biochemistry
Academic Year 2007

Student's Signature_____
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Co-advisor's Signature_____

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Absorbance |
| bp | Base pairs |
| BSA | Bovine Serum Albumin |
| °C | Degrees Celsius |
| cDNA | Complementary deoxynucleic acid |
| DNA | Deoxyribonucleic acid |
| g | Gravitational acceleration |
| (m, μ)g | (milli, micro) Gram |
| hr | Hour |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| kb | Kilo base pairs |
| kDa | Kilo Dalton |
| LB | Lysogeny broth |
| (m, μ) L | (milli, micro) Liter |
| (m, μ)M | (milli, micro) Molar |
| min | Minute |
| (μ)mol | (micro) Mole |
| mRNA | Messenger ribonucleic acid |
| Mr | Molecular weight |
| OD | Optical density |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| *p*NP | *p*-Nitrophenol |
| *p*NPG | *p*-Nitrophenyl-β-D-glucoside |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| SDS | Sodium dodecyl sulfate |
| Sec | Second |
| SSC | Saline sodium citrate |
| TEMED | Tetramethylenediamine |
| Tris | Tris-(hydroxymethyl)-aminoethane |
| UV | Ultraviolet |
| v/v | Volume/volume |
| w/v | Weight/volume |

# CHAPTER I

# INTRODUCTION

## 1.1 Introduction

Glycosyl hydrolases (GH; EC 3.2.1-3.2.3) are a widespread group of enzymes that catalyze the hydrolysis of glycosidic bonds between sugars and other moieties. GH intervene in many essential steps of life, such as hydrolysis of structural or storage polysaccharides, defense against pathogens, turnover of cell surface carbohydrates, etc. GH have been grouped and classified into more than 100 families families based on amino acid sequence similarities (Henrissat and Bairoch, 1993, 1996; Henrissat and Davies, 1997) (for up-to-date information see Carbohydrate-Active Enzymes database (CAZY) at http://www.cazy.org /CAZY/index.html). The three-dimensional structures of the enzymes recently been found to be more strongly conserved than the sequence. It is possible that several GH families share similar folds (Henrissat *et al*., 1995). Based on their 3-dimensional structures, GH can be grouped into clans of related structures (Henrissat and Davies, 1997). Clan A is the largest group and contains 17 families, the structures of which contain a core $(\beta/\alpha)_8$ barrel with two catalytic amino acid residues, an acid/base and a nucleophile, on the ends of strands 4 and 7 of the barrel, respectively (Jenkins *et al*., 1995; Henrissat *et al*., 1995; Coutinho and Henrissat, 1999).

β-Glucosidases (EC 3.2.1.21) that have been studied are the members of GH families 1 and 3 (Henrissat and Bairoch, 1993, 1996) which are classified into clan A β-Glucosidases are found widely in all types of organisms (bacteria, archaea, and eukaryote), and play important roles in fundamental biological processes (Esen, 1993). Recently, forty rice genes homologous to GH 1 β-glucosidase have been identified from Rice Genome Databases and at least 31 of these appear to be expressed in many tissues and plant developmental stages (Opassiri *et al*., 2006). This suggests their general importance to plants. Although the occurrence of β-glucosidases in rice may correlate with growth and development, fundamental information about their structure, physiological function, their natural substrate and the regulation of their expression is lacking.

GH family 5 (GH5) is one clan A family, originally identified as cellulase family A (Gilkes *et al*., 1991), that contains enzymes with a wide range of catalytic activities, including cellulases, chitosanases, endoglucanases, exoglucanases, exoxylanases, endoxylanases, β-mannanases, and endoglycoceramidase. The GH5 enzymes that have been investigated are primarily from microorganisms (Tsai *et al*., 2003; Tanabe *et al*., 2003; Reinhold-Hurek *et al*., 2006; Cutfield *et al*., 1999; Mitreva-Dautova *et al*., 2006; Perret *et al*., 2004; Caines *et al*., 2007), though a β-mannanase from a plant was recently described (Hrmova *et al*., 2006). There has been no previous report of characterization of a GH5 exoglucanase from plant. However, several genes encoding proteins similar to fungal exo-1,3-β-glucanses are found in the genomic sequences from rice (Opassiri *et al*., 2007).

## 1.2 Significance of the study

The vast number of GH1 β-glucosidases and GH5 exo-β-glucanases are only just being explored by genomic sequences in rice. However, a small number of these enzymes have been studied. A comprehensive approach is needed to study rice β-glucosidases and β-glucanases in order to associate each of them with the physiological role that it plays in the rice, and to determine the precise substrate specificity in relation to the enzyme sequences and structures. The understanding of the biochemistry and mechanistic fundamentals of these enzymes can provide the knowledge base, which could be used to apply the enzymes for the agronomic benefit, such as conversion of cellulosic biomass, an inexpensive renewable resource, into sugar derived high-value products.

## 1.3 Research objectives

1. To functionally express recombinant rice β-glucosidase (GH1-Os4bglu12) and exo-β-glucanase (GH5BG) in *Escherichia coli*.
2. To purify the recombinant GH1-Os4bglu12 and GH5BG in order to characterize the activities of specific enzyme in terms of substrate-specificity and other important biochemical properties.
3. To study kinetics parameters of the recombinant rice GH1-Os4bglu12 and GH5BG for hydrolysis of synthetic and natural substrates.
4. To develop GH1-Os4bglu12 and GH5BG overexpressing transgenic rice in order to study the biological functions of these enzymes.

## 1.4 Scope and limitations of the study

The recombinant rice β-glucosidase (GH1-Os4bglu12) and exo-β-glucanase (GH5BG) were expressed in *E. coli*. Biochemical characteristics, such as optimal pH and temperature, substrate specificity against the artificial and natural substrates, and the enzyme activity inhibition were studied. Transformation of rice for overexpressing of GH1-Os4blue12 and GH5BG cDNAs in one or two cultivars of rice were preliminarily studied.

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# CHAPTER II

# LITERATURE REVIEW

## 2.1 β-glucosidases

### 2.1.1 Overview of β-glucosidases

β-glucosidases (3.2.1.21) are glycosyl hydrolases which hydrolyze the β-O-glycosidic bond at the anomeric carbon of glucose moiety at the nonreducing end of a carbohydrate or glycoside molecule. These enzymes are found in many kinds of organisms, including fungi, bacteria, animals and plants, indicating their general importance to life. β-glucosidases have a variety of functions in different organisms, such as biomass conversion in microorganisms (Fowler, 1993), activation of defense compounds (Poulton, 1990; Duroux *et al*., 1998), phytohormones (Brzobohaty *et al*., 1993; Falk and Rask, 1995), lignin precursors (Dharmawardhana *et al*., 1995), aromatic volatiles (Mizutani *et al*., 2002), and metabolic intermediates by releasing glucose blocking groups from the inactive glucosides in plants (Barleben *et al*., 2005). To achieve specificity for these various functions, they must bind to a wide variety of aglycones, in addition to the glucose of the substrate.

β-Glucosidases that have been studied are the members of glycosyl hydrolase (GH) families 1 and 3 (EC 3.2.1-3.2.3) (Henrissat and Bairoch, 1993, 1996). The most of β-Glucosidases in both families present the same tertiary structures which are $(\beta/\alpha)_8$ barrel. In GH family 1, the two glutamic acid residues which serve as catalytic amino acids are located in the highly conserved peptide

motifs Thr-Phe-Asn-Glu-Pro (TFNEP) and Ile-Thr-Glu-Asn-Gly (ITENG) of β-stands 4 and 7, respectively. These amino acid residues are important in catalysis and make up part of a crater-shaped active site (Czjzek *et al*., 2000; Henrissat *et al*., 1995). The substrate binding site lies in a funnel-shaped pocket on the C-terminal end of the β-barrel, and residues found in the region dictate the substrate-specificity of the enzyme (Henrissat *et al*., 1995; Burmeister *et al*., 1997; Czjzek *et al*., 2000; 2001).

Although the enzymes in a family have similar structures and the same stereospecificity and the same mechanism of glycoside hydrolysis, they may have different specificities and modes of the action (Warren *et al*., 2000). The fundamental substrate specificity of these enzymes depends on the conformation of the active site amino acids that are important for the substrate recognition and binding, as well as the structure of the aglycone and glycone moieties of the substrate (Czjzek *et al.*, 2000; Verdoucq *et al*., 2004).

### 2.1.2 Catalytic mechanism of β-glucosidases

β-glycosidases and other glycosidases can be divided into two classes, inverting and retaining glycosidases, by whether the anomeric configuration of the released monosaccharide or oligosaccharide is inverted or retained compared to the substrate (McCarter and Withers, 1994). The mechanisms of both enzymes differ in that inverting glycosidases catalyze via a direct displacement of the aglycone (leaving group) by water (which acts as a nucleophile), whereas retaining glycosidases catalyze through a glycosylenzyme intermediate in a double displacement mechanism.

Catalysis by retaining β-glucosidases proceeds via a two step double displacement mechanism involving the formation and hydrolysis of a covalent

glycosyl enzyme intermediate, both steps again proceeding through oxocarbenium ion-like transition states (Esen, 1993). As shown in figure 2.1, the retaining glycosidases have a pair of conserved carboxylic amino acids, which may be aspartate (Asp) or glutamate (Glu) (McCarter and Withers, 1994). Two active site carboxylic acids residues are involved in this mechanism with different roles. One residue acts as the nucleophile by attacking at the sugar anomeric center to form the glycosyl-enzyme species, the other residue acts as an acid/base catalyst, protonating the glycosidic oxygen in the first step (general acid catalysis) and deprotonating the water in the second step (general base catalysis) (Esen, 1993).

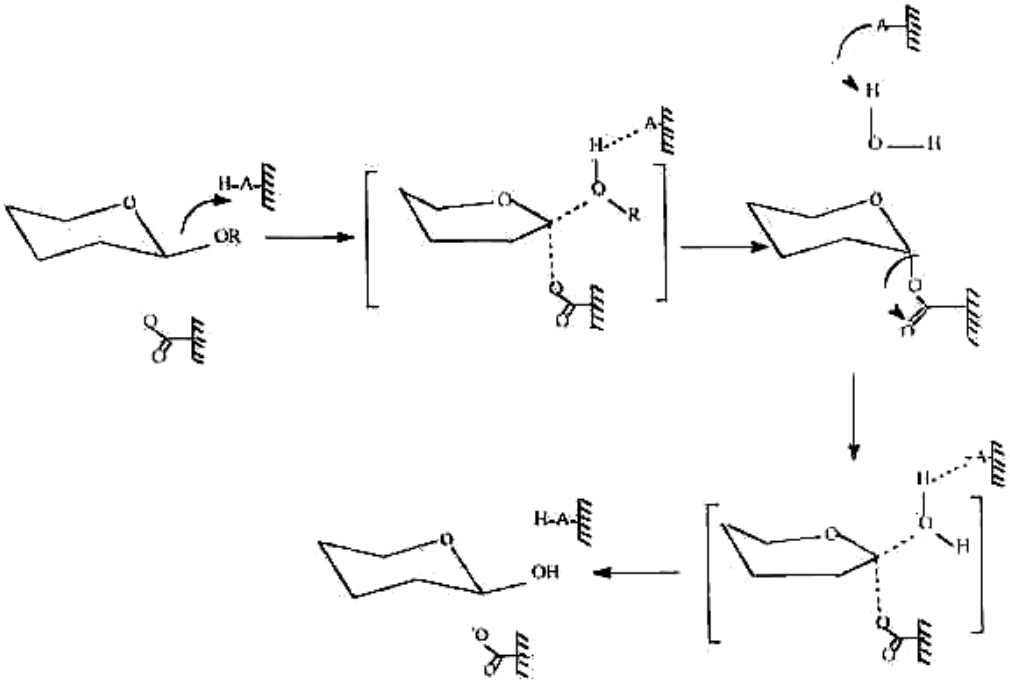


**Figure 2.1** Generally accepted endocyclic pathway of the double displacement mechanism proposed for retaining β-glucosidases (Esen, 1993).

### 2.1.3 Plants β-glucosidases

Based on a large number of reports about β-glucosidases and their glycoside substrates, the physiological functions of these enzyme and substrate combinations based on the activities of the aglycone moieties of substrate include 1) defense against pathogens and herbivores (Poulton, 1990; Dakora and Phillips, 1996; Mizutani *et al*., 2002), 2) phytohormone activation (Smith and van Staden, 1978; Zouhar, 1999; Schliemann, 1984), 3) lignification (Dharmawardhana *et al*., 1995), 4) cell wall catabolism (Leah *et al*., 1995; Akiyama *et al.*, 1998; Opassiri *et al*., 2003), and 5) release of active intermediate molecules (Barleben *et al*., 2005; Esen, 1993).

GH family 1 (GH1) contains a wide range of β-glycosidases, including β-galactosidases, β-mannosidases, phospho-β-galactosidases, phospho-β-glucosidases, and thioglucosidases, in addition to β-glucosidases. Plant GH1 β-glucosidases tend to show high specificity for their aglycones, though many hydrolyze synthetic, nonphysiological substrates, like *p*-nitrophenol (*p*NP)-β-glycosides (Esen, 1993). The aglycones span a wide range of structures, including sugars (Leah *et al*., 1995; Akiyama *et al*., 1998; Opassiri *et al*., 2004), hydroxaminic acids (Babcock and Esen, 1994), isoflavanoids (Hösel and Barz, 1975; Chuankhayan *et al*., 2005), rotenoids (Svasti *et al*., 1999), alkaloids (Geerlings *et al*., 2000; Warzecha *et al*., 2000) hydroxyquinones (Duroux *et al*., 1998), and cyanogenic nitriles (Poulton, 1990), etc., and it is the specificity for these aglycones which is thought to specify the function of most of these enzymes (Esen, 1993). Since many β-glucosidase function in plants, it is important that these enzymes specifically hydrolyze their own substrates and not other substrates with which they may come into contact. It seems evident that the substrate specificity, localization of the enzymes with respect to

potential substrates, and the activities of the substrates and products will determine the roles of these enzymes.

β-glucosidases have been isolated and characterized at the biochemical and DNA levels in many species of plants, both from dicots and monocots. Most β-glucosidases isolated from dicots so far, including *Trifolium repens* (Kakes, 1985), *Brassica napus* (Hoglund *et al*., 1992) and black cherry (Poulton and Li, 1994) are localized in the cell wall or vacuole, while the enzymes isolated from *Polygonum tinctorium* are localized in the plastid (Minami *et al*., 1997). In addition, most dicot β-glucosidases have been shown to be glycosylated (Esen, 1993). Initially, most β-glucosidases isolated from monocots, including sorghum (Thayer and Conn, 1981), oat (Nisius, 1988) and maize (Esen, 1992) were localized in the plastid. Later, several monocot β-glucosidases, including the endosperm specific β-glucosidase from barley and two β-glucosidases found in germinating rice, were found to have signal peptides for endoplasmic reticulum (ER) targeting (Leah *et al*., 1995; Opassiri *et al*., 2003) and another was found to localize to rice cell walls (Akiyama *et al*., 1998).

Comparison of amino acid sequences of plant β-glucosidases has shown that their similarity ranged from 35-90% depending, in some cases, on evolutionary distances among taxa. For instance, the deduced amino acid sequence of maize β-glucosidase shows 70% identity with dhurrinase from sorghum (Cicek and Esen, 1998), 55% identity with oat, and 47% identity with *Prunus serotina*, respectively. However, the similarity between the sequences of plant β-glucosidases cannot be used to infer the specific functions of enzymes, because only small sequence differences affect substrate specificity of the enzymes (Esen, 1993).

### 2.1.4 Rice β-glucosidases

With the completion of high quality drafts of the rice genome, a thorough analysis of glycosyl hydrolase family 1 can be conducted in rice. Opassiri *et al.* (2006) identified forty β-glucosidase genes in the rice genome by a bioinformatic approach, and the gene structures and protein gene products were predicted. Phylogenetic analysis revealed that many members that contain closely related sequences are located on the same chromosome and clustered in the same subfamilies. At least 31 family 1 genes are expressed in a broad range of tissues in various stages of rice, based on the sources cDNA and EST sequences in public databases. The source libraries for rice β-glucosidase gene matching ESTs included seedling (shoot and root), immature plant parts (stem, root, leaf), mature leaf, panicle at flowering and ripening stages, immature seeds, and callus, reflecting its expression in many tissues during plant development. Some rice β-glucosidase genes have ESTs represented in stressed plant tissue libraries, including salt, drought, cold, and blast/fungus infection.

To date, only a few rice β-glucosidase isozymes have been characterized for the possible function. The first experiment of rice β-glucosidase activity against the synthetic substrate *p*NP-β-D-glucoside was reports by Palmiano and Juliano (1973). Partially purified β-glucosidases from rice were described that hydrolyze both gibberillin glucosides and pyridoxine glucosides (Schlieman, 1984; Iwami and Yasumoto, 1986). Menegus *et al.* (1995) reported the isolation of the derivative of pantoic acid, *R(-)* pantoyllactone-β-D-glucopyranoside from rice seedlings and a β-glucosidase that could hydrolyze this compounds.

Thorough studies of rice β-glucosidases have been described for a cell wall-bound β-glucosidase and BGlu1 and BGlu2 β-glucosidases cloned from rice seedlings (Akiyama *et al*., 1998; Opassiri *et al*., 2003; 2004). Akiyama *et al*. (1998) determined the N-terminal sequence of the cell wall-bound β-glucosidase. This enzyme showed high hydrolytic activity against cello- and laminari-oligosaccharides. Opassiri *et al.* (2003) reported the cloning and characterization of expression for two rice β-glucosidase genes, *bglu1* and *bglu2*. Both genes were highly expressed in germinating shoots, but the *bglu1* gene was also highly expressed in flower. The *bglu1* cDNA was used to express a thioredoxin-β-glucosidase fusion protein, designated BGlu1, which was purified and characterized. The enzyme could hydrolyze a variety of *p*-nitrophenyl β-D-glycosides, demonstrating some flexibility in sugar binding. It also hydrolyzed a variety of natural glycosides at low levels and showed strong hydrolysis and glucotransferase activity with β-1,3- and β-1,4-linked glucooligosaccharides.

Using microarrays, the transcript of the EST contig BE607353 and BG101702, whose sequences are homologous to rice *bglu1* and GenBank indica rice entry *Os4bglu12* β-glucosidase genes, respectively, were upregulated in response to high salinity stress in salt-tolerant rice (var Pokkali), but not in the salt-sensitive cultivar IR29 (Kawasaki *et al*., 2001). Results from subtractive hybridization cDNA library screening indicated that the transcript level of the EST contig BPHiw028, corresponding to *Os4bglu12*, was upregulated in response to brown planthopper (Wang *et al.*, 2005). The presence of tricin-O-glucoside, a probing stimulant for planthopper (Adjei-Afriyie, *et al*., 2000), might imply a role of this enzyme in releasing an active flavonol for defense. However, most of these studies did not

indicate the roles of these enzymes in rice cells in response to such stresses. So, identification of natural substrate for the enzymes still remains.

## 2.2 β-glucanases

### 2.2.1 Overview of β-glucanases

β-glucanases or glucan-β-glucosidases produced by a wide variety of plants and microorganisms that catalyze the first step in the hydrolysis of plant polymers such as cellulose and other β-glucans. The primary substrates of the plant β-glucanases are β-(1,3)-, β-(1,4)-, β-(1-3),(1-4)- and β-(1,3),(1,6)-glucans found in the cell walls of certain plant tissues as well as plant fungal pathogens (Stone and Clarke, 1992; Akiyama and Pillai, 2001). There are 2 types of β-glucanases: endo-glucanases and exo-glucanases. The endo-glucanases cleave cellulose internally to generate oligosaccharides. The exo-glucanase cleavage yields glucose from the oligosaccharides produced by the endoglucanase (Huber and Nevins, 1981).

Plant β-glucanases belong to several subfamilies of GH families that occur widely in many plant species (Simmons, 1994). In plants, β-glucanase gene expression is regulated by certain environmental stresses and plant hormones (Thalmair *et al*., 1996; Akiyama and Pillai, 2001). It was found out that abscisic acid (ABA) treatment of tobacco seeds inhibited the induction of β-glucanase gene in the micropylar endosperm and greatly delayed endosperm rupture during germination (Leubner *et al*., 1995). β-glucanases also plays an indirect role in plant defense by releasing oligosaccharide elicitors from cell walls of fungal pathogens, which induce production of anti-fungal substances (Yamaguchi *et al*., 2000).

Recently, the vast number of β-glucanases only just being explored by genomic sequences in rice. Analysis of gene sequence in rice genome database revealed that there are at least 120 β-glucanases, which are classified into 8 GH families. Among these subgroups, the endo-glucanases primarily belong to GH families 9, 10, 16, 17, 51 and 81, while the exo-glucanases belong to GH families 3 and 5. A small number of rice β-glucanases have been studied, and most of which belong to the enzymes in GH families 3 and 17 (Thomas *et al*., 2000; Yamaguchi *et al.*, 2002; Nishizawa *et al*., 2003; Zheng *et al*., 2003; Akiyama *et al*., 2004).

### 2.2.2 Distinct substrate preferences of β-glucanases

Distinct substrate preferences of β-glucanase isozymes have been documented in a number of studies (Hrmova and Fincher, 2001a; 2001b). Hrmova and Fincher (2001a) compared the protein structures of plant β-glucanases and explained how their structures can be related to the substrate specificity. The differences of substrate specificity between endo-glucanases and exo-glucanases is derived from the shape of their binding sites. Endo-glucanases usually have substrate binding grooves or depressions that extend across their surface. The endo-glucanases can bind anywhere along the polymeric substrate and hydrolyze internal linkages by the catalytic amino acid residues which located in the substrate-binding cleft. In contrast, an exo-glucanase has a substrate-binding site in the shape of a dead-end tunnel, slot, or funnel shape. Substrate specificity depends on fit binding of the substrates into the substrate-binding pockets that contact the catalytic amino acid residues at the bottom of funnel (Hrmova and Fincher, 2001a; 2001b). This structural

analysis indicated the impact of β-glucanase structures on their difference in substrate specificity.

## 2.3 Glycosyl hydrolase family 5

GH family 5 (GH5) is one clan A family, originally identified as cellulase family A (Gilkes *et al*., 1991). The structures of members in GH5 contain a core $(\beta/\alpha)_8$ barrel with two catalytic amino acid residues, an acid/base and a nucleophile, on the ends of strands 4 and 7 of the barrel, respectively (Cutfield *et al*., 1999). Although these enzymes may have similar $(\beta/\alpha)_8$ barrel structures, differences in the loops at the ends of the β-strands of this barrel result in active site clefts ranging from long grooves to slot-like pockets (Cutfield *et al*., 1999; Dominguez *et al*., 1995).

GH5 contains enzymes with a wide range of catalytic activities, including cellulases, chitosanases, endoglucanases, exoglucanases, exoxylanases, endoxylanases, β-mannanases, and endoglycoceramidase (Tsai *et al*., 2003; Tanabe *et al*., 2003; Reinhold-Hurek *et al*., 2006; Cutfield *et al*., 1999; Mitreva-Dautova *et al*., 2006; Perret *et al*., 2004; Caines *et al*., 2007; Hrmova *et al*., 2006). Since the variation in the protein sequences is high, the endoglucanse families were split into 6 subfamilies, A1-A6 (Béguin, 1990; Lo Leggio *et al*., 1997), and 2 more subfamilies of mannanases, A7 and A8, were subsequently added (Hilge *et al*., 1998) with sequence identity between subfamilies generally being 20% or less. The exo-β-(1,3)-glucanases, have been noted to form a distinct subfamily, though no number was designated (Cutfield *et al*., 1999). Despite the high variation in the protein sequences and enzyme activities of the family members, they all possess eight conserved residues (including two glutamate residues acting as catalytic acid/base and

nucleophile) around the active site, which distinguish GH5 from other GH families (Cutfield *et al*., 1999; Sakon *et al*., 1996).

There are now twenty-two known GH5 3-D crystal structures: endoglucanases from *Acidothermus cellulolyticus*, *Bacillus agaradhaerens*, *Bacillus* sp., *Clostridium cellulyticum*, *Clostridium thermocellum, Erwinia chrysanthemi*, *Pseudoalteromonas haloplanktis*, *Thermobifida fusca*, and *Thermoascus aurantiacus*; exo-β-(1,3)-glucanases (Exg) from *C. albicans* and *Saccharomyces cervisiae*; mannanases from *Bacillus* sp., *Cellvibrio mixtus*, *T. fusca*, *Thermotoga maritim*a, *Hypocrea jecorina*, *Solanum lycopericum* and *Mytilus edulis*; xylanase from *H. jecorina* and *E. chrysanthemi*; xyloglucanase from *Paenibacillus pabuli*; and endoglycoceramidase from *Rhodococcus* sp. (Coutinho and Henrissat, 1999; http://www.CAZY.org/ CAZY/index. html). This abundance of structural data is necessary, since the similar overall structure of GH5 has resulted in several distinct activities, as indicated (Opassiri *et al*., 2007).

## 2.4 Plant gene transformation

The rapid development of biotechnology particularly plant genetic engineering offers an alternative approach for the control of plant diseases and plants pests. Genetic engineering offers an advantage over conventional breeding in a way that only one or two characters will be introduced into crop species. Tissue culture and gene transformation techniques has become important way to study gene functions, achieve genetic modification and improve plant breeding programs.

### 2.4.1 *Agrobacterium* mediated transformation

*Agobacterium tumefaciens* is exploited by many plant biologists in molecular and genetic studies to introduce DNA into plants. *Agrobacterium* carries three genetic components which are required for plant cell transformation, 1) the transferred DNA (T-DNA) on the Ti (tumor-inducing) plasmid that is transferred from *Agrobacterium* to the plant cell; 2) the virulence (*vir*) region on the Ti plasmid that is essential for the efficiency of plant cell transformation (Figure 2.2) (Binns and Thomashow, 1988); 3) the virulence loci resides in the *Agrobacteriutn* chromosome, *chvA* and *chvB* and *pscA*, which encode products involved in the binding of *Agrobacterium* to plant cells during the infection process (Zambryski, 1988).

During infection by *Agrobacterium,* any DNA between the 25-bp direct repeats that flank the T-DNA will be transferred to a plant cell (Walden, 1993). The processing and transfer of T-DNA are mediated by products encoded by the *vir* region the expression of which are tightly regulated (Stachel and Nester, 1986). The *vir* region is organized into six complementation groups, *virA, virB, virD* and *virG, virC* and *virE*. Control of gene expression is mediated by the VirA and VirG proteins, a two-component regulatory system. VirA detects the small phenolic compounds released by wounded plants resulting in autophosphorylation (Figure 2.2, step 1). VirA phosphoryloation of VirG then leads to activation of *vir* gene transcription (Winans, 1992).

Following *vir* gene induction, the production of a transfer intermediate begins with the generation of single strand copy of the T-DNA by VirDl and VirD2 (Stachel *et al.*, 1986). VirDl / VirD2 recognize the 25-bp border sequence and produce a single strand endonucleolytic cleavage in the bottom strand of each border

(Figure 2.2, step 2). These nicks are used as the initiation and termination sites for T-strand production. VirD2 remains tightly associated with the 5' end of the T-strand. (Zupan and Zambryski, 1995).

The T-strand must travel through numerous membranes and cellular spaces before arrival in the plant nucleus. Thus, to preserve its integrity, it was hypothesized that the T-strand likely travels as a single strand DNA-protein complex with VirE2 which might help prevent the degradation by nucleases (Figure 2.2, step 3). Finally, binding of VirE2 unfolds and extends single strand DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T-strand along with VirD2 and VirE2 are termed the T-complex (Zupan and Zambryski, 1995).

Subsequently, the T-complex must exit the bacterial cell (Figure 2.2, step 4) passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane (Figure 2.2, step 5). Once inside the plant cell, the T-complex targets itself to the plant cell nucleus and crosses the nuclear membrane (Figure 2.2, step 6), after which the T-strand becomes integrated into a plant chromosome (Figure 2.2, step 7).

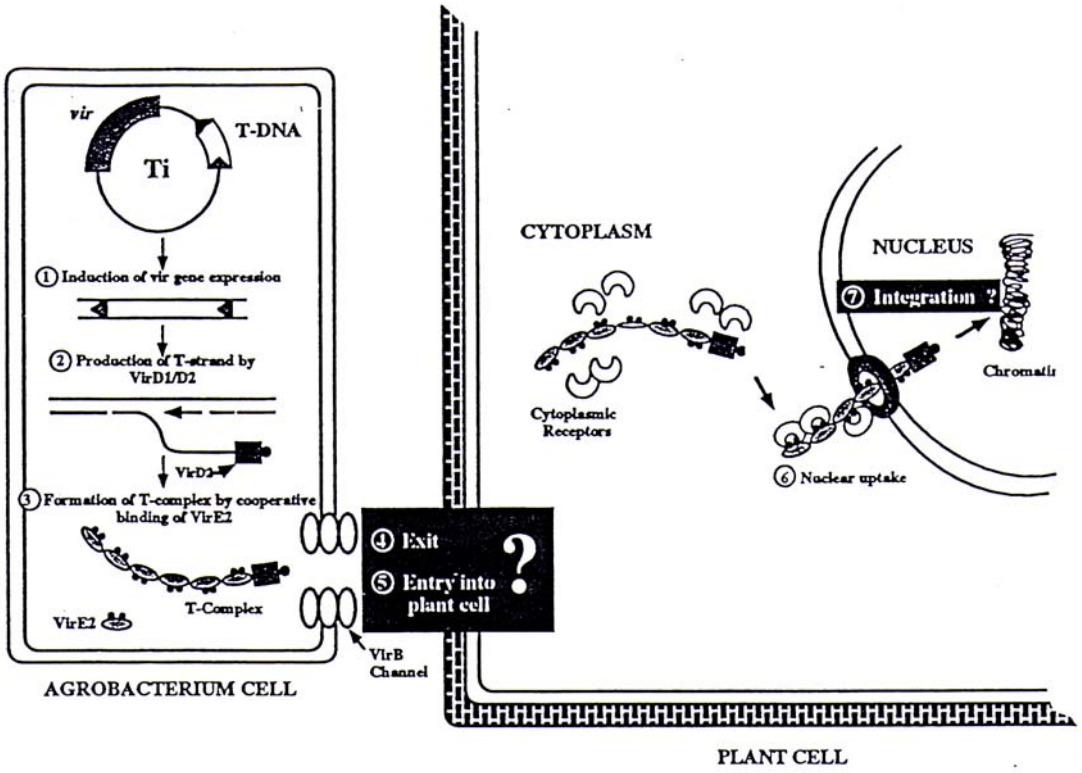


**Figure 2.2** A diagram that illustrates relevant genes and events that occur in *Agrobacterium* during the transformation process (Zupan and Zambryski, 1995).

### 2.4.2 Selectable marker genes

Selectable markers are used to select for the specific growth of transformed cells amongst a background of non-transformed individuals. Such markers allow growth, or at least viability, in the presence of the selective agent. Routinely, resistance to antibiotics or phytotoxins, such as herbicides, has been used. In the former case, antibiotic resistance genes derived from bacteria have been utilized (e.g. kanamycin and hygromycin), whereas in the latter case genes encoding products which are more tolerant to herbicides, for example glyphosate and phosphinothricin have been used (Walden, 1993).

The gene *hpt* (or *hph)* was isolated from *E. coli.* It codes for the enzyme hygromycin phosphotransferase. This gene therefore causes resistance to the antibiotic compound hygromycin. The *hpt/*hygromycin B combination was successfully employed in the genetic transformations of tobacco, *Arabidopsis,* maize and rice (Schrott *et al*., 1995). Hygromycin allows clear discrimination between transformed and non-transformed tissues and problems with albinos or the ferility of regenerants have not been reported (Ayres *et al*., 1994).

### 2.4.3 Reporter genes in plants

Upon expression in the transgenic plant, reporter genes produce gene products that provide a clear indication that genetic transformation did take place (Galun and Breiman, 1997). The product of the reaction catalyzed by the reporter gene product should be stable, easily detectable, and quantifiable (Crazzolara *et al*., 1995). The four most widely used systems of reporter genes are chloramphenicol acetyltransferase (CAT), luciferase, β-glucuronidase (GUS) and Green fluorescent protein (GFP).

GUS is encoded by the *Eschetichia coli uidA* gene (Jefferson *et al*., 1986; 1987). The protein has a molecular weight of 68.2 kDa. The best substrate currently available for histochemical localization of β-glucuronidase activity in tissues and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The indolyl derivative produced from glucuronidase activity on X-Gluc must undergo an oxidative dimerization to form an insoluble and highly colored indigo dye which can be stimulated by atmospheric oxygen, or using an oxidation catalyst such as a potassium ferricyanide/ferrocyanide mixture. However, the substrate for dectection of GUS

activity (X-Gluc) is expensive and the X-Gluc-stained plant material is killed by the GUS assay, so it not a vital staining (Jefferson *et al*., 1986; 1987).

Green fluorescent proteins are a unique class of proteins also involved in the bioluminescence of many jellyfish (Gilroy, 1997). The GFP from *Aequarea victoria* is a 26.9-kDa protein (238 amino acid) that fluoresces green upon excitation with blue light (Prasher *et al*., 1992). Native GFP from *A. victoria* absorbs optimally at 395 nm (with a weaker absorbance at 470 nm) and emits at 509 nm (Gilroy, 1997).

### 2.4.4 Rice transformation mediated *Agrobacterium*

Systems of *Agrobacterium*-mediated transformation have been well established for many dicotyledonous plants. However, *Agrobacterium*-mediated gene transfer has been less successful in cereals and other monocots (Bevan, 1984 and De Cleen and De Ley, 1985). In rice, callus induction and efficient plant regeneration for *O. sativa* L. *japonica* varieties has been reported widely (Abe and Futsuhara, 1986; Mikami and Kinoshita, 1985). However, plant regeneration from the major cultivated *indica* varieties is generally poor (Kavi Kishor and Reddy, 1986).

Hiei *et al*. (1994) subsequently reported a method for efficient production of transgenic rice plants from calli of *japonica* cultivars (cv. Tsukinohikari, cv. Asanohikari and cv. Kosihikari) that had been co-cultivated with *A. tumefaciens* containing *nptII, hpt* and GUS genes. Addition of acetosyringone (100 μM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. A large number of morphologically normal, fertile, transgenic rice plants were obtained by co-cultivation of rice tissues with *A. tumefaciens.* The efficiency of transformation was between 12.8 and 28.6%

similar to that obtained by the methods used routinely for transformation of dicotyledons with the bacterium. Stable integration, expression and inheritance of transgenes were demonstrated by molecular and genetic analysis of transformants in the R0, R1 and R2 generations. Sequence analysis revealed that the boundaries of the T-DNA in transgenic rice plants were essentially identical to those in transgenic dicotyledons.

Transformation of *indica* rice has been successful in a few laboratories using protoplast transformation with electroporation (Zhang *et al*., 1988) or PEG (Datta *et al*., 1990), or particle bombardment (Christou *et al*., 1991). In recent years, many researchers have developed reproducible transformation of monocot plants, such as *japonica*, *indica* and *javanica* rice cultivars by Agrobacterium with high efficiency and stability (Hiei *et al*., 1994; Rashid *et al*., 1996; Dong *et al*., 1996; Pipatpanukul, 1999; Rattana, 2001; Qian *et al*., 2004). These recent studies on the transformation of monocot plants by Agrobacterium have provided evidence for the hypothesis that T-DNA is transferred to dicots and monocots by an identical molecular mechanism. However, *indica* rice is still considered difficult to transform, especially by Agrobacterium mediated delivery (Qian *et al*., 2004).

Pipatpanukul (1999) was successful in *indica* rice, RD6, transformation via *Agrobacterium* strains LBA4404 and EHA105 which harboured pCAMBIA1301 containing *hpt* and *gus* genes. Under the optimal conditions, the GUS expression in infected calli was more than 90 percent after antibiotic selection. The resistant plants were found to have GUS activity in whole plant organs of young plants. In order to improve the effectiveness of *indica* rice transformation via *Agrobacterium* mediated delivery, a well established tissue culture system, particularly an improved procedure

for callus induction, is a prerequisite. Qian *et al*. (2004) evaluated some of these factors for rice transformation and improved efficiency of GUS transient expression to a relatively high level.

Imjongjirak (2000) transferred green fluorescent protein (GFP) reporter gene into indica rice variety KDML 105. Embryogenic calli derived from scutella calli of mature embryo were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 carrying pCAMBIA1301 (GUS as the reporter gene), pCAMBIA5305 (GFP as the reporter gene) and pCAMBIA1306IC (GFP and GUS as the reporter gene). Each was driven under the 35S promoter and contained *hpt* as a selectable marker. Hygromycin resistant calli were obtained after 4 weeks selection with 50 mg hygromycin. GUS expression from pCAMBIA1301 after 3 days of co-cultivation revealed 13.5% transformation efficiency. With EHA105 which contained pCAMBIA5305 and pCAMBIA1306IC, GFP-transformed calli were obtained with detectable of GFP fluorescence. The transformation frequency obtained by plants that stably expressed GFP for pCAMBIA5305 and expressed both GFP fluorescence and GUS activity for pCAMBIA1306IC was 9.5% and 5.2%.

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# CHAPTER III

# RECOMBINANT PROTEIN EXPRESSION AND CHARACTERIZATION OF RICE GLYCOSYL HYDROLASE FAMILY 1 OS4BGLU12 β-GLUCOSIDASE

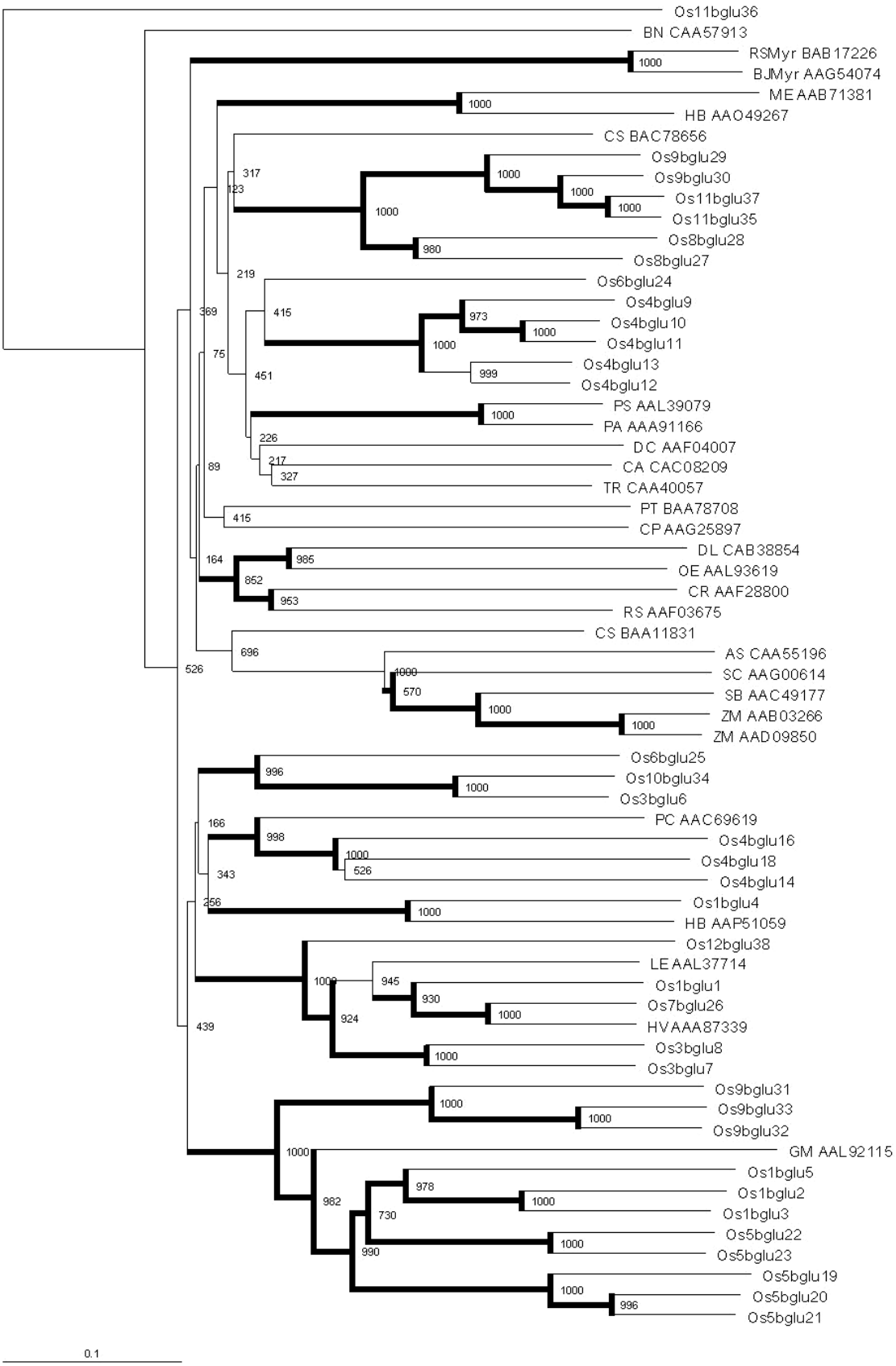

## Abstract

The full-length cDNA of the GH1 gene, *Os4bglu12* β-glucosidase, was previous cloned from rice seedlings by RT-PCR and the recombinant thioredoxin-Os4bglu12 fusion protein was functionally expressed in *E. coli*. The *Os4bglu12* cDNA encoded a 510 amino acid long precursor protein. Os4bglu12 protein was predicted to secreted outside the cells. Os4bglu12 enzyme efficiently hydrolyzed β-(1,4)-linked oligosaccharides of 3-6 glucose residues and β-(1,3)-linked disaccharide. The assay in which the Os4bglu12 was 10 min pre-incubated with cellobiose for 10 min indicated that cellobiose could inhibit Os4bglu12 enzyme in the hydrolysis of cellooligosaccharides and laminaribiose. Hydrolysis of β-(1,3)-linked oligo-saccharides with degree of polymerization more than 2, laminarin and 1,3-, 1,4-β-glucans by the enzyme could not be detected. Os4bglu12 hydrolyzed many kinds of *p*NP-β-glycosides which indicates the low stringency at the -1 subsite of the enzyme. The substrate specficity of Os4bglu12 was similar to those reported for rice BGlu1, and barley β-glucosidases, but not identical, which suggests that small sequence differences likely determine specific functional properties.

