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นางสาวจันทร์จิรา มณีสาร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# STUDY OF THE NATURAL SUBSTRATE STRUCTURE OF RICE β-GLUCOSIDASE

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# STUDY OF THE NATURAL SUBSTRATE STRUCTURE OF RICE β-GLUCOSIDASE

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

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จันทร์จิรา มณีสาร : การศึกษาโครงสร้างสารตั้งต้นธรรมชาติของบีตากลูโคซิเดสของข้าว (STUDY OF THE NATURAL SUBSTRATE STRUCTURE OF RICE β-GLUCOSIDASE) อาจารย์ที่ปรึกษา : ดร. รจนา โอภาสศิริ, 150 หน้า

บีตากลูโคซิเดส ไอโซไซม์ Os4bglu12 เป็นเอนไซม์ในไกลโคซิลไฮโดรเลสกลุ่มที่ 1 ก่อนหน้านี้ดีเอ็นเอซึ่งบรรจุรหัสพันธุกรรมของเอนไซม์นี้ได้ถูกแยกออกมาจากต้นอ่อนข้าว และได้นำมาใช้ผลิตโปรตีนซึ่งอยู่ในสภาพที่ทำงานได้ใน *Escherichai coli* การศึกษาการแสดงออกของยีนพบว่า *Os4bglu12* ถูกสังเคราะห์มากที่ส่วนยอดของต้นอ่อนข้าว และที่กาบใบและลำต้นของข้าวที่อยู่ในระยะออกดอก การแสดงออกของยีนเพิ่มขึ้นในต้นอ่อนข้าวที่เกิดบาดแผลและที่ได้รับฮอร์โมนเมทิลจัสโมเนทและอีเทฟอน การตรวจหาสารตั้งต้นในธรรมชาติของ Os4bglu12 ได้นำมาใช้อธิบายถึงบทบาทของเอนไซม์นี้ในข้าว พบว่า Os4bglu12 ที่ถูกผลิตขึ้นใน *E. coli* สามารถย่อยผลิตภัณฑ์ที่ได้จากการย่อยผนังเซลล์ข้าวด้วย endo-1,3;1,4-β-glucanase ได้ นอกจากนี้เอนไซม์ยังย่อยสารประกอบที่สกัดจากต้นอ่อนข้าว และต้นข้าวที่อยู่ในระยะออกดอก สารตั้งต้นในธรรมชาติของ Os2bglu12 ที่พบที่ส่วนใบและลำต้นของข้าวระยะออกดอกซึ่งได้ให้ชื่อว่า C1/sD ถูกแยกออกมาให้บริสุทธิ์ด้วยวิธีการต่างๆ จากการวิเคราะห์โครงสร้างด้วย $^{1}$H-NMR พบว่ามีอะตอมไฮโดรเจนของหมู่อโรมาติกและอโนเมอร์คาร์บอนของน้ำตาลในโครงสร้างของสารประกอบดังกล่าว จากการวิเคราะห์น้ำหนักโมเลกุลของสารประกอบ C1/sD1 พบว่ามีน้ำหนักโมเลกุล 492 ซึ่งตรงกับน้ำหนักโมเลกุลของ flavonoid glycosides หลายชนิด

สาขาวิชาชีวเคมี ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

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β-GLUCOSIDASE/RICE/NATURAL SUBSTRATES/STRESS RESPONSE

Os4bglu12 is a glycosyl hydrolase family 1 β-glucosidase. The full-coding cDNA of *Os4glu12* was previously isolated from rice seedlings by RT-PCR and its recombinant protein was expressed as soluble active form in *Escherichai coli*. Northern blot analysis revealed that *Os4bglu12* is highly expressed in rice seedling shoot and in the leaf sheath and stem of rice at flowering stage. *Os4bglu12* transcript levels increased in rice seedlings in response to wounding, methyl jasmonate and ethephon. The function of Os4bglu12 was confirmed by identifying its natural substrates. Recombinant Os4bglu12 protein expressed in *E. coli* could hydrolyze the products of rice cell wall hydrolysis by endo-1,3-1,4-β-glucanase. In addition, the enzyme could hydrolyze compounds extracted with methanol from 7-day old rice seedlings and rice plants at flowering stage. The substrates of Os4bglu12 found in rice leaf and stem at flowering stage, namely C1/sD, were purified using various chromatographic techniques. $^{1}$H-NMR analysis indicated the presence of aromatic groups and anomer protons of sugar molecules in C1/sD structures. The mass of one compound C1/sD1 glycoside was estimated to be 492, which matches many flavonoid glycosides.

School of Biochemistry Student's Signature_____

Academic Year 2007 Advisor's Signature_____

Co-advisor's Signature_____

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Absorbance |
| bp | Base pairs |
| BSA | Bovine Serum Albumin |
| °C | Degree celsius |
| cDNA | Complementary deoxynucleic acid |
| DNA | Deoxyribonucleic acid |
| g | Gravitational acceleration |
| (m, μ)g | (milli, micro) Gram |
| hr | Hour |
| HPLC | High performance liquid chromatography |
| 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 |
| μmol | micro Mole |
| mRNA | Messenger ribonucleic acid |
| Mr | Molecular weight |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| OD | Optical density |
| 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/vol |
| w/v | Weight/volume |

# CHAPTER I

# INTRODUCTION

## 1.1 β-Glucosidases and glycosides

β-Glucosidases (EC. 3.2.1.21) are glycosyl hydrolases (GH) which are found widely in all types of organisms (bacteria, archaea, and eukaryote), and play important roles in fundamental biological processes (Esen, 1993). These enzymes hydrolyze the β-O-glycosidic bond at the anomeric carbon of glucose moieties at the nonreducing end of carbohydrate or glycoside molecules. β-Glucosidases that have been studied are the members of glycosyl hydrolase families 1 and 3 (EC 3.2.1-3.2.3) (Henrissat and Bairoch, 1993, 1996). β-Glucosidases in both families present the same tertiary structure which are $(\beta/\alpha)_8$ barrel with the catalytic acid/base and nucleophile carboxylic acid residues on the C-terminal end of β-strands 4 and 7, respectively (Jenkins et al., 1995; Henrissat et al., 1995). 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 fundamental substrate specificity of these enzymes depends on the conformation of active site amino acids in the enzymes which 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). Until recently, there was little information describing how β-glucosidases recognize their substrates and the mechanism of aglycone binding (Verdoucq et al., 2003).