## 3.1 Introduction

β-Glucosidases catalyze the hydrolysis of alkyl- and aryl-β-glucosides, as well as disaccharides and oligosaccharides. In plants, these enzymes have been implicated in a variety of processes, such as release of glucose from cell-wall-derived oligosaccharides, defense against herbivores and fungi by release of toxic compounds from inactive glycosides, activation of lignin precursors in lignification, release of phytohormones from inactive glycosides, and general activation of glucose-blocked intermediates in metabolism (Esen, 1993). It seems evident that the substrate specificity, localization of the enzymes with respect to potential substrates will determine the roles of these enzymes.

Recently, forty rice genes homologous to GH 1 β-glucosidase have been identified from rice genome databases by Opassiri *et al*. (2006). Phylogenetic analysis revealed that many members that contain closely related sequences are located on the same chromosome and clustered in the same subfamilies. The relationship between rice and other plant GH1 protein sequences described by a phylogenetic tree rooted by Os11bglu36 (Opassiri *et al*., 2006) is shown in Figure 3.1. An initial homology search with β-glucosidase sequences, identified 31 genes have corresponding ESTs or full-length cDNAs (Opassiri *et al*., 2006). The source libraries for rice β-glucosidase gene matching ESTs included seedling (shoot and root), immature plant parts (stem, root, leaf), mature leaf, panicle at flowering and ripening stages, immature seeds, and callus, reflecting expression in many tissues during plant development. Some rice β-glucosidase genes have ESTs represented in stressed plant tissue libraries, including salt, drought, cold, and blast/fungus infection (Opassiri *et al*., 2006).


Os11bglu36
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RSMyr BAB17226
BJMyr AAG54074
ME AAB71381
HB AAO49267
CS BAC78656
Os9bglu29
Os9bglu30
Os11bglu37
Os11bglu35
Os8bglu28
Os8bglu27
Os6bglu24
Os4bglu9
Os4bglu10
Os4bglu11
Os4bglu13
Os4bglu12
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CA CAC08209
TR CAA40057
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CP AAG25897
DL CAB38854
OE AAL93619
CR AAF28800
RS AAF03675
CS BAA11831
AS CAA55196
SC AAG00614
SB AAC49177
ZM AAB03266
ZM AAD09850
Os6bglu25
Os10bglu34
Os3bglu6
PC AAC69619
Os4bglu16
Os4bglu18
Os4bglu14
Os1bglu4
HB AAP51059
Os12bglu38
LE AAL37714
Os1bglu1
Os7bglu26
HV AAA87339
Os3bglu8
Os3bglu7
Os9bglu31
Os9bglu33
Os9bglu32
GM AAL92115
Os1bglu5
Os1bglu2
Os1bglu3
Os5bglu22
Os5bglu23
Os5bglu19
Os5bglu20
Os5bglu21
0.1

**Figure 3.1** rRelationship between rice and other plant GH1 protein sequences described by a phylogenetic tree rooted by Os11bglu36. The sequences were aligned with ClustalX, then manually adjusted, followed by removal of N-terminal, C-terminal and large gap regions to build the data model. The tree was produced by the neighbor joining method and analyzed with 1000 bootstrap replicates. The internal branches supported by a maximum parsimony tree made from the same sequences are shown as bold lines. The sequences other than rice include: ME AAB71381, *Manihot esculenta* linamarase; RSMyr BAB17226, *Raphanus sativus* myrosinase; BJMyr AAG54074, *Brassica juncea* myrosinase; BN CAA57913, *Brassica napus* zeatin-O-glucoside-degrading β-glucosidase; HB AAO49267, *Hevea brasiliensis* rubber tree β-glucosidase; CS BAA11831, *Costus speciosus* furostanol glycoside 26-O-β-glucosidase (F26G); PS AAL39079, *Prunus serotina* prunasin hydrolase isoform PH B precursor; PA AAA91166, *Prunus avium* ripening fruit β-glucosidase; TR CAA40057, *Trifolium repens* white clover linamarase; CA CAC08209, *Cicer arietinum* epicotyl β-glucosidase with expression modified by osmotic stress; DC AAF04007, *Dalbergia cochinchinensis* dalcochinin 8'-O-β-glucoside β-glucosidase; PT BAA78708, *Polygonum tinctorium* β-glucosidase; DL CAB38854, *Digitalis lanata* cardenolide 16-O-glucohydrolase; OE AAL93619, *Olea europaea* subsp. europaea β-glucosidase; CR AAF28800, *Catharanthus roseus* strictosidine β-glucosidase; RS AAF03675, *Rauvolfia serpentina* raucaffricine-O-β-D-glucosidase; CP AAG25897, *Cucurbita pepo* silverleaf 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; LE AAL37714,

*Lycopersicon esculentum* β-mannosidase; HV AAA87339, barley BGQ60 β-glucosidase; HB AAP51059, *Hevea brasiliensis* latex cyanogenic β-glucosidase; PC AAC69619, *Pinus contorta* coniferin β-glucosidase; GM AAL92115, *Glycine max* hydroxyisourate hydrolase; CS BAC78656, *Camellia sinensis* β-primeverosidase (Opassiri *et al*., 2006).

To date, only a few rice β-glucosidase isozymes have been cloned and fully characterized for the possible function. Rice β-glucosidases have been described that hydrolyze gibberellin glucosides, pyridoxine glucosides and oligosaccharides (Schlieman, 1984; Akiyama *et al*., 1998; Opassiri *et al*., 2003; 2004). Tassanee Onkoksoong isolated the full-length cDNA of one of these genes, *Os4bglu12* β-glucosidase, by RT-PCR from rice seedlings. The isolated *Os4bglu12* cDNA encoded a protein that was identical at 40 of 44 amino acid residues with the N-terminal amino acid sequence of a cell wall-bound enzyme previously purified from germinating rice (Akiyana *et al*., 2006). In this study, the biochemical characterization of recombinant Os4bglu12 β-glucosidase expressed in *E. coli* was assessed.

## 3.2 Materials and methods

### 3.2.1 Materials and chemicals

#### 3.2.1.1 Chemicals and reagents

Tryptone and yeast extract were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Sodium dodecyl sulfate (SDS), acrylamide, *N,N',N'',N'''*-tetramethylethylenediamine (TEMED), *N,N′*–methylene–bis–acrylamide, ammonium persulfate, Triton X-100, and lysozyme were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Sodium hydroxide (NaOH), sodium phosphate, sodium

acetate, sodium chloride (NaCl), sodium carbonate ($Na_2CO_3$), disodium ethylenediamine tetraacetate (EDTA), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), bromophenol blue, sulfuric acid, glacial acetic acid, methanol, distilled water, ethanol, n-butanol and ethyl acetate were purchased from Carlo ERBA (Rodano, Milano, Italy). β-alanine, 2,2'-azinobis(3-ethylbenthaiazolinesulfonic acid) (ABTS), isopropyl thio-β-D-galactoside (IPTG), 2-mercaptoethanol, *p*-nitrophenol β-D-glucoside (*p*NPG), *p*NP-fucoside, *p*NP-arabinoside, *p*NP-galactoside, *p*NP-xyloside, *p*NP-mannoside, *p*NP-cellobioside and cellobiose were purchased from Sigma (St. Louis, MO, USA). Cellooligosaccharides of degree of polymerization (DP) 3-6 and laminari-oligosaccharides of DP 2-6 were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Coomassie brilliant blue R250, phenylmethylsulfonylfluoride (PMSF), calcium chloride, and ethydium bromide were purchased from Fluka (Steiheim, Swizerland). Taq DNA polymerase was purchased from Promega (Madison, USA). Thin-layer chromatography silica gel 60 $F_{254}$ plates were purchased from Merck (Darmstadt, Germany). Other chemicals and molecular reagents used but not listed here were purchased from a variety of suppliers.

#### 3.2.1.2 Bacterial strains

Origami B(DE3) host strain is a K-12 derivatives mutated in both the thioredoxin reductase *(trxB)* and glutathione reductase *(gor)* genes, which greatly enhances disulfide bond formation in the *E. coli* cytoplasm. The *lon* and *ompT* deficiencies of BL21 presented in the strain help increase protein stability (Novagen 2002-2003 catalog).

DH5α is a recombination-deficient suppressing strain used for cloning plasmids and cosmids. The $\phi$ 80 *lac*ZΔM15 permits α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors (Sambrook *et al*., 1989).

### 3.2.2 Putative Os4bglu12 protein sequence analysis

The protein sequence of glycosyl hydrolase family 1 *Os4bglu12* β-glucosidase (*Os4bglu12*) was aligned with various plant β-glucosidases with the ClustalX implementation of ClustalW (Jeanmougin *et al*., 1998; Thompson *et al*., 1994) and manually adjusted with the Gendoc program (Nicholas and Nicholas, 1997). Protein analyses were done at the Expasy proteomics server (http://www.expasy.org/), and the signal sequence and cellular location were predicted with SignalP (Bendtsen *et al*., 2004) and PSORT (Nakai and Horton, 1999), respectively.

### 3.2.3 Transformation of recombinant plasmids into expression host cells

A full-coding cDNA of glycosyl hydrolase family 1 *Os4bglu12* β-glucosidase was previously cloned and inserted into pENTR-D/TOPO Gateway entry vector and then subcloned into the pET32a+/DEST Gateway expression vector by LR Clonase recombination as described in Opassiri *et al*. (2006) (see section 1 in Appendix A).

A recombinant pET32a+/DEST-*Os4bglu12* plasmids was amplified by transformation of plasmids into $CaCl_2$-treated DH5α *E. coli* competent cells and the recombinant plasmids were isolated from selected clones by alkaline lysis, as described in section 3-5 in Appendix A. The recombinant pET32a+/DEST-*Os4bglu12*

plasmid was transformed into Origami B(DE3) *E. coli* by the $CaCl_2$ method, and positive clones were selected on a 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 12.5 μg/mL tetracycline LB agar plate (see section 6 in Appendix A).

### 3.2.4 Recombinant protein expression in Origami B(DE3) *E. coli*

To produce recombinant thioredoxin-Os4bglu12 fusion protein (Trx-Os4bglu12), the selected pET32a+/DEST-*Os4bglu12*/Origami B(DE3) clones were grown overnight in 3-5 mL LB broth containing 100 μg/mL ampicillin, 15 μg/mL kanamycin and 12.5 μg/mL tetracycline at 37°C with shaking at 200 rpm. The fresh starter cultures were diluted to a ratio of 1:100 with LB broth containing the same antibiotics and then the culture was grown at 37°C with shaking at 200 rpm for 3-5 hr until the optical density at 600 nm reached at 0.5-0.6. To induce the expression of recombinant protein, different concentrations of IPTG was added to each culture flasks to a final concentration varied from 0.05 to 0.5 mM. The cultures were shaken at 200 rpm at 20, 25 and 30°C with different induction times of 4, 8, 12 and 16 hr. The induced cultures were chilled on ice for 10 min and then centrifuged at 5,000 x g for 10 min at 4°C. The cell pellets were kept at -70°C until use.

To monitor the protein expression levels, 1 mL aliquots of the cultures were sampled at the indicated periods of time and precipitated by centrifugation at 14,000 x g for 10 min. The cell pellet was resuspended in 5X sample buffer (2.5 M Tris-HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 20% 2-mercaptoethanol) and boiled for 5 min to denature proteins. Then, the 8-12 μl aliquots were loaded onto 12% SDS polyacrylamide gels (SDS-PAGE), as described in section 3.2.8

### 3.2.5 Extraction of recombinant protein from induced cells

The IPTG-induced bacterial cell pellets were thawed on ice and then resuspended in freshly prepared extraction buffer (50 mM phosphate buffer, pH 8.0, 200 µg/mL lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonylfluoride (PMSF), 4 µL of 0.25 mg/mL DNase I) in a ratio of 5 mL extraction buffer per gram fresh weight of cell pellets. The resuspended cells were incubated at room temperature for 30 min. Then, the soluble proteins were recovered by centrifugation at 12,000 x g at 4°C for 10 min. The soluble protein fractions were kept on ice for protein purification in the next step. An aliquot of the supernatant (10 µL) was subjected to protein analysis by SDS-PAGE (see section 3.2.8).

### 3.2.6 Purification of recombinant protein

The soluble protein fractions extracted from above step were purified by immobilized metal affinity chromatography (IMAC) on BD TALON cobalt resin (BD Clonetech, Palo Alto, USA). A 5-10 mL of soluble protein fractions was loaded onto a 2 mL bed volume of cobalt resin which was pre-equilibrated with 8 bed volumes of equilibration buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0). The column containing bound recombinant proteins was washed with 4 bed volumes of equilibration buffer and then with 8 bed volumes of washing buffer (10 mM immidazole in an equilibration buffer). The bound protein fractions were eluted from a column with 4 bed volumes of elution buffer (250 mM immidazole in an equilibration buffer). The 0.5 mL fractions with *p*-nitrophenol β-D-glucoside (*p*NPG) β-glucosidase activity were pooled. The protein fractions were kept at 4°C.

### 3.2.7 Protein concentration determination

Purified protein concentration was determined by Bio-RAD protein assay kit (Bio-RAD, Richmond, CA) using bovine serum albumin (BSA) as the standard (2-10 μg BSA). 0.8 mL of diluted protein samples were mixed with 0.2 mL Bio-RAD protein assay solution. The reaction was allowed to stand at room temperature for 10 min and the absorbance at 595 nm was measured.

### 3.2.8 Determination of the protein expression profiles by SDS-PAGE

SDS-PAGE was done by the method first described by Laemmli (1970). The composition and preparation of a 12% SDS-PAGE separating gel as described in Appendix B were mixed and poured into the gap between a pair of glass plates assembled in a Hoefer vertical gel cassette (South San Francizco, CA, USA). The polyacrylamide gel plates were assembled in a Hoefer gel electrophoresis apparatus and the reservoir for electrodes was filled with 1X Tris-glycine electrode buffer (pH 8.3) (see Appendix B).

Protein samples were mixed with 1/4 volume of 5X sample buffer (2.5 M Tris-HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 20% 2-mercaptoethanol) and boiled for 5 min to denature proteins. Aliquots of 8-12 μL were loaded into sample wells, and electrophoresed at a constant voltage of 150 V from the cathodic (-) to anodic (+) end until the dye front reached the bottom of the gel plate. Then, the gels were stained in staining solution (0.1% (w/v) coomassie brilliant blue R250, 30% (v/v) methanol, 7% (v/v) acetic acid) for 30 min and destained in destaining solution (30% (v/v) methanol and 10% (v/v) acetic acid) for 1-2 hr. The molecular masses of protein bands were estimated by comparision to Bio-RAD low

molecular weight protein markers, which include phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and bovine α-lactalbumin (14.0 kDa).

### 3.2.9 pH and temperature profiles of activity and stability

The pH optimum for Trx-Os4bglu12 enzyme activity was determined. In a reaction volume of 100 μL, 0.4 μg of purified Trx-Os4bglu12 was mixed with 0.2 mM final concentration of *p*NPG in different buffers ranging from pH 3.5 to 10.5 (formate pH 3.5-4; sodium acetate pH 4.5-5.5; sodium phosphate pH 6-8.5; CAPS pH 9-10.5), at 0.5 pH unit intervals at the same buffer concentration of 50 mM. The reactions were allowed to proceed for 10 min at 37°C and the amount of product released was determined as described in section 3.2.10.

The pH stability for the Os4bglu12 was determined by incubating the enzymes in the same buffers ranging from pH 4 to 10 as above at increments of 1.0 pH unit for 10 min, 1, 3, 6, 12 and 24 hr. at room temperature (~25°C). After incubation, the enzyme was diluted 20 fold in 50 mM sodium acetate, pH 5.0, and the aliqouts of enzyme were assayed for activity with 0.35 mM *p*NPG in 50 mM sodium acetate, pH 5.0, in a reaction volume of 100 μL for 10 min at 37°C, and the product released was measured.

The temperature maximum was determined by incubating the Trx-Os4bglu12 with a final concentration of 0.2 mM *p*NPG in 50 mM sodium acetate, pH 5.0, in a reaction volume of 100 μL at temperatures ranging from 5°C to 90°C at 5° increments for 10 min. The amount of product released was determined. Thermostability of the enzyme was measured by incubating 0.4 μg of enzyme in 50

mM sodium acetate, pH 5.0, at different temperatures in the range of 20°C to 70°C at 10°C intervals for 15, 30, 45 and 60 min. Then, in a reaction volume of 100 μL, the enzyme samples were assayed with 0.35 *p*NPG in 50 mM sodium acetate, pH 5.0, at 37°C for 10 min.

### 3.2.10 Activity assays and kinetics study

The enzyme activity on various substrates was determined by either (1) the *p*-nitrophenol (*p*NP) liberated from the *p*NP derivatives of monosaccharides or disaccharides, or (2) glucose released from natural substrates. All substrate solutions were prepared in 50 mM sodium acetate, pH 5.0. The glycon specificity of Os4bglu12 β-glucosidase was tested with synthetic substrates, *p*NP-glycosides. In a 100 μL reaction, 0.05 μg (0.72 pmol) Trx-Os4bglu12 enzyme was incubated with 1 mM *p*NP-glycoside substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C. Then, 50 μL of 0.4 M sodium carbonate was added to stop the reaction, and the absorbance of the liberated *p*NP was measured at 405 nm. A reaction mixture of the same composition except without the enzyme was used as a blank.

Substrate specificity of Os4bglu12 enzyme was tested with various disaccharides and oligosaccharides. In a 50 μL reaction, 0.05 μg (0.72 pmol) Trx-Os4bglu12 enzyme was incubated with 1 mM substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C and the reaction was stopped by boiling. The release of the glucose was assayed with a coupled peroxidase/glucose oxidase (PGO) assay method, as previously described (Babcock and Esen, 1994; Opassiri *et al*., 2003). For this, the enzyme assay reaction was mixed with 50 μL of 1 mg/mL 2,2'-azinobis (3-ethyl benzthiazolinesulfonic acid, ABTS) and 100 μL of 10 mg/mL PGO enzyme (Sigma).

The absorbance of the product from the PGO assay was measured at 405 nm. A suitable blank containing buffer and substrate were set up at every substrate concentration to correct for glucose background.

The enzyme was also tested with polysaccharides. In the assay, 1-5 μg enzyme was incubated separately with 0.5% (w/v) laminarin and barley β-glucans in 50 mM sodium acetate, pH 5.0, at 37°C for 30-60 min. The reaction was stopped by the addition of *p*-hydroxybenzoic acid hydrozide reagent as described by Lever (1972), and the increase in reducing sugars was measured colorimetrically.

The products of Trx-Os4bglu12 hydrolysis of cello- and laminari-oligosaccharides were detected by TLC. In a 50 μl reaction mixture, 7.4 pmol enzyme was incubated with 5 mM substrate in 50 mM sodium acetate, pH 5.0, for 30 min at 37°C. A 5 μl of the reaction mixture was spotted on silica-gel 60 $F_{254}$ plates (Merck, Darmstadt, Germany) and chromatographed vertically with solvent consisting of ethyl acetate, acetic acid and water (2:1:1, by vol.). The products were detected by spraying with developer solution (ethanolic 10% $H_2SO_4$) and baked at 120°C for 5 min to visualize the sugar.

Kinetic parameters, $K_m$ and $V_{max}$ (at pH 5.0, at 37°C) of purified Trx-Os4bglu12 with *p*NP-glycosides and oligosaccharides were determined in triplicate reactions. The initial velocity of hydrolysis for each substrate was initially determined using various protein concentrations and incubation times (5-20 min) to find conditions that yield 0.1 to 1.0 absorbance units. The rate versus time was plotted and an appropriate time and enzyme amount were chosen for kinetic studies. The reactions containing buffer at 5-7 different substrate concentrations ranging from 0.1-4 fold of apparent $K_m$ value were pre-incubated at 37°C, for 10 min, and then the

reaction was started by addition of appropriately diluted of enzyme. The assays were done as above at 37°C for 5 to 15 min, depending on each substrate, to establish the initial velocity ($V_0$).

The 405 absorbance was measured using an iEMS Reader MF microtiterplate photometer (Labsystems iEMS Reader MF, Finland) and quantified by comparison to standards. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of product per min. Note that the activity values for disaccharides were determined by dividing the amount of glucose released by two, since two glucose molecules are released per molecule of disaccharides hydrolyzed. The micromoles of the product formation for oligosaccharides is defined in terms of total glucose released, though oligosaccharides may also have more than one glucose released per substrate molecule due to sequential cleavage. The kinetic parameters were calculated by the method of Lineweaver and Burk with the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). The enzyme molarity was calculated based on the molecular weight of 69 kDa for Trx-Os4bglu12.

### 3.2.11 The cellobiose inhibition studies

The inhibition of Os4bglu12 activity by cellobiose was studied. In a 25 μL mixture, 0.2 μg of Trx-Os4bglu12 enzyme was mixed with a final concentration of 0.5 mM cellobiose with and without 10 min incubation. The reaction was started by adding 25 μL of cello-oligosaccharides and laminaribiose substrates (0.5 mM final concentration) in each well and the reactions were incubated at 37°C for 15 min. The reactions were stopped by heating at 100°C for 5 min and the glucose released was determined by the PGO assay, as described in method 3.2.10.

## 3.3 Results

### 3.3.1 Sequence analysis of rice Os4bglu12

The full-coding sequence and 3' untranslated sequence of the *Os4bglu12* cDNA is shown in Figure 3.2. The 1635 bp-full-length cDNA sequence of *Os4bglu12* included a 1530-nucleotide long open reading frame (ORF) encoding a 510 amino acid long precursor protein. The Signal P program predicted the protein to contain a 24 amino acid signal sequence and a 486 amino acid mature protein. The deduced Os4bglu12 N-terminal amino acid sequence was identical to the N-terminal amino acid sequence of the previously purified cell-wall-bound rice β-glucosidase at 40 of 44 residues (Akiyama *et al*., 1998). By the PSORT program, the Os4bglu12 protein is predicted to be secreted outside the cell, or located in vacuole or peroxisome. There are 6 possible N-linked glycosylation sites on the Os4bglu12 protein sequence. The pI of the enzyme is predicted to be 8.9 (Table 3.1).

**Table 3.1** Predicted properties and location of rice Os4bglu12 β-glucosidase

| Gene name | Mature protein | | | Possible destination[c] |
|---|---|---|---|---|
| | Mw (kDa)[a] | pI[a] | N-linked[b] | |
| *Os4bglu12* | 55.3 | 8.9 | 6 | Extracellular, vacuole, peroxisome |

[a] determined by ProtParam, [b] predicted by NetNGlyc at the Expasy proteomics server, [c] cellular locations predicted by PSORT.

The Os4bglu12 polypeptide contains Glu residues at positions 203 and 417 that lie within the sequences TFNEP and ITENG, respectively, which matches the consensus motifs in family 1 β-glucosidases. The Os4bglu12 protein sequence had

the highest sequence identity (68%) with *Leucaena leucocephala* β-glucosidase (AC ABY48758). It also had sequence identity ranging from 60-64% with other β-glucosidases of many plant species, such as isoflavone β-glucosidases of *Medicago truncatula* (AC ABW76288), *Glycine max* (AC BAF34333), *Dalbergia nigrescens* (AC AAV34606) and *Camellia sinensis* (AC BAC78656), prunasin hydrolase (AC AAF34650) and amygdalin hydrolase (AC AAA93234) of *Prunus serotina* (Figure 3.3). The sequence alignment between Os4bglu12 with plant enzymes listed above revealed that rice Os4bglu12 contains the conserved glucose binding residues found in other GH1 β-glucosidases including Q38, H142, E191, E406, E464 and W465 in maize Bglu1 (Czjzek *et al*., 2000) (Figure 3.3). Its sequence is somewhat more similar to isoflavone β-glucosidases than rice BGlu1 and barley βII β-glucosidases /exoglucanases. In addition, Os4bglu12 has some amino acid residues similar to the residues lining the active site of the white clover linamarase, 1CBG, structure (P26205), such as Q33, H137, N190, F197, W369, W446, E453 and W454 in 1CBG (Barrett *et al*., 1995).

```
ATGGCGGCAGCAGGGGCAATGCCCGGTGGCCTTCTCCTCACGTTCCTCCTCCTTGCTGTCGTCGCTTCCGGC
M  A  A  A  G  A  M  P  G  G  L  L  L  T  F  L  L  L  A  V  V  A  S  G
445-1matNcoI_f primer
GCCTACAATAGCGCCGGCGAGCCGCCGGTCAGCCGGAGAAGCTTCCCCAAGGGGTTCATCTTCGGGACAGCC
A  Y  N  S  A  G  E  P  P  V  S  R  R  S  F  P  K  G  F  I  F  G  T  A
TCGTCGTCGTATCAGTACGAGGGTGGCGCAGCGGAGGGCGGCAGAGGACCAAGCATCTGGGACACCTTCACA
S  S  S  Y  Q  Y  E  G  G  A  A  E  G  G  R  G  P  S  I  W  D  T  F  T
CATCAGCACCCAGAGAAAATCGCCGACAGAAGCAACGGGGATGTGGCTTCGGATTCCTACCATCTCTACAAG
H  Q  H  P  E  K  I  A  D  R  S  N  G  D  V  A  S  D  S  Y  H  L  Y  K
GAAGATGTGCGCCTCATGAAGGATATGGGAATGGATGCATACAGGTTCTCCATCTCATGGACAAGAATCCTT
E  D  V  R  L  M  K  D  M  G  M  D  A  Y  R  F  S  I  S  W  T  R  I  L
CCAAATGGAAGTCTGAGGGGTGGAGTCAACAAAGAAGGCATAAAGTACTACAATAATTTGATCAATGAGCTA
P  N  G  S  L  R  G  G  V  N  K  E  G  I  K  Y  Y  N  N  L  I  N  E  L
TTATCGAAAGGGGTGCAACCGTTTATTACCCTTTTCCACTGGGACTCACCTCAGGCGTTGGAAGATAAATAT
L  S  K  G  V  Q  P  F  I  T  L  F  H  W  D  S  P  Q  A  L  E  D  K  Y
AACGGATTTCTTAGCCCTAATATCATAAATGACTTTAAGGACTATGCTGAAATCTGCTTCAAGGAGTTTGGT
N  G  F  L  S  P  N  I  I  N  D  F  K  D  Y  A  E  I  C  F  K  E  F  G
GACAGAGTGAAAAATTGGATCACCTTCAATGAGCCTTGGACTTTCTGCTCTAATGGCTATGCAACTGGCCTG
D  R  V  K  N  W  I  T  F  N  E  P  W  T  F  C  S  N  G  Y  A  T  G  L
TTTGCACCAGGCCGTTGTTCGCCCTGGGAGAAGGGAAATTGCAGTGTTGGAGATTCAGGAAGGGAGCCTTAC
F  A  P  G  R  C  S  P  W  E  K  G  N  C  S  V  G  D  S  G  R  E  P  Y
ACTGCATGCCATCATCAACTACTTGCCCACGCGGAAACTGTTCGGTTGTACAAAGCGAAATATCAGGCCTTA
T  A  C  H  H  Q  L  L  A  H  A  E  T  V  R  L  Y  K  A  K  Y  Q  A  L
CAAAAAGGGAAGATTGGAATAACTCTGGTCTCGCACTGGTTTGTTCCCTTCTCCCGCTCCAAATCCAACAAT
Q  K  G  K  I  G  I  T  L  V  S  H  W  F  V  P  F  S  R  S  K  S  N  N
GATGCTGCAAAGCGTGCTATAGACTTCATGTTTGGATGGTTTATGGATCCCCTCATTAGAGGCGACTACCCC
D  A  A  K  R  A  I  D  F  M  F  G  W  F  M  D  P  L  I  R  G  D  Y  P
CTAAGCATGAGAGGATTGGTTGGGAATCGCTTGCCACAGTTCACTAAAGAACAGTCTAAGTTGGTCAAGGGT
L  S  M  R  G  L  V  G  N  R  L  P  Q  F  T  K  E  Q  S  K  L  V  K  G
GCATTTGACTTTATTGGACTTAACTACTACACTGCGAACTATGCTGATAACCTTCCTCCATCAAATGGCCTT
A  F  D  F  I  G  L  N  Y  Y  T  A  N  Y  A  D  N  L  P  P  S  N  G  L
AACAACAGCTATACCACCGATTCTCGAGCTAATCTTACCGGTGTACGAAATGGCATCCCCATAGGACCGCAG
N  N  S  Y  T  T  D  S  R  A  N  L  T  G  V  R  N  G  I  P  I  G  P  Q
GCTGCTTCACCTTGGCTTTACGTCTACCCTCAAGGCTTCCGTGACTTGCTACTTTATGTTAAGGAGAACTAT
A  A  S  P  W  L  Y  V  Y  P  Q  G  F  R  D  L  L  L  Y  V  K  E  N  Y
GGCAATCCTACCGTCTACATCACTGAAAATGGCGTTGATGAATTCAACAATAAGACCTTACCACTCCAAGAA
G  N  P  T  V  Y  I  T  E  N  G  V  D  E  F  N  N  K  T  L  P  L  Q  E
GCCTTGAAGGATGACGCTAGGATAGAATACTACCACAAGCACCTCCTTTCCCTGCTAAGTGCTATAAGGGAT
A  L  K  D  D  A  R  I  E  Y  Y  H  K  H  L  L  S  L  L  S  A  I  R  D
GGAGCAAACGTGAAGGGATACTTTGCATGGTCGCTGCTTGATAACTTCGAGTGGTCGAACGGCTACACAGTT
G  A  N  V  K  G  Y  F  A  W  S  L  L  D  N  F  E  W  S  N  G  Y  T  V
CGATTTGGGATAAACTTCGTAGATTACAATGACGGAAGGAAGAGATACCCCAAGAACTCTGCCCATTGGTTC
R  F  G  I  N  F  V  D  Y  N  D  G  R  K  R  Y  P  K  N  S  A  H  W  F
445-1stop_r primer
AAGAAGTTCCTCCTGAAATGATAAAAAAGCACCATTGCACTCATTGCTTTTCTCATTGAATAATAAAAGAGT
K  K  F  L  L  K  *
ATATGTTTTGCGTGAATTTGGTACAAGAGATGGAAGTAATCCAGTTAAATAATAAATCCCTCTCTTACTGC
```

**Figure 3.2** The full-length cDNA sequence and deduced amino acid sequence of rice *Os4bglu12*. Underlined letters represent the regions of DNA sequence matching the PCR primers used for cloning the mature protein encoding cDNA for recombinant protein production.

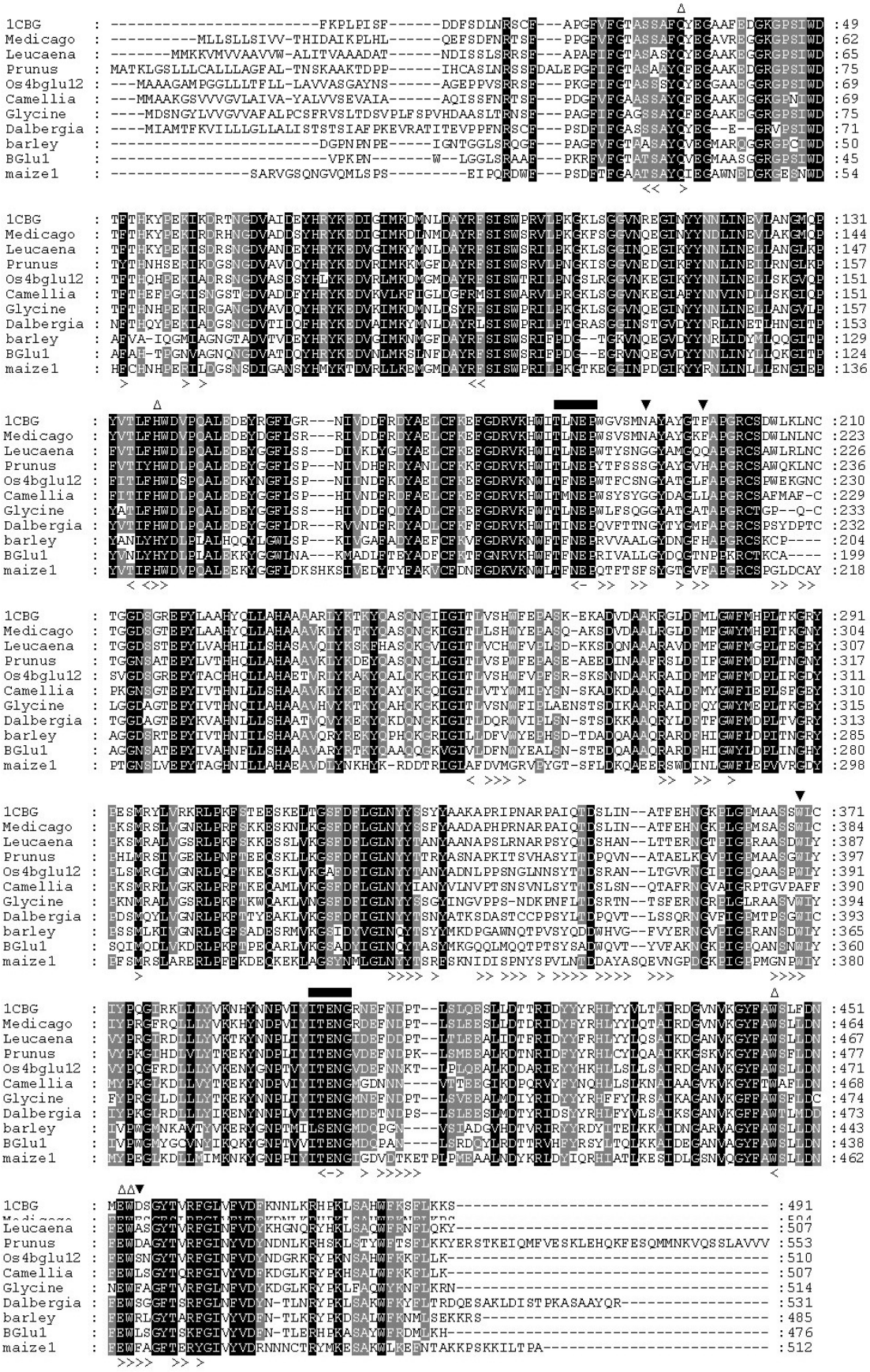
1CBG
Medicago
Leucaena
Prunus
Os4bglu12
Camellia
Glycine
Dalbergia
barley
BGlu1
maize1

**Figure 3.3** Alignment of the predicted mature protein sequences of rice Os4bglu12 with related β-glucosidases. The rice cDNA derived sequence is labeled as Os4bglu12; BGlu1 is rice β-glucosidase 1 (AC U28047); maize1 is maize β-glucosidase 1 (accession no. U33816); barley is deduced from barley BGQ60 nucleotide sequence (AC AAA87339), which was previously determined to be the same as βII β-glucosidase (Hrmova *et al*., 1998; Leah *et al*., 1995); 1CBG is white clover linamarase (P26205); Medicago is *Medicago truncatula* isoflavone β-glucosidase (AC ABW76288) (Naoumkina *et al*., 2007); Leucaena is *Leucaena leucocephala* β-glucosidase (AC ABY48758); Prunus is *Prunus serotina* amygdalin hydrolase (AC AAA93234); Camellia is *Camellia sinensis* isoflavone β-glucosidase (AC BAC78656); Glycine is *Glycine max* isoflavone β-glucosidase (AC BAF34333); and Dalbergia is *Dalbergia nigrescens* isoflavone β-glucosidase (AC AAV34606). Sequences corresponding to the catalytic acid/base and nucleophile consensus sequences are marked by a thick line over the top. Residues shown by Czjzek *et al*. (2000) to be in contact with the DIMBOA aglycone in the maize β-glucosidase are indicated by: ▼, while those conserved residues making contacts with the sugar are marked by: Δ above the column. Residues lining the active site of the 1CBG structure are marked with a "<" or ">" under their column in the alignment for residues appearing to reside inside the two catalytic carboxylic acids and those appearing to be outside these residues, respectively (Barrett *et al*., 1995; Opassiri *et al*., 2004). The alignment was generated using the Clustal X implementation of Clustal W (Jeanmougin *et al*., 1998; Thompson *et al*., 1995), analyzed and manually adjusted by Genedoc (http://www.psc.edu/biomed/genedoc/).

### 3.3.2 Expression of recombinant rice Os4bglu12 β-glucosidase

The soluble active recombinant Trx-Os4bglu12 β-glucosidase fusion protein was produced in Origami B(DE3) *E. coli* (Opassiri *et al*., 2006). In this study, the conditions for biosynthesis of functional Trx-Os4bglu12 in Origami B(DE3) was optimized by varying the IPTG concentrations, expression temperature and incubation time. The soluble crude protein fraction of each expression condition was assayed for the β-glucosidase activity with *p*NPG. The Trx-Os4bglu12 activity in cell lysates tended to be constant as the IPTG concentration increased from 0.1 to 0.5 mM (Figure 3.4). There were no significant difference in the activity of Trx-Os4bglu12 expressed in *E. coli* in the presence of 0.3 mM IPTG at 20, 25 and 30°C, for 8-16 hr (Figure 3.5). According to above results, the optimal expression condition for Trx-Os4bglu12 could be obtained by inducing the cells with 0.1-0.5 mM IPTG and culture the induced cells at 20 or 25°C for 8 or 12 hr.

Comparison of the protein profiles by SDS-PAGE showed that the induced cultures of pET32a+/DEST-*Os4bglu12*/Origami B(DE3) highly expressed the Trx-Os4bglu12 and an intense band at approximately 69 kDa was seen on the SDS-PAGE of the total cell lysates and soluble fractions (Figure 3.6). This band was absent from the profiles of induced cultures containing pET32a+ plasmid without a cDNA insert (negative control).

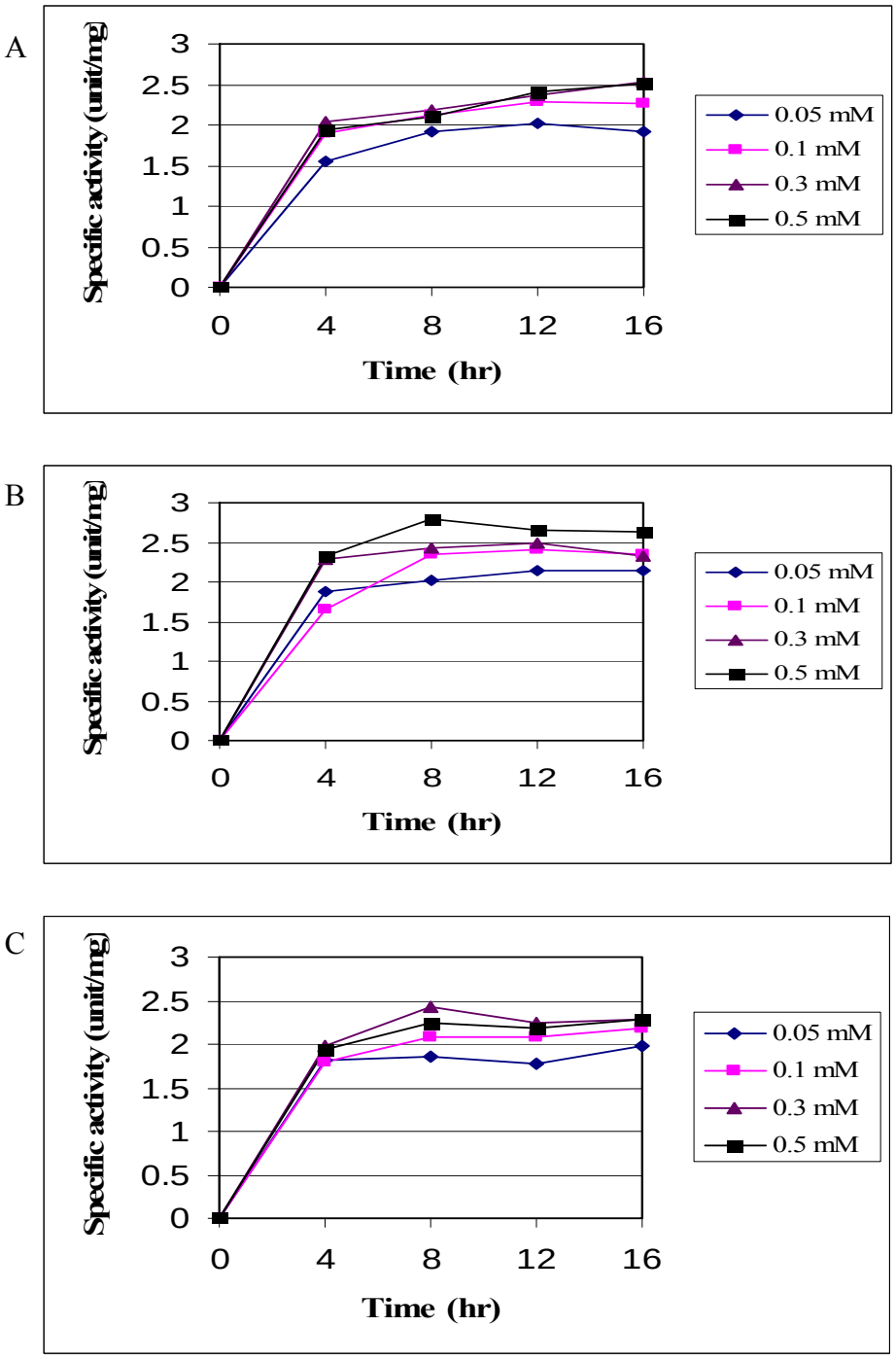


**Figure 3.4** Comparison of specific activity of crude lysates of pET32a+/DEST-*Os4bglu12* expressed in Origami B(DE3) after incubation in the presence of various IPTG concentrations and at times ranging from 0 to 16 hr at A, 20°C; B, 25°C and C, 30°C. The assays were done with 0.5 mM *p*NPG at 37°C for 10 min.

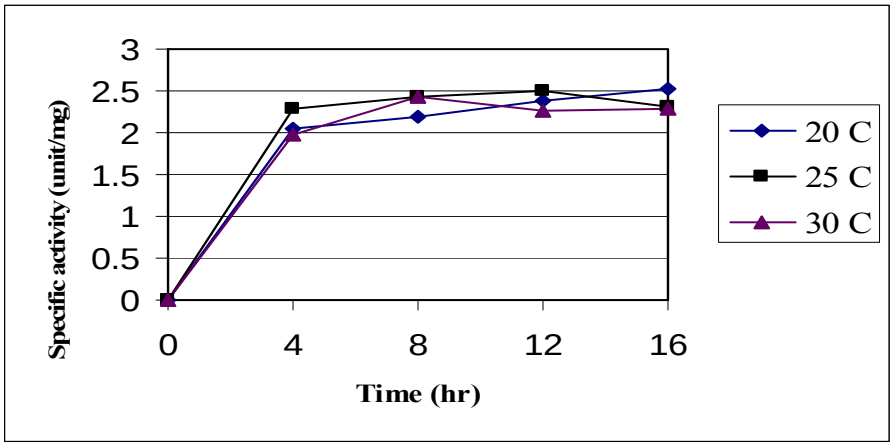


**Figure 3.5** Comparison of specific activity of Trx-Os4bglu12 expressed in Origami B(DE3) after incubation in the presence of 0.3 mM IPTG at different temperature for 0 to 16 hr. The assays were done with 0.5 mM *p*NPG at 37°C for 10 min.

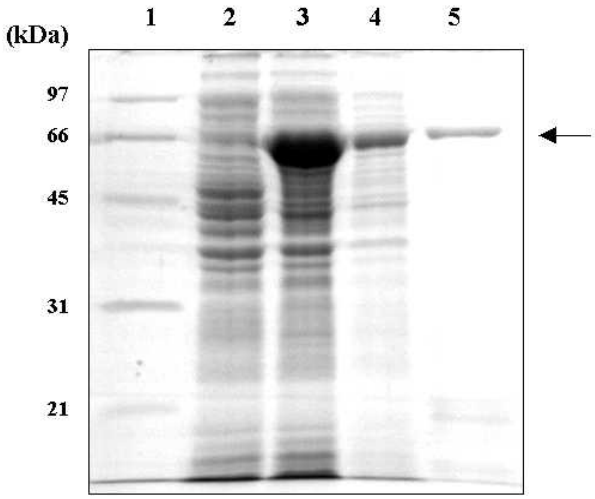


**Figure 3.6** SDS-PAGE profiles of Trx-Os4bglu12 recombinant protein expressed in Origami B(DE3) after incubation in the presence of 0.3 mM IPTG, at 20°C for 8 hr. Lanes: 1, standard marker (Bio-RAD); 2, total protein in *E. coli* cells containing pET32a+ without an insert; 3 and 4, total protein and soluble fraction of *E. coli* cells containing pET32a+/DEST-*Os4bglu12*, respectively; 5, IMAC purified Trx-Os4bglu12 protein. The arrow points to the position of Trx-Os4bglu12 monomer.

The Trx-Os4bglu12 fusion protein was purified by immobilized metal affinity chromatography (IMAC) with $Co^{2+}$ column, which allows the $His_6$.tag portions of the protein bind to the $Co^{2+}$ resin. The recombinant protein was eluted from the column as a single band (approximately 95% pure) and a band corresponding to 69 kDa was observed on SDS-PAGE (Figure 3.6). Approximately 3 mg of purified protein could be obtained per liter of bacterial expression culture. The enzyme activity with *p*NPG still remained after the TRX-Os4bglu12 was cleaved with enterokinase. The purified Trx-fusion enzyme was used to characterize the biochemical properties.

### 3.3.3 Effect of pH and temperature on the activity and stability of Os4bglu12

To define an optimum pH of Os4bglu12 activity, the enzyme activity was assayed with *p*NPG in different pH buffers with a pH range of 3.5-10.5. Os4bglu12 was most active at pH 5.0 (Figure 3.7). The activity dropped dramatically at pH higher values than 6.5 and lower than 4.5. To study the pH stability, the enzyme was incubated in different pH buffers over the range of 4.0 to10 up to 24 hr at room temperature (25°C). Os4bglu12 was relatively stable over the pH range 5.0 to 7.0, when incubated for up to 24 hr (Figure 3.8). The activity of Os4bglu12 decreased dramatically at acidic pH (4.0) within 10 min incubation. The enzyme was less stable after incubation more than 6 hr at pH 7.0 and 8.0. However, the enzyme was more stable at pH 9.0 and 10.0 than pH 7.0 and 8.0.

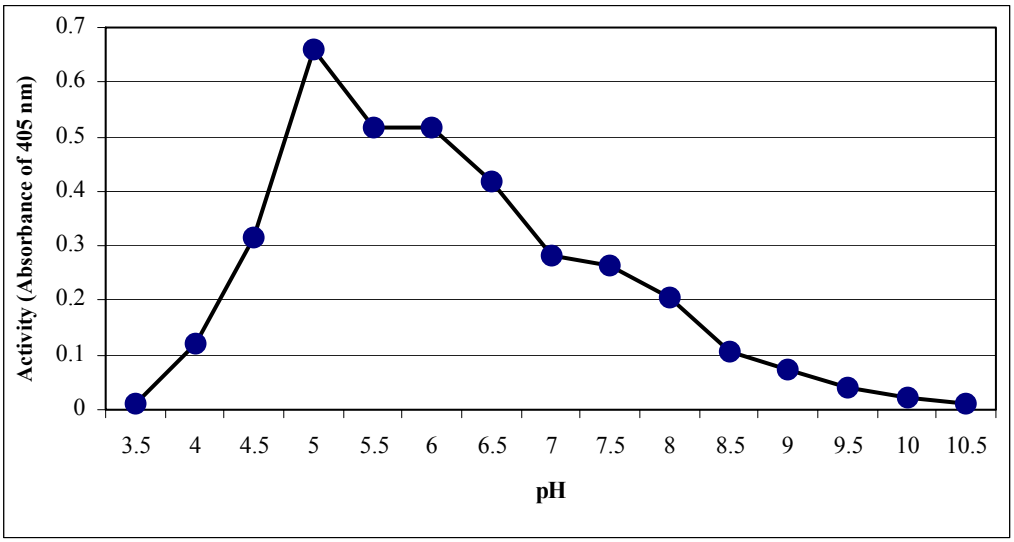


**Figure 3.7** pH profile of activity for Os4bglu12 over the pH range of 3.5 to 10.5 (formate pH 3.5-4; sodium acetate pH 4.5-5.5; sodium phosphate pH 6-8.5; CAPS pH 9-10.5). The enzyme was assayed with 0.2 mM *p*NPG at 37°C for 10 min.

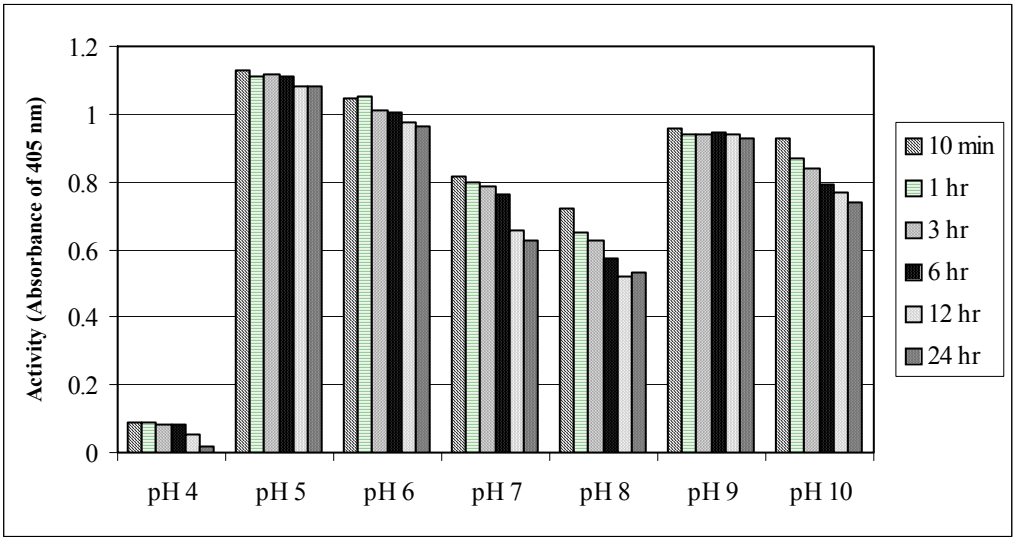


**Figure 3.8** The pH stability of Os4bglu12 after incubation for 10 min to 24 hr at room temperature (25°C) over the pH range of 4.0 to 10.0 (formate pH 4; sodium acetate pH 5; sodium phosphate pH 6-8; CAPS pH 9-10). Aliquots of enzyme in each pH buffer were removed at different times and diluted 20 fold and assayed in 50 mM sodium acetate, pH 5.0, and incubated with 0.35 mM *p*NPG at 37°C for 10 min.

The temperature optimum for Os4bglu12 activity was determined by incubating the enzyme with 0.2 mM *p*NPG for 10 min at different temperatures that ranged from 5 to 90°C. The temperature optimum for Os4bglu12 activity covers a wide range of 25-70°C (Figure 3.9). The enzyme activity decreased dramatically at temperature above 70°C. At 70°C and 80°C the enzyme activity dropped about 17% and 59%, respectively, from the maximun activity at 37°C in a 10 min assay. It was found that the Trx-Os4bglu12 was stable at 20-40°C, when incubated for up to 60 min. The enzyme was less stable at 50°C (Figure 3.10).

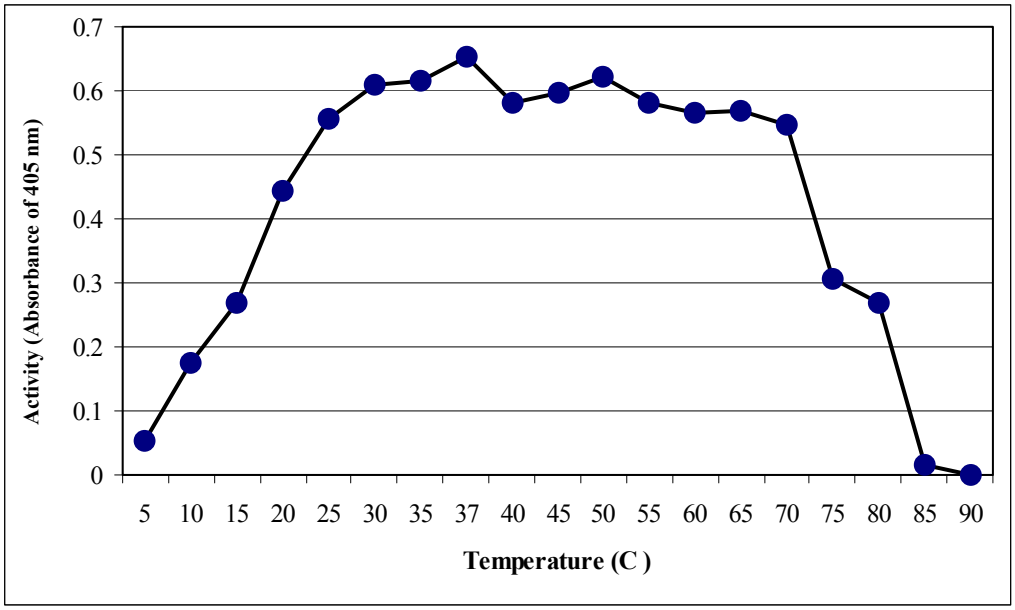


**Figure 3.9** Activity of Os4bglu12 over temperatures ranging from 5 to 90°C. The enzyme was assayed with 0.2 mM *p*NPG at 37°C for 10 min.

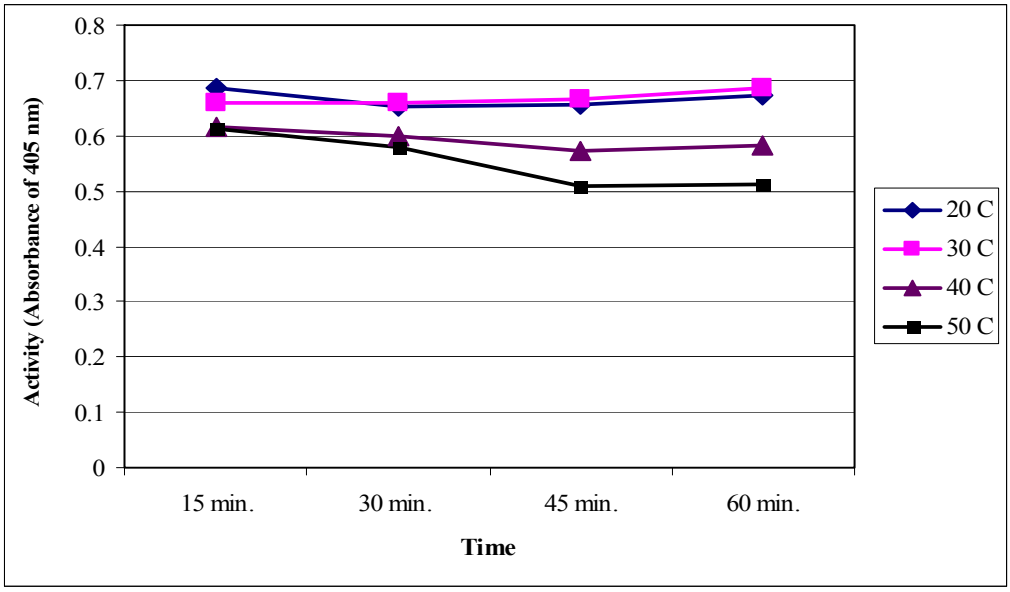


**Figure 3.10** Thermostability of Os4bglu12 after incubation for 15 to 60 min at temperatures in the range of 20-50°C. The enzyme (0.2 µg) was assayed with 0.35 mM *p*NPG at 37°C for 10 min.

### 3.3.4 Substrate specificity and kinetic parameters of Os4bglu12

The activity of the purified rice Trx-Os4bglu12 β-glucosidase towards natural and artificial glycosides was characterized. Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess glycone specificity of Os4bglu12. The enzyme activity was assayed by incubating 0.05 µg (0.72 pmol) enzyme with 1 mM *p*NP-glycoside substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C. The activity of the purified rice Trx-Os4bglu12 towards *p*NP-glycosides is summarized in Table 3.2. Among the artificial *p*NP-glycosides, Os4bglu12 hydrolyzed the *p*NP-β-D-glucoside and *p*NP-β-D-fucoside with relatively high efficiency. It hydrolyzed *p*NP-β-D-galactoside, and *p*NP-β-D-xyloside and *p*NP-α-L-arabionoside, at 45%, 45% and 26% the rate of *p*NPG, respectively. Hydrolysis of

*p*NP-β-D-manoside, *p*NP-β-D-cellobioside, *p*NP-α-D-glucoside, and *p*NP-β-L-fucoside was not detectable.

**Table 3.2** Substrate specificity of the purified rice Trx-Os4bglu12 in the hydrolysis of *p*NP-glycosides

| **Substrate** | **Specific activity[a] (μmol/min/mg)** | **Relative activity[b] (%)** |
|---|---|---|
| *p*NP-β-D-glucoside | 8.82 ± 0.7 | 100 |
| *p*NP-β-D-fucoside | 10.6 ± 0.6 | 120.4 ± 3.3 |
| *p*NP-β-D-galactoside | 4.12 ± 0.1 | 46.7 ± 2.3 |
| *p*NP-β-D-xyloside | 4.11 ± 0.1 | 46.7 ± 2.3 |
| *p*NP-α-L-arabionoside | 3.02 ± 0.8 | 34.8 ± 3 |
| *p*NP-β-D-mannoside | n.d.[c] | n.d.[c] |
| *p*NP-β-D-cellobioside | n.d.[c] | n.d.[c] |
| *p*NP-α-D-glucoside | n.d.[c] | n.d.[c] |
| *p*NP-β-L-fucoside | n.d.[c] | n.d.[c] |

[a]The assay contained 1 mM substrate in 50 mM sodium acetate, pH 5.0, at 37°C for 10 min. [b] Percent activity relative to *p*NP released from *p*NP-β-D-glucoside. [c] means not detectable.

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of Os4bglu12 enzyme in the hydrolysis of various *p*NP-β-D-glycosides was determined and the data are summarized in Table 3.3. The hydrolysis of *p*NP-β-D-glucoside and *p*NP-β-D-fucoside by the enzyme was performed with low $K_m$ and high $k_{cat}$ values. The enzyme hydrolyzed *p*NP-β-D-xyloside and *p*NP-α-L-arabinoside with lower $K_m$ and $k_{cat}$ values than *p*NP-β-D-glucoside and *p*NP-β-D-fucoside hydrolysis. The enzyme

hydrolyzed *p*NP-β-D-fucoside with the highest catalytic efficiency ($k_{cat}/K_m$ value) at 46.6 ± 1.6 $s^{-1}$ $mM^{-1}$, whereas *p*NP-β-D-glucoside, *p*NP-β-D-xyloside and *p*NP-α-L-arabionoside were hydrolyzed with 2-fold less efficiency as *p*NP-β-D-fucoside hydrolysis. Os4bglu12 hydrolyzed *p*NP-β-D-galactoside with 35% of efficiency of *p*NP-β-D-glucoside hydrolysis.

**Table 3.3** Kinetic parameters of rice Os4bglu12 in the hydrolysis of *p*NP-glycosides.

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| *p*NP-β-D-glucoside | 19.6 ± 0.8 | 0.80 ± 0.08 | 24.3 ± 0.8 |
| *p*NP-β-D-fucoside | 18.2 ± 0.6 | 0.39 ± 0.04 | 46.6 ± 1.6 |
| *p*NP-β-D-galactoside | 11.96 ± 0.9 | 1.42 ± 0.21 | 8.45 ± 1.3 |
| *p*NP-β-D-xyloside | 5.95 ± 0.26 | 0.24 ± 0.04 | 24.7 ± 0.4 |
| *p*NP-α-L-arabionoside | 2.26 ± 0.09 | 0.12 ± 0.03 | 23.25 ± 3.9 |

Substrate specificity of Os4bglu12 towards various kinds of disaccharides and oligosaccharides was determined to study the roles of this enzyme in cell wall metabolism. The enzyme activity was assayed by incubating 0.05 μg enzyme with 1 mM substrates in pH 5.0 buffer, at 37°C for 5 min. The Os4bglu12 hydrolyzed the β-1,3-linked glucose disaccharide laminaribiose, but not cellobiose (β-1,4) or gentiobiose (β-1,6). It showed high hydrolytic efficiency with β-(1,4)-linked oligosaccharides with DP of 3-6 (Table 3.4). The rate of hydrolysis of these oligomeric substrates trended to remain approximately constant with increasing DP. Hydrolysis of β-(1,3)-linked oligosaccharides with DP > 2, laminarin and barley 1,3;1,4-β-glucans by this enzyme could not be detected. On the TLC, Os4bglu12

showed hydrolytic activity towards 5 mM laminaribiose and cello-oligosaccharides, but no measurable transglycosylation activity (Figure 3.11).

**Table 3.4** Substrate specificity of the purified rice Trx-Os4bglu12 in the hydrolysis of oligosaccharides and polysaccharides.

| **Substrate** | **Specific activity[a] (μmol/min/mg)** | **Relative activity[b] (%)** |
|---|---|---|
| *p*NP-β-D-glucoside | 8.82 ± 0.7 | 100 |
| Laminaribiose | 19.52 ± 3.9 | 221 ± 44 |
| Laminaritriose | n.d.[c] | n.d.[c] |
| Laminaritetraose | n.d.[c] | n.d.[c] |
| Laminaripentaose | n.d.[c] | n.d.[c] |
| Cellobiose | n.d.[c] | n.d.[c] |
| Cellotriose[d] | 23.07 ± 3.6 | 291 ± 1 |
| Cellotetraose[d] | 23.58 ± 6.4 | 294 ± 34 |
| Cellopentaose[d] | 22.13 ± 5.7 | 277 ± 29 |
| Cellohexaose[d] | 22.11 ± 7.8 | 274 ± 54 |
| Gentiobiose | n.d.[c] | n.d.[c] |
| Laminarin | n.d.[c] | n.d.[c] |
| Barley 1,3;1,4-β-glucans | n.d.[c] | n.d.[c] |

[a] The assay contained 1 mM substrate in 50 mM sodium acetate, pH 5.0, at 37°C for 10 min. [b] Percent activity relative to glucose or *p*NP released from *p*NPG. [c]means not detectable. [d] The values for oligosaccharides are in terms of total glucose released.

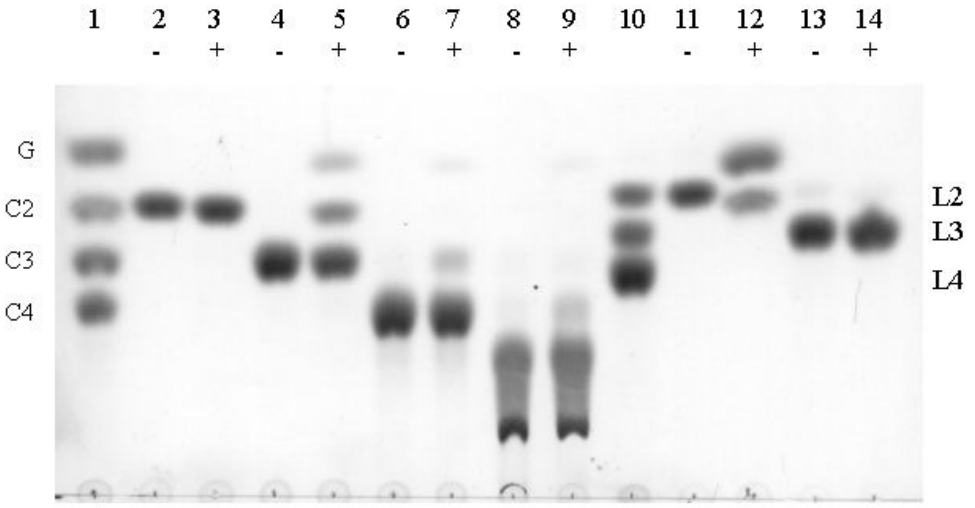


**Figure 3.11** Hydrolysis of oligosaccharide substrates by Os4bglu12 detected by TLC. The Os4bglu12 was incubated with 5 mM substrates for 30 min and the products were detected after TLC by the carbohydrate staining. Samples were incubated with (+) or without (-) enzyme. Lanes: 1, glucose (G) and cello-oligosaccharides of DP 2-4 (C2-C4) marker; 2 and 3, cellobiose; 4 and 5, cellotriose; 6 and 7, cellotetraose, 8 and 9, cellopentaose, 10 laminari-oligosaccharides of DP 2-4 (L2-L4) marker; 11 and 12, laminaribiose; 13-14 laminaritriose.

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of Os4bglu12 enzyme in the hydrolysis of various cello-oligosaccharides and laminaribiose was determined and the data are summarized in Table 3.5. The enzyme hydrolyzed cello-oligosaccharides and laminaribiose with higher $K_m$ and $k_{cat}$ values than *p*NP-glycosides. The $k_{cat}/K_m$ values for the hydrolysis cello-oligosaccahrides with DP of 4-6 tended to remain approximately constant. The enzyme hydrolyzed cellotetraose with the highest efficiency at 47 ± 8.7 $mM^{-1}$ $s^{-1}$. Os4bglu12 hydrolyzed laminaribiose (β-1,3) with less efficient than cell-oligosaccharides.

**Table 3.7** Kinetic parameters of rice Os4bglu12 in the hydrolysis of laminaribiose and cellooligosaccharides

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| Cellotriose | 87 ± 10 | 2.94 ± 0.54 | 29.7 ± 0.15 |
| Cellotetraose | 89.3 ± 4.6 | 1.9 ± 0.2 | 47 ± 8.7 |
| Cellopentaose | 96 ± 11 | 2.7 ± 0.5 | 35.3 ± 3.0 |
| Cellohexaose | 73 ± 9.4 | 1.8 ± 0.4 | 41.2 ± 3.2 |
| Laminaribiose | 87 ± 13 | 5.7 ± 1.2 | 15.1 ± 3.1 |

### 3.3.5 The cellobiose inhibition on the Os4bglu12 activity

The inhibition effect of cellobiose on Os4bglu12 hydrolysis of oligosaccharides was studied. The inhibition pattern from cellobiose was determined in the presence of 0.5 mM cellobiose in the enzyme assay containing 0.05 μg enzyme and 0.5 mM substrates at pH 5.0 and 37°C. The activity was compared between the control reaction without cellobiose, and the reaction with and without 10 min pre-incubation of the enzyme with cellobiose. It was found that in the assay with 10 min pre-incubation of the enzyme with cellobiose, the hydrolysis of cellooligosaccharides of DP 3-5 and laminaribiose by Os4bglu12 was inhibited by cellobiose with the obviously decrease in activity of 50-70%. Among the substrates tested, the hydrolysis of laminaribiose was most inhibited by cellobiose with 70% loss of activity. For the assay in which the enzyme was not pre-incubated with cellobiose, however, there was no inhibitory effect by cellobiose on the hydrolysis of cellooligosaccharides, while it showed inhibitory effect on laminaribiose hydrolysis with 20% loss of activity.

**Table 3.6** The cellobiose inhibitory effect on the oligosaccharide hydrolysis of Os4bglu12 β-glucosidase.

| | **Specific activity (umol/min/mg)[a]** | | |
|---|---|---|---|
| Substrate | Control without cellobiose | Assay with cellobiose with no pre-incubation | Assay with cellobiose with 10 min pre-incubation |
| Cellotriose | 1.35 | 1.60 | 0.66 |
| Cellotetraose | 2.19 | 2.16 | 0.84 |
| Cellopentaose | 2.13 | 2.31 | 1.06 |
| Laminaribiose | 4.91 | 4.04 | 1.37 |

[a] Os4bglu12 enzyme was assayed with the substrate in the presence of 0.5 mM of cellobiose. The reaction was assayed with 0.5 mM oligosaccharide substrates in 50 mM sodium acetate, pH 5.0, at 37°C for 15 min.

## 3.4 Discussion

### 3.4.1 Sequence analysis of rice Os4bglu12

The full length cDNA of glycosyl hydrolase family 1 β-glucosidase Os4bglu12 was previously isolated by RT-PCR from rice seedlings (Opassiri *et al.*, 2006). The cDNA sequence of *Os4bglu12* included a 1530-nucleotide long open reading frame encoding a 510 amino acid long precursor protein. The SignalP program predicted the protein to contain a 24 amino acid signal sequence and a 486 amino acid mature protein. Like many putative rice β-glucosidases, this enzyme appeared to enter the secretory pathway, as judged by their N-terminal signal sequences. There are 6 possible N-linked glycosylation sites on the Os4bglu12 protein sequence. Sequence analysis among GH family 1 β-glucosidases has revealed

that two Glu residues are highly conserved and appear within the consensus sequences: W-X-T/I-F/L/I/V/S/M-N/A/L/I/D/G-E/Q-P/I/Q and V/I/L-T/S-E-N-G, respectively (Czjzek *et al*., 2001; Opassiri *et al*., 2006). The two conserved Glu residues are found in the loop regions close to the carboxy terminal ends of β-strands 4 and 7, respectively. The Os4bglu12 polypeptide contains Glu residues at positions 203 and 417 that lie within the sequences TFNEP and ITENG, respectively, which matches the consensus motifs in family 1 β-glucosidases (Czjzek *et al*., 2001). Rice Os4bglu12 contains the conserved glucose binding residues found in other GH1 β-glucosidases including Q38, H142, E191, E406, E464 and W465 in maize Bglu1 (Czjzek *et al*.,2000).

The protein product for *Os4bglu12* gene has highest sequence similarity to the previously described cell wall-bound β-glucosidase purified from rice seedlings (Akiyama *et al*., 1998). The Mr of the rice Os4bglu12 mature protein is approximately 55 kDa, which is similar to the Mr (56 kDa) of cell-wall-bound rice β-glucosidase, which is a monomeric protein (Akiyama *et al*., 1998). The predicted pI of Os4bglu12 is 8.85, which indicates that the enzyme is a basic protein. The basic pI of the rice Os4bglu12 is similar to that described for the other enzymes involved in oligosaccharide hydrolysis and purified from the cell wall of rice seedlings (pI 10) (Akiyama *et al*., 1998), and germinated barley seeds (pI 9-9.5) (Leah *et al*., 1995; Hrmova *et al*., 1996).

### 3.4.2 Expression of recombinant rice Os4bglu12 β-glucosidases

The function of the *Os4bglu12* gene as expressing a β-glucosidase was confirmed by characterization of the biochemical and catalytic properties of the

enzyme expressed as a recombinant protein in *E. coli*. Similar to rice BGlu1 β-glucosidase (Opassiri *et al*., 2003), Os4glu12 which is targeted to secretion and likely be glycosylated in the plant, could be functionally expressed in *E. coli*. Expression in *E. coli* of other grass β-glucosidase has been successful in producing active and soluble enzymes from maize and sorghum form using a pET21a(+)/BL21(DE3) pLys system (Cicek and Esen, 1998b). Note that the other enzymes from grasses expressed outside of our lab are chloroplast enzymes, so they are unlikely to be glycosylated.

In this study, the conditions for biosynthesis of functional Trx-Os4bglu12 in Origami B(DE3) was optimized. There were no significant differences in the activity of Trx-Os4bglu12 expressed in *E. coli* in the presence of 0.1-0.5 mM IPTG at 20, 25 and 30°C, for 8-16 hr. Cicek and Esen (1998) expressed the maize β-glucosidases, rGlu1 and rGlu2, from the pET21 vector in *E. coli* strain BL21 (DE3)pLys and indicated that a higher percentage of total expressed β-glucosidase was soluble when the cultures were grown and induced at room temperature (25°C) than at 37°C. The recombinant linamarase of cassava (Keresztessy *et al*., 1996) and primeverosidase of tea leaves (Mizutani *et al*., 2002) in *E. coli* were also not soluble and directed to inclusion bodies when cultures were grown and induced at 37°C. This suggested that the recombinant proteins tend to form inclusion bodies when synthesis was induced at higher temperature.

Purification of the recombinant protein was designed to allow easy and fast, single-step purification. An affinity-fusion system was used as the recombinant protein purification strategy, which facilitates the use of affinity chromatography. The recombinant Trx-Os4bglu12 could be purified by IMAC on BD Talon$^{TM}$ (immobilized cobalt) metal affinity column. Approximatly 95% purity of Trx-fusion

Os4bglu12 was obtained from elution fractions 1-6 when using 250 mM immidazole elution.

### 3.4.3 Effect of pH and temperature on the activity and stability

The pH optimum of the purified Os4bglu12 enzyme was pH 5.0, which is similar to other β-glucosidases (Hrmova and Fincher, 1998; Esen, 1993 and Opassiri, 2003). This pH is related to the pH environment of the catalytic glutamic acids. Os4bglu12 was relatively stable over the pH range of 5.0 to 7.0 up to 24 hr at 25°C, which indicated the reversible inactivation of the enzyme in this pH range. Os4bglu12 was highly active over a wide temperature range of 25-70°C with maximum activity at 37°C in a 10 min assay. However, the enzyme was not stable at temperatures higher than 50°C when incubated more than 10 min. This indicated that irriversible inactivation of the enzyme occurred at temperatures higher than 50°C. Therefore, the temperature optimum for the activity of Os4bglu12 is in the range of 25-40°C, but not up to 70°C, which is similar to rice BGlu1 (Opassiri, 2003). Many β-glucosidases have temperature optima near 50°C (Akiyama *et al*., 1998; Konno *et al*., 1996 and Riou *et al*., 1998), but some β-glucosidases have higher optimal temperatures, such as the Thai rosewood and *Dalbergia nigrescens* β-glucosidases which has a temperature optimum of 60 and 65°C, respectively (Srisomsap *et al*., 1996; Chuankhayan, 2004). Dixon and Webb (1979) stated that the maximum temperature for a given enzyme depends on a balance between the rate of the catalytic reaction and denaturation. β-glucosidases from different organisms might have different optimum temperature and stability, which would reflect different interactions stabilizing enzyme tertiary and quaternary structures.

### 3.4.4 Substrate specificity of Os4bglu12 β-glucosidases

The Os4bglu12 showed high cleavage efficiency at different rates with β-(1,4)-linked oligosaccharides with degrees of polymerization (DP) of 3-6. However, this enzyme could not hydrolyze the β-1,4-linked glucose disaccharide cellobiose or β-1,6-linked disaccharide gentiobiose. Among β-1,3-linked laminari-oligosaccharides, Os4bglu12 hydrolyzed only the laminaribiose, but hydrolysis of those with DP more than 2 could not be detected. It showed high hydrolytic efficiency at approximately constant rates with β-(1,4)-linked oligosaccharides with DP of 4-6, which is a characteristic often observed with β-glucosidases (Reese *et al*., 1968). The preference of the rice Os4bglu12 for (1,4)-β-oligosaccharides suggested that it has an extensive subsite binding region where each subsite binds a single glycosyl residue of oligosaccharide, as has been described for barley β-glucan glucohydrolases and β-glucosidase/1,4-β-glucan exohydrolase exohydrolases (Hrmova *et al*., 1996; 1998; 2001).

Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess glycone specificity of Os4bglu12. It hydrolyzed *p*NPG and *p*NP-β-D-fucoside with 2-3 fold lower efficiency than oligosaccharides. The enzyme hydrolyzed various kinds of *p*NP-β-D-glycosides with catalytic efficiency ($k_{cat}/K_m$) values that varied 8 fold from *p*NP-β-D-galactoside to *p*NP-β-D fucoside, which suggests that the sugar moiety has a moderately high effect on the rate of hydrolysis, like maize and rice BGlu1 β-glucosidases (Babcock and Esen, 1994; Opassiri *et al*., 2004). In contrast, the hydrolysis of *p*NP-β-D-mannoside, *p*NP-β-D-cellobioside, *p*NP-α-D-glucoside, and *p*NP-β-L-fucoside could not be detected. High hydrolysis of

β-xyloside is similar to white clover β-glucosidase, but otherwise rare in GH1 enzymes that have been characterized to date (Marana, 2006). The enzyme could not hydrolyze *p*NP-cellobiose, so, the rapid hydrolysis of cellotriose to cellobiose and glucose may reflect binding to the active site in a manner different from *p*NP-β-D-cellobiose.

Rice Os4bglu12 showed hydrolysis activity towards various substrates which are β-linked glucose oligosaccharides, but not polysaccharides, which is similar to rice BGlu1 (Opassiri *et al*., 2003), cell wall-bound β-glucosidases (Akiyama *et al*., 1998) and barley βII β-glucosidase (Hrmova *et al*., 1998). However, the specificity for glycones and substrate chain lengths of these enzymes are different. In contrast to barley and rice cell wall-bound enzyme, Os4bglu12 did not hydrolyze β-(1,3)-linked oligosaccharides longer than laminaribiose, but hydrolyzed various *p*NP-derivatives of monosaccharides. The substrate preference of Os4bglu12 is somewhat similar to rice BGlu1, in that they both show slightly faster hydrolysis of *p*NP-β-D-fucoside than *p*NP-β-D-glucoside and hydrolyze laminaribiose and cello-oligosaccharides. However, there were many differences between these enzymes. For example, in contrast to rice BGlu1, Os4bglu12 hydrolyzed β-(1,4)-linked oligosaccharides and laminaribiose at higher rates than *p*NP-β-D-glucoside, and did not hydrolyze cellobiose, gentiobiose, *p*NP-β-D-mannoside, and *p*NP-β-D-cellobioside.

Their sequence differences are likely to reflect the differences in substrate binding to the active site between these enzymes. Different from rice BGlu1 and barley BGQ60, some of substrate binding residues, such as F198 and F466, that line the active site cleft and interact with the substrate aglycone of maize Bglu1

Czjzek *et al*. (2000), were not conserved in the Os4bglu12 sequence, which may account for the different substrate specificities for some oligosaccharides and glycones. For instance, BGlu1 and barley BGQ60 cluster with tomato and *Arabidopsis* β-mannosidases in sequence-based phylogenetic analysis and can hydrolyze β-mannoside, while Os4bglu12 does not, and they also hydrolyze longer chain β-1,3-linked oligosaccharides (Opassiri *et al*., 2004; Hrmova *et al*., 1998). All three enzymes prefer shorter 1,3-linked oligosaccharides, with Os4bglu12 being the most extreme, only hydrolyzing the dimer with this linkage. This likely reflects the bent shape of oligosaccharides with the 1,3-linkage, which is somehow incompatible with the active site for longer chains.

In addition, Os4bglu12 could hydrolyze cell wall oligosaccharides released from 1,3;1,4-β-glucans of rice cell wall by rice endo-1,3;1,4-β-glucanase which implies that these enzymes may play cooperative roles in hydrolysis of 1,3;1,4-β-glucans of cell walls (Maneesan, 2007). This mode of action of Os4bglu12 in the hydrolysis of cell wall oligosaccharides released from 1,3;1,4-β-glucans of rice cell wall by rice endo-1,3;1,4-β-glucanase is somewhat similar to barley BGQ60 β-glucosidase (Leah *et al*., 1995). In addition, the study from Hrmova and Fincher (2001) indicated that (1-3),(1-4)-β-D-pentasaccharide has a very similar shape to cellopentaose, particularly at the nonreducing end. Since Os4bglu12 preferentially hydrolyzed cello-oligosaccharide and is able to hydrolyze laminaribiose, (1,3);(1,4)-β-D-oligosaccharides could be completely depolymerized to glucose by Os4bglu12. As Os4bglu12 could not hydrolyze laminari-oligasaccharides with DP higher than 2, it could be speculated that the laminaribiase activity of this enzyme might specifically hydrolyze a single (1-3)-β-D-glucosyl residue that was originally at reducing terminus

of a (1,3),(1,4)-oligosacchride. Elucidation of the tertiary structures of this enzyme would help to clarify the enzyme-substrate binding mechanism leading to these preferences.

### 3.4.5 Inhibitory effect of cellobiose on Os4bglu12 enzyme activity

The hydrolysis of cellooligosaccharides with DP 3-5 and laminaribiose by Os4bglu12 was inhibited by 0.5 mM cellobiose by 50-70% when the enzyme was pre-incubated with cellobiose for 10 min. However, there was no significant difference in the hydrolysis of these substrates, except laminaribiose, when cellobiose was put in the assay reaction at the same time as the substrates. The inhibitory effect of cellobiose could indicate that it tightly binds in the substrate binding pocket at the productive or nonproductive subsites resulting in it interfering with the binding of other substrates at the active site.

### 3.4.6 Possible biological functions of rice Os4bglu12 β-glucosidase

It was previously showed that *Os4bglu12* mRNA is highly expressed in shoots of rice seedlings, and in leaf sheaths and stems of 6-week-old mature rice plants (Maneesan, 2007). The high preference of Os4bglu12 to hydrolyze cello-oligosaccharides and laminaribiose suggested Os4bglu12 may play a role in the cell-wall regeneration process by completing the depolymerization to release glucose. At least two major cell wall polysaccharides present in rice tissues, (1,4)-β-glucan (cellulose) and (1,3)-β-glucan, may be sources of oligosaccharide hydrolyzed by Os4bglu12 thereby promoting reconstruction of cell wall. The reorganization and alteration of specific β-linked polysaccharides involves selective cleavage of

monosaccharides derived from β-linked polysaccharide to re-enter the sugar-nucleotide interconversion pathways and contribute to the synthesis of new β-linked polysaccharides (Gibeaut and Carpita, 1991; Carpita and Gibeaut, 1993). The mRNA levels of both endo-1,3;1,4-β-glucanase (Akiyama T., unpublished data) and *Os4bglu12* β-glucosidase gene in rice seedlings are induced within 5 to 10 hr after wounding, methyl jasmonate and ethephon treatments (Maneesan, 2007). Consistent with this study, Wang *et al.* (2005) reported that the transcript level of EST contig BHPiw028, homologous to *Os4bglu12*, increased in response to brown planthopper attack, as observed from subtractive hybridization cDNA library screening. Os4bglu12 might be involved in the cell wall cross-linking process to restore damaged tissues, which may be mediated by the methyl jasmonate and ethylene signal transduction pathways (Maneesan, 2007). Lee *et al*. (2007) reported that the expression of three *Arabidopsis* secreted proteins including a GH35 β-galactosidase, a GH3 β-xylosidase and a GH1 β-glucosidase, in suspension cells are induced by sugar starvation. In addition, the decrease in the amount of monosaccharide in pectin and hemicellulose could be detected in detached leaves in response to sugar starvation, suggesting the roles the cell wall as a storage reserve of carbon in addition to providing physical support for the plant body (Lee *et al*., 2007).

Maneesan (2007) also reported that Os4bglu12 enzyme could hydrolyze putative flavonoid glycosides extracted from leaves and stem of rice plants at flowering stage. According to the previous evidence in the induction of Os4bglu12 mRNA levels in response to wounding stress and brown planthopper attack (Maneesan, 2007; Wang *et al*., 2005), Os4bglu12 might play another role in releasing active flavonols for defense against herbivore attack or pathogenic microorganisms

infection after wounding. Recently, Naoumkina *et al*. (2007) reported the induction of four flavone/isoflavone β-glucosidases transcript levels in cell suspensions of a legume, *Medicago truncatula*, within 30 min to 2 hr as confirmed by DNA microarray analysis, and the accumulation of the free isoflavonoid phytoalexin medicarpin released from its glycosides in response to methyl jasmonate. They suggested that methyl jasmonate might play a role as a signal for rapid hydrolysis of preformed, conjugated intermediates for the biosynthesis of defensive compounds during wound responses.

Suzuki *et al*. (2006) reported that soybeans (*Glycine max*) excrete isoflavones released from their glycosides by β-glucosidase from their roots, which participate in plant-microbe interactions. They also reported that soybean β-glucosidase is a glycoprotein and was found to be localized in the cell wall and the apoplast of seedling roots. The pI value of the mature form of the enzyme was predicted to be 8.1, providing the possibility that this cationic protein binds to pectic polysaccharides that are also present in plant cell walls and the intercellular space. In addition to the high protein sequence identity (60%) with soybean isoflavone β-glucosidase, Os4bglu12 enzymes appeared to enter the secretory pathway and contains 6 possible N-linked glycosylation sites. The predicted pI of Os4bglu12 is 8.9, indicating that the enzyme is a basic protein. It is possible that rice Os4bglu12 might function and be localized in similar manner to the soybean enzyme.