Glycosides are widely distributed throughout the plant kingdom. An overwhelming number of plant secondary products is reported to occur in glucosylated forms, which increases their water solubility, allows their high vacuolar accumulation, stores metabolic compounds in inactive forms, and stabilizes intermediate molecules during metabolic processes (Esen, 1993). In addition to their enhanced solubility, glucosides may have other important features for plant metabolism, but the functions of only a few compounds have been elucidated. To date, a number of plant β-glucosidases that have hydrolytic activities towards various glycosides have been reported, including cyanogenic glucosides (Poulton, 1990; Cicek et al., 2000), hydroxamic acid glucoside (Sue et al., 2000, Cicek et al., 2000), β-linked oligoglucosides (Hrmova et al., 1996; Akiyama et al, 1998), isoflavonoid glycosides (Hsieh and Graham, 2001; Chuankhayan et al., 2005) and furostanol glycosides (Nisius, 1988; Arthan et al., 2006). For example, grape β-glucosidase releases flavor compounds, namely geraniol, nerol and linalol from their corresponding monotepenyl-β-D-glucosides (Bitteur et al., 1989). Soybean β-glucosidase hydrolyzes isoflavone glucosides, genistin and daidzin, to release the corresponding aglycones, genistein and daidzein (Hsieh and Graham, 2001; Suzuki et al., 2006). Mayapple (*Podophyllum peltatum* L.) β-glucosidase converts podophyllotoxin-4-*O*-β-D-glucoside to its corresponding lignan aglycone (Dayan et al., 2003). Maize β-glucosidase hydrolyzes DIMBOAGlc (2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) and sorghum β-glucosidase catalyzes

the hydrolysis of dhurrin (*p*-hydroxy-(S)-mandelonitrile-β-D-glucose (Cicek et al., 2000), to release active defense compounds. *Solanum torvum* family GH3 β-glucosidase hydrolyzes the steroid glucosides torvoside A and H to release toxic aglycone compounds (Arthan et al., 2006).

## 1.2 Physiological functions of plant 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).

In defense against pathogens and herbivores, β-glucosidases act by releasing toxic defense compounds, such as hydrogen cyanide, isoflavones, and flavonoids produced by hydrolysis of their glucosides (Poulton, 1990; Dakora and Phillips, 1996; Mizutani et al., 2002) and by releasing induced factors, such as volatiles that attract parasitoids of the herbivores to attack them (Mattiacci et al., 1995). In white clover, β-glucosidase is responsible for the cleavage of cyanogenic glucosides from leaf tissue after mechanical damage (Barrett et al., 1995).

In maize leaves, β-glucosidase hydrolyzes 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-O-glucoside (HDMBOA-Glc) to release HDMBOA, which was subsequently degraded into 6-methoxy-2-benzoxazolinone (MBOA), upon infection by pathogens (Oikawa et al., 2004). In olive leaf and fruit, β-glucosidase cleaves the oleuopein, a bitter phenol glucoside, to release an active aglycone in defense against pathogens (Amiot et al., 1989; Soler-Rivas et al., 2000).

β-glucosidases regulate the biological activity of plant phytohormones, such as cytokinins, gibberellins, auxins and abscisic acids by releasing active forms from inactive hormone glucoside conjugates (Smith and Van Staden, 1978; Zouhar et al., 1999; Schliemann, 1984). β-Glucosidases are also critical for activation of lignin precursors by the hydrolysis of coniferin glycoside to the monolignol coniferyl alcohol (Dharmawardhana et al., 1995; Escamilla-Treviño et al., 2006). β-Glucosidases have also been reported to act in the depolymerization process of plant cell wall. Rice and barley β-glucosidases could hydrolyze cell wall hydrolysis products, including cellobiose, laminaribiose and other short-chain gluco-oligosaccharides (Leah et al., 1995; Akiyama et al., 1998; Opassiri et al., 2003). β-Glucosidases release molecules that act as intermediates during metabolic processes, such as of strictosidine, an intermediate in the synthesis of monoterpenoid indole alkaloids (Barleben et al., 2005; Esen, 1993). 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 in living cells.

## 1.3 Rice glycosides

To date, a number glycosides have been purified from rice and their structures characterized. The major glycosides found in various tissues of rice include glycosylsterols, flavonoid glucosides, hormone glucosides, a vitamin glucoside, and pantonic acid glucoside. There are many kinds of diglycosylsterols, and triglycosylsterols present in rice, such as glycosyl-sitosterol, - campesterol and -stigmasterol, which are found in rice bran. The major species of glycosylsterol in rice bran are D-glucopyranosyl-β-(1,4)-D-glucopyranosyl-β-(1,3)-sitosterol and D-glucopyranosyl-β-(1,4)-D-gluco-pyranosyl-β-(1,4)-D-glucopyranosyl-β-(1,3)-sitosterol (Fujino and Ohnishi, 1979). β-sitosterol-3-O-β-D-glucoside was also isolated from rice hulls (Chung et al., 2005). Peng et al. (2002) reported that sitosterol-β-D-glucoside acts as a primer to provide a glucose unit for cellulose synthesis in plants.

Flavonoid glucosides, one of the major groups of compounds found in rice, have been extensively studied. The major flavonoid glucosides present in rice are 1) anthocyanins, such as cyanidin-O-β-D-glucoside, peonidin-O-β-D-glucoside, and cyanidin 3-gentiobioside, malvidin, pelargonidin-3,5-diglucoside, cyanidin-3-glucoside and cyanidin-3,5-diglucoside in black rice (Hu et al., 2003; Ichikawa et al., 2001; Fossen et al., 2002; Zhang et al., 2006), 2) tricin-O-glucoside, a probing stimulant for planthopper, in rice hulls, rice bran, leaf and stem (Adjei-Afriyie et al., 2000; Chung et al., 2005; Cai et al., 2005; Adjei-Afriyie et al., 2000), 3) hydroxycinnamate sucrose esters, such as 6'-O-feruloylsucrose and 6'-O-sinapoylsucrose in germinated brown rice (Tian et al., 2005). The phenolic compounds addressed above posses antioxidant, anticancer and antiviral biological activities as free radical scavengers and protein

kinase C inhibitors (Hu et al., 2003; Ichikawa et al., 2001; Cai et al., 2005; Tian et al., 2005). Furthermore, many flavone-*C*-glycosides were found in rice plants including schaftoside, neoschaftoside, carlinoside, isoorientin 2″-glucoside and neocarlinoside, and isoscoparin 2″-glucoside. These compounds act as probing stimulants for planthopper (Besson et al., 1984).

Some hormone glucosides have been found in rice, including 1) gibberellic glucosides, such as $GA_8$-O-2-glucoside and $GA_3$-O-2-glucoside in ungerminated seeds (Schliemann, 1984), 16α,17-dihydroxy-16,17-dihydrogibberellin $A_4$-17-O-β-D-glucopyranoside and 16α,17-dihydroxy-16,17-dihydrogibberellin $A_7$-17-*O*-β-D-glucoside in rice anther (Hasegawa et al., 1994), 2) salicylic glucoside (Chern et al., 2005), and 3) IAA-glucoside (Marino et al., 2005). The only a vitamin glucoside reported so far to be present in rice is pyridoxine-β-D-glucoside found in rice bran, callus and seedling (Yasumoto et al., 1977; Suzuki et al., 1986a; 1986b). 5′-O-(β-glucosyl) pyridoxine is the main pyridoxine metabolite in rice bran, which also has small amounts of 4′-O-(β-glucosyl) pyridoxine (Suzuki et al., 1986a). Another type of glycoside, namely R(-) pantoyllactone-β-D-glucopyranoside, was found in shoot but not in root of rice seedlings (Menegus et al., 1995). Many compounds (including glycosides) present in rice tissues in response to environmental stresses and in transgenic rice plants have been studied in many research groups. Recently, it was found that there is a high accumulation of indole-3-acetic acid (IAA)-glucoside in tryptophan-overproducing transgenic rice (Marino et al., 2005) and of salicylic glucoside in rice overproducing NH1, a key regulator of salicylic acid mediated systematic acquired resistance (Chern et al., 2005). There has been a report on the level of pyridoxine glucoside increased by the application of pyridoxine to rice callus

and germinating seeds (Suzuki et al., 1986a; 1986b). Markham et al. (1998) reported that exposing UV-tolerant rice to high UV-B levels increased the levels of flavone glucosides, including isoorientin-2”-O-β-D-glucopyranoside, isoorientin-2”-O-β-[6-O-*E*-ρ-coumaroylglucopyranoside] and isoorientin-2”-O-β-[6-O-*E*-feruloylglucopy ranoside]. These results suggest that the presence of high amounts of some metabolic compounds may be corrected with the accumulation of the glucoside conjugated forms.

## 1.4 Rice β-glucosidases

In contrast to the occurrence of a number of glycosides in rice, few rice β-glucosidases have been studied. The first reports of rice β-glucosidase activity against the synthetic substrate *p*NP-β-D-glucoside was by Palmiano and Juliano (1973). To date, only a few rice β-glucosidase isozymes have been characterized for their possible functions. 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). The N-terminal sequence of the cell wall-bound β-glucosidase and the full-length deduced amino acid sequences of BGlu1 were

described. In addition, both enzymes showed high hydrolytic activity against cello- and laminari-oligosaccharides.

A few reports described the expression patterns of β-glucosidases in rice plants. Based on enzyme activity, gibberellic glucoside and pyridoxine glucoside β-glucosidases were found in rice bran (Schlieman, 1984; Iwami and Yasumoto, 1986), and the cell-wall bound enzyme found in seedling (Akiyama et al., 1998). The results from northern blot analysis showed that rice *bglu1* and *bglu2* β-glucosidase genes were highly expressed in seedling shoots, but only *bglu1* was expressed in flowers of mature plants (Opassiri et al., 2003). The BGlu1 β-glucosidase could hydrolyzed pyridoxine-5'-O-β-D-glucoside to release pyridoxine. 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, homologous to a gene in GeneBank indica rice contig *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 the 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.

Recently, forty rice genes homologous to GH 1 β-glucosidase have been identified from Rice Genome Databases (Opassiri et al., 2006). An initial homology search with β-glucosidase sequences, identified 33 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 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 (Opassiri et al., 2006).

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 mode of regulation of their expression is lacking. Rice β-glucosidases may play functions similar to those attributed to β-glucosidases from other plants, but any such a function is speculative until it is demonstrated *in vivo*, the natural substrate(s) of each enzyme is isolated from rice tissues, and the conditions under which each substrate is hydrolyzed are determined.