## 3.5 Conclusions

The rice *Os4bglu12* β-glucosidase cDNA encoding a 510 amino acid long precursor protein with an amino acid sequence that was most similar to the previously purified and characterized cell wall-bound β-glucosidase. Recombinant Os4bglu12 protein was most active at pH 5.0 and stable over the pH range 5.0 to 7.0, when incubated for up to 24 hr. The temperature optimum for Os4bglu12 activity is in the range 25-40°C. Os4bglu12 β-glucosidase hydrolyzed the β-1,3-linked glucose disaccharide laminaribiose, but not laminarioligosaccharides with higher DP than 2. It showed high hydrolytic efficiency at approximately constant rates with β-(1,4)-linked oligosaccharides with DP of 4-6. The enzyme hydrolyzed various kinds of *p*NP-β-D-glycosides with catalytic efficiency ($k_{cat}/K_m$) values that varied 8-fold from *p*NP-β-D-galactoside (lowest) to *p*NP-β-D fucoside (highest). The assay in which the Os4bglu12 was pre-incubated with cellobiose for 10 min indicated that cellobiose could inhibit the hydrolysis of cellooligosaccharides and laminaribiose by Os4bglu12. The specificity of Os4bglu12 for oligosaccharides and *p*NP-glycosides was different from the previously characterized GH1 β-glucosidases/exoglucanases, cell-wall bound rice β-glucosidase, rice BGlu1, and barley βII β-glucosidase**.**

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# CHAPTER IV

# RECOMBINANT PROTEIN EXPRESSION AND CHARACTERIZATION OF RICE GLYCOSYL HYDROLASE FAMILY 5 GH5BG β-GLUCOSIDASE


## Abstract

The full-length cDNA of glycosyl hydrolase family 5 β-glucosidase was previously cloned from indica rice cv. KDML105 seedlings by RT-PCR, and was designated *GH5BG*. The *GH5BG* cDNA included an open reading frame encoding a 510 amino acid precursor protein predicted to consist of a 19 amino acid long prepeptide and a 491 amino acid long mature protein. The protein was predicted to be extracellular. The mature protein contains two major domains, a β-1,3-exoglucanase-like domain and a fascin-like domain, which is not commonly found in plant enzymes. A thioredoxin fusion protein produced from the *GH5BG* cDNA in *E. coli* hydrolyzed various *p*-nitrophenyl-glycosides. The GH5BG exhibited a marked preference for β-(1,4)-linked oligosaccharides and β-(1,3)-linked disaccharide. The catalytic efficiency ($k_{cat}/K_m$) for hydrolysis of β-(1,4)-linked oligosaccharides by the enzyme remained constant as the degree of polymerization (DP) increased from 3 to 5. This substrate specificity is significantly different from fungal GH5 exoglucanases, such as *Candida albicans* exo-β-(1,3)-glucanase, which may correlate to a marked reduction in a loop that makes up the active site wall in the *Candida* enzyme.

## 4.1 Introduction

GH family 5 (GH5) is one clan A GH family, originally identified as cellulase family A (Gilkes *et al*., 1991). The structures of GH5 enzymes contain a core $(\beta/\alpha)_8$ barrel with two catalytic amino acid residues, an acid/base and a nucleophile, on the ends of strands 4 and 7 of the barrel, respectively (Cutfield *et al*., 1999). GH5 contains the enzymes with a wide range of catalytic activities, including cellulases, chitosanases, endoglucanases, exoglucanases, exoxylanases, endoxylanases, β-mannanases, and endoglycoceramidases. The GH5 enzymes have mainly been investigated in microorganisms (Tsai *et al*., 2003; Tanabe *et al*., 2003; Reinhold-Hurek *et al*., 2006; Cutfield *et al*., 1999; Mitreva-Dautova *et al*., 2006; Perret *et al*., 2004; Caines *et al*., 2007). Recently, β-mannanase from a plant was described by Hrmova *et al*. (2006). Although there are high variation in the protein sequences and enzyme activities of the family members, they all possess eight conserved residues (including two glutamate residues acting as catalytic acid/base and nucleophile) around the active site, which distinguish GH5 from other GH families (Cutfield *et al*., 1999; Sakon *et al*., 1996).

Exoglucanases are generally secreted enzymes with both hydrolase and transferase activities on β-glucans (Mackenzie *et al*., 1997). Exo-β-(1,3)-glucanases may act in the metabolism of cell wall glucan by cleaving a single glucose from the nonreducing end of β-1,3-glucans (Stubbs *et al*., 1999). Most GH5 exoglucanases that have been studied are fungal exo-β-(1,3)-glucanases, including those from *C. albicans* (Cutfield *et al*., 1999; Chambers *et al*., 1993), *S. cervisiae* (Vazquez de Aldana *et al*., 1991), *Agaricus bisporus* (van de Rhee *et al*., 1996), *Lentinula edodes* (Sakamoto *et al*., 2005), and *Pichia pastoris* (Xu *et al*., 2006). Cutfield *et al*. (1999)

reported the structure of *C. albicans* Exg to be a distorted $(\beta/\alpha)_8$ barrel structure with a deep active-site pocket. The geometry of the pocket fits for cleavage of β-1,3- but not β-1,4-glycosidic linkages. According to active site labeling and mutagenesis experiments, Glu-192 and Glu-292 in the mature *C. albicans* Exg protein were identified as the proton donor and nucleophile, respectively (Chambers *et al*., 1993; Mackenzie *et al*., 1997).

There has been no previous report of characterization of a GH5 exoglucanase from a plant. As reported in CAZY homepage (http://www.cazy.org/CAZY/), there are twenty GH5 genes putatively encoding 7 cellulases, 9 endo-β-mannanases, 3 glucan 1,3-β-glucosidases, and one 1,3-β-glucanase in rice genome databases. There appear to be four putative rice genes encoding putative glucan 1,3-β-glucanases including Genbank accession numbers (AC) AAM08614, AAM08620, AAV43969 and BAD10703 (Opassiri *et al*., 2007). A BLAST comparison revealed that AAM08620 and AAV43969 protein sequences have 71% and 69% identity to AAM08614, respectively. The amino acid sequence of the putative 1,3-β-glucanase BAD10703 is also 49% identical to AAM08620, which has only 28-33% identity with glucan-1,3-β-glucosidases of fungi (Opassiri *et al*., 2007).

In this study, the cDNA of one of the putative GH5 glucan-1,3-β-glucosidase containing a fascin-like domain was cloned into an expression vector and its function assessed by recombinant protein expression. The catalytic activity indicated the enzyme is a β-glucosidase. This is the first report of a GH5 β-glucosidase from a plant that contains a fascin-like domain.

## 4.2 Materials and methods

### 4.2.1 Materials and chemicals

#### 4.2.1.1 Chemicals and reagents

The chemicals and reagents used in this experiment is the same as the list in section 3.2.1.1

#### 4.2.1.2 Bacteria strains

Origami B(DE3) *E. coli* host strain was used for recombinant protein expression. DH5α *E. coli* host strain was used for used for cloning plasmids.

#### 4.2.1.3 GH5BG cDNA template

A full-coding sequence cDNA of glycosyl hydrolase family 5 (GH5) β-glucosidase, *GH5BG,* used to cloned into expression vector in this study was previously cloned and inserted into pBlueScript II SK+ plasmid by Tassanee Onkoksoong, as described in section 2 in Appendix A.

### 4.2.2 Putative GH5BG protein sequence analysis

Protein sequence alignments were done with the ClustalX implementation of ClustalW (Jeanmougin *et al*., 1998; Thompson *et al*., 1994) and manually adjusted with the Gendoc program (Nicholas and Nicholas, 1997). The protein sequence alignment of GH5BG was made with exo-β-(1,3)-glucanase from *Candida albicans* (AC CAA39908), *Lentinula edodes* (AC AB192344), *Pichia pastoris* (AC AY954499), and endo-β-(1,4)-glucanase from *Clostridium thermocellum* (AC AAA23220). Protein sequence analyses were done at the Expasy

proteomics server (http://www.expasy.org/), and the signal sequence and cellular location were predicted with SignalP (Bendtsen *et al*., 2004) and PSORT (Nakai and Horton, 1999), respectively.

### 4.2.3 Construction of recombinant plasmid for protein expression in *E. coli*

The cDNA encoding the predicted mature protein of the *GH5BG* was PCR amplified with the cloned full-length cDNA as the template, the AAM08614matNcoIf (5'-CACCATGGTCTCCGATGGGAGGACG-3') and AAM08614XhoIstopr (5'-CCCTCGAGCTAGCTTTTGAGAGAGATGATCC-3') primers to introduce an *Nco*I site at the 5' end and an *Xho*I site at the 3' end. The amplification was performed with 30 cycles of 94°C, 30 sec, 45°C 30 sec and 72°C 4 min using *Pfu* DNA polymerase (Promega, Madison, USA). The cDNA product was digested with *Nco*I and *Xho*I, ligated into pENTR4 Gateway entry vector (Invitrogen) digested with the same restriction enzymes, and transformed into DH5α *E. coli* by heat shock method (Appendix A). The positive clones were selected on 30 μg/mL kanamycin LB-agar. The cDNA product was subcloned into the pET32a+/DEST Gateway expression vector by LR Clonase recombination by the recommended protocol (Invitrogen). In brief, the *GH5BG* cDNA in pENTR4 Gateway vectors flanked by attL at both termini of the cDNA was used as the entry clone, while pET32a+/DEST, which contains attR was used as the destination vector. The LR recombination reaction was performed by mixing equal amounts of entry clone and destination vector (150 ng) in a 5 μL total reaction volume that contained 1 μL of LR clonase buffer and 1 μL of LR clonase enzyme mix. After 4-16 hr incubation at

25°C, 0.5 μL Proteinase K was added to stop the reaction. The recombination reaction solution was transformed into DH5α by heat shock method, and the plasmid was isolated from ampicillin-resistant clones thoroughly sequenced. The recombinant pET32a+/DEST-*GB5BG* plasmid was transformed into *E. coli* strain Origami B(DE3), and positive clones were selected on 15 μg/ml kanamycin, 12.5 μg/ml tetracycline and 50 μg/ml ampicillin LB-agar.

### 4.2.4 Recombinant protein expression in Origami B(DE3) *E. coli*

To produce the protein, selected clones were grown in the selection media at 37°C until the optical density at 600 nm reached 0.5-0.6. Then IPTG was added to the culture medium to concentrations of 0.1-1 mM to induce the expression of the target proteins at different temperatures in the range of 15-25°C for 4-20 hr. The optimal condition for recombinant protein expression was obtained by inducing the cells with 0.5 mM IPTG at 20°C for 12 hr. Induced cultures were harvested by centrifugation at 3000 × g at 4°C for 10 min. The cell pellets were resuspended in freshly prepared extraction buffer (50 mM sodium phosphate, pH 8.0, 200 μg/ml lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonyl-fluoride, 40 μg/ml DNase I), and incubated at room temperature for 30 min. The soluble protein was recovered by centrifugation at 12,000 x g at 4°C for 10 min as described in section 3.2.5. The expressed thioredoxin-GH5BG fusion protein (Trx-GH5BG) was purified by immobilized metal affinity chromatography (IMAC) with BD TALON cobalt resin according to the manufacturer's instructions (Clontech, Palo Alto, CA), as described in section 3.2.6. The fractions with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) hydrolysis activity were pooled and concentrated with 10 kDa-cut off centrifugal

ultrafiltration membranes (YM-10, Amicon, Beverly, MA). All of the protein samples were analyzed by SDS-PAGE as described in section 3.2.8. The protein concentration was determined by Bio-RAD protein assay kit (see section 3.2.7)

### 4.2.5 pH and temperature profiles of activity and stability

pH and temperature profiles of activity and stability for Trx-GH5BG enzyme were determined using the same method described for Trx-Os4bglu12 (see section 3.2.9).

### 4.2.6 Activity assays and kinetics study

The initial velocity of hydrolysis for each substrate was initially determined using various protein concentrations and incubation times (5-20 min) to find conditions that yield a 0.1 to 1.0 absorbance units. Kinetic parameters were calculated from triplicate assays of 5-7 substrate concentrations done at 37°C in 50 mM sodium acetate, pH 5.0. The purified Trx-GH5BG recombinant protein was tested against *p*NP-derivatives of monosaccharides and cellobioside to determine sugar specificity. In a 100 μL reaction assay volume, 1.47-2.94 pmol enzyme was incubated with substrate in 50 mM sodium acetate, pH 5.0, at 37°C, except for the assay with *p*NP-β-D-cellobioside, in which 29.4 pmol enzyme was used. At the end of the reaction time, 50 μL of 0.4 M sodium carbonate was added to stop the reaction, and the absorbance of the liberated *p*NP was measured at 405 nm.

The enzyme was tested with oligosaccharides including cello-oligosaccharides with DP of 2-6, laminari-oligosaccharides with DP of 2-5 and gentiobiose. In a 50 μL reaction volume, 0.74 pmol enzyme was incubated with

substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C, except for the assay with cellobiose, in which 14.7 pmol enzyme was used. The reactions were stopped by boiling, and the glucose released was quantified by the PGO assay method (see section 3.2.10).

Kinetic parameters, $K_m$ and $V_{max}$ (at pH 5.0 and 37°C), were calculated by linear regression of Lineweaver and Burk plots with the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). Note that the activity values for disaccharides were determined by dividing the amount of glucose released by two, since two glucose molecules are released per molecule of disaccharides hydrolyzed. The amount of products for oligosaccharides is given in terms of total glucose released, since release of more than one glucose per substrate molecule due to sequential cleavage should be negligible for $V_0$. The enzyme molarity was calculated based on the molecular weight of 68 kDa of Trx-GH5BG.

The enzyme was also tested for hydrolysis of polysaccharides. In the assay, 1-5 μg enzyme was incubated separately with 0.5% (w/v) laminarin and barley β-glucans in 50 mM sodium acetate, pH 5.0, at 37°C for 30-60 min. The reaction was stopped by the addition of *p*-hydroxybenzoic acid hydrozide reagent and the increase in reducing sugars was measured colorimetrically, as described by Lever (1972).

The products of GH5BG hydrolysis of cello- and laminari-oligosaccharides were detected by thin layer chromatography (TLC). In a 50 μL reaction, 7.4 pmol enzyme was incubated with 5 mM substrate in 50 mM sodium acetate, pH 5.0, for 30 min at 37°C. Five microliters of the reaction mixture was spotted on silica gel 60 $F_{254}$ plates (Merck, Darmstadt, Germany) and

chromatographed vertically with ethyl acetate-acetic acid-water (2:1:1) as solvent. The products were detected by spraying with developer solution (ethanolic 10% $H_2SO_4$) and baking at 120°C for 5 min to visualize the sugar.

### 4.2.6 Effects of metal ions on enzyme activity

The effects of selected cations on enzyme activity were determined by adding to 10 mM final concentration the following metal ion salts: $BaCl_2$, $CdCl_2$, $CoCl_2$, $FeSO_4$, $HgCl_2$, $LiCl_2$, $MnCl_2$, $MgCl_2$, $SrCl_2$, NaCl, KCl, $ZnSO_4$, and $CuSO_4$, as well as L-histidine and EDTA. The purified Trx-GH5BG enzyme (0.2 μg) was incubated with 10 mM metal ions. The enzyme samples were aliqouted at 0 and 10 min incubation and diluted 20 times in 50 mM sodium acetate buffer, pH 5.0, and assayed for activity with 1 mM *p*NPG as described in section 3.2.10.

## 4.3 Results

### 4.3.1 Rice GH5BG protein sequence analysis

A putative GH5 glucan-1,3-β-glucosidase cDNA, designated *GH5BG*, was cloned from rice seedlings by RT-PCR with KDML105 rice seedling RNA. The primers used to amplify the *GH5BG* cDNA were designed from the GenBank accession number AC107314 (the rice genomic contig from which AAM08614 is derived) and AK065000 (full-length cDNA, Kikuchi *et al*., 2003) sequences. A specific PCR product of 1680 bp was produced, and its sequence overlapped those of AC107314 and AK065000. The full-length cDNA sequence contains a 1530-nucleotide open reading frame (ORF) encoding a 510-amino-acid precursor protein (Figure 4.1). The protein sequence was predicted to contain a 19-amino-acid-long

prepeptide and a 491-amino-acid-long mature protein. The protein was predicted to be secreted out of the cell by PSORT. Its predicted pI is 5.28.

AAM08614matNcoIf primer

```
ATGCGCCATTTTGAGCTCCTCCTCTTCCTCCTCCTCTGCTCGCCGTGCCTCTTCTCAGTCTCCGATGGGAGGACGGTG
M  R  H  F  E  L  L  L  F  L  L  L  C  S  P  C  L  F  S  V  S  D  G  R  T  V

CGGCCCGCGAAACGGGGCGCCCCGTCGCCCCCCATTCGGGCGGTGAACCTTGGAGGTTGGCTCGTCACGGAGGGCTGG
R  P  A  K  R  G  A  P  S  P  P  I  R  A  V  N  L  G  G  W  L  V  T  E  G  W

ATCCTTCCCTCCCTCTTCGACGACATTCCAAACAAAGACCTCCTGGATGGAACCCAGCTGCAATTCAAGTCGGTGACG
I  L  P  S  L  F  D  D  I  P  N  K  D  L  L  D  G  T  Q  L  Q  F  K  S  V  T

CAGAACATGTACCTGTGCGCCGAGCAGGGCGGCGGCACCATCCTGGTGGCGAACCGGACGAGCGCCTCGGGGTGGGAG
Q  N  M  Y  L  C  A  E  Q  G  G  G  T  I  L  V  A  N  R  T  S  A  S  G  W  E

ACCTTCAAGCTGTGGAGGATCGACGAGGACACGTTCGACCTCAGGGTGTTCGACAACCTGTTCGTCACCGTCGCCGGC
T  F  K  L  W  R  I  D  E  D  T  F  D  L  R  V  F  D  N  L  F  V  T  V  A  G

GACGGGGTCACCGTCGTGGCGACGGTGGCGTCGCCGGGGCCCGGGGAGGCGTTCCAGATCGTGCGCAACGGCGACAAG
D  G  V  T  V  V  A  T  V  A  S  P  G  P  G  E  A  F  Q  I  V  R  N  G  D  K

ACTCGCGCGCGCATCAGGGCACCCAATGGCATGTTCCTGCAGGCGAAGACAAGCGACTCAGTTACAGCAGATTATGAT
T  R  A  R  I  R  A  P  N  G  M  F  L  Q  A  K  T  S  D  S  V  T  A  D  Y  D

GGGGAGACAAATTGGGGCGACGATGACCCCTCTGTGTTTGTGGTCACTAGGGTAGGCGGTCTACAAGGGGAGTACCAA
G  E  T  N  W  G  D  D  D  P  S  V  F  V  V  T  R  V  G  G  L  Q  G  E  Y  Q

ATATGCAATGGATATGGTAAAGCAAAGGCCACACAAGTGCTCAGGGAACACTGGCGCACATACATAGTTGAGAGCGAC
I  C  N  G  Y  G  K  A  K  A  T  Q  V  L  R  E  H  W  R  T  Y  I  V  E  S  D

TTCAAGTTCATATCAACAAGTGGGCTAAACGCAGTGAGAATTCCAGTTGGATGGTGGATTGCTAGCGACCCCAATCCT
F  K  F  I  S  T  S  G  L  N  A  V  R  I  P  V  G  W  W  I  A  S  D  P  N  P

CCTGCTCCTTTCGTTGGAGGATCCCTCCAAGCCTTGGATAATGCATTCAAATGGGCAGAGAAGTATAATTTGGGAGTC
P  A  P  F  V  G  G  S  L  Q  A  L  D  N  A  F  K  W  A  E  K  Y  N  L  G  V

ATTGTGGACCTGCACGCTGCACCTGGGTCGCAGAACCCGTTTGAGCACAGTGCCTCCAGAGATGGTTCACAGGACTGG
I  V  D  L  H  A  A  P  G  S  Q  N  P  F  E  H  S  A  S  R  D  G  S  Q  D  W

GGCACCACGGATGCCAACATCGCCCAAACTGTCCAAGTCATCGATTTCCTCACACACAGATACGCGAGTAGCCCCAGC
G  T  T  D  A  N  I  A  Q  T  V  Q  V  I  D  F  L  T  H  R  Y  A  S  S  P  S

CTCCTGGCCGTGGAGCTCCTGAACGAGCCACTGGCACCCGGGGTGACTCTCCCGGCTCTCATGAGATACTACAAGGAC
L  L  A  V  E  L  L  N  E  P  L  A  P  G  V  T  L  P  A  L  M  R  Y  Y  K  D

GGCTACAACGCCGTCCGCAAGTACACCTCGACGGCGTACGTGGTCATGTCCAACCGCCTCTCCGCCAGCAACACGGAG
G  Y  N  A  V  R  K  Y  T  S  T  A  Y  V  V  M  S  N  R  L  S  A  S  N  T  E

CTCCTCGGCTTCGCCGCCGGCTTCCCCGGCGCCGTCCTCGACGTCCACTACTACAACCTCTTCACCAGCAGCTTCAAC
L  L  G  F  A  A  G  F  P  G  A  V  L  D  V  H  Y  Y  N  L  F  T  S  S  F  N

GGCCTCACCGTCGACCAGAACATCGACTACGTCAGGACCAACCGCTCCGACGAACTCAGCACCGTCACAAGGCCGAAT
G  L  T  V  D  Q  N  I  D  Y  V  R  T  N  R  S  D  E  L  S  T  V  T  R  P  N

GGGCCGCTGACGTTCGTGGGAGAGTGGGTGGCTGAGTGGAATGTGCAGGGAGCATCTAACCAGGATTATCAGAGGTTT
G  P  L  T  F  V  G  E  W  V  A  E  W  N  V  Q  G  A  S  N  Q  D  Y  Q  R  F

GCACAAGCCCAGCTAGATGTTTATGGACGAGCTACCTTCGGATGGGCCTATTGGACTTACAAGAACGTCAACAACCAC
A  Q  A  Q  L  D  V  Y  G  R  A  T  F  G  W  A  Y  W  T  Y  K  N  V  N  N  H
```

AAM08614XhoIstopr

```
TGGAGCATGCAGTGGAATATCCAGAATGGGATCATCTCTCTCAAAAGCTAG
W  S  M  Q  W  N  I  Q  N  G  I  I  S  L  K  S  *
```

**Figure 4.1** The full-length cDNA sequence and deduced amino acid sequence of rice *GH5BG*. Underlined letters represent the regions of DNA sequence of PCR primers used for cloning the mature-protein-encoding cDNA for recombinant protein production.

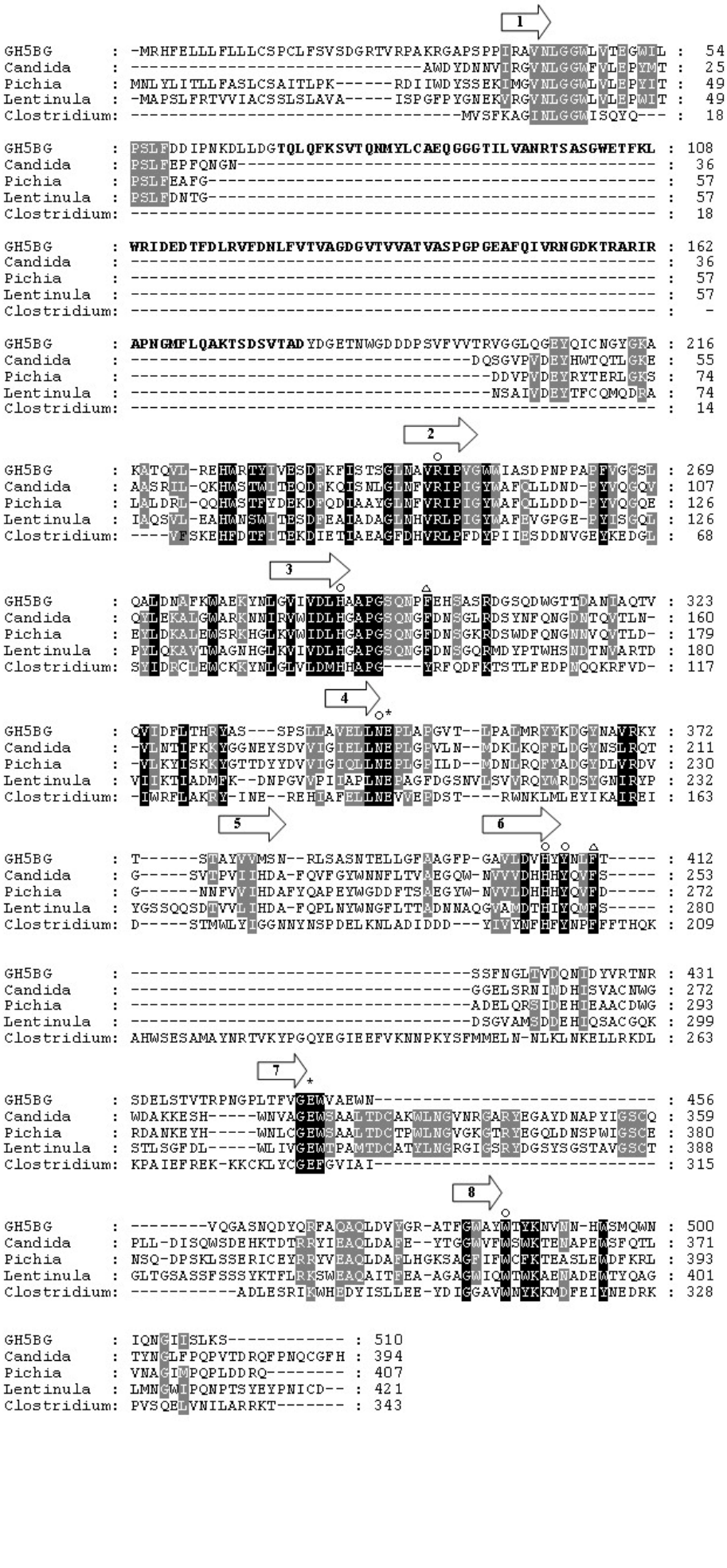
GH5BG : -MRHFELLLFLLLCSPCLFSVSDGRTVRPAKRGAPSPPIRAVNLGGWLVTEGWIL : 54
Candida : ----------AWDYDNNVIRGVNLGGWFVLEPYMT : 25
Pichia : MNLYLITLLFASLCSAITLPK------RDIIWDYSSEKIMGVNLGGWLVLEPYIT : 49
Lentinula : -MAPSLFRTVVIACSSLSLAVA-----ISPGFPYGNEKVRGVNLGGWLVLEPWIT : 49
Clostridium: ----------MVSFKAGINLGGWISQYQ--- : 18
GH5BG : PSLFDDIPNKDLLDGTQLQFKSVTQNMYLCAEQGGGTILVANRTSASGWETFKL : 108
Candida : PSLFEPFQNGN---------- : 36
Pichia : PSLFEAFG---------- : 57
Lentinula : PSLFDNTG---------- : 57
Clostridium: ---------- : 18
GH5BG : WRIDEDTFDLRVFDNLFVTVAGDGVTVVATVASPGPGEAFQIVRNGDKTRARIR : 162
Candida : ---------- : 36
Pichia : ---------- : 57
Lentinula : ---------- : 57
Clostridium: ---------- : -
GH5BG : APNGMFLQAKTSDSVTADYDGETNWGDDDPSVFVVTRVGGLQGEYQICNGYGKA : 216
Candida : ----------DQSGVPVDEYHWTQTLGKE : 55
Pichia : ----------DDVPVDEYRYTERLGKS : 74
Lentinula : ----------NSAIVDEYTFCQMQDRA : 74
Clostridium: ---------- : 14
GH5BG : KATQVL-REHWRTYIVESDFKPISTSGLNAVRIPVGWWIASDPNPPAPFVGGSL : 269
Candida : AASRIL-QKHWSTWITEQDFKQISNLGLNFVRIPIGYWAFQLLDND-PYVQGQV : 107
Pichia : LALDRL-QQHWSTFYDEKDFQDIAAYGLNFVRIPIGYWAFQLLDDD-PYVQGQE : 126
Lentinula : IAQSVL-EAHWNSWITESDFEAIADAGLNHVRLPIGYWAFEVGPGE-PYISGQL : 126
Clostridium: ----VFSKEHFDTFITEKDIETIAEAGFDHVRLPFDYPIIESDDNVGEYKEDGL : 68
GH5BG : QALDNLFKWAEKYNLGVIVDLHAAPGSQNPFEHSASRDGSQDWGTTDANIAQTV : 323
Candida : QYLEKALGWARKNNIRVWIDLHGAPGSQNGFDNSGLRDSYNFQNGDNTQVTLN- : 160
Pichia : EYLDKALEWSRKHGLKVWIDLHGAPGSQNGFDNSGKRDSWDFQNGNNVQVTLD- : 179
Lentinula : PYLQKAVTWAGNHGLKVIVDLHGAPGSQNGFDNSGQRMDYPTWHSNDTNVARTD : 180
Clostridium: SYIDFCLEWCKKYNLGLVLDMHHAPG----YRFQDFKTSTLFEDPNQQKRFVD- : 117
GH5BG : QVIDFLTHRYAS---SPSLLAVELLNEPLAPGVT--LPALMRYYKDGYNAVRKY : 372
Candida : -VLNTTFKKYGGNEYSDVVIGIELLNEPLGPVLN--NDKLKQFFLDGYNSLRQT : 211
Pichia : -VLKYISKKYGTTDYYDVVIGIQLLNEPLGPILD--NDNLRQFYADGYDLVRDV : 230
Lentinula : VILKTLADMFK--DNPGVVPIIAPLNEPAGFDGSNVLSVYRQYWRDSYGNIRYP : 232
Clostridium: -IWRFLAKRY-INE--REHIAFELLNEVVEPDST----RWNKLMLEYIKAIREI : 163
GH5BG : T------STAYVYMSN--RLSASNTELLGFAAGFP-GAVLDVHYYNIFT----- : 412
Candida : G------SVTPVIIHDA-FQVFGYWNNFLTVAEGQW-NVVVDHHHYQVFS----- : 253
Pichia : G------NNFVVIHDAFYQAPEYWGDDFTSDEGYW-NVVLDHHHYQVFD----- : 272
Lentinula : YGSSQQSDTVVLIHDA-FQPLNYWNGFLTTADNNAQGYAMDTHIYQMFS----- : 280
Clostridium: D-----STMWLYIGGNNYNSPDELKNLADIDDD---YIVYNFHFYNPFFFTHQK : 209
GH5BG : ----------SSFNGLTVDQNIDYVRTNR : 431
Candida : ----------GGELSRNINDHISVACNWG : 272
Pichia : ----------ADELQRSIDEHIEAACDWG : 293
Lentinula : ----------DSGVAMSDDEHIQSACGQK : 299
Clostridium: AHWSESAMAYNRTVKYPGQYEGIEEFVKNNPKYSFMMELN-NLKLNKELLRKDL : 263
GH5BG : SDELSTVTRPNGPLTFVGEWVAEWN---------- : 456
Candida : WDAKKESH-----WNVAGEWSAALTDCAKWLNGVNRGARYEGAYDNAPYIGSCQ : 359
Pichia : RDANKEYH-----WNLCGEWSAALTDCTPWLNGVGKGTRYEGQLDNSPWIGSCE : 380
Lentinula : STLSGFDL-----WLIVGEWTPAMTDCATYLNGRGIGSRYDGSYSGSTAVGSCT : 388
Clostridium: KPAIEFREK-KKCKLYCGEFGVIAI---------- : 315
GH5BG : --------VQGASNQDYQFFAQAQLDVYGR-ATFGWAYWTYKNVNN-HWSMQWN : 500
Candida : PLL-DISQWSDEHKTDTRRYIEAQLDAFE--YTGGWVFWSWKTENAPEWSFQTL : 371
Pichia : NSQ-DPSKLSSERICEYRRYVEAQLDAFLHGKSAGFIFWCFKTEASLEWDFKRL : 393
Lentinula : GLTGSASSFSSSYKTFLRKSWEAQAITFEA-AGAGWIQWTWKAENADEWTYQAG : 401
Clostridium: ----------ADLESRIKWHEDYISLLEE-YDIGGAVWNYKKMDFEIYNEDRK : 328
GH5BG : IQNGIISLKS---------- : 510
Candida : TYNGLFPQPVTDRQFPNQCGFH : 394
Pichia : VNAGIWPQPLDDRQ-------- : 407
Lentinula : LMNGWIPQNPTSYEYPNICD-- : 421
Clostridium: PVSQELVNILARRKT------- : 343

**Figure 4.2** Alignment of the protein sequences of rice GH5BG with exo-β-1,3-glucanases and endo-β-1,4-glucanase. GH5BG is rice GH5BG, Candida is exo-β-(1,3)-glucanase from *Candida albicans* (AC CAA39908), Lentinula is exo-β-(1,3)-glucanase from *Lentinula edodes* (AC AB192344), Pichia is exo-β-(1,3)-glucanase from *Pichia pastoris* (AC AY954499), and Clostridium is endo-β-(1,4)-glucanase from *Clostridium thermocellum* (AC AAA23220). The alignment was generated with the ClustalX implementation of ClustalW (Jeanmougin *et al*., 1998; Thompson *et al*., 1994) and analyzed and manually adjusted with Gendoc (Nicholas and Nicholas, 1997). Alignment of the *C. thermocellum* sequence relied on the structural alignment of the 1CEC structural model with the *C. albicans* Exg 1CZ1 structure. The positions of the β-strands of the central $(\beta/\alpha)_8$ barrel are indicated by arrows above the alignment. The asterisks identify the two catalytic glutamate residues, the invariant GH family 5 residues are marked by the symbol O above the column, and the black and grey shading highlight other identities between sequences. The two phenylalanine found at the +1 subsite of *C. albicans* Exg are marked by △ above the column. The region of rice GH5BG homologous to fascin is indicated by bold text.

The mature protein includes two domains, a fascin-like domain (amino acids 70-180) and a glucan-1,3-β-glucosidase domain (amino acids 37-60 and 208-496) (Figure 4.2). The fascin-like domain found at the N-terminus of this enzyme is not commonly found in plant enzymes, but it aligned well with the N-terminus of fascin found in sea urchin, *Drosophilia*, *Xenopus*, mouse, and human (Kane 1976; Paterson and O'Hare, 1991; Holthuis *et al.*, 1994; Edwards *et al*., 1995; Ono *et al*., 1997). BLAST search analysis in GenBank revealed that the fascin-like domain has

been found in 5 plant enzymes, at the N-terminus of a putative *Medicago truncatula* endoglucanase, AC ABE91799, and the previously described putative rice GH5 glucan-1,3-β-glucosidases, AC AAM08614 (GH5BG), AAM08620, AAV43969, and BAD10703.

The sequence alignment between the deduced amino acid sequence of rice GH5BG with with those of fungal GH5 exoglucanases revealed that Glu-347, which lies in the conserved NEP motif, is likely to be the catalytic acid/base and Glu-450, which lies in the conserved GEW motif, is likely the catalytic nucleophile (Cutfield *et al*., 1999; Sakatomo *et al*., 2005; Xu *et al*., 2006). Similar to other GH5 members, rice GH5BG contains eight invariant residues that contributed hydrogen-bond interactions to the nonreducing terminal sugar residue at the -1 subsite found in *C. albicans* endo-1,3-β-glucanase (Exg), these being Arg-247, His-291, Asn-346, Glu-347, His-406, Tyr-408, Glu-450, and Trp-486 in GH5BG. However, the amino acid residues seen at the +1 subsite of *C. albicans* Exg, including Trp-229, Leu-304, and Asn-305, are not conserved in GH5BG. GH5BG does contain Phe-300 and Phe-411, corresponding to Phe-144 and Phe-258 in *C. albicans* Exg, which were found to be located at the +1 subsite near the entrance to the active-site pocket (Cutfield *et al*., 1999). Mackenzie *et al*. (1997) reported that the *C. albicans* Exg residues Leu-304 and Asn-305 are located in the extended loop after strand 7 of the β-barrel and surround Glu-292 (catalytic nucleophile) together with Ala-296, Asp-299, and Gly-306. These residues are conserved among fungal GH5 exo-1,3-β-glucanases and many GH5 members with this extended loop, but not GH5BG.

The sequence alignment between GH5BG with Exg from *C. albicans* and a GH5 endo-1,4-β-glucanase (cellulase) from *C. thermocellum* (Dominguez *et al*.,

1995) in Figure 4.2 indicated that though rice GH5BG is somewhat more similar to *C. albicans* Exg, it shares some features more similar to the cellulase. The amino acid residues 301-366 seen at *C. albicans* Exg are not present in GH5BG sequence (Figure 4.2). In addition, the amino acid residues 205-245 found in the *C. thermocellum* endo-1,4-β-glucanase are not found in GH5BG (Figure 4.2).

### 4.3.2 Functional expression of recombinant GH5BG

The cDNA encoding the mature protein of AAM08614 β-glucanase (designated *GH5BG*) was cloned and inserted into pET32a+/DEST. The construct was transformed into Origami B(DE3) *E. coli.* The *GH5BG* cDNA was expressed in redox-deficient, Origami B(DE3), as a catalytically active thioredoxin fusion protein (Trx-GH5BG). Trx-GH5BG was highly expressed and had intense bands at 68 kD in total cell lysates in SDS-PAGE (Figure 4.3). This band was absent from extracts proteins of pre-induced culture and induced cultures containing pET32a+ plasmid without insert (negative control). There is no significant difference in the activity of the Trx-GH5BG protein expressed in *E. coli* system at 20, 25 and 30°C.

The expressed fusion protein from 12 hr induction could be purified by immobilized metal affinity chromatography (IMAC) with both $Co^{2+}$ column and Ni-NTA resins. The Trx-GH5BG were eluted with elution buffer containing 250 mM immidazole and the protein band with approximately 95% purity was obtained (Figure 4.4). The profile of purification with both types of column was not shown significantly different in purity and activity of GH5BG. However, the flowthrough and wash fractions from purification with Ni-NTA had lower GH5BG activity than those from the $Co^{2+}$ column, indicating the Ni-NTA column has more binding

efficiency with Trx-fusion protein. This purified enzyme was characterized for its biochemical properties.

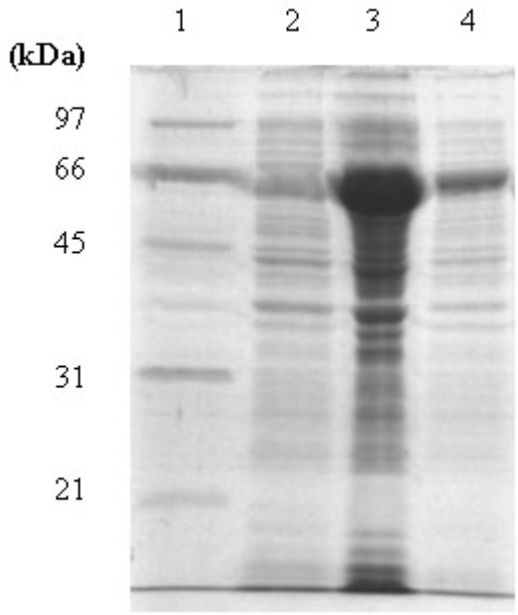


**Figure 4.3** SDS-PAGE of recombinant GH5BG protein expressed in the pET32a+/DEST/ Origami B(DE3) system. The profiles of proteins in extracts of Origami B(DE3) *E. coli.* that contained the recombinant pET32a+/DEST-GH5BG after incubation in the presence of 0.5 mM IPTG, at 25°C for 16 hr. Lane 1, standard marker (Bio-RAD); 2, total protein of *E. coli* cells containing pET32a(+); 3, total protein of *E. coli* cells containing pET32a(+)/DEST-*GH5BG*; 4, soluble fraction of *E. coli* cells containing pET32a(+)/DEST-*GH5BG*.

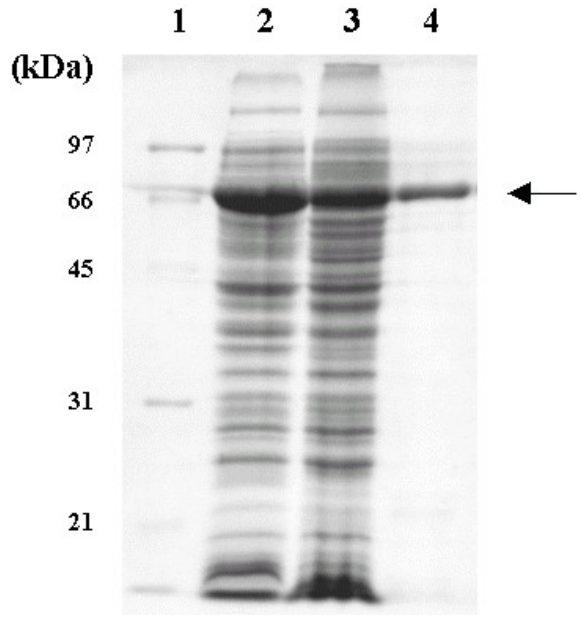


**Figure 4.4** SDS-PAGE of Trx-GH5BG fusion protein expressed in *E. coli* strain OrigamiB (DE3) after incubation in the presence of 0.5 mM IPTG at 20°C for 12 hr. Lanes: 1, standard marker (Bio-RAD); 2, total protein of *E. coli* cells containing pET32a(+)/DEST-*GH5BG*; 3, soluble fraction of *E. coli* cells containing pET32a(+)/DEST-*GH5BG*; 4, purified thioredoxin-GH5BG. The arrow points to the thioredoxin-GH5BG.

The purified Trx-fusion GH5BG was cleaved with enterokinase to remove the Trx portion from the Trx-GH5BG fusion protein. The *p*NPG hydrolysis activity of nonfusion GH5BG protein was much lower than the fusion Trx-GH5BG protein when the equal amount of enzyme (1 pmol) was assayed with 0.2 mM *p*NPG in 0.5 mM sodium acetate, pH 5.0, for 10 min at 37°C (Figure 4.5).