## 1.5 Wound signaling in plant

Plants have evolved many mechanisms for prevent themself from wound and pathogen/herbivore attack. A whole range of evidence points to the existence of cross-talk between wound- and pathogen-activated signaling pathways (LeÓn et al., 2001).

These wound- and pathogen/herbivore attack-activated responses are associated with the healing of the damaged tissues and the activation of defense mechanisms that prevent further damage. Most of the induced responses occur in a few minutes to several hours after wounding, and include the generation/release, perception and transduction of specific signals for the subsequent activation of wound-related defense genes. Proteins encoded by those wound- and pathogen/herbivore attack- inducible genes may play one of the following functions 1) repairing of damaged plant tissue, 2) producing substances that inhibit growth of the predator insect, i.e. protease inhibitor or toxin, 3) participating in the activation of wound defense signaling pathways, and 4) adjusting plant metabolism to the imposed nutritional demands (LeÓn et al., 2001).

Wounding signals in plant include several chemical compounds, including the oligopeptide systemin (Pearce et al., 1991), oligosaccharides released from the damaged cell wall (Bishop et al., 1981), and hormones such as jasmonate (Farmer and Ryan, 1990), ethylene (O'Donnell et al., 1996), salicylic acid and abscissic acid (Peňa-Cortés et al., 1989). These chemical signaling compounds function together in the defense/stress response.

Arimura et al. (2005) proposed that at the early step of the signal transduction pathway caused by mechanical damage, there is an accumulation of reactive oxygen species generated near cell walls of the damaged cells and the resulting $H_2O_2$ was shown to act as a second messenger for the activation of defensive genes (Orozco-Cá rdenas et al., 2001). A depolarization of the membrane potential ($V_m$) and intracellular calcium influx were also observed only at the site of damage in Lima bean leaves infested with *Spodoptera littoralis* larvae (Maffei et al., 2004). Simple mechanical

wounding of the leaves also caused $V_m$ depolarization, but a concomitant influx of calcium could not be detected. Thus, both wounding and the introduction of herbivore-specific elicitors appear to be essential for the induction of defense responses.

The cDNA encoding a mitogen-activated protein kinase (MAPK) was isolated from tobacco. The MAPK transcript begins to accumulate in the leaves within 1 min after a mechanical wounding event (Seo et al., 1995). The mutant tobacco plant in which the WIPK protein kinase gene has been silenced show impaired accumulation of JA and MeJA after wounding. Therefore, WIPK is considered to be involved in jamonic acid (JA) formation and JA-induced responses (Seo et al., 1995; Kodama et al., 2000). In contrast, the loss of *WIPK* function in this transgenic line resulted in an increased accumulation of salicylic acid (SA) and its sugar conjugate salicylic acid β-glucoside after wounding, which was not observed in wild-type plants. The signaling pathways associated with JA and SA are generally thought to cross-communicate antagonistically to control and coordinate induced defenses. WIPK and the SA-induced protein kinase (SIPK) were found to share an upstream MAPKK, NtMEK2 (Liu et al., 2003), and to interact with the calmodulin (CaM)-binding MAPK phosphatase (NtMKP1) (Yamakawa et al., 2004). Both kinases are commonly involved in wound-mediated defence responses in tobacco (Liu et al., 2003; Zhang et al., 1998) and (Seo et al., 1999). Ethylene was assumed to be involved in plant defence responses mediated by the NtMEK2-SIPK/WIPK pathway. ABA has also been shown to increase levels of JA after mechanical damage to potato leaf tissues (Dammann et al., 1997), whereas ABA did not regulate any defence-related genes by itself (Birkenmeier et al., 1998). Thus, the wound signaling may be due to the

interactions of protein kinase/phosphatase cascades, and the cross-talk between the JA-, SA-, ethylene and ABA-signaling pathways.

Altogether, such responses, when initiated by the recognition of physical and chemical signals of the feeding herbivores, activate subsequent signal transduction cascades to activate the genes involved in defense responses, including 1) the reception of an extracellular signal(s), 2) a depolarization of the membrane potential ($V_m$) and an intercellular calcium influx, 3) the activation of protein kinase/ phosphatase cascades, and 4) the synthesis of JA and other hormones. (Arimura et al., 2005).

## 1.6 The (1,3),(1,4)-β-D-glucans and endo-1,3-1,4-β-glucanases

The composition of grass cell walls different from those of other flowering plants (Carpita and Gibeaut, 1993; Carpita, 1996). The (1,3),(1,4)-β-D-Glucans are found only in the Poales family that includes cereals and other grass species (Dahlgren et al., 1985; Smith and Harris, 1999). (1,3),(1,4)-β-D-Glucans is unbranched, and about 90% of the polymer consists of cellotriosyl and cellotetraosyl units connected by single (1,3)-β-linkages (Figure 1.1). The remainder comprises higher oligosaccharides of up to 10 or more (1→4)-β-D-glucosyl residues with a single reducing terminal (1→3)-β-D-glucosyl residue found in the longer regions of adjacent (1→4)-linkages (Woodward et al., 1983; Wood et al., 1994). 1,3-1,4-β-Glucan are a major component of endosperm cell wall. They are also found in young leaves and roots, but are difficult to detect in elongating coleoptiles (Slakeski and Fincher, 1992). (1,3),(1,4)-β-D-Glucans are not synthesized in dividing cells, but accumulate specifically during cell enlargement and are then hydrolyzed extensively when

growth ceases (Carpita and Gibeaut, 1993). Lee et al. (1967) demonstrated that the walls of grasses were capable of autolysis observed *in vitro* and release of glucose into an incubation medium.