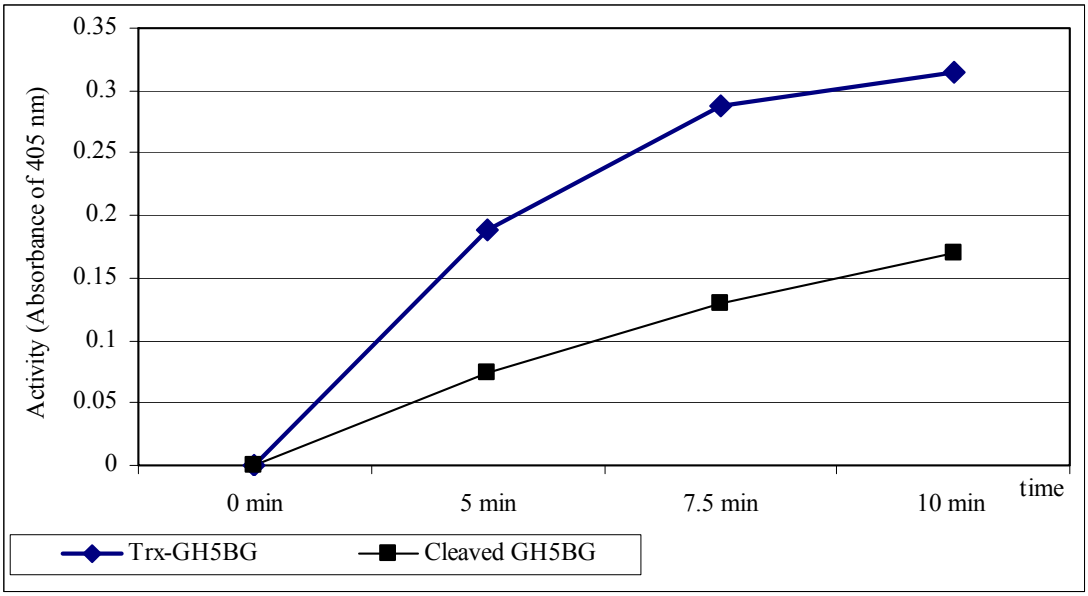


**Figure 4.5** The comparision of enzyme activity between fusion Trx-GH5BG and nonfusion GH5BG. The enzyme (0.1 pmol) were assayed with 0.2 mM *p*NPG in 0.5 mM sodium acetate, pH 5.0, for 10 min at 37°C.

### 4.3.3 pH and temperature profile of activity and stability of GH5BG

GH5BG was most active at pH 5.0 when assayed with 0.2 mM *p*NPG for 10 min (Figure 4.6). There was no *p*NPG activity when the enzyme was assayed in pH 3.5 and 4.0 buffer. The enzyme activity with *p*NPG at pH above 6.5 dropped more than 50% from the optimal activity at pH 5.0. GH5BG was relatively stable over the pH range 5.0 to 7.0, when incubated for up to 24 hr (Figure 4.7). The GH5BG dramatically lost activity at acidic pH (4.0) within 10 min incubation, and at pH 7.0 and 8.0 the enzyme was less stable after incubation more than 3 hr. The enzyme was more stable in pH 9.0 and 10.0 buffers than at other pH.

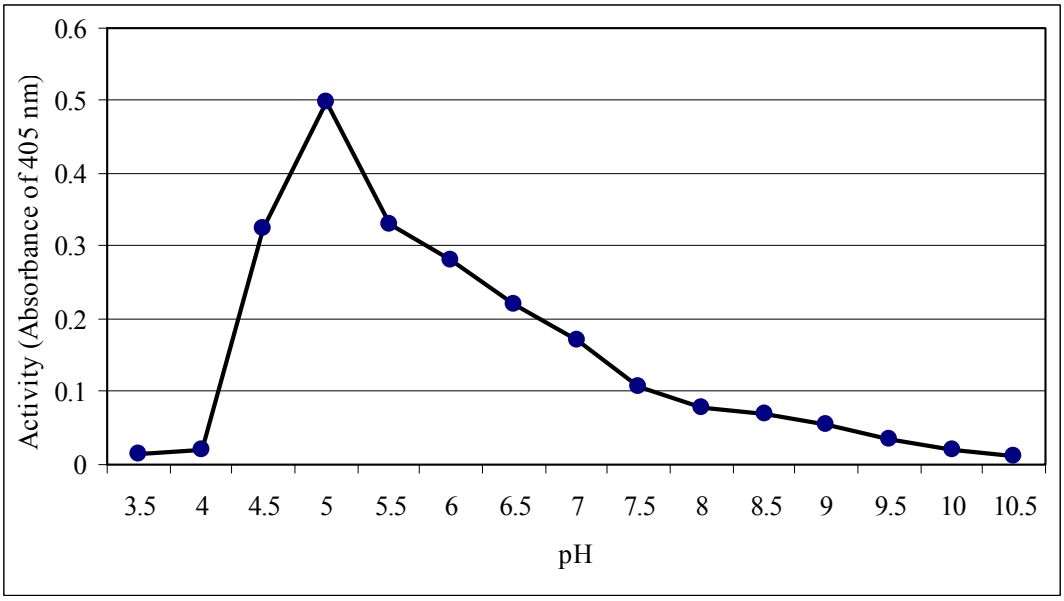


**Figure 4.6** pH profile of activity for GH5BG over the pH range 3.5 to 10.5. The activity assayed with 0.35 mM *p*NPG at 37°C for 10 min.

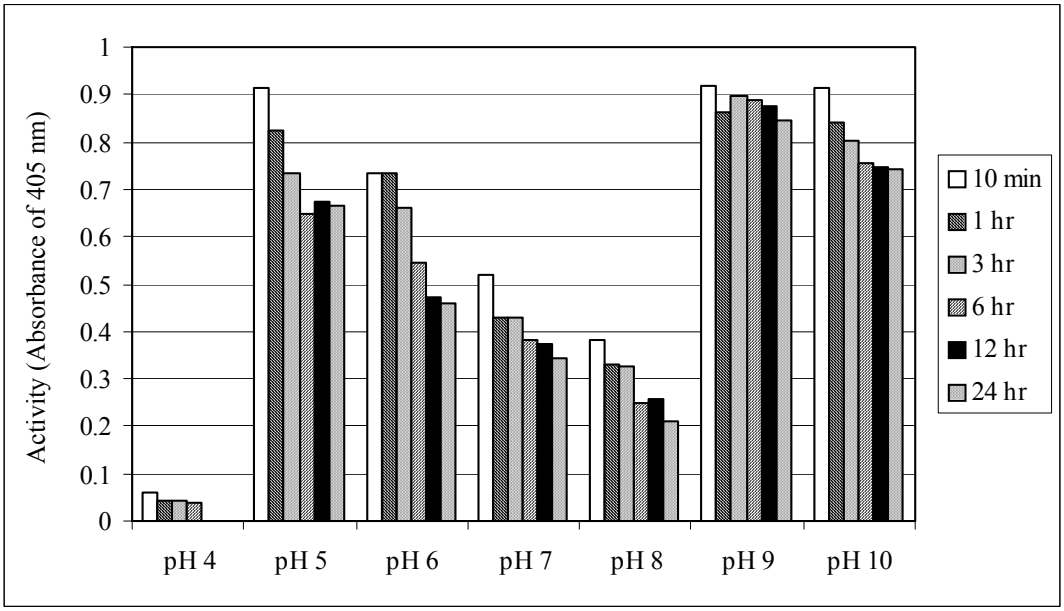


**Figure 4.7** The pH stability of GH5BG after incubation for 10 min to 24 hr at room temperature (25°C). The aliquots of enzyme in each pH buffer at different time course was diluted 20 fold and assayed in 50 mM sodium acetate, pH 5.0, and incubated with 0.35 mM *p*NPG at 37°C for 10 min.

The optimum temperature profile of GH5BG in the hydrolysis of 0.2 mM *p*NPG indicated the enzyme was active over a wide temperature range between 35-65°C (Figure 4.8). The activity decreased dramatically at temperature above 70°C. When compare activity with the optimal activity at 37°C, the enzyme activity dropped about 29% and 55% at 70°C and 80°C, respectively.

The thermostability study was performed by incubating the enzyme at temperatures in the range from 20-70°C up to 90 min, and then assaying the activity with 0.35 mM *p*NPG at pH 5.0 for 10 min (Figure 4.9). The enzyme was stable at 20 and 30°C, when incubated for up to 90 min. The enzyme was less stable at 40 and 50°C at longer incubation times. GH5BG was very unstable at 60 and 70°C, with complete loss of activity within the first 15 min of incubation.

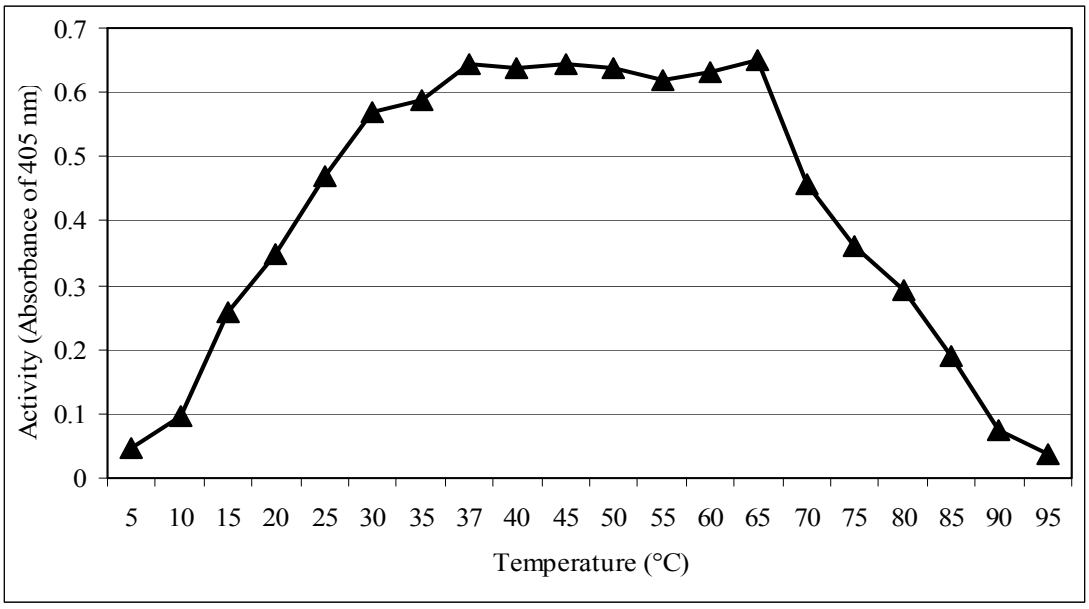


**Figure 4.8** Activity of GH5BG over the temperature range from 5 to 95°C. Activity was assayed with 0.2 mM *p*NPG at 37°C for 10 min.

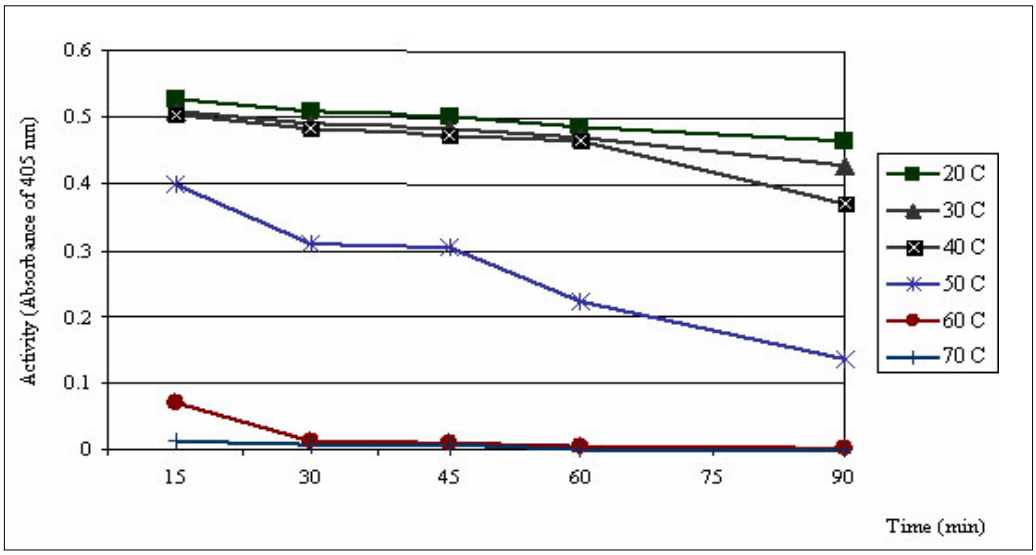


**Figure 4.9** Thermostability of GH5BG for 15 to 90 min at 20-70°C. After incubation the enzyme (0.2 μg) was assayed with 0.35 mM *p*NPG at 37°C for 10 min.

### 4.3.4 Substrate specificity and kinetic parameters of GH5BG

The activity of the purified rice Trx-GH5BG β-glucanase towards natural and artificial glycosides was characterized. Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess glycone specificity of GH5BG. The enzyme activity was assayed by incubating 0.05 μg (0.72 pmol) enzyme with 1 mM *p*NP-glycoside substrate in 50 mM sodium acetate, pH 5.0, for 5 min at 37°C. The activity of the purified rice Trx-GH5BG towards *p*NP-glycosides is summarized in Table 4.1. Among the artificial *p*NP-glycosides, GH5BG hydrolyzed the *p*NP-β-D-glucoside with relatively high efficiency. It hydrolyzed *p*NP-β-D-fucoside, *p*NP-β-D-galactoside, *p*NP-α-L-arabionoside and *p*NP-β-D-xyloside at 77%, 50%, 10.3% and 10% the rate of *p*NPG, respectively. Hydrolysis of *p*NP-β-D-cellobioside was relatively low at 1.04%, while hydrolysis of *p*NP-β-D-manoside was not detectable.

**Table 4.1** Substrate specificity of the purified rice Trx-GH5BG in the hydrolysis of *p*NP-glycosides

| Substrate | Specific activity[a] (μmol/min/mg) | Relative activity[b] (%) |
|---|---|---|
| *p*NP-β-D-glucoside | 19.6 ± 2.25 | 100 |
| *p*NP-β-D-fucoside | 15.7 ± 0.9 | 80.2 ± 4.6 |
| *p*NP-β-D-galactoside | 9.15 ± 2 | 46.4 ± 5.1 |
| *p*NP-β-D-xyloside | 3.45 ± 1.9 | 18.3 ± 11.7 |
| *p*NP-α-L-arabionoside | 2.9 ± 1 | 15.4 ± 7.2 |
| *p*NP-β-D-mannoside | n.d.[c] | n.d.[c] |
| *p*NP-β-D-cellobioside | 0.22 ± 0.03 | 1.04 ± 0.12 |

[a]The assay contained 1 mM substrate in 50 mM sodium acetate pH 5.0 buffer at 37°C.

[b] Percent activity relative to *p*NP released from *p*NP-β-D-glucoside.

[c] means not detectable

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of GH5BG enzyme in the hydrolysis of various *p*NP-β-D-glycosides was determined and the data are summarized in Table 4.2. The hydrolysis of *p*NP-β-D-glucoside and *p*NP-β-D-fucoside by the enzyme was performed with low $K_m$ and high $k_{cat}$ values. The enzyme hydrolyzed *p*NP-β-D-xyloside and *p*NP-α-L-arabionoside with lower $K_m$ and $k_{cat}$ values than *p*NP-β-D-glucoside hydrolysis. The enzyme hydrolyzed *p*NP-β-D-fucoside with the highest catalytic efficiency ($k_{cat}/K_m$ value) at 114 ± 3 $s^{-1}$ $mM^{-1}$, whereas *p*NP-β-D-glucoside was hydrolyzed with 2-fold less efficiency. GH5BG hydrolyzed *p*NP-β-D-galactoside and *p*NP-α-L-arabionoside with 27%, while *p*NP-β-D-xyloside was hydrolyzed at 15% of the efficiency of *p*NP-β-D-glucoside hydrolysis.

**Table 4.2** Kinetic parameters of rice GH5BG in the hydrolysis of *p*NP-glycosides.

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| *p*NP-β-D-glucoside | 36.1 ± 0.7 | 0.47 ± 0.03 | 77 ± 4 |
| *p*NP-β-D-fucoside | 24.5 ± 0.5 | 0.17 ± 0.07 | 144 ± 3 |
| *p*NP-β-D-galactoside | 27 ± 3 | 1.30 ± 0.10 | 20.7 ± 0.5 |
| *p*NP-β-D-xyloside | 3.2 ± 0.3 | 0.27 ± 0.05 | 11.9 ± 1.5 |
| *p*NP-α-L-arabionoside | 2.88 ± 0.08 | 0.14 ± 0.02 | 21 ± 3 |

The substrate specificity of GH5BG towards various kinds of disaccharides and oligosaccharides was determined to study the possible roles of this enzyme in cell wall metabolism. The enzyme activity was assayed by incubating 0.05 μg enzyme with 1 mM substrates in pH 5.0 buffer, at 37°C for 5 min. The GH5BG hydrolyzed the β-1,3-linked glucose disaccharide laminaribiose, but not cellobiose (β-1,4) or gentiobiose (β-1,6). It showed high hydrolytic efficiency with β-(1,4)-linked oligosaccharides with DP of 3–6 (Table 4.3). The rate of hydrolysis of these oligomeric substrates tended to remain approximately constant with increasing DP. Hydrolysis of β-(1,3)-linked oligosaccharides with DP > 2, laminarin and barley 1,3;1,4-β-glucans by this enzyme could not be detected. On the TLC, GH5BG showed hydrolytic activity towards 5 mM laminaribiose and cello-oligosaccharides, but no measurable transglycosylation activity (Figure 4.10).

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of GH5BG enzyme in the hydrolysis of various cello-oligosaccharides and laminaribiose were determined and the data are summarized in Table 4.4. The enzyme hydrolyzed cello-oligosaccharides and laminaribiose with higher $K_m$ and $k_{cat}$ values than *p*NP-glycosides. The $k_{cat}/K_m$ values for the hydrolysis cello-oligosaccahrides with DP of 3-5 tended to remain

approximately constant. The enzyme hydrolyzed cellopentaose with the highest efficiency at 10.4 ± 0.4 $mM^{-1}$ $s^{-1}$, while it hydrolyzed cellohexaose with 2-fold less efficiency than cellopentaose. GH5BG hydrolyzed laminaribiose (β-1,3) less efficiently than cell-oligosaccharides.

**Table 4.3** Substrate specificity of the purified rice Trx-GH5BG in the hydrolysis of oligosaccharides and polysaccharides.

| **Substrate** | **Specific activity[a] (μmol/min/mg)** | **Relative activity[b] (%)** |
|---|---|---|
| *p*NP-β-D-glucoside | 19.6 ± 2.2 | 100 |
| Laminaribiose | 4.78 ±1.8 | 24.4 ± 9 |
| Laminaritriose | n.d.[d] | n.d.[d] |
| Laminaritetraose | n.d.[d] | n.d.[d] |
| Laminaripentaose | n.d.[d] | n.d.[d] |
| Cellobiose | 0.07 ± 0.02 | 0.3 ± 0.1 |
| Cellotriose[d] | 6.29 ± 1.3 | 32.1 ± 6.7 |
| Cellotetraose[d] | 5.61 ± 1.2 | 28.6 ± 5.93 |
| Cellopentaose[d] | 6.64 ± 0.2 | 33.8 ± 1 |
| Cellohexaose[d] | 2.68 ± 0.04 | 13.7 ± 0.2 |
| Gentiobiose | n.d.[d] | n.d.[d] |
| Laminarin | n.d.[d] | n.d.[d] |
| Barley 1,3;1,4-β-glucans | n.d.[d] | n.d.[d] |

[a] The assay contained 1 mM *p*NPG in 50 mM sodium acetate pH 5.0 buffer at 37°C.

[b] Percent activity relative to glucose or *p*NP released from *p*NPG.

[c]means not detectable. [d] The values for oligosaccharides are in terms of total glucose released.

**Table 4.4** Kinetic parameters of rice GH5BG in the hydrolysis of laminaribiose and cellooligosaccharides

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| Cellobiose | 4.3 ± 0.8 | 16.4 ± 1.9 | 0.27 ± 0.02 |
| Cellotriose | 41 ± 5 | 4.53 ± 0.01 | 9.1 ± 1.2 |
| Cellotetraose | 38 ± 2 | 4.09 ± 0.17 | 9.3 ± 0.9 |
| Cellopentaose | 35.5 ± 0.4 | 3.4 ± 0.4 | 10.4 ± 0.4 |
| Cellohexaose | 9.7 ± 0.8 | 2.2 ± 0.5 | 4.5 ± 0.5 |
| Laminaribiose | 36 ± 5 | 7.0 ± 1.1 | 5.05 ± 0.07 |

The hydrolyzed products of cello-oligosaccharides and laminaribiose with were analyzed by TLC. The mechanism of the hydrolysis of oligosaccharides by GH5BG was further analyzed. There products of oligosaccharides hydrolysis were seen by TLC after hydrolysis with GH5BG for 1hr (Figure 4.10).

### 4.3.5 The effect of metal ions and histidine on the GH5BG activity

The inhibition effect of metal ions on GH5BG hydrolysis with *p*NPG was studied. The inhibition pattern from metal ions was determined in the presence of 10 mM final concentration of $BaCl_2$, $CdCl_2$, $CoCl_2$, $FeSO_4$, $HgCl_2$, $LiCl_2$, $MnCl_2$, $MgCl_2$, $SrCl_2$, NaCl, KCl, $ZnSO_4$, $CuSO_4$, L-histidine and EDTA in the enzyme assay containing 0.2 μg enzyme and 1 mM substrates at pH 5.0 and 37°C. Comparison of the activity were made between the control reaction without metal ions, and the reaction with and without 10 min pre-incubation of the enzyme with metal ion. It was found that in the assay with 10 min pre-incubation of the enzyme with metal ions, the hydrolysis of *p*NPG by GH5BG was inhibited by metal ions with the obviously decreases in activity of 39-87% (Table 4.5). Among the metal ions tested, the

reaction in the presence of 10 mM $SrCl_2$, $HgCl_2$ and $ZnSO_4$ exhibited 2-fold less activity, indicated the high inhibitory effect, while less inhibited by $CoCl_2$ (87% of activity remaining). For the assay in which the enzyme was not pre-incubated with metal ions, however, there was no inhibitory effect by metal ions on the hydrolysis of *p*NPG, except in the presence $HgCl_2$, in which 77% activity remained.

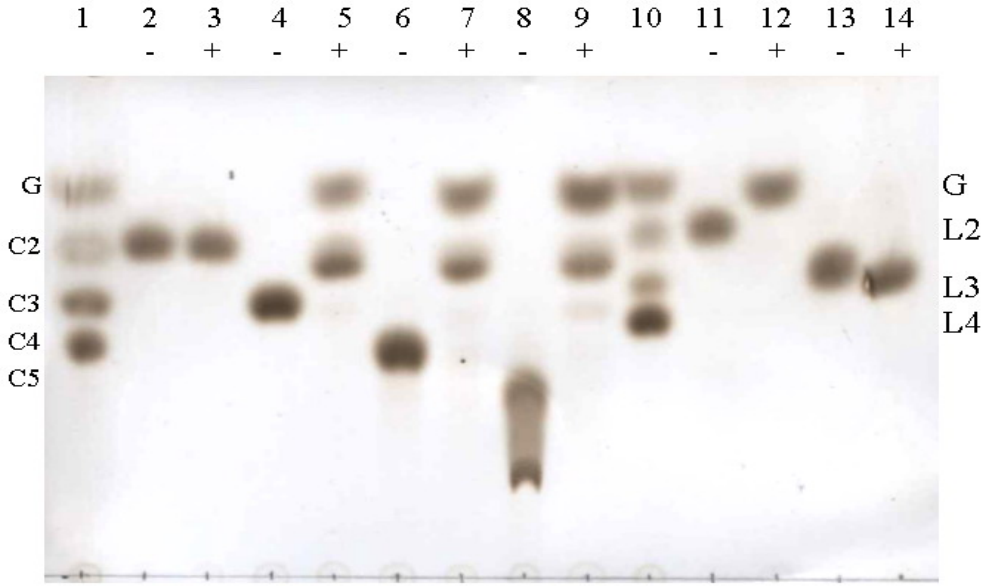


**Figure 4.10** Hydolyzed product of rice GH5BG as assayed with cello-oligosaccharides and laminarioligosaccharides and detected by TLC. Purified GH5BG (0.05 nmol) was incubated with 5 mM oligosaccharides at 37°C for 1 hr. The plate was detected with staining method as described in method 4.2.6. Samples were incubated with (+) and without (-) enzyme. Lane: 1 is glucose and cellooligosaccharide marker; 2 and 3, hydrolysis of cellobiose; 4 and 5, hydrolysis of cellotriose; 6 and 7, hydrolysis of cellotetraose; 8 and 9, hydrolysis of cellopentaose; lane 10 is glucose and laminarioligosaccharide marker; 11 and 12, hydrolysis of laminaribiose; 13 and 14, hydrolysis of laminaritriose.

**Table 4.5** The relative activity of GH5BG when 10 mM of various cation were present in the assay reaction with and without 10 min pre-incubation with the enzyme. The reaction was assayed with 1 mM *p*NPG in 50 mM sodium acetate, pH 5.0, at 37°C.

| | **Relative activity** | |
|---|---|---|
| **Inhibitor** | **No incubation** | **10 min incubation** |
| Control | 100 | 91 |
| $BaCl_2$ | 102 | 50 |
| $CdCl_2$ | 104 | 53 |
| $CuSO_4$ | 111 | 68 |
| $CoCl_2$ | 101 | 87 |
| $FeSO_4$ | 97 | 66 |
| $HgCl_2$ | 77 | 43 |
| LiCl | 102 | 64 |
| $MnCl_2$ | 98 | 54 |
| $SrCl_2$ | 94 | 39 |
| $ZnSO_4$ | 96 | 43 |
| NaCl | 96 | 52 |
| KCl | 93 | 55 |
| $MgCl_2$ | 92 | 57 |
| EDTA | 96 | 60 |
| L-His | 96 | 66 |

## 4.4 Discussion

### 4.4.1 Sequence analysis of rice GH5BG

A putative rice GH5 glucan-1,3-β-glucosidase cDNA, designated GH5BG, was cloned from rice seedlings by RT-PCR. A specific PCR product of 1680 bp was produced, and its sequence overlapped those of contig AC107314 and AK065000. The 1530 bp open reading frame of GH5BG cDNA encoded 510-amino-acid-long precursor protein. GH5BG protein contains two domain, a glucan-1,3-β-glucanase domain (amino acid residues 37-60 and 208-496) and a fascin domain at its N-terminus (amino acid residues 70-180).

There are two highly conserved catalytic Glu residues found in the GH5BG sequence. Glu-347 appeared within the consensus NEF motif, which indicates it might act as the catalytic acid/base, and Glu-450 lies in the conserved GEW motif, so it likely to act as a catalytic nucleophile. Glu-192 and Glu-292 in the mature *C. albicans* Exg protein were identified as the proton donor and nucleophile, respectively (Chambers *et al*., 1993; Mackenzie *et al*., 1997). Such conserved glutamic acid residues with these features are also present in enzymes representing the other Clan A families, such as 1, 2 and 30 (Henrissat *et al*., 1995). It should be noted that Grabnitz *et al*. (1991) had already suggested a possible similarity between family 1 and family 5 enzymes around this residue. The other highly conserved residues in *C. albicans* Exg, Arg-247, His-291, Asn-346, Glu-347, His-406, Tyr-408, Glu-450, and Trp-486 are found in rice GH5BG and other GH5 members. These residues contribute hydrogen-bond interactions to the nonreducing terminal sugar residue at the -1 subsite found in *C. albicans* Exg.

The fascin-like domain found at the N-terminus of this enzyme is not commonly found in plant enzymes, but it aligned well with the N-terminus of fascin found in sea urchin, *Drosophilia*, *Xenopus*, mouse, and human (Kane *et al.*, 1976; Paterson *et al.*, 1991; Holthuis *et al.*, 1994; Edwards *et al.*, 1995; Ono *et al.*, 1997). All the plant sequences with the fascin-like domain, except AAM08620, contain signal sequences for secretion. However, fascin contributes to the bundle of F-actin located in the cytoplasm in human (Adam, 2004). So, it is unlikely that the rice fascin containing enzymes interact with intracellular actin molecules.

### 4.4.2 Expression of recombinant rice GH5BG

The GH5BG mature cDNA were cloned, sequenced and constructed into pET32a+/DEST vectors in ordered to produce the rice GH5BG protein in a recombinant *E. coli* system. The soluble and catalytically functional rice GH5BG was obtained when the protein was produced by N-terminal fusion with thioredoxin (Trx) and expressed in the special *E. coli* strain Origami B(DE3). Previously, recombinant rice β-glucanase expression was successfully obtained in the pGEX4T plasmid/DH5α *E. coli* system (Akiyama and Pillai, 2001). The fusion rice GST-OsGLN1 (GH family 17) recombinant protein was produced in the DH5α cultured when induced with 0.1 mM IPTG at 30°C for 2.5 hr. Palumbo *et al.* (2003) expressed three recombinant β-glucanases (GH family 16) of *Lysobacter enzymogenes*. The *GluA* gene was constructed in pET3C and its protein expressed in BL21 (DE3) *E. coli*, while GluB and GluC were expressed in TOP10 *E. coli* cells.

Fusion of enzymes to soluble fusion partners, such as TRX, GST and MBP, is thought to help to overcome the aggregation of insoluble protein forms by

promoting the proper folding of the attached protein into its biologically active conformation (Kapust and Waugh, 1999). Some recombinant fusion enzymes maintain their activity after proteolytic cleavage of the fusion part, but in some cases the enzyme activity was loss. In this study, the cleaved GH5BG protein exhibited a dramatic decrease in GH5BG activity of approximately 50%, after proteolytic cleavage of the Trx fusion part. Removal of the calmodulin-binding-protein (CBP) tag from glutamate 1-semialdehyde aminotransferase resulted in 60% loss of specific activity (Tsang *et al*., 2003). However, rice BGlu1 showed no loss of β-glucosidase activity after Trx cleavage (Opassiri, 2003).

### 4.4.3 Effect of pH and temperature on the activity and stability

The pH optimum of the purified GH5BG enzyme was pH 5.0, which is similar to the Os4bglu12 and the purified rice exo-β-glucanases from rice bran which are highly active at pH between 3.5-6.0 (Akiyama *et al*., 1997). For the *lysobacter* β-glucanases, GluA, GluB and GluC, activity was highest in the range of pH 4.0 to 7.0 and the optimal pH was 4.5 (Palumbo *et al.,* 2003). The enzyme was relatively stable over pH range 5.0 to 6.0, when incubated for up to 24 hr, and less stable after incubation more than 6 hr at pH 7.0 and 8.0 or pH lower than 5.0. Similar results were reported by Opassiri (2003), who showed that rice BGlu1 was stable at 20-30°C, and over the pH range of 5.0 to 7.0, when incubated up to 24 hr.

The temperature optimum of Os4bglu12 was at 37°C and high enzyme activity was observed over a wide range of 30-65°C. This optimum temperature range was a little bit higher and wider than rice BGlu1 (10 to 35°C) (Opassiri, 2003). The GH5BG enzyme was stable over the temperature range of 20-40°C, when

incubated for up to 60 min, but was less stable at temperatures above 50°C. Similar to the purified rice β-glucanase from rice bran showed no loss of activity after incubate at 40°C for 10 min, but rapidly lost its activity at 60°C (Akiyama *et al*., 1997).

### 4.4.4 Substrate specificity and kinetic parameters of GH5BG

Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess the glycone specificity of GH5BG. The GH5BG hydrolyzed *p*NP-β-D-fucoside twice as efficiently as *p*NPG (based on $k_{cat}/K_m$ values), while *p*NP-β-D-galactoside and *p*NP-α-L-arabinoside were hydrolyzed with 27% and *p*NP-β-D-xyloside with 15% the efficiency of *p*NPG. These results suggest that GH5BG is low stringency at the -1 subsite, in which the non-reducing glycosyl moiety is bound. This is similar to many GH1 and GH3 β-glucosidases, such as the rice BGlu1 (Opassiri, 2003) and rice Os4bglu12 enzymes. In contrast, *p*NP-β-D-mannoside, *p*NP-β-D-cellobioside, *p*NP-α- D-glucoside, and *p*NP-β-L-fucoside were not detectably hydrolyzed, which should be due to the substrate comformation of the different epimers.

The GH5BG showed relatively high efficiency ($k_{cat}/K_m$) for hydrolysis of β-(1,4)-linked oligosaccharides with degrees of polymerization (DP) of 3-5 and a drop to half for cellohexaose, due to a drop in the $k_{cat}$ for this substrate. The rates of hydrolysis of cellooligosaccharide substrates increased with oligosaccharide length. However, this enzyme showed relatively low efficiency when it hydrolyzed the β-1,4-linked glucose disaccharide (cellobiose) and β-1,6-linked gentiobiose hydrolysis could not detected. For the β-1,3-linked polymers, laminarioligosaccharide laminarin,

and barley 1,3-1,4-β-glucans, GH5BG hydrolyzed only laminaribiose, but hydrolysis of polymers with DP more than 2 could not be detected. Similar to yeast exo-β-glucanase (EXG1), a high rate was obtained in hydrolysis of laminaribiose, where relatively low efficient in hydrolysis of cellobiose and gentiobiose (Suzuki *et al*., 2000).

Although GH5BG was designated a putative glucan exo-β-(1,3)-glucanase based on sequence homology, its catalytic activity is somewhat like GH1 β-glucosidases, which show similar oligosaccharide preferences. Rice GH5BG exhibited a hydrolysis preference for β-1,4-linked oligosaccharides and β-1,3-linked disaccharide but it cannot hydrolyze long chain β-1,3-linked oligosaccharides. In contrast, this hydrolysis preference is different with fungal GH5 exo-β-1,3-glucanases, which prefer to hydrolyze 1,3-glucans (laminarin) (Cutfield *et al*., 1999; Sakamoto *et al*., 2005; Xu *et al*., 2006). This difference in substrate specificity must be the result of differences in the structures and/or positions of amino acid residues in the active site between rice GH5BG and the fungal enzymes. The pocket geometry of *C. albicans* Exg allows hydrolysis of longer β-1,3-linked oligosaccharides and is not well suited for cleavage of 1,4-glycosidic linkages (Cutfield *et al*., 1999).

Opassiri *et al.* (2007) compared the difference in the loop around the active site of *C. albicans* Exg structure and rice GH5BG and showed that GH5BG lacks the extended loop at the end of strand 7 of the β-barrel, which forms a wall along one side of the active site in the fungal exoglucanases, as shown in Figure 4.11. Opassiri *et al.* (2007) suggested that the lack of this loop might result in a more open active site, allowing GH5BG to accept β-1,4-linked glucan oligosaccharides, as well as laminaribiose.

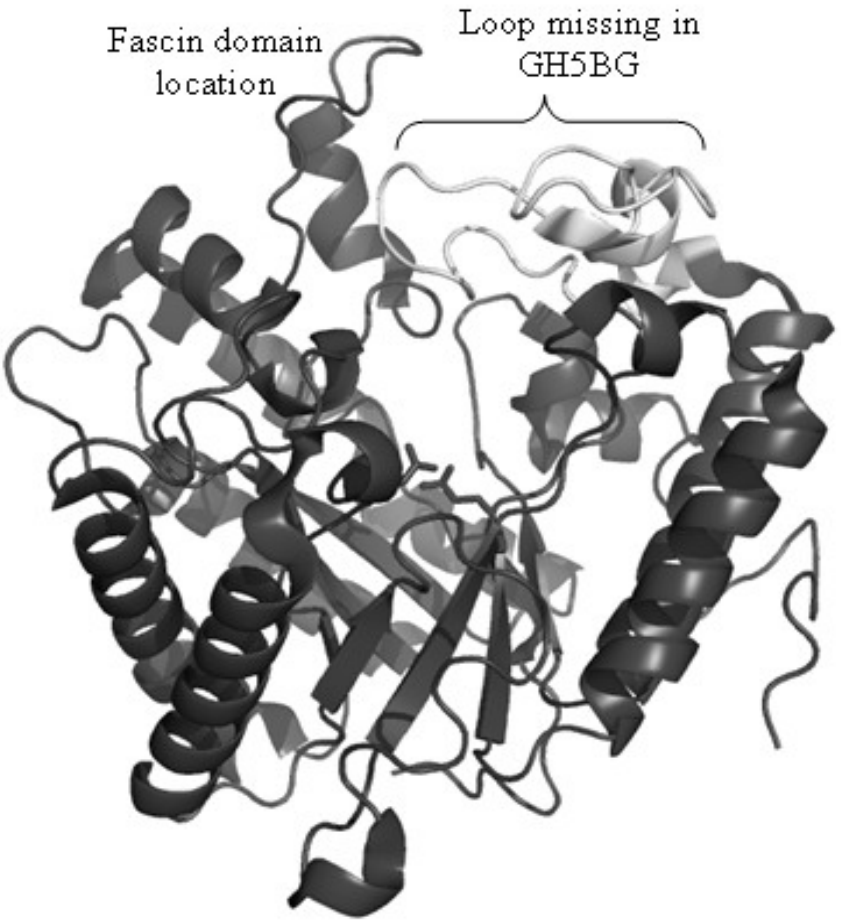


**Figure 4.11** The active site of *Candida albicans* Exg structural model with differences in the loops around the active site found in rice GH5BG highlighted was done by Opassiri *et al*. (2007). The 1CZ1 structure (Cutfield *et al*., 1999) is shown as a ribbon diagram colored in dark grey, with the loop after β-strand 7 of the $(\beta/\alpha)_8$ barrel shown in white and labeled to draw attention to its absence in rice GH5BG. The insertion of the fascin-like domain after the first helix of the extended loop after strand 1 of the β-barrel is indicated by the label. The catalytic acid/base (left) and catalytic nucleophile (right) are displayed in stick representation to indicate the location of the active site. The image was generated with Pymol (DeLano, 2002).

### 4.4.5 The effect of metal ion on the GH5BG activity

With 10 min pre-incubation of the enzyme with metal ions, the hydrolysis of *p*NPG by GH5BG was inhibited by metal ions with obvious decreases in activity, whereas there was no inhibitory effect of metal ions on the hydrolysis in the assay in which the enzyme was not pre-incubated with metal ions. In this study, the metal ion which exhibited high inhibitory effects were $Sr^{2+}$, $Hg^{2+}$ and $Zn^{2+}$, which gave 2-fold less activity than the control reaction. A inhibitory effect was found in rice BGlu1 (Opassiri, 2003) and maize β-glucosidases (Esen, 1992) in which the copper and mercury inhibited the enzyme activity. The exo-β-(1,3)-glucosidases from *Aspergillus*, exoG-I and exoG-II, are not inhibited by divalent metal ions, such as $Cu^{2+}$, $Hg^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ (Fontaine *et al.*, 1997). However, the rice BGlu1, maize β-glucosidases and exo-β-(1,3)-glucosidases from *Aspergillus* were not pre-incubated with metal ions as did for Os4bglu12

### 4.4.6 Possible function of rice GH5BG

Takashi Akiyama determined the expression of *GH5BG* in rice tissues and in response to environmental conditions (Opassiri *et a*l., 2007). The transcript level of *GH5BG* was high in the shoot of 7-d-old seedlings and in leaf sheaths of 6-week-old mature plant (Figure 4.12). The transcript levels of *GH5BG* in 9-d-old seedlings exposed to salt stress, submergence stress, $10^{-4}$ M methyl jasmonate, and $10^{-4}$ M abscissic acid for 2 days were higher than the rice seedlings grown at 28°C (control) (Figure 4.12). The up-regulation of *GH5BG* for various environmental conditions may correlate with recycling of cell wall oligosaccharides in these

processes, or to the function of other, as yet unidentified, substrates (Opassiri *et al*., 2007).

Opassiri *et al.* (2007) also reported the evidence for the localization of GH5BG in the cell. They reported that a C-terminal fusion of a GUS reporter with GH5BG (GH5BG-GUS) was secreted to the apoplast of onion cells transformed with pMDC139-*GH5BG-GUS*, as observed by β-glucuronidase activity (Figure 4.13). This result is consistent with the prediction of the PSORT program. Together with the preference of GH5BG to hydrolyze cello-oligosaccharides and laminaribiose, its extracellular location implies it may play a role in cell wall recycling.

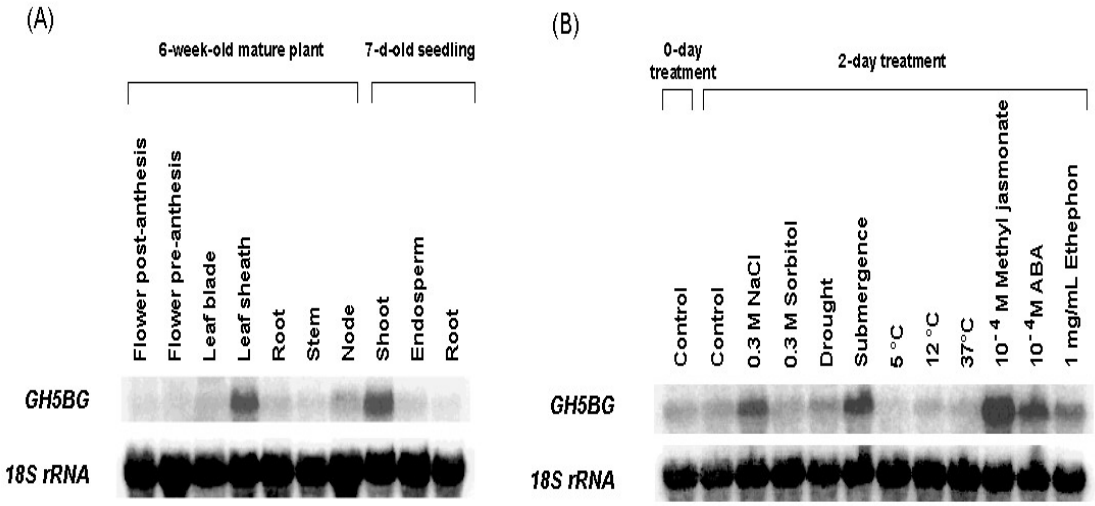


**Figure 4.12** Northern-blot analysis of *GH5BG* transcript levels in (A) 7-d-old rice seedlings and 6-wk-old mature plant tissues and (B) 7-d-old rice seedlings grown a further 2 days with various abiotic stresses and plant hormones (Opassiri *et al*., 2007). *GH5BG*, RNA blots were probed with α-$^{32}$P-labeled *GH5BG* gene-specific probe, *18S rRNA* indicates the same blot probed with an α-$^{32}$P-labeled *18S rRNA* cDNA probe. Twenty micrograms of total RNA from the appropriate tissues were loaded in each lane.