Because the (1,3),(1,4)-β-D-glucans accumulate during cell elongation and become the major cellulose cross-linking glycan, the hydrolysis of the (1,3),(1,4)-β-D-glucans by the seedling endoglucanases and exoglucanases has been considered to be necessary for wall expansion in the grasses (Hoson and Nevins, 1989). The endoglucanase and exoglucanase activities are located in the seedling cell walls (Huber and Nevins, 1981). 1,3-1,4-β-Glucanase (EC 3.2.1.73) also called lichenases hydrolyse (1→4)-β-glucosidic linkages where these linkages are adjacent to a (1→3)-β-D-glucosyl residue (Anderson and Stone, 1975; Woodward and Fincher, 1982) (Figure 1.1). Cleavage of the polymer into smaller fragments facilitates the complete hydrolysis to glucose by the seedling exoglucanase. The exoglucanase hydrolyzes both (1,4)-β- and (1,3)-β-linkages from the non-reducing end of β-D-poly- and oligosaccharides (Huber and Nevins, 1982).

Two 1,3-1,4-β-D-glucan 4-glucanohydrolase isoenzymes El and Ell were isolated from barley (*Hordeum vulgare* L.). The transcription level of the El isoenzyme is expressed in a wider range of tissues during seedling development, whereas the Ell isoenzyme gene's expression appears to be restricted to the germinating grain (Slakski and Fincher, 1992). Endo-1,3-1,4-β-glucanases have functions in plant cell wall metabolism (Stewart et al., 2001), and hydrolyze cell wall 1,3-1,4-β-glucan during distinctly different physiological processes, such as endosperm degradation during kernel germination (Fincher et al.,1989), vegetative cell wall elongation (Carpita et al., 1984) and turnover of cell wall β-glucans in oat

(Yun et al., 1993). In coleoptiles of rice and maize, endo-1,3-1,4-β-glucanases are involved in control of plant growth (Thomas et al., 2000), since hydrolysis of the glucan polymer by these glucanases may loosen the cell wall and allow incorporation of newly synthesized glucan polymers during cell wall expansion (Inouhe et al., 1997).

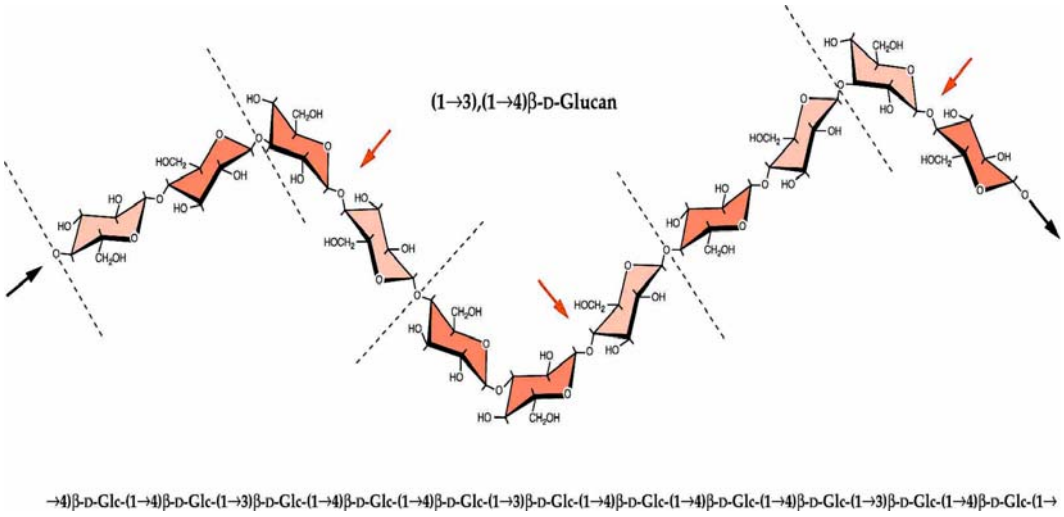



**Figure 1.1** The mixed-linkage (1,3),(1,4) β-D-glucan unique to the Poales. The arrows indicate sites cleaved by the *Bacillus subtilis* endoglucanase. The dotted lines demonstrate cellobiose units within the polymer. (Carpita and McCann, 2000).

## 1.7 Research objectives

1. To study the expression pattern of the *Os4bglu12* β-glucosidase gene in different rice organs, and developmental stages.

2. To investigate the effects of environmental conditions on rice *Os4bglu12* β-glucosidase gene expression during germination.

3. To extract and isolate the natural substrates of rice β-glucosidase.

4. To determine the structure of natural substrates of rice β-glucosidase.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Plant materials

Rice (*Oryza sativa* L. spp. *indica*) seeds of cultivar KDML 105 were obtained from the Seed Center, Nakhon Ratchasima, and rice plants at flowering stage of cultivar KDML 105 including leaves, stems and flowers were obtained from the Rice Research Center, Pathumthani.

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

### 2.1.2 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'''*-tetramethylethyllenediamine (TEMED), *N,N´*–methylene–bis–acrylamide, ammonium persulfate, Triton X-100, and lysozyme were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Redivue $^{32}$P dCTP radioisotope was purchased from GE healthcare (Buckinghamshire, UK). Sodium hydroxide (NaOH), sodium phosphate, sodium acetate, sodium chloride (NaCl), sodium carbonate ($Na_2CO_3$), disodium ehtylenediamine tetraacetate (EDTA) , bromophenol blue, sulfuric acid, glacial acetic acid, methanol, distilled water, ethanol, n-butanol, hexane, ethyl acetate, chloroform,

and Sephadex LH-20 were purchased from Carlo ERBA (Rodano, Milano, Italy). Isopropyl thio-β-D-galactoside (IPTG), 2-mercaptoethanol, abscissic acid (ABA,), 2,4-dichlorophenoxyacetic acid (2,4-D), giberellin $A_3$ (GA), and *p*-nitrophenol β-D-glucoside (*p*NPG) were purchased from sigma (St. Louis, MO, USA). Methyljasmonic acid (MeJA), and orcinol were purchased from Wako Pure Chemicals (Osaka, 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). Si-60 gel resin, Lichroprep – RP 18, Thin-layer chromatography and preparative thin-layer chromatography silica gel 60 $F_{254}$ were purchased from Merck (Darmstadt, Germany). Other chemicals and molecular reagents used but not listed have were purchased from a variety of suppliers.

### 2.1.3 Bacteria 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. This host strain derived from the *lacZY* mutant of BL21 enables accurate control of expression levels by adjusting the concentration of IPTG. The *trxB* and *gor* mutations are selectable on kanamycin and tetracycline, respectively. It is recommended to use with pET plasmids carrying the ampicillin resistance marker *bla*. This strain includes the *lon* and *ompT* deficiencies of BL21, which increase protein stability (Novagen 2002-2003 catalog).

DH5α is a recombination-deficient suppressing strain used for cloning plasmids and cosmids. The $\phi$ 80 *lacZ*ΔM15 permits α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors (Sambrook et al., 1989).

## 2.2 Methods for recombinant protein expression

### 2.2.1 Competent cell preparation for DH5α *E. coli*

DH5α *E. coli* cells from a glycerol stock were streaked on LB plate, and incubated at 37°C overnight (~16-18 hr). A single colony was picked up from LB plate and inoculated into 3 mL of LB medium, and incubated at 37°C with shaking at 220 rpm 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 supernatant was discarded. 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. The cell pellets were resuspended again in 10 mL of ice-cold calcium chloride solution by gently pipeting. Then, the resuspended cells were kept on ice for 30 min. This step is necessary 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. An ice-cold calcium chloride solution (1.2 mL) and 70 μL DMSO were added to the tube and the cell pellets were gently resuspended by

pipeting. The cell suspension was dispensed into pre-cooled tubes as 70 µL aliquots and kept immediately at -70°C.

### 2.2.2 Transformation of DH5α cells with a recombinant plasmid

DH5α competent cells were used for amplification of a pET 32a+/DEST-*Os4bglu12* plasmid (see Appendix A), which was used to express a recombinant protein. A 70 µL aliquot of frozen DH5α competent cells was thawed on ice for 5 min. One microliter of pET32a+/DEST-*Os4bglu12* plasmids (containing 100-500 ng plasmids) was added to thawed DH5α competent cells, mixed gently 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 cell were incubated in 450 µL of LB medium at 37°C by shaking at 200 rpm for 45 min. After centrifugation at 3000 x g for 5 min, precipitated cells were resuspended in 200 µL of LB medium and spread on LB agar plates containing 100 µg/mL ampicillin. After spreading, the plates was set upright at room temperature for 15-20 min to let the spread cells and medium absorb into the agar, then the plate was inverted and incubated at 37°C overnight (~16-18 hr).

### 2.2.3 Isolation of pET32a+/DEST-*Os4bglu12* plasmid

To isolate pET32a+/DEST-*Os4bglu12* recombinant plasmid, a single colony grown on an LB plate containing 100 µg/mL ampicillin was selected and inoculated in a 3 mL LB medium containing 100 µg/mL ampicillin and incubated at 37°C with shaking at 200 rpm for 9-10 hr. The cell culture 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). Two hundred microliters of 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. One hundred microliters of ice cold 3 M potassium acetate (pH 4.8) was added to the cell lysates to neutralize. Precipitated material was removed by centrifugation at 14,000 x g for 5 min at 4°C and supernatant was transferred into new tube. Four hundred microliters (1 volume) 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. Nine hundred microliters (2 volumes) 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. The plasmid DNA pellet was resuspended in 100 µL TE (pH 8.0) buffer (10 mM Tris pH 8.0, 1 mM EDTA). Two microliters of 1 mg/mL RNase solution was added to the plasmid solution and incubated at 37°C for 15 min. The plasmid DNA solution, was mixed with 70 µL of ice cold 20% PEG 6000 solution containing 2.5 M NaCl, incubated on ice for 1 hr, and then centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was removed carefully and the pellet was rinsed with 500 µL 70% ethanol and centrifuged at 14,000 x g for 5 min at 4°C. After centrifugation, the supernatant was removed carefully and the DNA pellet was dried under vacuum and redissolved in 22 µL TE (pH 8.0) buffer and kept at -20°C until use.

The purity of plasmid DNA solution was analyzed on 1% agarose gel electrophoresis (Sambrook et al., 1989). One microliter of 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyonol FF, 30% glycerol) was mixed with 5 µL plasmid DNA solution and applied to the gel wells. Electrophoresis was performed at

constant 95 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, 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 DNA fragments were estimated by comparing with a 1 kb ladders (New England Biolabs, Beverly, MA).

**2.2.4 Transformation of Origami B(DE3) *E. coli* host cells with a recombinant plasmid**

Origami B(DE3) *E. coli* was used as host strain for expression of recombinant Os4bglu12 β-glucosidase from pET32a+/DEST-*Os4bglu12*. The competent cells was prepared according to the same procedures as described for DH5α cells (see method 2.2.1), but the medium for Origami B(DE3) culture is LB medium containing 15 μg/mL kanamycin and 12.5 μg/mL tetracycline.