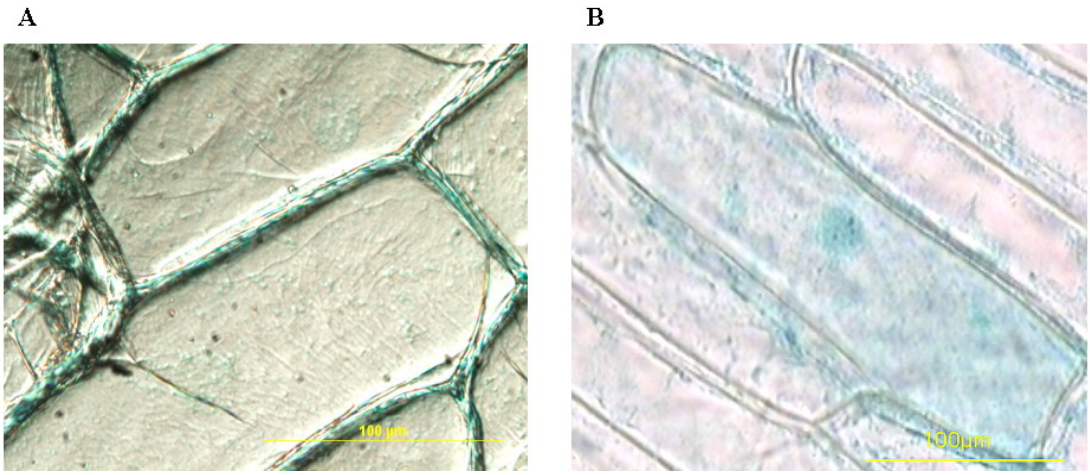


**Figure 4.13** Extracellular localization of the GH5BG protein (Opassiri *et al.*, 2007). The epidermal layers of onion bulbs were transformed with (A) pMDC139-*GH5BG* and (B) control pMDC139 plasmid without insert by particle bombardment. After 48 h incubation at 25°C in complete darkness, the transformed onion cells were stained overnight with X-Glu at 37°C, and the location of blue color product was observed by light microscopy.

## 4.5 Conclusions

The rice *GH5BG* cDNA encodes a 510 amino acid long precursor protein, which contains a 19-amino-acid-long prepeptide and a 491-amino-acid-long mature protein, and appears to be secreted out of the cell. Recombinant GH5BG protein was most active at pH 5.0 and stable over pH in the range of 5.0 to 6.0, when incubated for up to 24 hr. The temperature optimum for Os4bglu12 activity is in the range 30-65°C. Although GH5BG was designated a putative glucan exo-β-(1,3)-glucanase based on sequence homology, its catalytic activity is somewhat like GH1 β-glucosidases, which show similar oligosaccharide preferences. The GH5BG enzyme hydrolyzed the β-1,3-linked glucose disaccharide laminaribiose, but not

laminarioligosaccharides with DP higher than 2. It showed high hydrolytic efficiency at approximately constant rates with β-(1,4)-linked oligosaccharides with DP of 4-6. The enzyme hydrolyzed various kinds of *p*NP-β-D-glycosides with different catalytic efficiency ($k_{cat}/K_m$) values. The assay in which the GH5BG was pre-incubated with metal ion for 10 min indicated that most of metal ion could inhibit the hydrolysis of *p*NPG by GH5BG. The substrate specificity of GH5BG is different from fungal GH5 exo-β-(1,3)-glucanases, which is likely due to differences in the structures of the loops and types of amino acids around the active site, indicating GH5BG along with 3 closely related rice enzymes could be considered a new subfamily of GH5.

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# CHAPTER V

# OVER EXPRESSION OF OS4BGLU12 AND GH5BG IN RICE USING *AGROBACTERIUM*-MEDIATATED TRANSFORMATION


## Abstract

The induction of calli from rice seeds of KDML105, RD6 and Yukihikari cultivars was performed in the N6D medium supplemented with 2 mg/L 2,4-D, and more than 90% callus induction efficiency was obtained except for RD6. The induced callus was pale yellow, opaque and compact with a rough surface and exhibited good secondary calli proliferation. Regeneration medium, RM5 and RM6 containing 1 mg/L NAA and 2 mg/L or 2.5 mg/L kinetin, respectively, gave the highest regeneration efficiency for all rice cultivars. Although the transgenic KDML105 and RD6 could not be produced, relatively high transformation efficiency was obtained at more than 30% of the calli. Successful *Agrobacterium*-mediated transformation was obtained in Yukihikari calli transformation with 4 different plasmids, pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. Putative transgenic rice plants could be regenerated and specific 35S PCR amplification indicated that these 4 transgenic pMDC139-*Os4bglu12* and 3 transgenic pMDC139-*GH5BG* plants were true transgenic rice plants.

## 5.1 Introduction

Rice *(Oryza sativa* L.) is one of the most important crops in the world and is the staple food for about three billion people, predominantly in developing countries (Toenniessen, 1996). Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed (David, 1991). Rice (*Oryza sativa* L.) is a grass (Gramineae) that is classified in the genus *Oryza* Linn. Of which two species are cultivated, *O. sativa* and *O. glaberrima*. *O. sativa* is more important than *O. glaberrima*, which is locally grown in West Africa (Grist, 1975). Generally, there are three types of *O. sativa*, the indica, japonica and javanica genotypes. Eighty percent of cultivated rice is identified in the indica type, the long grain rice that originated in the tropical area. The rest is japonica type, the short and round grain rice which can grow well in temperate areas (Morishima and Oka, 1981; Swaminathan, 1982). The javanica, the broad and thick grain rice is only grown in Indonesia (Oka, 1991). Rice is a homologous diploid. The chromosome number is 2N=2X=24, and it has a relatively small genome ($4.2 \times 10^8$ base pairs per haploid genome) (Arumuganathan and Earle, 1991). The normal life span of rice is 3-7 months, depending on variety and climate.

KDML105 and RD6 are the most important cultivars in the food markets of Thailand. KDML105 has the best cooking and eating quality of the non glutinous rice group, while RD6 has among the best eating quality of the glutinous rice group. The japonica rice, Yukihikari cultivar, a Hokkaido variety, is one of the important rice cultivars in Japan and belongs to the japonica rice. In this experiment, the above three cultivars were used for functional study of β-glucosidases and exo-β-glucanases by

producing the transgenic rice β-glucosidases and exo-β-glucanases lines with *Agrobacterium*-mediated transformation approach.

Gene transformation has become important method in gene function research and can be used to improve plant breeding programs. Transformation of *indica* rice has been successful in a few laboratories using protoplast transformation with electroporation (Zhang *et al*., 1988) or PEG (Datta *et al*., 1990), or particle bombardment (Christou *et al.*, 1991). In recent years, many researchers have been developed reproducible transformation of monocot plants, such as *japonica*, *indica* and *javanica* rice cultivars by *Agrobacterium* with high efficiency and stability (Hiei *et al*., 1994; Rashid *et al*., 1996; Dong *et al*., 1996; Pipatpanukul, 1999; Rattana, 2001; Qian *et al*., 2004). These recent studies on the transformation of monocot plants by *Agrobacterium* have provided evidence for the hypothesis that T-DNA is transferred to dicots and monocots by an identical molecular mechanism. However, *indica* rice is still considered difficult to be transformed, especially by *Agrobacterium* mediated delivery (Qian *et al*., 2004).

## 5.2 Materials

### 5.2.1 Plant materials

Rice (*Oryza sativa* L. spp. *indica*) seeds of cultivar KDML105 and RD6 were obtained from the Rice Seed Center, Khon Kaen.

Rice (*O. sativa* L. spp. *japonica*) seeds of Yukihikari cultivar, a Hokkaido variety, were obtained from Hokuren Co. Ltd., Sapporo, Japan.

### 5.2.2 *Agrobacterium tumefaciens* strain

*Agrobacterium tumefaciens* is a gram-negative soil bacterium that causes the crown gall disease in plants by infecting cells through wound sites. *A. tumefaciens* strain EHA 105 is a virulent strain with high transformation efficiency. EHA 105 strain, which does not carry any resistance markers on its Ti plasmid, is designated from disarming derivative of supervirulent strain A281.

### 5.2.3 Chemicals and reagents

Ammonium sulphate ($(NH_4)_2SO_4$), potassium dihydrogen sulphate ($KH_2PO_4$), manganese sulphate ($MnSO_4$), zinc sulphate heptahydrate ($ZnSo_4 \cdot 7H_2O$), potassium iodide (KI), boric acid ($H_3BO_3$), cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), disodium molybdrate dehydrate ($Na_2MoO_4 \cdot 2H_2O$), calcium chorile dihydrate ($CaCl_2 \cdot 2H_2O$), magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), iron sulphate hepta hydrate ($FeSO_4 \cdot 7H_2O$), disodiumehtylenediamine tetraacetate ($Na_2 \cdot$EDTA), dipotassium hydrogen phosphate ($K_2HPO_4$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), ammonium chloride ($NH_4Cl$), potassium chloride (KCl), potassium nitrate ($KNO_3$), nicotinic acid, thiamine, pyridoxine, myoinositol, sucrose, glucose, proline, casein hydrolysate, glutamine, aspartic acid, arginine, glycine, gelrite, 2,4-D, NAA, kinetin, sorbitol, acetosyringone, hygromycin, cefotaxime, tryptone, and yeast extract were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Other chemicals and molecular reagents used but not listed were purchased from a variety of suppliers.

### 5.2.4 Plant expression vectors

Plant gateway expression vectors, pMDC83 and pMDC 139 (Figure 5.1) were provided by Mark Curtis, University of Zürich (Curtis M.D. and Grossniklaus U., 2003). Both plasmids contain a double set of 35S promoters and a hygromycin-B resistance gene for expression and selection in plant. The pMDC139 contains a *gus* gene, while pMDC83 contains a green fluorescence protein (*gfp*) gene as the reporter genes. The pMDC83 also contains a histidine tag for protein purification.

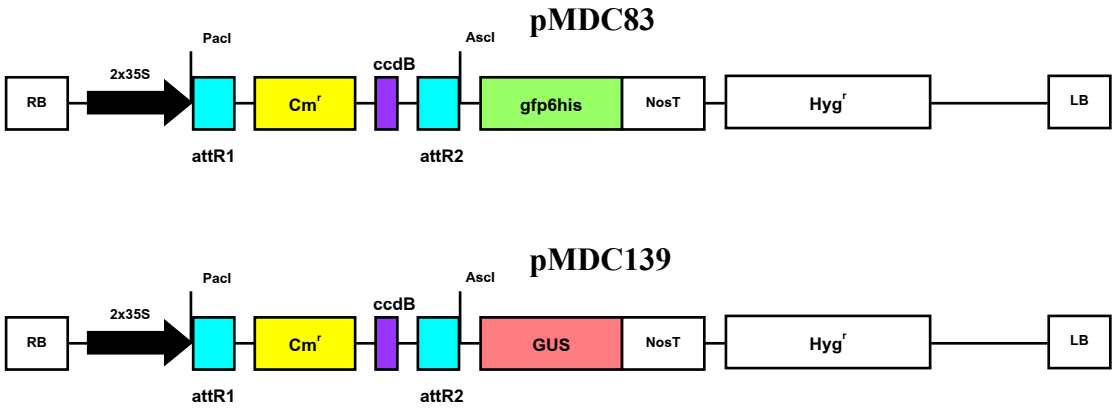


**Figure 5.1** Schematic diagrams of the T-DNA of the plant gateway expression plasmids pMDC83 and pMDC139. LB and RB represented the left border and right border of the vectors which contain two copies of the 35S promoter and *nosT* terminater. The attR1 and attR2 in both plasmids represented the recombination sites into which the target genes were recombined to replace the ccdB gene used for negative selection. Both plasmids contain the *Hyg*$^r$, hygromycin resistance gene as a selection gene and a reporter gene, which is gfp6his (green fluorescence with a histidine tag fusion) in pMDC83 and *gus* in pMDC139.

## 5.3 Methods

### 5.3.1 Construction of recombinant plant expression vectors

The full-length cDNAs of rice *GH1-Os4bglu12* and *GH5BG* were used as the template in the PCR reactions to amplify the full-length cDNAs flanked by attB1 and attB2 sites. The forward primer 445-1full-attB1-F (5'-GGGGACAAGT TTGTACAAAAAAGCAGGCTCCATGGCGGCAGCAGGGGCAAT-3'), and the reverse primer 445-1full-attB2-R (5'-GGGGACCACTTTGTACAAGAAAGCTGG GTATTTCAGGAGGAACTTCTTGAAC-3') were used to amplify the full-length coding region of *Os4bglu12*. The forward primer 8614full-attB1-F (5'-GGGGACA AGTTTGTACAAAAAAGCAGGCTTCATGCGCCATTTTGAGCTCCTC-3'), and the reverse primer 8614 full-attB2-R (5'-GGGGACCACTTTGTACAAGAAAGCT GGGTAGCTTTTGAGAGAGATGATCC-3') were used to amplify the full-length coding region of *GH5BG*. The amplifications were performed with 5 cycles of 94°C 30 sec, 45°C 1 min and 72°C 2 min, and 25 cycles of 94°C 30 sec, 50°C 1 min and 72°C 2 min using *Pfu* DNA polymerase (Promega, Madison, USA). The full-length PCR products were purified by the QIAQuick gel purification kit (QIAGEN) with the recommended protocol.

The purified attB-full-length PCR products were subjected to a BP recombination reaction with a donor vector that contains attP sites, pDONR-Zeo vector, to generate an entry clone, according to the Gateway Technology protocols (Invitrogen). Equal amounts of entry PCR product (~300 μg) and destination vector (300 μg) were used in 10 μL total reaction volume that contains 2 μL of BP clonase buffer and 2 μL of BP clonase enzyme mix. After 8 hr incubation at 25°C, 1 μL of Proteinase K was added and incubated at 37°C for 10 min to stop the reaction. The

recombination reaction mixtures were transformed into DH5α *E. coli* competent cells $CaCl_2$-mediated transformation as described in Appendix A and positive clones were selected with 50 μg/mL zeocin LB-agar plates in the dark. Plasmid size determination and amplification with gene specific primers were used to identify the recombinant pDONR-Zeo vectors containing the attB-full-length gene insert.

The inserts from the recombinant pDONR-Zeo plasmids were subcloned into the plant gateway expression vectors pMDC83 and pMDC139 by LR recombination (Invitrogen). A 1 μL (~300 μg) of entry clone (recombinant pDONR-Zeo vectors) and 2 μL (300 μg) of destination vector were used in 5 μL total reaction volume that contains 1 μL of LR recombinase buffer and 1 μL of LR recombinase enzyme mix. After 6 hr incubation at 25°C, 0.5 μL of Proteinase K was added and the reaction was incubated at 37°C for 10 min to stop the reaction. The recombination reactions were transformed into DH5α *E. coli* competent cell by $CaCl_2$-mediated transformation and positive clones were selected with 30 μg/mL kanamycin LB-agar plates. Plasmid size determination and PCR amplification with gene specific primers were used to identify the recombinant pMDC plasmids containing the attR-full-length gene inserts and the sequences were determined to confirm the correct reading frame of the DNA insert in the plasmid.

### 5.3.2 *Agrobacterium* transformation by electroporation

The recombinant pMDC plasmids were transformed into *Agrobacterium tumefaciens* strain EHA 105 by electroporation. The EHA 105 cells from a glycerol stock were streaked on LB plate, and incubated at 28°C overnight. A single colony was picked up and incubated in 2 mL of LB medium at 28°C with

shaking at 220 rpm overnight. Then, 0.5 mL of overnight starter culture was transferred into 500 mL LB medium, which was shaken at 250 rpm at 28°C until the $OD_{600}$ of the culture reached 0.5. The bacterial culture was transferred into pre-chilled centrifuge tubes and placed on ice for 15-30 min prior to centrifugation. The chilled bacterial culture was centrifuged at 4000 x g at 4°C for 15 min and supernatant was discarded as much as possible. The cell pellets were resuspended gently in 250 mL of ice-cold 1 mM HEPES, pH 7.4 and centrifuged again at same speed. The supernatant was discarded and the cell pellets were resuspended in 10 mL of ice-cold 1 mM HEPES, pH 7.4 and centrifuged as above. After discarding the supernatant, the cell pellets were gently resuspended again in 2 mL ice-cold 10% glycerol by pipeting. The cell suspension was dispensed into pre-cooled tubes as 100 µL aliquots and kept immediately at -70°C.

The competent cells were thawed on ice and mixed with 1-2 µL (~100 µg) recombination pMDC plasmids. The competent cell mixture was transferred to a pre-chilled electroporation cuvette and place on ice 10-15 min. The electroporation machine (Bio-RAD) was set at 130-200 Ω and 1.44 kV of charging voltage with a 1 mm gap. After placing the cuvettes in the electroporation chamber, the activated pulse was performed at approximately 5 ms pluses length. Electroporated cells were transferred immediately to LB broth and incubated at room temperature for 1 hr without agitation. The transforming cells were plated on 30 µg/mL kanamycin LB-agar for selection and incubated at 28°C for 2-3 days.

The selected colonies were grown in the LB medium containing 30 µg/mL kanamycin for 16 hr and then plasmid purification was performed by the alkaline lysis method, as described in section 3.3.5. Plasmid size determination and

PCR amplification with gene specific primers were used to identify the recombinant pMDC plasmids containing the attR-full-length gene inserts.

### 5.3.3 Surface-sterilization of rice seeds in preparation for tissue culture and callus induction

Dehulled rice seeds of KDML105, RD6 and Yukihikari cultivars were surface-sterilized with detergent for 5 min and rinsed with sterile water. The seeds were soaked with 70% ethanol for 5 min and rinsed with sterile water again. The seeds were cleaned with 30-60% Clorox (commercial bleach) for 15-30 min with shaking and then washed 3-5 times with sterile water. After blotting with sterile tissue paper, the sterile seeds were plated on callus induction medium (N6 medium supplemented with 2 mg/L 2,4-D, 300 mg/L casein hydrolysate, 10 mM proline, 30 g/L sucrose and 4 g/L gelrite, and the pH adjusted to 5.8). To study the appropriate concentration of Clorox and cleaning time, the seeds were cultured for 5 days under 12-16 hr photoperiod at 25°C. For callus induction, the seeds were cultured in the same conditions for 3-5 weeks, and the calli were subcultured into the same fresh medium every 3 weeks, until the small secondary calli had 2-3 mm diameters.

### 5.3.4 Screening of the appropriate concentration of NAA and kinetin for plant regeneration

After culturing the calli in the callus induction medium for 4 weeks, the 2-3-mm-diameter secondary calli were transfered to regeneration medium. The regeneration media were prepared with 6 different concentrations of NAA and kinetin (Table 3.1) in MS medium supplemented with 2 g/L casein hydrolysate, 30 g/L

sorbitol, 30 g/L sucrose and 4 g/L gelrite, with the pH adjusted to 5.8. The calli were cultured for 6 weeks under 12-16 hr photoperiod at 25°C and subcultured at 3-weeks intervals. Regeneration ability was scored after 6 weeks cultivation on the regeneration medium. Multiple shoots induced on a single callus were counted as a single regeneration callus. In this experiment, twenty starter calli were used for each experiment with 3 replicates. Regeneration ability was calculated as the number of regenerated calli/number of calli inoculated x 100%.

**Table 5.1** Regeneration media supplemented with 6 different concentrations of NAA and kinetin

| Regeneration medium | Hormone concentration (mg/L) | |
|---|---|---|
| | NAA | Kinetin |
| RM 1 | 0.5 | 1.0 |
| RM 2 | 0.5 | 2.0 |
| RM 3 | 0.5 | 2.5 |
| RM 4 | 1.0 | 1.0 |
| RM 5 | 1.0 | 2.0 |
| RM 6 | 1.0 | 2.5 |

### 5.3.5 Rice transformation by *Agrobacterium*

Transformations of indica rice, KDML105 and RD6 cultivars were performed with 2 types of rice tissues. The first type of rice tissue that was tried was the dehulled seeds that were sterilized with 50% commercial bleach for 30 min and then used as target tissues for transformation. For the second type, calli 2-3 mm in

diameter from 4-5 weeks culturing were used for transformation. *Agrobacterium* strain EHA 105 transformed with the individual recombinant pMDC plasmids was used for transformation. A single colony of transformed *Agrobacterium* from the glycerol stock which had grown on a 30 mg/L kanamycin LB-agar plate was streaked on AB medium plates supplemented with 30 mg/L kanamycin. After incubation at 28°C for 2-3 days, the *Agrobacterium* were resuspended in 20 mL N6D liquid medium supplemented with 10 mg/L acetosyringone until the $OD_{600}$ reached 1.0.

Both target tissues were soaked with *Agrobacterium* suspension, for 15-60 min for the seeds transformation and 5-20 min for the calli transformation. The excess *Agrobacteria* on the surfaces of inoculated tissues were removed by blotting twice on sterile tissue paper. The inoculated tissues were co-cultivated on sterile Whatman No. 1 paper that was overlaid on callus induction medium supplemented with 10 mg/L acetosyringone at 28°C in the dark for 3 days. The infected tissues were washed, for removal all of surface *Agrobacterium*, 6-7 times with sterile water or until the turbidity was not found, and 1 time with sterile water supplemented with 400 mg/L cefotaxime for 15 min. The washed tissues were blotted on sterilized tissue paper 2 times. Then, the cleaned tissues were cultured in the callus induction medium supplemented with 250 mg/L cefotaxime selection medium for 2 weeks. The healthy calli were transferred to the selection medium, which is the callus induction medium supplemented with 250 mg/L cefotaxime and 20 mg/L hygromycin. The calli were cultured for 3-4 weeks under 12-16 hr light at 25°C and subcultured at 2-weeks intervals.

In Japonica rice, Yukihikari cultivar, healthy and yellowish secondary calli 1.5-2.5 mm in diameter were used for *Agrobacterium*-mediated transformation.

Single colonies of EHA 105, containing the individual recombinant pMDC plasmids were streaked on AB medium plates supplemented with 30 mg/L kanamycin. After incubation at 28°C for 2-3 days, a full loop, approximately 2 mm diameter, of *Agrobacterium* colony were resuspended homogeneously in 20 mL AA medium (B5 medium supplemented with 68.5 g/L sucrose, 3.6 g/L glucose, 500 mg/L casein hydrolysate and 10 mg/L acetosyringone). Then, the calli were inoculated by soaking in the *Agrobacterium* suspension for 2 min and blotted with sterile tissue paper, as described above. The inoculated calli were co-cultivated on the callus induction medium supplemented with 30 g/L sucrose, 10 g/L glucose and 10 mg/L acetosyringone at 28°C in the dark for 3 days. The infected tissues were washed for 4-5 times with sterile water and 1 time with sterile water supplemented with 250 mg/L cefotaxime for 5-10 min. The washed tissues were blotted on sterilized tissue paper twice. Then, the cleaned tissues were transferred to selection medium, callus induction medium supplemented with 200 mg/L cefotaxime and 20 mg/L hygromycin. The infected calli were cultured in selection medium for 4 weeks under a 12-16 hr photoperiod at 25°C and subcultured at 2-week intervals.

After 4-6 weeks, surviving calli from all rice cultivars and target tissues were transfered to the regeneration medium, MS medium supplemented with 30 g/L sucrose, 30 g/L sorbitol 2 g/L casein hydrolysate, 4 g/L gelrite, 200 mg/L cefotaxime, 20 mg/L hygromycin and 1 mg/L NAA and 2 mg/L kinetin for indica rice and 1 mg/L NAA and 2.5 mg/L kinetin for japonica rice. The calli were cultured under the same conditions, until the regenerated shoots and roots were produced, and subcultured at 3 weeks intervals. Subsequently, the regenerated plants with perfected shoot and root systems were transferred to hormone-free MS medium for plant

elongation and the plants were allowed to grow for an additional 2-3 weeks. Well-developed plantlets were established in plastic pots filled with sterile soil and grown in the greenhouse under under a 12-16 hr photoperiod at room temperature.

### 5.3.6 Gus enzyme activity assay in transformed tissues

Histochemical assay of β-glucuronidase (GUS) and measurement of GUS enzyme activity were performed by a standard method (Dong *et al*., 1996). The selected tissues, callus, seeds or a shorten part of leaf or stem, were rinsed with sterile water to make sure that there was no *Agrobacterium* contamination on the tissue surface. The cleaned tissues were placed into 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution containing 0.5 mg/mL X-gluc in phosphate buffer, pH 7.0, supplemented with 0.1% Triton X 100 and 0.4% ethanol, overnight (12-16 hr) at 37°C. To determine the GUS activity, which produces blue spots on the calli and the green tissues such as the stem or leaf, the color of internal chlorophyll that hides the blue signal was removed by washing several time with 70% ethanol.

### 5.3.7 Genomic DNA isolation and PCR amplification

Genomic DNA was extracted from transgenic rice stem and leaf by using the modified rapid DNA extraction method (Yu and Pauls, 1992). Approximately 200 mg of leaflet or stem were ground to a fine powder with a mortar and pestle cooled in liquid nitrogen. The frozen homogenate was placed in 400 µL of extraction buffer (made by mixing 10 mL of 1M Tris-HCl, pH 7.4, 2.5 mL of 5 M NaCl, 2.5 mL of 0.5 M EDTA, pH 8.0, 2.5 mL of 10% SDS, 200 µL of β-mercaptoethanol, and 32.5 mL of distilled water), and incubated at 55°C for 10 min.

The sample was centrifuged at 10,000 x g for 5 min and the supernatant (~300 μL) transfered into a new tube. Then 300 μL of 25:24:1 phenol/chloroform/isoamyl alcohol was added and centrifuged at 12,000 x g for 5 min. The supernatant was transfered into a new tube and then one volume of isopropanol was added to the DNA solution. The DNA was pelleted by centrifugation at 12,000 x g for 5 min and the DNA pellet washed with 500 μL 70% ethanol. The DNA pellet was dissolved in 50 μL TE buffer. The DNA solution was treated with 0.5 μL of 10 mg/mL RNase and incubated at 37°C for 15 min. DNA was kept at -30°C until use in next experiment.

Specific gene amplification was performed with primer pairs which were specific for Os4bglu12 and GH5BG. The amplification were performed with 5 cycles of 94°C 30 sec, 45°C 1 min and 72°C 2 min, and 25 cycles of 94°C 30 sec, 50°C 1 min and 72°C 2 min with *Taq* DNA polymerase (Promega, Madison, USA). The transgenic rice DNA was also amplified with 35S-promoter-specific primer pairs under the same condition.

## 5.4 Results

### 5.4.1 Optimal condition for surface sterilization rice seeds

To study the appropriate conditions for rice seed surface sterilization, the experiment was performed with 4 concentrations of Clorox, 30%, 40%, 50% and 60% with 2 different sterilization times. After culturing the seed in the callus induction medium for 5 days, the contaminated seeds were scored from the 50 starter seeds. For KDML105, 98% of seeds survived after 15 min sterilization with 60% Clorox solution, while after 30 min sterilization with 50% and 60% Clorox solutions 100% of seeds survived. For the RD6 seeds sterilization, 98% and 100% of seeds

survived after 30 min sterilization with 50% and 60% Clorox solutions, respectively. For Yukihikari, the appropriate sterilization conditions that gave 100% seed survival were found with 60% Clorox solution for 15 min, 50% Clorox for 30 min, and 60% Clorox for 30 min (Table 5.2).

**Table 5.2** Percentage of contaminated rice seeds in the presence of different sterilization conditions

| Sterilization Condition | | KDML105 | | RD6 | | Yukihikari | |
|---|---|---|---|---|---|---|---|
| Clorox (% ) | Time (min) | Contaminated seeds | Surviving seeds (%) | Contaminated seeds | Surviving seeds (%) | Contaminated seeds | Surviving seeds (%) |
| 30 | 15 | 20.3 ± 3 | 59.3 | 25 ± 3.6 | 50 | 7.3 ± 2.5 | 85.3 |
| 40 | 15 | 11 ± 2.7 | 78 | 12.3 ± 2.5 | 75.3 | 6 ± 2.6 | 88 |
| 50 | 15 | 2.7 ± 1.5 | 94.7 | 3.7 ± 1.5 | 92.7 | 1.3 ± 1.5 | 97.3 |
| 60 | 15 | 1 ± 1 | 98 | 3 ± 1 | 94 | 0 | 100 |
| 30 | 30 | 10 ± 2 | 80 | 18 ± 3 | 64 | 8.7 ± 2.1 | 82.7 |
| 40 | 30 | 3.7 ± 1.5 | 92.7 | 6 ± 2.7 | 88 | 2 ± 1 | 96 |
| 50 | 30 | 0 | 100 | 1 ± 1 | 98 | 0 | 100 |
| 60 | 30 | 0 | 100 | 0 | 100 | 0 | 100 |

### 5.4.2 Determination of suitable condition for rice callus induction

The callus induction was performed in callus induction medium, N6 medium supplemented with 2 mg/L 2,4-D, 300 mg/L casein hydrolysate, 10 mM proline, 30 g/L sucrose and 4 g/L gelrite. Three replicates of each experiment were started with 50 sterile seeds. The seeds were cultured in the callus induction medium for 4 weeks and the numbers of induced calli were scored. The highest callus induction ability was found in KDML105 with 92.7 ± 2.8%, while RD6 and Yukihikari had the callus induction ability at 80.9 ± 3.9% and 90.7 ± 1.9%, respectively. Ten healthy calli from each experiment were randomly selected to measure the calli diameters. KDML105 calli had the largest average diameter at 13.4 ± 2 mm. The sizes of RD6 and Yukihikari calli were smaller than KDML105 with average diameters of 11.7 ± 2 and 11.1 ± 2.1, respectively.

For all rice cultivars, after 1-2 days culture of the seeds in callus induction medium, there were the 1-3 cm green shoots protruding germinated from the rice seeds. The embryos of the seeds were swollen and callus formation could be observed within 4 days. Then, the calli were enlarged and appeared pale yellow, opaque and compact with rough surface, while the green shoots still had the same size as observed in a beginning (Figures 5.2, 5.3 and 5.4). Among the indica rice, KMDL105 calli appeared to have a faster growth rate than RD6 calli in the first two weeks of growing in callus induction medium. The RD6 calli appeared to be more compact and harder than KMDL105 calli and RD6 exhibited the good small secondary calli formation ability. The secondary callus of RD6 started to separate from mature callus after 3 weeks culturing and they had larger and more healthy secondary calli than KMDL105 calli. Although the KDML105 produced secondary

calli after 4 weeks of culture, which is slower than RD6, the numbers of secondary calli were higher than RD6. For Yukihikari, secondary calli started to separate from the mature calli after 3 weeks of culturing in callus induction medium and then an increasing number for secondary calli were observed in the fourth week. The Yukihikari secondary calli appeared to be healthy and the number of secondary calli were more than KDML105. Sub-culture was performed at 2-week intervals to prevent callus browning.

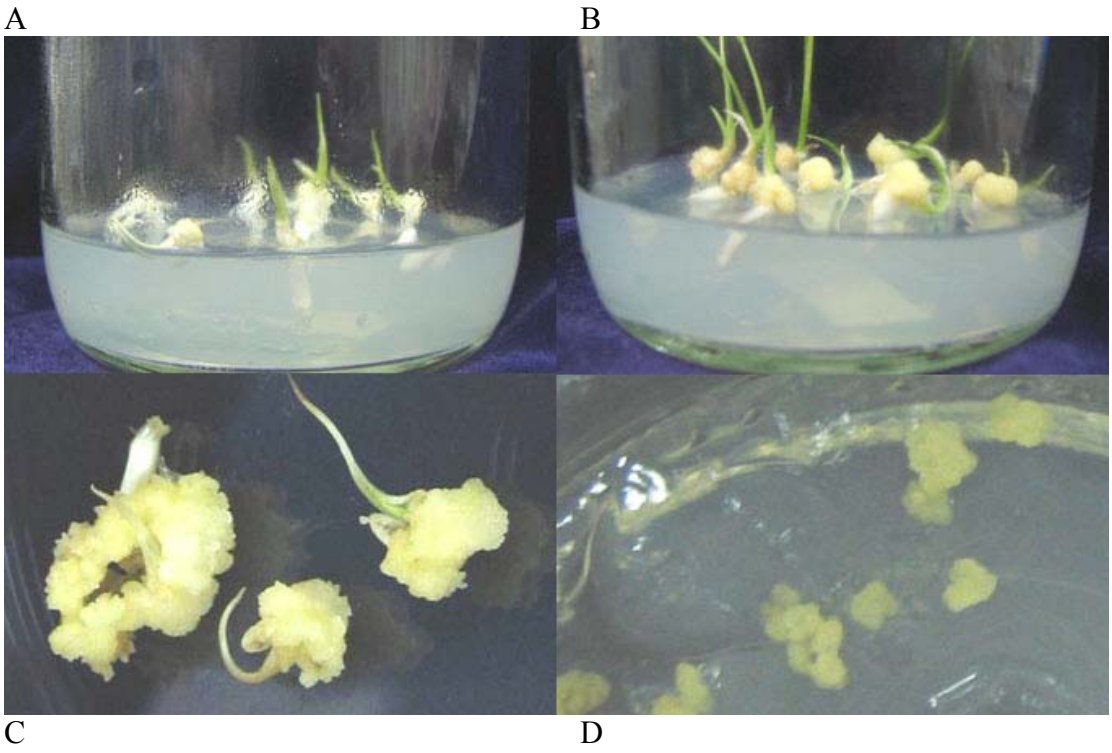


**Figure 5.2** Different stages of callus induction of KDML105. A, 1-week-old calli; B, 2-week-old calli; C, 4-week-old calli; and D, secondary calli.

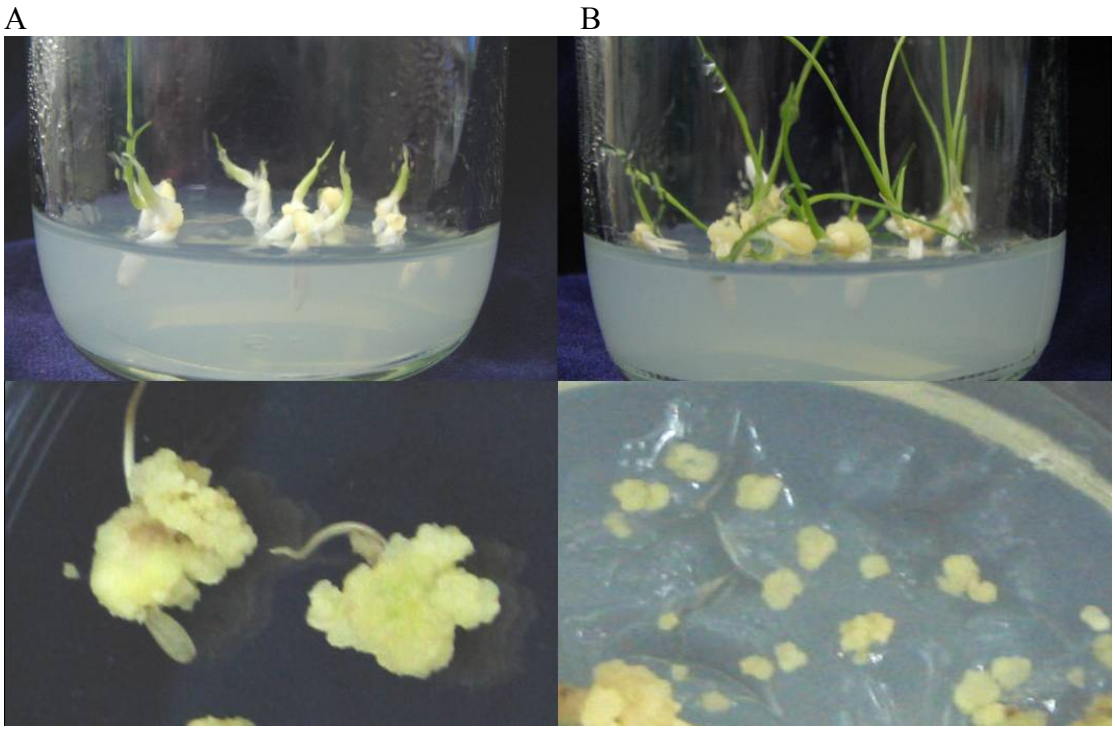


**Figure 5.3** Different stages of callus induction of RD6. A, 1-week-old calli; B, 2-week-old calli; C, 4-week-old calli; and D, secondary calli.

### 5.4.3 The effect of NAA and kinetin in plant regeneration

The secondary calli of KDML105, RD6 and Yukihikari cultivars cultured in callus induction medium for 5-6 weeks with 2-3 mm diameters were used in plant regeneration experiments with 6 different concentrations of NAA and kinetin. The green spots were observed on the calli after 2-3 weeks and shoot regeneration was observed after 4 weeks of culturing in the regeneration medium. Multiple roots, 2-5 roots per a single shoot, were observed within 4-5 weeks, and increasing numbers of the regenerated roots were found after 7-8 weeks cultivation in the same medium. The 2.5-month-old regenerated rice plants were transferred to MS hormone-free medium for plant elongation (Figure 5.5).

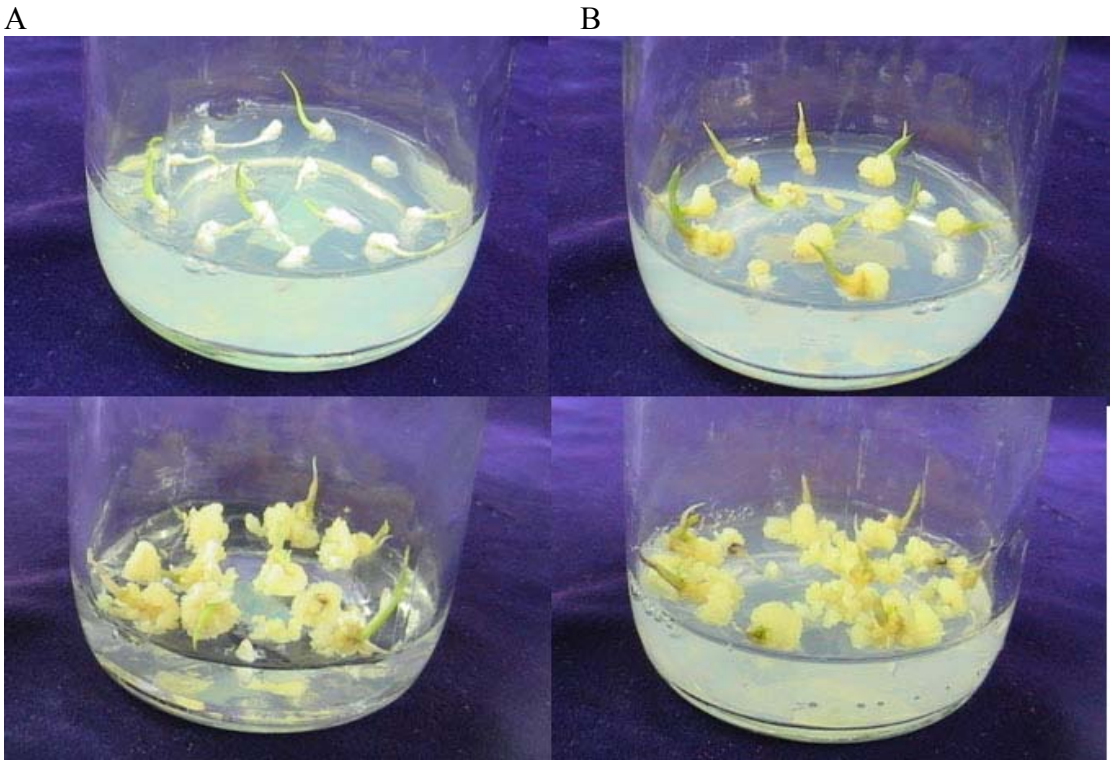


**Figure 5.4** Different stages of callus induction of Yukihikari cultivar. A, 3-day-old calli; B, 2-week-old calli; C, 3-week-old calli; and D, 4-week-old calli which produced the secondary calli.

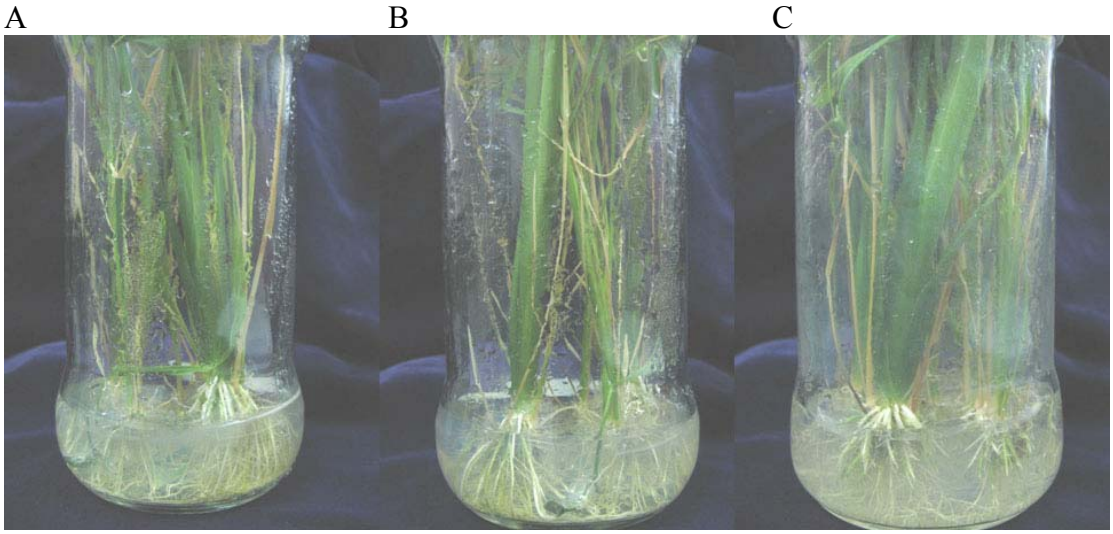


**Figure 5.5** The 3-month-old regenerated plants on MS medium. A, KDML105; B, RD6; and C, Yukihikari regenerated rice plants.

The plant regeneration was performed with 3 replications and started with 20 calli for each experiment. Regeneration ability was scored after 6 weeks cultivation on the regeneration medium. Multiple shoots induced on a single callus were still regarded as a single regeneration callus. Regeneration ability was calculated as the number of regenerated calli/number of calli inoculated x 100%. The numbers of multiples shoots produced were scored from the regenerated shoots that were produced from one callus of all 3 replication experiments (Table 5.3).