A 70 μL aliquot of frozen Origami B(DE3) competent cells was thawed on ice for 5 min. One microliter of pET32a+/DEST-*Os4bglu12* (100-500 ng) was added to a thawed competent cells and mixed gently, then the tube was incubated on ice for 30 min. The cells were transformed by heat shock at 42°C for 60 sec and chilled immediately on ice for 2 min. The transformed cells were incubated in 450 μL of LB medium with shaking at 200 rpm at 37°C for 45 min. The cells were precipitated by centrifugation at 3000 x g for 5 min. The cell pellet was resuspended in 200 μL LB medium and 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 2.2.3.

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

To express recombinant Os4bglu12 protein, a selected colony containing pET32a+/DEST-*Os4bglu12* recombinant plasmid was 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 inoculum cultures were diluted to a ratio of 1:100 with LB broth containing the same antibiotics. The cultures were grown at 37°C with shaking at 200 rpm for 3-5 hr until an optical density at 600 nm reached at 0.5-0.6. To induce the expression of Os4bglu12 recombinant protein, IPTG was added to the culture to a final concentration of 0.2 mM and the cultures were shaken at 200 rpm at 20°C for an additional 8-16 hr. The induced cultures were transferred to 50 mL centrifuge tubes and 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. One milliter aliquot of the cultures was sampled at indicated periods of time and precipitated by centrifugation at 14,000 x g for 10 min. Aliquots of cell pellets were used to monitor the expression levels of recombinant protein by SDS PAGE (see method 2.2.10).

### 2.2.6 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-X100, 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 and the soluble protein was recovered by centrifugation at 12,000 x g for 10 min at 4°C. The supernatant containing soluble protein was then transferred into new tube. The cell pellets were extracted again with the same extraction buffer without DNase I in order to increase a recovery of recombinant protein. The soluble protein fractions were kept on ice for protein purification in next step. An aliquot of the supernatant (10 μL) was subjected to protein analysis by SDS-PAGE (see method 2.2.10).

### 2.2.7 Purification of recombinant protein by immobilized metal affinity chromatography (IMAC)

The expressed thioredoxin-Os4bglu12 fusion protein was purified by immobilized metal affinity chromatography (IMAC) on BD TALON cobalt resin (BD Clonetech, Palo Alto, USA), in which the affinity between the his-tag on the protein and $Co^{2+}$ ion of a matrix is used for purification of recombinant protein. A cobalt-column (2 mL bed volume) was equilibrated with 8 bed volumes of equilibration buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0). Eight milliliters of soluble protein fraction was mixed with a cobalt resin, and the tube was gently shaked horizontally at 4°C for 30 min. A protein solution was allowed to flow through the column. The

column containing bound recombinant proteins was washed with 4 bed volumes of equilibration buffer (Appendix C) and then with 8 bed volumes of a washing buffer 1 (5 mM immidazole in an equilibration buffer). The column was washed further with 4 bed volumes of washing buffer 2 (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) and the fractions (1 mL protein solution/fraction) were collected. The protein fractions were kept at 4°C and subjected to enzyme assay in next step (see method 2.2.8).

### 2.2.8 Os4bglu12 activity assay

To determine enzyme activity of *Os4bglu12*, *p*-nitrophenyl-β-D-glucoside (*p*NPG) was used as a substrate. A reaction mixture containing 80 µL 50 mM sodium acetate, 10 µL 10 mM *p*NPG and 10 µL (0.8 µg/mL) Os4bglu12 protein solution was incubated in the wells of a microtiter plate at 37°C for 10 min. The reaction was stopped by addition 50 µL 0.4 M $Na_2CO_3$ and the amount of the *p*-nitrophenol (*p*NP) released was measured at 405 nm. A control reaction mixture of the same composition as that of the standard reaction mixture except without Os4bglu12 enzyme was used as a blank.

### 2.2.9 Protein determination

The protein concentration of purified recombinant protein was estimated by Bio-RAD protein assay kit using bovine serum albumin (BSA) as the standard (2-10 µg BSA). Appropriate dilutions of protein samples were prepared in 0.8 mL and 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.

### 2.2.10 Determination of the expression profiles of Os4bglu12 recombinant protein by SDS-PAGE

The method for SDS-PAGE was done by the method first described by LaemmLi (1970). The composition of a 12% SDS-PAGE separating gel is shown in Table 2.1

**Table 2.1** 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 |

Solutions shown in Table 1 were mixed in a beaker and poured into a gap of a pair of glass plates assembled in a Bio-Rad gel cassette up to a level of three fourths of the height point of the lower plate. Two hundred microliters of n-butanol was layered over the separating gel solution, which was allowed to polymerize for 20 min at room temperature. After that, the n-butanol was discarded and the surface of the gel was rinsed with distilled water.

The composition of a 5% stacking SDS-PAGE gel is shown in Table 2.2

**Table 2.2** 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 |

Solutions for stacking gels were mixed and layered on the separating gels. A plastic comb used to prepare wells for the samples was inserted into the stacking gel solution. After polymerization for 20 min, the comb was removed from the stacking gel and the sample wells of the gel were rinsed with distilled water. The polyacrylamide gel plate was assembled in a Bio-Rad gel electrophoresis apparatus and the reservoir for electrodes was filled with 1X Tris-glycine electrode buffer (pH 8.3) (Appendix C). 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) and boiled for 5 min to denature proteins. Aliquots of 8-12 μL were loaded into sample wells in the stacking gel, and electrophoresed at constant voltage of 150 V from cathodic (-) to anodic (+) end for 60 min or until the dye front reached the bottom of the gel plate. After electrophoresis, the gel was stained in staining solution (0.1% (w/v) Coomassie Brilliant Blue R250, 30% (v/v) methanol and 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. Molecular mass of the protein band were estimated by composition 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).