**Table 5.3** Regeneration ability of KDML105, RD6 and Yukihikari calli in different regeneration medium

| | KDML105 | | RD6 | | Yukihikari | |
|---|---|---|---|---|---|---|
| Medium | Regenera-tion ability (%) | Number of regenerated shoots | Regenera-tion ability (%) | Number of regenerated shoots | Regenera-tion ability (%) | Number of regenerated shoots |
| RM 1 | 31.7 ± 7.6 | 3.8 ± 1.5 | 36.7 ± 5.8 | 3.9 ± 1.3 | 43.3 ± 12.6 | 3.7 ± 1.7 |
| RM 2 | 28.3 ± 10.4 | 2.6 ± 0.9 | 35.0 ± 13.2 | 3.2 ± 1.1 | 58.3 ± 7.6 | 3.4 ± 1.6 |
| RM 3 | 33.3 ± 7.6 | 2.9 ± 1.1 | 30.0 ± 10.0 | 2.5 ± 0.9 | 46.7 ± 7.6 | 2.8 ± 1.2 |
| RM 4 | 41.7 ± 2.9 | 4.0 ± 1.0 | 31.7 ± 5.8 | 2.7 ± 1.0 | 35.0 ± 5.0 | 3.5 ± 1.3 |
| RM 5 | 56.7 ± 7.6 | 6.0 ± 1.7 | 58.3 ± 2.9 | 5.3 ± 1.9 | 60.0 ± 5.0 | 5.6 ± 1.7 |
| RM 6 | 61.7 ± 10.4 | 6.7 ± 1.6 | 55.0 ± 5.0 | 5.0 ± 1.6 | 66.7 ± 7.6 | 5.6 ± 1.7 |

KDML105 cultivar exhibited good regeneration ability with 56.7 ± 7.6% and 62 ± 10% regeneration when the calli were regenerated with RM 5 and RM 6 medium, respectively. Multiple shoots were regenerated from one callus with the highest numbers at 6.7 ± 1.6 shoots/calli when regenerated with RM 6 medium. RD6

also exhibited high regeneration ability with RM 5 and RM 6 medium at 58.3 ± 2.9% and 55.0 ± 5.0%, respectively. The highest multiple shoots numbers 5.3 ± 1.9 were found when the RD6 calli were regenerated with RM 5 medium. For the Yukihikari cultivar, high regeneration ability was observed when the calli were regenerated with RM 2, RM 5 and RM 6, at 58.3 ± 7.6%, 60.0 ± 5.0% and 66.7 ± 7.6%, respectively.

### 5.4.4 Determination of the appropriate inoculation time for *Agrobacterium*-mediated transformation of KDML105 and RD6

To produce transgenic indica rice, KDML105 and RD6 cultivars, sterile seeds and secondary calli were used for *Agrobacterium*-mediated transformation. The 2-3 mm diameter, healthy calli that were cultured in callus induction medium for 4-5 weeks and rice seeds sterilized with 50% Clorox were used to determine the efficiency of *Agrobacterium* strain EHA 105 containing pMDC139-*GH5BG*. To determine the appropriated inoculation time for transformation, four different inoculation times 5, 10, 15 and 20 min, were tested for calli and 15, 30, 45 and 60 min were tested for seed transformation with *Agrobacterium* cell suspension with $OD_{600}$ at 1.0 and 3 replicates of each experiment were done. After 3 days co-cultivation with *Agrobacterium*, the inoculated tissues were washed to remove excess *Agrobacterium* cells from the surfaces of the tissues. Since the GH5BG cDNA was inserted in-frame before the *GUS* gene in pMDC139 plasmid to form the GH5-GUS protein, β-glucuronidase activity was observed by staining the tissues with X-gluc solution overnight, and the blue color product was observed in the positive tissues. The tissue that showed multiple areas of the blue spots were regarded as a single

transformant. The transformation ability was calculated as the number of transformed calli or seeds /number of inoculated calli or seeds x 100%.

The result showed that the transformation ability of KDML105 secondary calli increased with increasing inoculation time, and the highest transformation ability of 34.4 ± 3.7% was obtained when the calli were inoculated with *Agrobacterium* for 20 min (Table 5.4). The transformation ability of RD6 calli was a little bit higher than KDML105 with the same inoculation times and the highest transformation ability at 37.8 ± 5.2% was observed with 15 min inoculation time. For sterile seed transformation, KDML105 exhibited a high transformation ability of approximately 40% when 30, 45 and 60 min inoculation times were used. RD6 seed transformation exhibited significantly lower transformation ability than KDML105 using the same inoculation times. The highest transformation ability, 31.1 ± 5.9%, was observed when the sterile seeds were inoculated with bacterial cells for 60 min. It was found that the increasing the inoculation time did not have much effect on the transformation ability of RD6 seed. For 60 min inoculation of both KDML105 and RD6 transformed calli, the presence of the blue spots could be observed in the embryo area and the seed surface, while the blue color could be observed only on the seed surface for the short inoculation times (Figure 5.6).

**Table 5.4** Transformation ability of KDML105 and RD6 in relation to inoculatation time

| Secondary callus transformation | | | Sterile seed transformation | | |
|---|---|---|---|---|---|
| inoculation time | KDML105 | RD6 | inoculation time | KDML105 | RD6 |
| 5 | 25.6 ± 5.2 | 27.8 ± 3.7 | 15 | 30.0 ± 6.7 | 18.3 ± 3.7 |
| 10 | 28.9 ± 1.5 | 31.1 ± 3.7 | 30 | 41.7 ± 5.9 | 25.6 ± 3.7 |
| 15 | 31.1 ± 3.7 | 37.8 ± 5.2 | 45 | 41.7 ± 3.7 | 25.6 ± 5.2 |
| 20 | 34.4 ± 3.7 | 36.7 ± 2.2 | 60 | 40.0 ± 4.4 | 31.1 ± 5.9 |

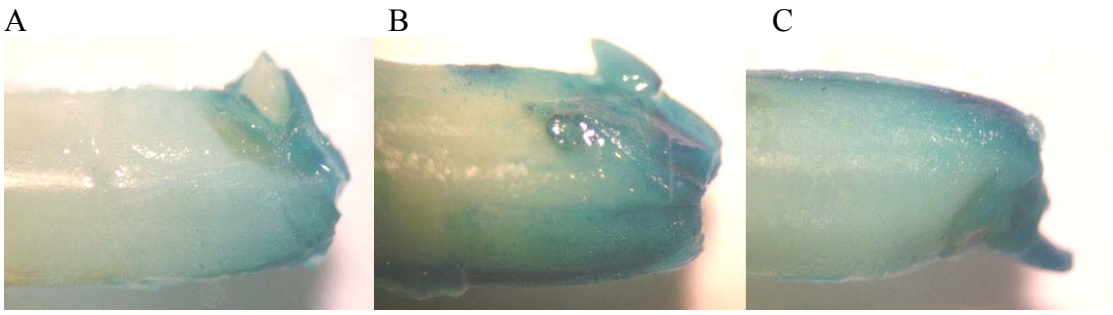


**Figure 5.6** GUS activity in GH5BG-GUS transgenic seeds. A, KDML105 seed with 15 min inoculation; B, KDML105 seed with 60 min inoculation; and C, RD6 seed with with 60 min inoculation with *Agrobacterium* strain EHA105 containing pMDC139-*GH5BG*. GUS activity was determined by staining with X-Gluc overnight.

### 5.4.5 Transformation of KDML105 and RD6 with *Agrobacterium* containing recombinant pMDC139 plasmids

Transformations of KDML105 and RD6 with *Agrobacterium* strain EHA 105 containing pMDC139-*Os4bglu12* and pMDC139-*GH5BG* were performed with both secondary calli and the sterile seeds. Approximately 200-300 4-5-week-old secondary calli were inoculated with *Agrobacterium* containing the recombinant pMDC plasmids which were diluted to $OD_{600}$ of 1.0 with N6D medium supplemented with 10 mg/L acetosyringone for 20 min. For the early experiment, after 3 days co-cultivation with *Agrobacterium*, the infected calli were directly transferred to the selection medium supplemented with 20 mg/L hygromycin. It was found that most infected calli became brown and died. So, 2 weeks pre-culture in the callus induction medium without hygromycin was performed in later experiment.

Infected calli that had been co-cultivated for 3 days with the recombinant *Agrobacterium* were randomly selected to determine the transient expression of GUS activity with X-Gluc staining. Both KDML105 and RD6 calli were found with transient expression demonstrated by the presence of blue spots on the surface that did not cover the whole calli, when transformed with the pMDC139 plasmid series. The transformed calli that showed dark blue spots were referred as those with highly expression, in pMDC139-*Os4bglu12* and pMDC139-*GH5BG* (Figure 5.7).

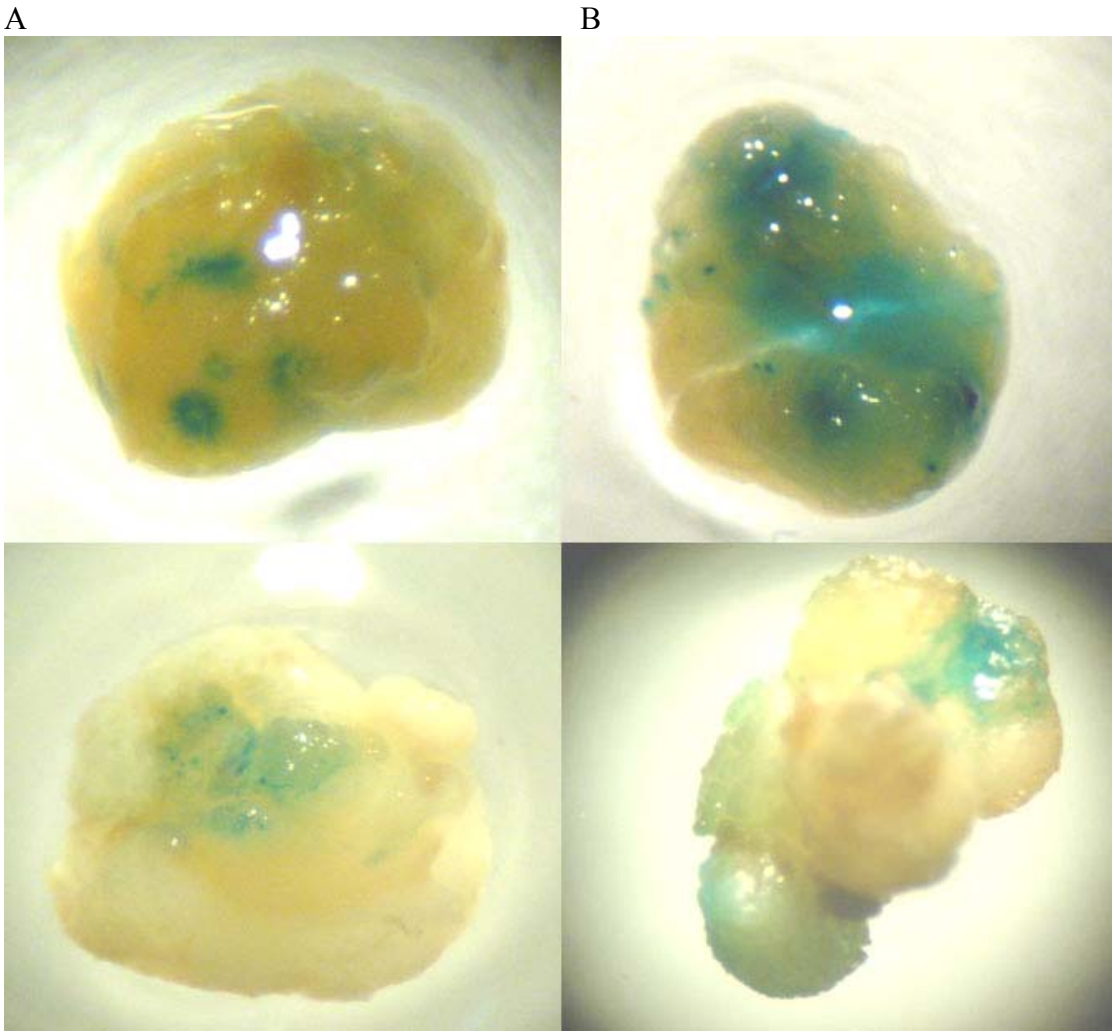


**Figure 5.7** The transient expression of *Os4bglu12* and *GH5BG* fusion proteins in transgenic calli determined by GUS staining. A and B, transgenic KDML105 calli transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively. C and D, transgenic RD6 calli which transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively.

After 5 times washing with sterile water and sterile water supplemented with 250 mg/L cefotaxime for 15 min, *Agrobacterium* contamination could be detected in 60% of one-week-old calli. So, this washing step was increased to 6-7 times to reduced *Agrobacterium* contamination. Although 250 mg/L of cefotaxime solution was used to wash the infected calli to prevent overgrow of *Agrobacterium*, *Agrobacterium* contamination could be observed in the later steps. For this reason, an increased cefotaxime concentration of 400 mg/L and 60 min wash in the last washing and 250 mg/L in the culture medium were also performed in later experiments.

Since most transformed calli selected in the selection medium supplemented with 50 mg/L hygromycin appeared to become dark brown color and died, in the later experiment, 20 mg/L of hygromycin was used to select the transformed calli that possess the hygromycin resistant character. In all experiments for both KDML105 and RD6 the surviving calli from the hygromycin selection step appeared weak and frail, so they lost their regeneration ability.

The seed transformation of KDML105 and RD6 was performed with approximately 100 sterile seeds per each experiment. The sterile seeds with 50% Clorox sterilization for 30 min were inoculated with *Agrobacterium* containing the recombinant pMDC139-*Os4bglu12* and pMDC139-*GH5BG* plasmids with N6D medium supplemented with 10 mg/L acetosyringone for 60 min. The early germinating seeds after the washing step with 400 mg/L cefotaxime solution were used for incubation with X-gluc solution to detect the transient expression of GUS. The positive signal of dark blue product from GUS staining could be observed on the seed surface in of both KDML105 and RD6 transformed seeds (Figure 5.8).

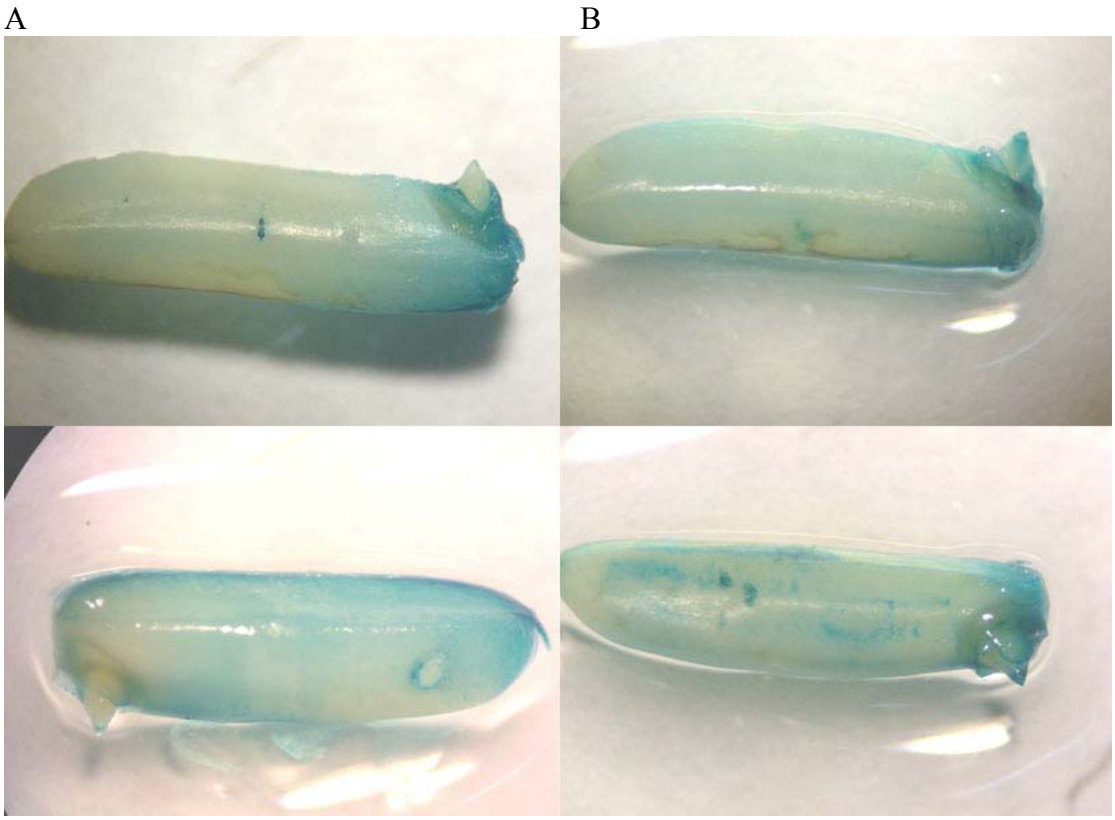


**Figure 5.8** Transient expression of Os4bglu12-GUS and GH5BG-GUS fusion proteins in transgenic KDML105 and RD6 seeds detected by GUS staining. A and B, KDML105 seeds transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively, C and D, RD6 seeds transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively.

Similar to callus transformation, after transfer of the infected seeds directly to the selection medium supplemented with 20 mg/L hygromycin, they could not germinate or generate the callus and eventually died. Therefore, 2 weeks pre-culture in the callus induction medium without hygromycin was also applied in the later experiments. Since the inoculation time for seed transformation was longer than

the callus transformation, the seeds were additionally washed by soaking with N6D liquid medium supplemented with 300 mg/L cefotaxime overnight.

The infected seeds were subjected to generate the transgenic plantlets by 2 ways, 1) induction of the infected seeds to be calli and regenerated the callus to the rice plants, 2) germination of the infected seeds to rice plants. For callus induction of infected seeds, the seeds were cultured on callus induction medium supplemented with 250 mg/L cefotaxime for 2-3 weeks and then transferred to the selection medium supplemented with 20 mg/L hygromycin. The callus formation from infected KDML105 and RD6 seeds indicated poor induction ability, with only approximately 30% of the total infected seeds regenerating calli. In addition, not only the callus-formation ability, but the diameter and healthiness of the calli were also decreased. Because of the poor quality of the calli and *Agrobacterium* contamination, transformed rice plants could not be generated from calli by using this method.

The direct germination of infected seeds to generated the rice seedlings was performed by culturing of the infected seeds 7-10 days in hormone-free MS medium supplemented with 250 mg/L cefotaxime. The infected seed germinated poorly and required a longer time to germinate when compared with normal seeds. Approximately 8-10 cm high seedlings were subcultured in the selection medium, hormone-free MS medium supplemented with 250 mg/L cefotaxime and 30 mg/L hygromycin for an additional 2-4 week. The transformed plants that survived on the selection medium were transferred to planting in the sterile soil in the greenhouse. In addition, some the plants surviving after selection were transferred to hygromycin-free MS medium for 1-2 weeks, to increase the healthiness of the transformed plants, after which they were transferred to soil and grown in the greenhouse. Since the

transformed plants exhibited thin leaf and blade sheet, and weak emaciated stems (Figure 5.9).

A B

**Figure 5.9** The 6-week-old KDML105 rice plants transformed with pMDC139-*GH5BG* cultured on the MS hormone-free medium (A), and then transferred to soil and grown in the greenhouse (B).

In this experiment the 11 transgenic KDML105 rice plants transformed with pMDC139-*Os4bglu12* and 24 transgenic KDML105 rice plants transformed with pMDC139-*GH5BG* were produced. Unfortunately, the transgenic rice plants could not survive in the greenhouse longer than 3 weeks. So, the rice plants were collected 2-3 weeks after planting in soil for Gus staining to identify the transformed plants. The blue product of the GUS staining was present in many parts of the transformed

plants (Figure 5.10, 5.11). Approximately 50% were positive with GUS staining, 2 transgenic pMDC139-*Os4bglu12* rice plants could exhibit the blue product at root, stem, leaf and leaf sheet, while another 3 plants exhibited the blue signal only in the leaf sheet. For transgenic pMDC139-*GH5BG* rice plants, approximately 25% were positive for GUS staining. Only 2 rice plants presented the blue product in all parts, root, stem, leaf and leaf sheet, while another 4 plants exhibited the blue signal only in the leaf sheet and root.

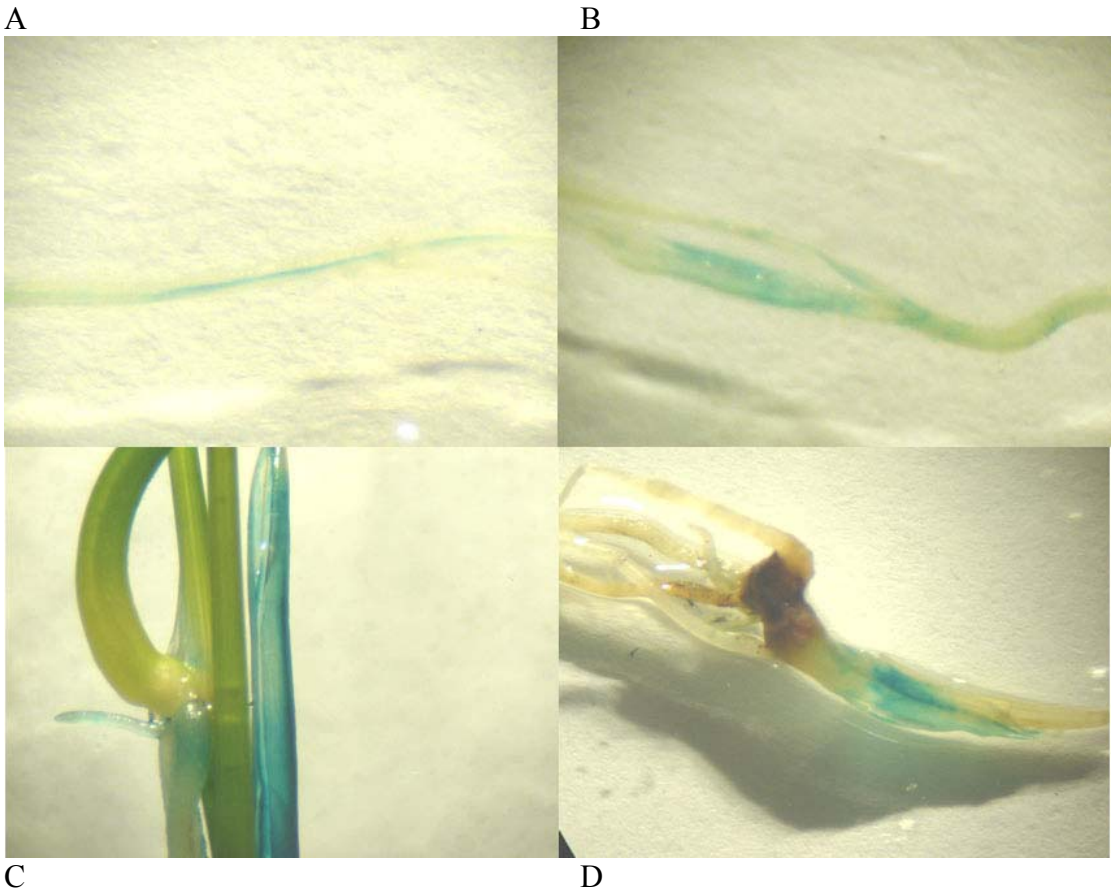


**Figure 5.10** The GUS expression in the transgenic KDML105 rice plants transformed with pMDC139-*Os4bglu12* in various tissue, A, root tip; B, root; C, stem and leaf sheet; and D, leaf sheet.

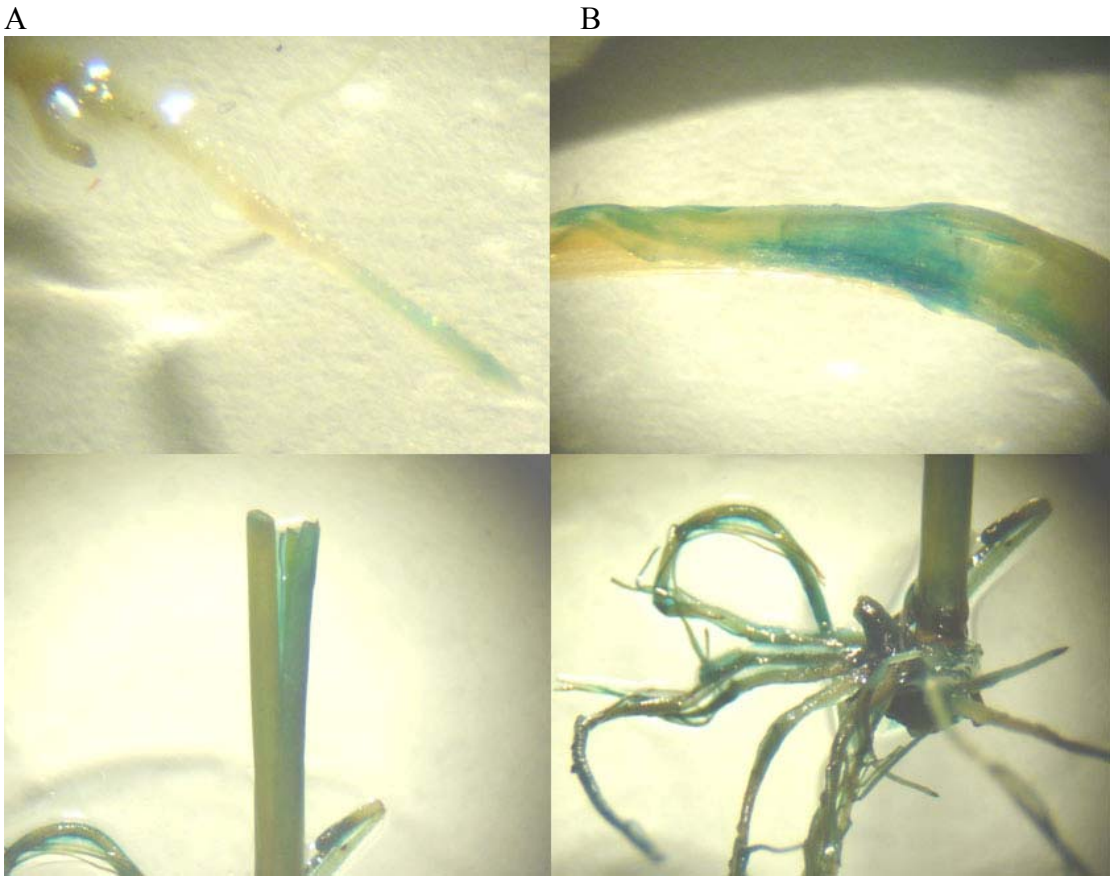


**Figure 5.11** The GUS expression in the transgenic KDML105 rice plants transformed with pMDC139-*GH5BG* in various tissues A, root tip; B, leaf sheet; C, stem and leaf; and D, roots.

### 5.4.6 Generation of transgenic Yukihikari rice plants by *Agrobaterium*-mediated transformation

Transformation of Yukihikari rice with *Agrobacterium* strain EHA 105 containing the 6 difference plasmids shown in the Table 5.5 was performed with the secondary calli. Approximately 300 secondary calli from 4-5 weeks cultivation were used in each experiment. However, the transient expression of GFP fusion protein, which is detected by the bright green signal upon lighting with 395 nm light, in

transgenic tissue with pMDC83 series could not be detected. Since the rice tissue exhibits auto-fluorescence, which is nearly the same wavelength with the detected emission wavelength, so it interfered the detection process. So, the transformation ability was performed by determination of the transient expression of GUS fusion protein of transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. To define the transformation ability, fifteen calli were randomly selected, 5 calli from each of 3 replicated experiments, and incubated in X-gluc solution. The percentage of GUS positive calli for the calli transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG* were 27 ± 8% and 36 ± 9% of the total calli, respectively. The pMDC139-transformed calli also exhibited transient expression with the presence of blue spots of GUS product (Figure 5.12). Transgenic calli exhibited dark blue spots on their surfaces, indicating high expression for both pMDC139-*Os4bglu12* and pMDC139-*GH5BG*.

**Table 5.5** The recombinant plasmids used for *Agrobaterium*-mediated transformation to generate transgenic Yukihikari rice plants

| Plasmid | Inserted gene | Reportor gene |
|---|---|---|
| pMDC83 | - | GFP-his tag |
| pMDC83-*Os4bglu12* | *Os4bglu12* | GFP-his tag |
| pMDC83-*GH5BG* | *GH5BG* | GFP-his tag |
| pMDC139 | - | GUS |
| pMDC139-*Os4bglu12* | *Os4bglu12* | GUS |
| pMDC139-*GH5BG* | *GH5BG* | GUS |

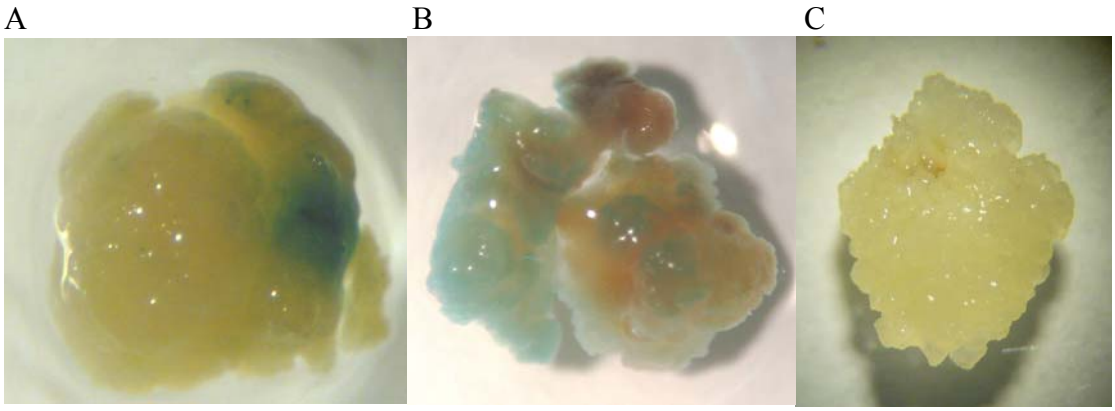


**Figure 5.12** The GUS positive signal of transgenic Yukihikari calli transformed with (A) pMDC139-*Os4bglu12*, (B) pMDC139-*GH5BG* and (C) GUS staining of Yukihikari (negative control).

In the selection step, the transformed calli were selected with 20 mg/L hygromycin and to prevent an overgrown of *Agrobacterium*, 200 mg/L cefotaxime was applied to the selection medium. The selection of the infected calli with 20 mg/L hygromycin was produced 28.4 ± 8.3% survival of calli in 5 replicate experiments. After 1-2 weeks selection, the unhealthy brown calli were observed on the medium, while the healthy calli proliferated new secondary calli. After about 4 weeks on the selection medium, transformed calli could be visually distinguished from the non-transformed calli. The transformed calli appeared white, opaque and compact, while the non-transformed calli had a yellow to brown color and were soft and watery (Figure 5.13).

A B

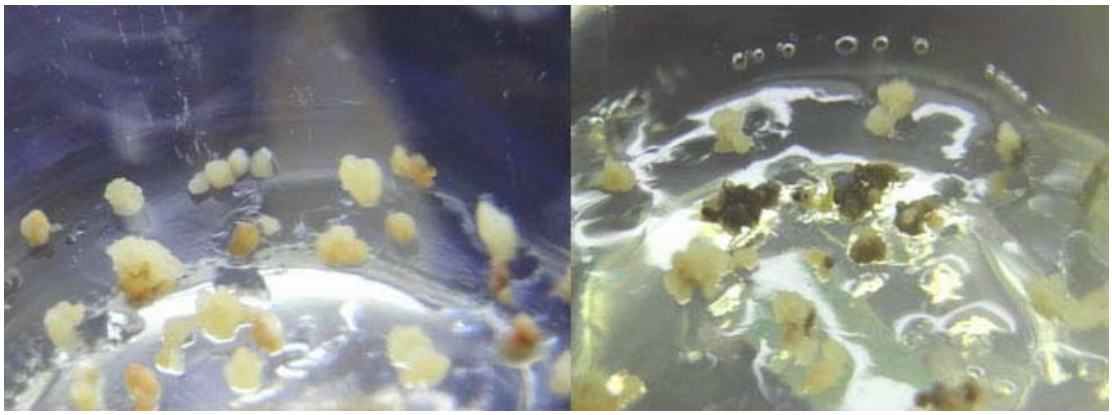

**Figure 5.13** The selection of infected calli transformed with pMDC139-*GH5BG*. The calli were selected with 20 mg/L hygromycin and are shown (A) after 5 days selection and (B) after 4 weeks selection.

The surviving calli from 4-5 weeks selection were regenerated with the regeneration medium supplemented with 20 mg/L hygromycin and 100 mg/L cefotaxime for 1-2 months. The regeneration ability was observed in approximately 25% of the total calli on the selection medium, which is lower than the non-transformed calli (66 ± 7.6%). Not only the transformation ability, but the number of regenerated shoots per a callus was also low, approximately 3 ± 1 shoots per callus, while the nontransformed calli had 5.6 ± 1.7 shoots per callus. The regenerated shoots and roots started to form in the fourth week after culture in the regeneration medium. Then, healthy plantlets that appeared 6 weeks after regeneration were transferred to the MS hormone free medium. The 2 months-old transgenic rice plants in Figure 5.14 exhibited the production of healthy shoots and roots systems in MS hormone-free medium. The clump of transgenic rice plant was found in several plants

that had 2-3 plants per a clump. The transgenic rice plants exhibited lower plants per clump and number of roots when compared with nontransgenic rice.

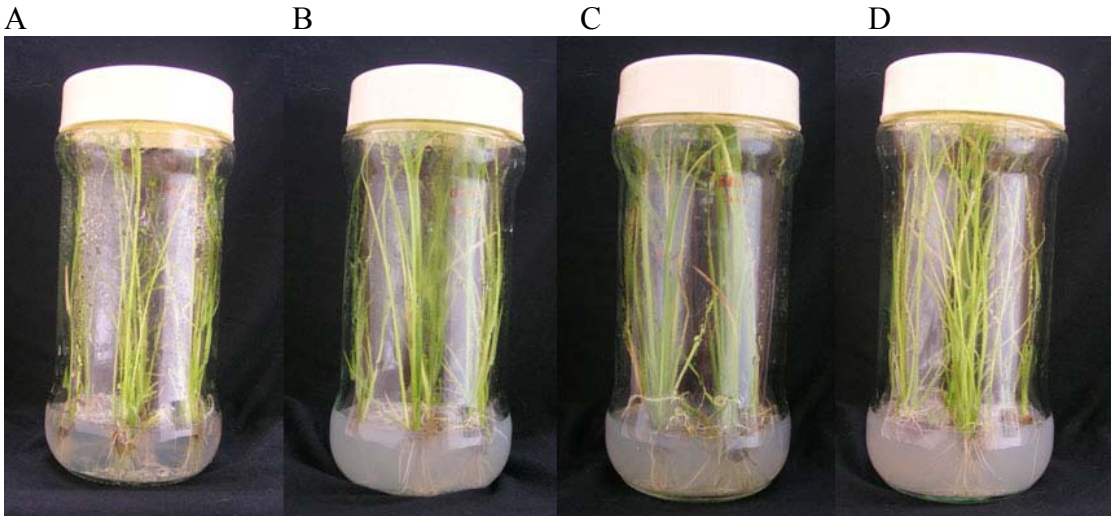


**Figure 5.14** The 2-month-old plantlets after regenerated in MS medium of Yukihikari transgenic plants transformed with (A) and (B) pMDC139-*Os4bglu12*, (C) pMDC139-*GH5BG*, and (D) pMDC139.

*Agrobacterium*-mediated transformation of secondary Yukihikari calli successfully produced putative *Os4bglu12* and *GH5BG* transgenic rice plants. The numbers of each expected transgenic plant obtained in each gene transformation experiment are presented in Table 5.6. Then, these putative transgenic rice plants were transferred to soil and grown in the greenhouse. An example of a putative transgenic rice plant that was transferred to the soil for a week is shown in the Figure 5.15. A few of these plants could survive for 2.5-3 month after planting and grew to approximately 50 cm high plants which produced a panicle (Figure 5.16).

Morphology comparison of transgenic rice plants indicated no significant different with nontransgenic rice plants at the same plant height.

A B

**Figure 5.15** The 1 week-old putative Yukihikari transgenic plants which were transformed with (A) pMDC139-*Os4bglu12,* and (B) pMDC139-*GH5BG*

The GUS staining assay was performed with shorten parts of the transformed stem (Figure 5.17). The GUS positive signal in the separated parts of stem indicated chimeric expression. This indicated that chimeric transgenic rice plants were also produced from this experiment.

**Table 5.6** The numbers of putative transgenic Yukihikari rice plants produced from each plasmid.

| The target plasmid transformation | Numbers of putative transgenic plants |
|---|---|
| Empty pMDC 83 | 0 |
| pMDC 83-*Os4bglu12* | 15 |
| pMDC 83-*GH5BG* | 0 |
| Empty pMDC 139 | 17 |
| pMDC 139-*Os4bglu12* | 26 |
| pMDC 139-*GH5BG* | 38 |

A B C

**Figure 5.16** The 2.5-month-old putative Yukihikari transgenic plants transformed with (A) pMDC139-*Os4bglu12*, (B) pMDC139-*GH5BG*, and (C) Empty pMDC139 plasmid.

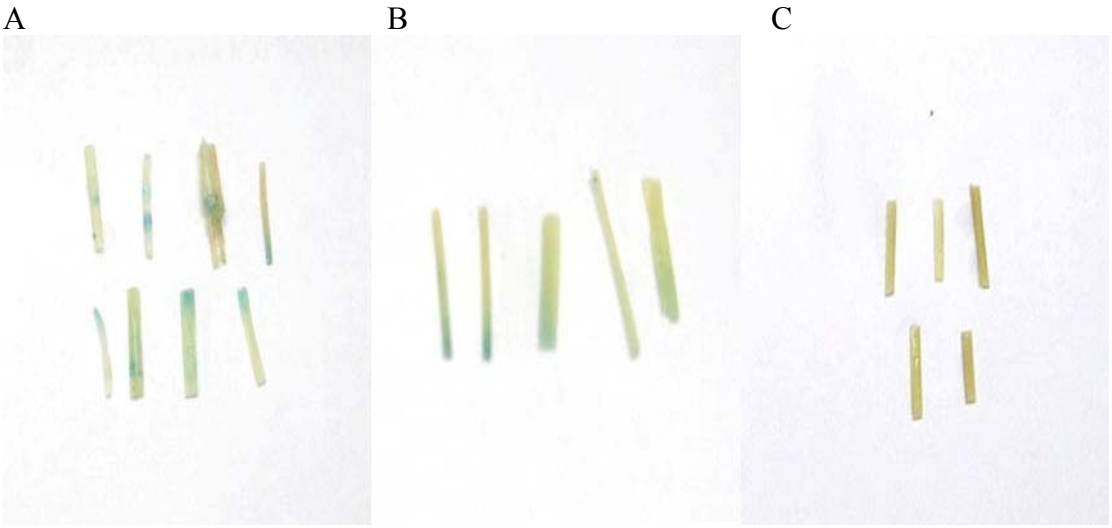


**Figure 5.17** The GUS positive signals of 2-month-old Yukihikari transgenic plants transformed with (A) pMDC139-*Os4bglu12*, (B) pMDC139-*GH5BG*, and (C) Negative control without transformation.

### 5.4.7 Molecular characterization of transgenic rice by PCR

Since the all of target plasmid contain a double set of 35S promoters, the amplification of specific 35S promoter was used to identify transgenic rice plant. The approximately 200 bp of PCR product amplified with a 35S promoter specific primer pair was found in 4 selected putative transgenic plants transformed with pMDC139-*Os4bglu12*, and 3 transgenic plants transformed with pMDC139-*GH5BG* (Figure 5.18). The positive control of this amplification was pMDC139 plasmid containing *Os4bglu12* and pMDC139 containing *GH5BG* as a DNA templates. A negative control was also performed with DNA extracted from a non-transformed Yukihikari callus as the DNA template.

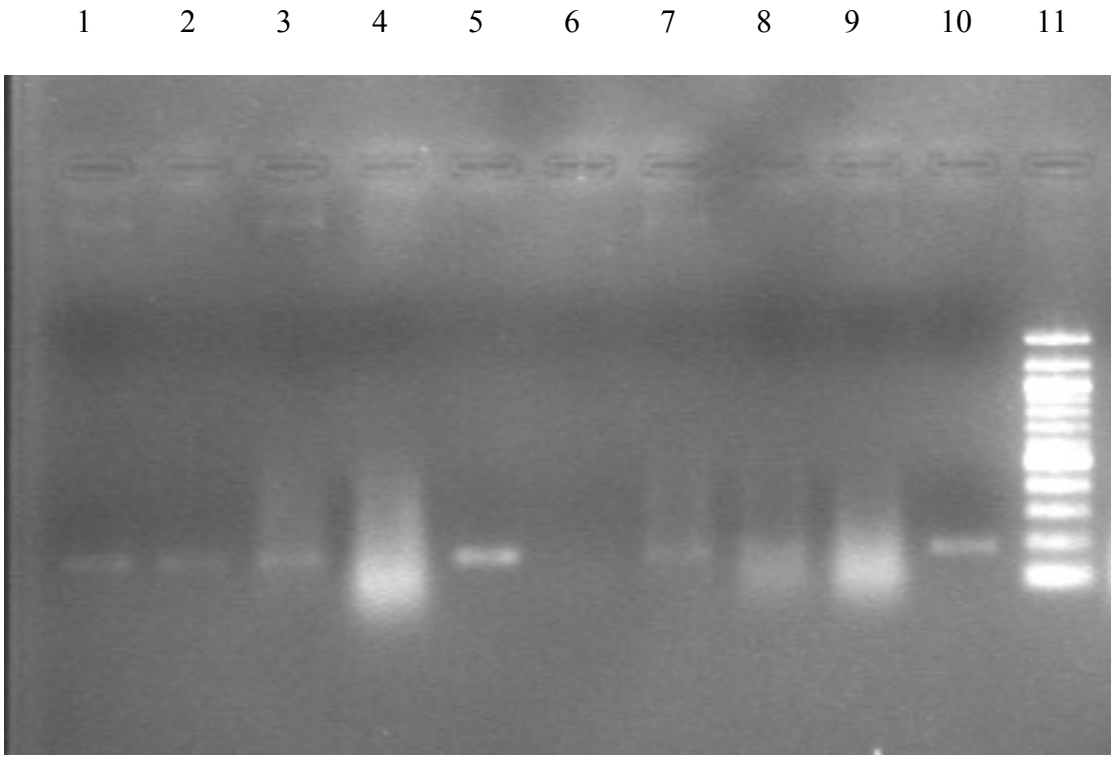


**Figure 5.18** Approximately 200 bp PCR product amplified with 35S specific primers from putative transgenic plant. Lanes 1-4, the 4 transgenic rice plants transformed with pMDC139-*Os4bglu12,* lane 5, pMDC139-*Os4bglu12* plasmid (positive control), lane 6, the negative control, lanes 7-9, the 3 transgenic rice plants transformed with pMDC139-*GH5BG*, and lane 10, pMDC139-*GH5BG* plasmid (positive control).

## 5.5 Discussions

### 5.5.1 Surface-sterilization of rice seeds

Bacterial and fungal contamination, is an important problem in tissue culture, which causes a decreases growth rate and usually plant death (Leifert *et al*., 1994). In this experiment, 50% commercial household bleach was used for surface sterilization of all rice cultivars, KDML105, RD6 and Yukihikari. The commercial bleach (Clorox) contains 6% active ingredient, calcium hypochlorite, so 3% calcium hypochlorite was used for surface sterilization in this experiment. Although sterilization time for more than 30 min reduced the number of contaminated seeds, it also decreased the callus induction ability. Therefore, the appropriate cleaning solution concentration and sterilization time for surface sterilization was very importance for successful tissue culture.

### 5.5.2 Callus induction of mature rice seeds

Callus formation is dependent on the presence in the culture medium of auxins and cytokinins. N6 medium supplemented with 2 mg/L 2,4-D, 300 mg/L casein hydrolysate, 10 mM proline and 30 g/L sucrose was used for rice callus induction. The callus induction ability was at 92.7 ± 2.8, 80.9 ± 3.9 and 90.7 ± 2.8 for cv. KDML105, RD6 and Yukihikari, respectively. The induced callus was compact, healthy callus, which is the same as the *indica* rice calli induced by Pipatpanukul (1999) and Rattana (2001). Bhaskaran and Smith (1990) reported that callus inducible tissue should be meristematic tissues, such as root tip, shoot apex or leave and stem in the dicotyledon plant, but these tissue are not suitable for monocotyledon plant. Most rice tissue culture experiments reported that the scutella tissue on the

embryo area of mature seed was the most efficient tissue for calli induction for both the *indica* rice (Burikam, 2001; Rattana, 2001; Maneewan, 2005) and *japonica* rice (Kucherenko, 1993; Rueb *et al*., 1994; Zhang *et al*., 1996).

The 2,4-dichlorophenoxy acetic acid (2,4-D), acting as the plant hormone auxin, induced the callus formation more efficiently than other plant hormones in the rice tissue culture, but the appropriate concentration depends on the type of induced tissue and cultivars (Bhaskaran and Smith, 1990; Yang *et al*., 1999; Chen and Zhu, 1999). In this study, 2 mg/L of 2,4-D was used for callus induction and relatively high callus induction ability of up to 90% was obtained for *indica* rice.

Siripun (1997) used 0.5, 1.0, 2.0 and 4.0 mg/L 2,4-D for callus induction of LPT 123 indica rice cultivar, and the best callus induction was observed with MS medium supplemented with 2 mg/L 2,4-D. Tinjuangjun (1996), Kuleung (1997) and Chaiwiratnukul (2000) induced the callus of KDML105 with MS medium supplemented with 2 mg/L 2,4-D and callus induction abilities of 44.4%, 76.2% and 86.5%, respectively, were obtained. For japonica rice, Wu *et al*. (1997) and Huang *et al*. (2001) induced the callus of TP 309 in the N6 medium supplemented with 2 mg/L 2,4-D and 3% sucrose. Although 2,4-D shows high efficiency in callus induction, in some cases, a combination of plant hormones was also used for rice callus induction. Suvarnalatha *et al*. (1994) induced Basmati seeds with N6 medium supplemented with 2 mg/L 2,4-D and 0.1 mg/L kinetin.

Imjongjirak (2000) studied the effect of culture medium, NB and MS medium supplemented with 2 mg/L 2,4-D, on KDML105 callus induction. On NB medium, the percentage of embryogenic calli induction (79.2 ± 3.4%) was higher than on 2MS medium. Both NB and MS media are composed of similar macronutrients

and micronutrients, which are essential for plant growth, but the concentrations of these nutrients differ. Moreover, NB medium contains L-proline, and Kuleung (1997) reported the use of 10 mM L-proline can promote embryogenic callus formation in KDML105. So, in this study 10 mM L-proline was used in the callus induction medium. KDML105 rice callus induction was also previously found in MS medium supplemented with 8 mM proline (Tinjuangjun, 1996) and 1 g/L proline (Chaiwiratnukul, 2000).

### 5.5.3 Effect of plant hormone in rice regeneration

To study the effects of NAA and kinetin in KDML105, RD6 and Yukihikari rice plant regeneration, it was performed with six different concentrations of NAA and kinetin. The highest regeneration ability 66.7 ± 7.64% was found in Yukihikari calli regenerated with MS medium supplemented with 1.0 mg/L NAA and 2.5 mg/L of kinetin. This hormone concentration also produced high regeneration efficiency 61.7 ± 10.4 and 55.0 ± 5.0 in the regeneration of KDML105 and RD6, respectively. In addition, although the RM 2 produced high regeneration ability for Yukihikari, the numbers of multiple shoots regeneration was appeared lower than RM 5 and RM 6 that exhibited 5.6 ± 1.7 and 5.6 ± 1.7 shoots per callus.

Efficiency of plant regeneration depended on the ratio of NAA and kinetin in the culture medium. Skoog and Miller (1957) reported the effect of the auxin and cytokinin ratio on tissue development. If the ratio of auxin to cytokinin was high in the regeneration medium, callus would be induced to form roots, whereas, when the ratio of auxin to cytokinin was low, shoots were formed. Intermediate ratios of auxins to cytokinins favored maintained callus growth (Walden, 1993). Zhang *et*

*al*. (1996) regenerated japonica rice cultivars Aikoku and Sen-ichi with 2.5 mg/L NAA and 8 mg/L kinetin and found more than 85% regenerated plants.

Another type of hormone was used by Pipatpanukul (1999) with 2.5 μM IAA and 18 μM BA in RD6 regeneration, which obtained 61.1% regeneration. Rattana (2000) obtained 83.3% regeneration for regeneration of RD6 with 9 μM NAA and 18 μM BA. Imjongjirak (2000) successfully regenerated KDML105 on NB-RE medium supplemented with 1, 2 and 4 mg/L 6-benzylaminopurine (BAP).

Recently, Lee *et al*. (1999) reported increasing rice plant regeneration efficiency with water stress treatment. However, there was variability among cultivars; Dongjinbyeo, IR43, Nagdongbyeo and Sinseonchalbyeo showed a considerable increase in the frequency of calli regeneration (15.3%-31.6%, 34%-48.7%, 44.1%-79.5% and l.l%-32.6%, respectively), whereas Nonganbyeo and Sangjubyeo cultivars responded poorly to shoot regeneration even after water stress (0%-0.2% and 6.5%-11.2%).

### 5.5.4 *Agrobacterium*-mediated transformation of KDML105 and RD6 rice

To generate transgenic KDML105 and RD6 rice plants, secondary calli and sterilized seeds were used to transform with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. Secondary calli transformation may have failed for many reasons, such as the *Agrobacterium* concentration in the inoculation step was high, which led to a contamination problem in later steps. In an early experiment, using an *Agrobacterium* solution with an OD600 of 1.0 for 30 min in the inoculation step, *Agrobacterium* contamination covered more than 60% of the transformed calli and spread out to cover all of the transformed calli within 10 days. So, a higher

cefotaxime concentration of 400 mg/L in the washing step and 300 mg/L for the first 2 weeks of selection was applied. For this reason, *Agrobacterium* contamination was decreased to approximately 40% of total transformed calli and some transformed calli remained for the regeneration step. However, the transformed calli that remained after the selection step could not be regenerated to plantlets. In the same way, in transformation of sterilized seeds, *Agrobacterium* contamination also found in approximately 75% of total transformed seeds in 7 days culture, which decreased to approximately 45% when the higher cefotaxime concentration was used.

The hygromycin concentration at 20 mg/L was used in the selection step of later experiments. For selection of both KDML105 and RD6, the surviving calli from the 20 mg/L hygromycin selection step appeared weak and frail, so they lost regeneration ability. However, in the seed transformation of both KDML105 and RD6, lower callus induction ability of infected seeds of approximately 30% of the total infected seeds was found, while noninfected seeds gave 92.7 ± 2.8% in KDML105 and 80.9 ± 3.9% in RD6. Not only the callus induction ability, but the diameter and healthiness of the calli produced were also decreased. Because of the *Agrobacterium* contamination and healthiness of the calli, transgenic rice plants could not produced by seed transformation method.

### 5.5.5 *Agrobacterium*-mediated transformation of Yukihikari

In this study, successful *Agrobacterium*-mediated transformation was obtained in Yukihikari calli with 4 different plasmids, pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. *Agrobacterium*-mediated transformation systems have been well established for many dicotyledonous plants. However, monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens.* The studies on rice, maize, wheat and barley transformation mediated by *A. tumefaciens* (Hiei *et al*., 1994; Ishida *et al*., 1996; Cheng *et al*., 1997; Tingay *et al*., 1997) have provided strong support that monocotyledons can be transformed like dicotyledons with *A. tumefaciens* by manipulating various factors, such as using appropriate starting material which is capable of active cell division leading to efficient plant regeneration, the addition of acetosyringone to the media, and the conditions of tissue culture and co-cultivation, as well as the *A. tumefaciens* strain.

The choice of a suitable explant source as starting material for infection of *Agrobacterium* is one of the most important factors. Hiei *et al*. (1994) compared various rice tissues, namely, shoot apices and segments of roots from young seedlings, scutella, immature embryos, calli induced from young roots and scutella and cells in suspension for *Agrobacterium*-mediated transformation. Early expression of GUS was detected in all of the tissues examined apart from the root segments. The calli derived from scutella gave the highest ratio of GUS-expressing tissue to inoculated tissue. GUS expression was also observed in tissues that included shoot apices and immature embryos, tissues that were successfully transformed in previous studies (Chan *et al*., 1993; Raineri *et al*., 1990). However, only a few transformants

were obtained from immature embryos and none were obtained from shoot apices. The results of this study clearly indicate that callus cultures initiated from scutella are excellent materials for transformation of rice *by Agrobacterium.* In this study, the calli initiated from scutella (secondary calli) of mature seeds were used as starting material for *Agrobacterium-mediated* transformation of Yukihikari rice.

Since acetosyringone in the co-cultivation medium is known to enhance T-DNA strand synthesis (Li *et al*., 1992) and T-strand production is reported to peak 12-24 hr after acetosyringone-mediated induction (Culianez-Macia and Hepburn 1988), induction of *vir* genes during co-cultivation and efficient release of T-DNA may be the cause of high frequency transformation. Use of exogenous acetosynngone has enhanced transformation efficiency in certain dicots and has been found to be one of the factors affecting transformation in monocots. In this study, 10 mg/L of acetosyringone was shown to be sufficient with *A. tumefaciens* strain EHA 105. This EHA 105 is an efficient strain for transformation of higher plants, as it contains a disarmed version of pTiBo542 (Hood *et al*., 1986). The strains that derived from pTiBo542, a super-virulent Ti plasmid, were reported to operate very efficiently in transformation (Konian, 1999).

Khanna and Raina (1999) reported the effect of acetosyringone concentration in preinduction and co-cultivation media of *indica* rice (ER-43 and Basmati cultivar Kainal Local). Its total absence, from the co-cultivation as well as preinduction media, resulted in complete absence of GUS expression. Increasing the levels of acetosyringone from 60 to 200 µM in the co-cultivation medium did not produce any significant enhancement of GUS expression in any explant of the cultivars tested.

The *Agrobacterium* contamination is a serious impediment to the success of any plant transformation. Shackelford and Chlan (1996) studied the effect of 10 antibiotics in the *Agrobacterium* growth found the best antibiotic was cefotaxime, which best prevented LBA 4404 and EHA 101 contamination. Cefotaxime is the beta-lactam antibiotic which classifies in cefalosporins that are specific for rod shape gram negative bacteria group. Although higher antibiotic concentration could prevent the over growth of *Agrobacterium* after co-cultivation, it affected the plant growth, so the suitable concentration must be optimized. Pipatpanukul (1999) reported the appropriate cefotaxime concentration for *Agrobacterium* strain LBA 4404 was 50 mg/L in culture of RD6 calli. Higher cefotaxime concentration was reported to be effective by Rattana (2001) who used 150 mg/L cefotaxime for *Agrobacterium* strain LBA 4404 in the RD15 rice culture. Cefotaxime concentrations up to 250 mg/L were reported to not affect the rice growth in transformation of japonica rice with *Agrobacterium* strain LBA 4404 (Zhang *et al*., 1997; Hiei *et al*., 1994).

Though Yukihikari shows as much as 60% regeneration of green plants from secondary calli, the regeneration efficiency of transformed calli was lower than those of nontransformed calli. This was similar to results from studies of transformation of KL and IR-64 cultivars (Khanna and Raina, 1999) and transformation of KDML105 (Imjongjirak, 2000). Khanna and Raina (1997) reported the regeneration efficiency of non-transformed KL and IR-64 show as much as 100% but the calli undergo a loss in regeneration potential which resulted in approximately 15% regeneration of transformed calli. The loss in regeneration potential in transformation procedure may have resulted from the long term cultures and the

length of time in culture in the presence of hygromycin, impaired plant regeneration (Rameri *et al*., 1990; Peng *et al*., 1992). Moreover, *indica* cultivars are known to be recalcitrant to in *vitro* manipulations, largely due to a rapid decline in regenerability with increasing age in culture.

The transformation efficiencies obtained in this work, determined by calli that expressed GUS activity relative to the number of calli that had been co-cultivated with *Agrobacterium* containing pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, was 27% and 36%, respectively, which are as high as those reported by others for rice (Hiei *et al*., 1994; Khanna and Raina, 1999; Zhang *et al*., 1997; Rashid *et al*., 1996; Imjongjirak, 2000). Imjongjirak (2000) transformed KDML105 with 15.8% transformation efficiency using *A. tumefaciens* EHA 105 carrying genes for kanamycin resistance and hygromycin resistance as selectable markers and GUS as a reporter gene.

Hiei *et al*. (1994) reported *Agrobactenum*-mediated transformation of *japonica* rice, Tsukmohikan, Asanohikan and Koshihikari using scutellum-derived calli as starting material. *A. tumefaciens* strain LBA 4404 (pTQK233), containing genes for kanamycin resistance and hygromycin resistance as selectable markers and GUS as a reporter gene, was used in transformation. Transformation efficiency determined by hygromycin-resistant plants that stably expressed GUS activity relative to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells was range between 10-30%.

Zhang *et al*. (1997) reported the transformation of calli induced from embryos of mature seeds with *Agrobacterium* strain LBA 4404 carrying the plasmid pTOK233 for *indica* rice cultivar, PusaBasmati 1 and *japonica* cultivars, E-yi 105, E-wan 5, and Zhong-shu-wan-geng (ZSWG), were used as targets for *Agrobacterium*-mediated transformation. Transformation frequencies were 13.5%, 13.0%, 9.1% and 9.3%, respectively.

These indicated that the frequency of transformation with both plant genotype and with the transformation system used. In general, *japonica* cultivars of rice are more tissue culture-responsive and give higher frequency of transformation than *indica* cultivars, and one finds variations even between different *japonica* cultivars (Imjongjirak, 2000).

Putative transgenic rice plants could be regenerated from hygromycin-resistant calli of Yukihikari. In PCR assays using 35S specific primers derived from the 35S promoter coding sequence, genomic DNA from 4 transgenic pMDC139-*Os4bglu12* and 3 transgenic pMDC139-*GH5BG* showed the expected size of the amplified DNA fragment, indicating that these plants are true transgenic plants.

## 5.6 Conclusion

High callus induction ability was obtained in this experiment for KDML105, RD6 and Yukihikari rice cultivars when using N6 medium supplemented with 2 mg/L 2,4-D. However, RD6 exhibited lower callus induction efficiency at ~81%, while KDML105 and Yukihikari showed more than 90% callus induction efficiency. The induced callus was pale yellow, opaque and compact with rough surface. The average diameter of KDML105 calli was larger, 13.4 mm, while RD6 and Yukihikari

produced 11.7 and 11.1 mm callus diameters, respectively. Different rice cultivars could be regenerated to the rice plant at not much different frequency, when regenerated with RM5 and RM6 medium containing 1 mg/L NAA and 2 mg/L or 2.5 mg/L kinetin, respectively. Generation of transgenic KDML105 and RD6 was not successful in this study; however, relatively high transformation efficiencies were obtained at more than 30% in the calli transformation. For the Yukihikari rice cultivar transformation with *Agrobacterium* strain EHA 105, 27.8% and 36.9% transformation were observed when calli were transformed with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively. The successful of *Agrobacterium*-mediated transformation was obtained in Yukihikari calli transformed with 4 different plasmids, pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12* and pMDC139-*GH5BG*. Putative transgenic rice plants could be regenerated and exhibited approximately 200 bp in PCR assays using 35S promoter specific primers. This indicated that these 4 transgenic pMDC139-*Os4bglu12* and 3 transgenic pMDC139-*GH5BG* plants was true transgenic rice plants. The successful *Agrobacterium*-mediated transformation of rice depends on many conditions, such as target tissues types, inoculation time, antibiotic concentration and other affected, which should be considered in future experiments.

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# CHAPTER VI

# CONCLUSION

The *Os4bglu12* cDNA encoding the protein with the amino acid sequence that was most similar to the previously purified and characterized cell-wall-bound β-glucosidase was cloned by RT-PCR and expressed in *E. coli*. Recombinant Os4bglu12 protein was most active at pH 5.0 and stable over a pH range of 5.0 to 7.0, when incubated for up to 24 hr. The temperature maximum for Os4bglu12 activity is in the range 25-40°C. Os4bglu12 enzyme efficiently hydrolyzed β-(1,4)-linked oligosaccharides of 3-6 glucose residues and also hydrolyzed the β-1,3-linked glucose disaccharide laminaribiose, but not laminarioligosaccharides with higher DP than 2. Os4bglu12 hydrolyzes many kinds of *p*NP-β-glycosides, which indicates the low stringency at the -1 subsite of the enzyme. The specificity of Os4bglu12 for oligosaccharides and *p*NP-glycosides was different from the previously characterized GH1 β-glucosidases/exoglucanases, cell-wall-bound rice β-glucosidase, Os3bglu7, and barley βII β-glucosidase. The assay in which the Os4bglu12 was pre-incubated with cellobiose for 10 min indicated that cellobiose could inhibit the hydrolysis of cellooligosaccharides and laminaribiose by Os4bglu12.

The cDNA of a putative GH5 glucan-1,3-β-glucosidase was cloned from rice seedlings. The mature protein is a member of a plant-specific subgroup of the GH5 exoglucanase subfamily that contain two major domains, a β-1,3-exoglucanase-like domain and a fascin-like domain, which is not commonly found in plant enzymes. A

thioredoxin-GH5BG fusion protein produced in *E. coli* showed high hydrolytic activity toward various kinds of *p*NP-glycosides and exhibited a marked preference for β-(1,4)-linked oligosaccharides and laminaribiose (β-(1,3)-linked disaccharide). Although GH5BG was designated a putative glucan exo-β-(1,3)-glucosidase based on sequence homology, its catalytic activity is somewhat like GH1 β-glucosidases, which show similar oligosaccharide preferences. The substrate specificity of GH5BG is different from fungal GH5 exo-β-(1,3)-glucanases, which is likely due to differences in the structures of the loops and types of amino acids around the active site, indicating GH5BG, along with 3 closely related rice enzymes, could be considered a new subfamily of GH5.

High callus induction ability was obtained in this experiment for KDML105, RD6 and Yukihikari rice cultivars when using N6 medium supplemented with 2 mg/L 2,4-D. KDML105 and Yukihikari showed more than 90% callus induction efficiency, while RD6 exhibited lower callus induction efficiency at ~81%. Different rice cultivars could be regenerated to the rice plant at similar frequencies, when regenerated with RM5 and RM6 medium which containing 1 mg/L NAA and 2 mg/L or 2.5 mg/L kinetin, respectively. Although relatively high transformation efficiencies were obtained (~30%) from the callus transformation of KDML105 and RD6, but generation of the transgenic rice did not succeed in this study because the decreasing regeneration ability of transformed calli. For Yukihikari rice cultivar transformation with *Agrobacterium* strain EHA105, the transformation of 27.8% and 36.9% was obtained with pMDC139-*Os4bglu12* and pMDC139-*GH5BG*, respectively. The successful *Agrobacterium*-mediated transformation of Yukihikari calli was obtained with 4 different plasmids, pMDC83-*Os4bglu12*, pMDC139, pMDC139-*Os4bglu12*

and pMDC139-*GH5BG*. Putative transgenic rice plants could be regenerated, and an approximately 200 bp in PCR assays of their genomic DNA with 35s-promoter-specific primers. This indicated that these 4 transgenic pMDC139-*Os4bglu12* and 3 transgenic pMDC139-*GH5BG* plants were true transgenic rice plants.

# APPENDIX A

# CLONING OF *OS4BGLU12* AND *GH5BG* β-GLUCOSIDASES

## 1. Cloning of rice *Os4bglu12* β-glucosidase cDNA

A full-coding sequence cDNA of rice *Os4bglu12* β-glucosidase gene was cloned and inserted into pET32a+/DEST by Tassanee Onkoksoong, as described in Opassiri *et al*. (2006). Total RNA was isolated from 100 mg of 6-day-old rice seedlings using Trizol Reagent (Invitrogen, Carlsbad, CA). The total RNA (5 μg) was used as the template to synthesize the first-strand cDNA with SuperScript II reverse transcriptase, according to the manufacturer's protocol (Invitrogen). Primers for amplifying the full-length coding sequence cDNA and a cDNA encoding the mature protein of rice Os4bglu12 β-glucosidase were designed from the GenBank indica rice genome contig number AAAA02014151 and AK100820 and AK105375 cDNA sequences (Kikuchi *et al*., 2006). The forward primer 445-1full (5'-TGTCCAT GGCGGCAGCAG-3'), and the reverse primer 445-1_3'UTRr (5'-AACTGGATT ACTTCCATCTC-3') were used to amplify the full protein coding region cDNA. The amplification was performed with 30 cycles of 94°C 30 sec, 53°C 30 sec and 72°C 4 min, using *Pfu* DNA polymerase (Promega, Madison, USA). A full-length product was cloned into the *Eco*R V site of pBlueScript II SK+ (Stratagene, La Jolla, USA), and sequenced.

The cDNA encoding mature protein of the Os4bglu12 was amplified by PCR using the full-length cDNA cloned as the template with a 445-1matNcoIf (5'CACCA TGGCCTACAATAGCGCCGGCGAG-3') and 445-1stop (5'-ATCATTTCAGGAG GAACTTCTTG-3') primers and *Pfu* DNA polymerase to introduce a directional cloning site at the 5' end. The amplification was done as above, but with 45°C annealing temperature. The PCR product was cloned into pENTR-D/TOPO Gateway entry vector, according to the supplier's directions (Invitrogen). The cDNA insert in the pENTR-D/TOPO vectors was subcloned into the pET32a+/DEST Gateway expression vector (Opassiri *et al*., 2006) by LR Clonase recombination, following the recommended protocols (Invitrogen), and thoroughly sequenced.

## 2. Cloning of rice *GH5BG* β-glucosidase cDNA

The full-coding cDNA of rice *GH5BG* β-glucosidase gene was cloned and inserted into pBlueScript II SK+ by Tassanee Onkoksoong. Rice (*Oryza sativa* L. spp. *indica* cv. KDML105) seeds were germinated in the dark from day 0 to day 3 and in 12 h light-12 h dark from day 4 to day 7 at 28°C on germinating paper moistened with sterile distilled water. Total RNA was isolated from 100 mg rice seedlings with Trizol Reagent, and 5 μg of total RNA was used as the template to synthesize the first-strand cDNA with SuperScript II reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The GenBank rice genome contig accession number AC107314 (deduced protein sequence GenBank AC AAM08614) and AK065000 cDNA sequences (Kikuchi *et al*., 2003) were used to design the primers to amplify a full-length coding sequence (CDS) cDNA and a cDNA encoding the mature protein of rice glycosyl hydrolase family 5 β-glucosidase (designated *GH5BG*). The

5' sense primer AK065000f (5'-GCTGAAAAATCTTC GTCTTCATC-3') and the antisense primer AAM08614EcoRIr (5'-CCATCCAACTG GAATTCTCAC TG-3') were used to amplify a 774 bp-5' PCR fragment. The 5' sense primer AM08614EcoRIf (5'-CGCAGTGAGAATTCCAGTTG-3') and the antisense primer AK065000r (5'-CTTCACAAGAGAAAGTTACACTC-3') were used to amplify a 1016 bp-3' PCR fragment. The amplification for 5' and 3' PCR fragments was done with *Pfu* DNA polymerase (Promega, Madison, WI) with the first-strand cDNA as the template. Finally, the AK065000f and AK065000r primers were used to amplify a full CDS cDNA with the 5' and 3' PCR cDNA fragments as template in overlapping PCR. A full-length product was cloned into the *Eco*R V site of pBlueScript II SK+ (Stratagene, La Jolla, CA), and sequenced.

**3. Competent cell preparation for DH5α *E. coli***

Competent DH5α *E. coli* cells was prepared by $CaCl_2$ method as described in Sambrook *et al*. (1989). A single colony of DH5α *E. coli* cells from a glycerol stock grown overnight on LB plate was picked up and inoculated into 3 mL of LB medium. This starter was incubated at 37°C with shaking at 220 rpm for overnight (~14-16 hr). A 0.5 mL of overnight starter culture was transferred into 50 mL LB medium (in a 250 mL flask) and shaken at 240-250 rpm at 37°C until $OD_{600}$ of the culture reached 0.4-0.6 (3 to 5 hr). The bacterial culture was transferred into pre-chilled centrifuge tubes and placed on ice for 10 min prior to centrifuge. The chilled bacterial culture was centrifuged at 3000 x g for 15 min at 4°C and discarded the supernatant. The cell pellets were resuspended gently in 10 mL of ice-cold calcium chloride solution (60 mM $CaCl_2$, 10 mM PIPES pH 7.0, 15% glycerol) until it looked

homogeneous. The cell suspension was centrifuged at 3000 x g for 10 min at 4°C and the supernatant was discarded. Then the cell pellets were gently resuspended again in 10 mL of ice-cold calcium chloride solution by pipeting. Incubation of resuspended cell on ice for 30 min was important step for the cells to be saturated with $Ca^{2+}$. The resuspended cells were centrifuged at 3000 x g for 10 min at 4°C and the supernatant was discarded. Then the cell pellets were gently resuspended again on ice-cold calcium chloride solution (1.2 mL) containing 70 µL DMSO by pipeting. After the cell look homogeneous, the cell suspension was dispensed into pre-cooled tubes as 70 µL aliquots and kept immediately at -70°C.

**4. Transformation of DH5α cells with a recombinant plasmid**

For amplification of the recombinant pET32a+/DEST plasmid, the DH5α competent cells were used as the host cells for increasing number of transformed plasmids. After the frozen competent cells were thawed on ice for 5 min, one microliter of the recombinant pET32a+/DEST plasmid (containing 100-500 ng plasmid DNA) was added and incubated on ice for 30 min. The cells were transformed by heat shock at 42°C for 60 sec and immediately chilled on ice for 2 min. The transformed cells were incubated in 450 µL of LB medium at 37°C by shaking at 200 rpm. After 60 min incubation the transformed cell were centrifuged at 3000 x g for 5 min, and resuspended in 200 µL of LB medium. These cells were spread on LB agar plates containing 100 µg/mL ampicillin. The plates was set upright at room temperature for 15-20 min to let the spread cells and medium absorb into the agar and then it was inverted and incubated at 37°C overnight (~16-18 hr).

**5. Isolation of recombinant pET32a+/DEST plasmids by alkaline lysis method**

Recombinant pET32a+/DEST-*Os4bglu12* and pET32a+/DEST-*GH5BG* plasmid were isolated from DH5α *E. coli* transformed with the recombinant plasmids by the alkaline lysis method (Sambrook *et al*., 1989). A single colony of each recombinant clone was grown in 3 mL LB medium containing 100 μg/mL ampicillin at 37°C with shaking at 200 rpm overnight (~14-16 hr.). The cell cultures were centrifuged at 5,000 x g at room temperature for 5 min and the supernatant was discarded. The cell pellet was resuspended in 100 μL lysis buffer (1 M Tris/HCl pH 8.0, 0.5 M EDTA pH 8.0, 1 M glucose). Then, 200 μL of lysis solution containing 1% SDS/0.2M NaOH was added to the cell lysates, mixed quickly by inverting the tube and placed on ice for 3 min. The cell lysates were neutralized by adding 100 μL of ice cold 3 M potassium acetate (pH 4.8) and mixed by inverting. Only the clear supernatant was collected from centrifugation at 14,000 x g for 5 min at 4°C and transferred into new tube. The equal supernatant volume (~450 μL) of phenol/ chloroform was added to the clear lysate, which was then mixed vigorously and centrifuged at 14,000 x g at room temperature for 5 min. Then, 2 volumes (approximately 900 μL) of absolute ethanol was added to the supernatant and mixed by inverting the tube. The plasmid DNA was precipitated by centrifugation at 14,000 x g for 5 min and supernatant was removed completely. In last step, the plasmid DNA pellet was resuspended in 100 μL TE (pH 8.0) buffer (10 mM Tris pH 8.0, 1 mM EDTA) containing 2 μL of 1 mg/mL RNase solution and incubated at 37°C for 15 min. The plasmid DNA solution, was kept at -20°C until use.

To estimate size and purity of the isolated plasmid the DNA solution was analyzed on 1% agarose gel electrophoresis (Sambrook *et al*., 1989). The 5 μL

plasmid DNA solution was mixed with 1 μL of 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and applied to the gel wells. Electrophoresis was performed at constant 100 V for 45-50 min in 1X TAE buffer (0.04 M Tris-HCl pH 8.0, 0.04 M acetic acid, 0.001 M EDTA pH 8.0). After electrophoresis, the gel was stained in 0.1 μg/mL ethidium bromide solution for 10 min and destained with distilled water for 20-30 min. The bands of plasmid DNA in the gel was visualized by using a UV light transillumination with Fluor-S$^{TM}$ MultiImager (Bio-RAD Laboratories, CA). The sizes of plasmid DNA were estimated by comparing with the exactly known size of other plasmids and a 1 kb ladders (New England Biolabs, Beverly, MA).

**6. Transformation of Origami B(DE3) *E. coli* host cells with recombinant plasmids**

Origami B(DE3) *E. coli* was used as host strain for expression of recombinant β-glucosidases from pET32a+/DEST-*Os4bglu12* and pET32a+/DEST-*GH5BG*. The competent cells was prepared according to the same procedures as described for DH5α cells as above, but the medium for Origami B(DE3) culture is LB medium containing 15 μg/mL kanamycin and 12.5 μg/mL tetracycline.

For transformation, 2 μL of recombinant pET32a+/DEST plasmids (100-500 ng) were added to a 70 μL aliquot of Origami B(DE3) competent cells. This mixture was then mixed gently and incubated on ice for 20 min. The cells were transformed by heat shock at 42°C for 60 sec and chilled immediately on ice for 10 min. The transformed cells were incubated in 450 μL of LB medium with shaking at 200 rpm at 37°C for 60 min. The cells were pelleted by centrifugation at 3000 x g for

5 min and the cell pellet was resuspended in 200 μL LB medium. These cell solutions were spread on LB agar plates containing 100 μg/mL ampicillin, 15 μg/mL kanamycin and 12.5 μg/mL tetracycline. The LB plates were placed right side up at room temperature for 15-20 min and then the plates were inverted and incubated overnight at 37°C (~16-18 hr). The positive colonies were selected and the recombinant plasmids were isolated, as described in method 4 above.

# APPENDIX B

# SOLUTION PREPARATION

## 1. Reagents for bacterial culture and competent cell transformation

### 1.1 LB broth containing antibiotics (1 L)

Dissolve 10 g Bacto tryptone, 5 g Bacto yeast extract and 5 g NaCl in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Autoclave the solution at 121°C for 15 min and then allow the medium to cool to 50°C before adding antibiotics with concentration recommended for each cloning system and store at 4°C.

### 1.2 LB plate with 100 µg/mL of ampicillin (1 L)

Dissolve 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl and 15 g Bacto agar in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Autoclave the solution at 121°C for 15 min and then allow the medium to cool to 50°C. Add ampicillin to a final concentration 100 µg/mL, then pour medium into petri-dishes. Allow the agar to harden, and store at 4°C.

### 1.3 LB agar plate with 15 µg/mL kanamycin, 12.5 µg/mL tetracycline (1 L)

Dissolve 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl and 15 g Bacto agar in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume

to 1 L with distilled water. Autoclave the solution at 121°C for 15 min and then allow the medium to cool to 50°C. Add kanamycin to a final concentration 15 µg/mL, and tetracycline 12.5 µg/mL. Then pour medium into petri-dishes, allow the agar to harden, and store at 4°C.

**1.4 LB agar plate with 100 µg/mL of ampicillin, 15 µg/mL kanamycin, 12.5 µg/mL tetracycline (1 L)**

Dissolve 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl and 15 g Bacto agar in 800 mL distilled water. Adjust pH to 7.2 with NaOH and the volume to 1 L with distilled water. Autoclave the solution at 121°C for 15 min and then allow the medium to cool to 50°C. Add ampicillin to a final concentration 100 µg/mL, kanamycin 15 µg/mL, and tetracycline 12.5 µg/mL. Then pour medium into Petri-dishes, allow the agar to harden, and store at 4°C.

**1.5 Antibiotics solution stock**

Ampicillin (100 mg/mL): dissolve 100 mg ampicillin in 1 mL sterile distilled water.
Kanamycin (30 mg/mL): dissolve 30 mg kanamycin in 1 mL sterile distilled water.
Tetracyclin (12.5 mg/mL): dissolve 12.5 mg tetracyclin in 1 mL sterile distilled water.
Filter sterile all antibiotics solution and keep at -20°C.

**1.6 100 mM IPTG stock solution**

Dissolve 0.12 g IPTG (isopropyl thio-β-D-galactoside) in distilled water and make to 5 mL final volume. Sterilize by filter sterile and store at -20°C.

## 2. Reagent for competent cell preparation

### 2.1 $CaCl_2$ Solution (60 mM $CaCl_2$, 10 mM PIPES pH 7.0, 15% glycerol)

To prepare 100 mL solution, mix the stock solution as follow:

- 6 mL of 1 M $CaCl_2$ (14.7 g/100 mL, filter sterile)
- 10 mL of 100 mM PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)) pH 7.0 (3.02 g/100 mL adjust pH with KOH, filter sterile)
- 15 mL of 100% glycerol (autocleave at 121°C, 15 min)

Add sterile distilled water to bring a volume up to 100 mL and store the solution at 4°C.

## 3. Reagent for agarose gel electrophoresis

### 3.1 0.5 M EDTA (pH 8.0) (100 mL)

Dissolve 18.61 g EDTA (disodium ethylene diamine tetraacetate $\cdot 2H_2O$) in 70 mL distilled water, then adjust pH to 8.0 with NaOH (about 20 g) and the volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min and store at room temperature.

### 3.2 50 X TAE for agarose gel electrophoresis (1 L)

Mix 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) and adjust the volume to 1 L with distilled water. Store at room temperature.

### 3.3 6 X DNA loading dye (10 mL)

Mix 0.025 g Bromophenol blue, 0.025 g xylene cyanol and 3 mL of 100% glycerol in distilled water to a 10 mL final volume and store at 4°C.

## 4. Solutions for protein

**4.1 SDS-gel loading buffer (5 X stock) (2.5 M Tris, 10% SDS, 0.5% Bromophenol blue, 50% glycerol)**

Dissolve 0.30 g Tris Base, 1 g SDS, 0.05 g bromophenol blue, 5 mL glycerol then adjust pH to 6.8 with HCl and the volume to 8 mL with distilled water. Before use add 20 μL of 2-mercaptoethanol to 80 μL of solution mixture. Store at room temperature.

**4.2 1.5 M Tris pH 8.8 (100 mL)**

Dissolve 18.17 g Tris Base in 80 mL distilled water, then adjust pH to 8.0 with HCl and the volume to 100 mL with distilled water. Store at 4°C.

**4.3 0.5 M Tris pH 6.8 (100 mL)**

Dissolve 6.06 g Tris Base in 80 mL distilled water, then adjust pH to 6.8 with HCl and the volume to 100 mL with distilled water and store at 4°C.

**4.4 30% Acrylamide solution (100 mL)**

Dissolve 29 g acrylamide and 1 g *N*,*N*'-methylene-bis-acrylamide in distilled water to a volume 100 mL. Mix the solution by stirring for 1 hr to be homogenious and filter though Whatman membrane No. 1. Store in the dark bottle at 4°C.

**4.5 Tris-Glycine electrode buffer (5 X stock) (1L)**

Dissolve 30.29 g Tris base, 144 g glycine, 5 g SDS in distilled water. Adjust pH to 8.3 with HCl and the volume to 1 L with distilled water.

**4.6 Staining solution with Coomassie brilliant blue for protein**

Mix 1 g Coomassie brilliant blue R-250, 400 mL methanol, 500 mL distilled water and 100 mL glacial acetic acid and filter through a Whatman No. 1.

**4.7 Destaining solution for Coomassie Stain**

Mix 400 mL methanol, 100 mL glacial acetic acid, and add distilled water to a final volume of 1000 mL.

**4.8 10% (w/v) Ammonium persulfate (1 mL)**

Dissolve 100 mg ammonium persulfate in 1 mL distilled water and store at -20°C.

**4.9 SDS-PAGE**

The composition of a 12% SDS-PAGE separating gel shown in Table 1 were mixed and poured into the gap between a pair of glass plates assembled in a Hoefer vertical gel cassette (South San Francizco, CA, USA) up to a level of three fourths of the height of the lower plate. Approximately 200 μL of n-butanol was layered over the separating gel solution, which was allowed to polymerize for 30 min at room temperature. Then, the surface of the gel was rinsed with distilled water. The components of a 5% stacking SDS-PAGE gel, shown in Table 2, were mixed and

layered on the separating gels. After polymerization for 30 min, the polyacrylamide gel plates were assembled in a Hoefer gel electrophoresis apparatus and filled with 1X Tris-glycine electrode buffer (pH 8.3).

**Table 1** Preparation of a 12% separating SDS-polyacrylamide gel

| **Solution components** | **Component volume (mL) for 10 mL** |
|---|---|
| 1.5 M Tris (pH 8.8) | 2.5 |
| Distilled water | 3.3 |
| 10% (w/v) SDS | 0.1 |
| 30% acrylamide solution | 4.0 |
| 10% (w/v) ammonium persulfate | 0.1 |
| TEMED | 0.004 |

**Table 2** Preparation of a 5% stacking SDS-polyacrylamide gel

| **Solution components** | **Component volume (mL) for 5 mL** |
|---|---|
| 1.5 M Tris (pH 6.8) | 1.26 |
| Distilled water | 2.77 |
| 10% (w/v) SDS | 0.05 |
| 30% acrylamide solution | 0.83 |
| 10% (w/v) ammonium persulfate | 0.05 |
| TEMED | 0.005 |

## 5. Buffers and reagents for enzyme assay

### 5.1 50 mM Sodium acetate pH 5.0 buffer (100 mL)

Dissolve 0.41 g sodium acetate in 80 mL distilled water, then adjust pH to 5.0 with glacial acetic acid and the volume to 100 mL with distilled water. Store at 4°C.

### 5.2 0.4 M $Na_2CO_3$ (100 mL)

Dissolve 0.42 g in $Na_2CO_3$ distilled water, and adjust the volume to 100 mL with distilled water. Store at 4°C.

### 5.3 10 mM *p*-Nitrophenol (10 mL)

Dissolve 0.0139 g *p*-nitrophenol in 50 mM sodium acetate pH 5.0 buffer and make to 10 mL final volume. Store at 4°C.

### 5.4 10 mM *p*-Nitrophenol (*p*NPG) (10 mL)

Dissolve 0.0301 g *p*-nitrophenol-β-D-glucopyranoside in 50 mM sodium acetate pH 5.0 buffer and make to 10 mL final volume. Store at 4°C in the dark.

## 6. Solution for protein extraction and purification by $Co^{2+}$ column

### 6.1 Lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl) (100 mL)

Dissolve 0.69 g $NaH_2PO_4$, 1.75 g NaCl with distilled water, then adjust pH to 8.0 with NaOH and the volume to 100 mL. Store at 4°C.

**6.2 10% Triton X-100 (100 mL)**

Dissolve 1 mL Triton X-100 with 9 mL distilled water and store at room temperature.

**6.3 100 mM PMSF (1 mL)**

Dissolve 0.0174 g PMSF (phenylmethanesulfonyl) with 1 mL isopropanol to final volume and store at -20°C in the dark.

**6.4 10 mg/mL Lysozyme (1 mL)**

Dissolve 0.010 g Lysozyme with distilled water to final volume 1 mL.

**6.5 Wash 1 buffer ((50 mM $NaH_2PO_4$, 300 mM NaCl, 5 mM immidazole) (100 mL)**

Dissolve 0.69 g $NaH_2PO_4$, 1.75 g NaCl and 0.034 g immidazole with distilled water, then adjust pH to 8.0 with NaOH and the volume to 100 mL. Store at 4°C.

**6.6 Wash 2 buffer ((50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM immidazole) (100 mL)**

Dissolve 0.69 g $NaH_2PO_4$, 1.75 g NaCl and 0.068 g immidazole with distilled water and adjust pH to 8.0 with NaOH and the volume to 100 mL. Store at 4°C.

**6.7 Elution buffer ((50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM immidazole) (100 mL)**

Dissolve 0.69 g $NaH_2PO_4$, 1.75 g NaCl and 1.7 g immidazole with distilled water, then adjust pH to 8.0 with NaOH and the volume to 100 mL. Store at 4°C.