## 2.3 Determination of *Os4bglu12* gene expression patterns in rice by northern blot analysis

### 2.3.1 Plant materials and growth conditions

Rice (*Oryza sativa* L. cv Yukihikari) seeds obtained from Hokuren Co., Ltd. (Sapporo, Japan) were surface sterilized in 2% sodium hypochlorite solution containing 0.01% (v/v) Tween 20 for 30 min with gentle shaking and then washed extensively in distilled water. Rice seeds were allowed to germinate in the dark for 4 days at 28°C and then in a 12 hr light-12 hr dark cycle from day 4 to day 10 at 28°C. Some rice seedlings were transferred to soil and grown for an additional 4-5 weeks to reach the flowering stage. Ten-day-old rice seedlings were exposed to environmental stresses for an additional 2 days, including cold stress at 5°C, heat stress at 37°C, drought stress (grown without irrigation up to approximately 50% decrease in fresh weight), salt stress (0.3 M NaCl), flooding stress (fully submerged in distilled water), high osmotic stress (0.5 M mannitol), and mechanical wounding (aerial parts, such as leaves and stems were crushed with a plastic ruler at intervals of 1 cm). Some rice seedlings were treated with phytohormones, including 10 μM abscissic acid (ABA), 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 10 μM giberellin $A_3$ (GA), 10 μM

methyljasmonic acid (MeJA) for an additional 2 days. The control rice plants in each experiment were grown in the dark for 4 days at 28°C and then in 12 hr light-12 hr dark cycle from day 4 to day 12 at 28°C. Some ten-day-old rice seedlings were treated with wounding at different time courses: 5 hr, 10 hr, 1 day, 2 days and 3 days. Some seedlings were treated with 10 μM methyl jasmonate and 10 μM 2,4-dichlorophenoxyacetic acid for 10 hr, 1 day, 2 days, 3 days and 4 days.

### 2.3.2 Extraction of total RNA from rice tissues

Total RNA was isolated from rice tissues by the SDS-phenol method as described by Bachem et al. (1996). Rice tissue materials (6-7 g fresh weight) were ground in pre-cooled motar and pestle in liquid nitrogen to a fine powder. Rice tissue powder was transferred to a cooled 50 mL plastic tube containing 8 mL RNA extraction buffer (100 mM Tris-HCl, 100 mM LiCl, 10 mM EDTA, 1% SDS, pH 8.0) and 8 mL saturated phenol, and mixed vigourously. Eight milliters of chloroform were added to the mixture and mixed vigourously for 1 min. The rice mixture was centrifuged at 10,000 x g at 4°C for 20 min and the upper phase was transferred to new 50 mL plastic tube. The chloroform extraction was repeated twice. The upper clear aqueous phase was transferred to a new 14 mL plastic tube. To precipitate the RNA, 1/3 volume of ice cold 10 M LiCl was added to the clear aqueous phase and mixed by inverting the tube until turbid solution became almost transparent and then kept at 5°C overnight. The mixture tube was centrifuged at 10,000 x g at 4°C for 40 min and supernatant discarded carefully. RNA pellets were resuspended in 1 mL sterile distilled water by shaking for 10 min. The RNA solution was mixed with 100 μL 3 M sodium acetate and 2.5 mL chilled absolute ethanol by inverting the tube

and kept at -20°C for at least 1 hr to overnight, and centrifuged at 10,000 x g at 4°C for 1 hr. The supernatant was carefully discanted and the tube was inverted to remove moisture for 5 min. Four milliliters of 80% ethanol was added to wash the pellet, and carefully discanted. Next, one hundred fifty microliters of sterile distilled water was added and the total RNA solution was transferred to a new 1.5 mL tube. The RNA was kept at -80°C until used. To analyze the concentration and purity of RNA, 2 μL total RNA solution were mixed with 998 μL sterile distilled water (x 500 dilution) and the $A_{260}/A_{280}$ ratio was measured with UV/VIS spectrometer. A $A_{260}/A_{280}$ ratio of approximately 1.8-2.0 indicates good purity of RNA. The amount of RNA was calculated as the following equation:

$$\mu g/mL \text{ of total RNA} = (A_{260} \text{ x dilution factor x } 40)$$

One $A_{260}$ unit equals 40 μg of single strand RNA/mL (Sambrook et al., 1989).

### 2.3.3 RNA gel electrophoresis and transferring RNA from an agarose gel to a nylon membrane

Total RNA samples were electrophoresed on a 1.2% (w/v) formaldehyde agarose gel and transferred to a Highbond-N+ nylon membrane using 20 X SSC (Sambrook et al., 1989). A 1.2% (w/v) formaldehyde agarose gel in 1X formaldehyde gel-running buffer was prepared (Appendix B) and the gel was allowed to set for at least 30 min at room temperature. Thirty microliters of RNA loading dye (Appendix C) was added to 30 μg total RNA samples, mixed well by vortexing for 10 min at room temperature and centrifuged at 10,000 x g for 1 min. The total RNA samples were incubated at 65°C for 10 min and then immediately put on ice for 10 min. The denatured RNA solutions were loaded on to 1.2% (w/v) formaldehyde agarose gel

which was submerged in 1X formaldehyde gel-running buffer (Appendix C), and electrophoresed at a constant 50 V for 4 hr. After running, the gel was stained in ethidium bromide solution (1 μg/mL) for 20 min and photographed by UV illuminator to document the RNA bands appearance before blotting.

Size separated RNA was transferred from the gel to Highbond-N+ nylon membrane (GE healthcare, Buckinghamshire, UK). Whatman 3 MM filter paper wick was soaked in 20 X SSC, and put on the plastic plate to make a bridge on the plastic container containing 200 mL 20 X SSC (Appendix C). Whatman 3 MM paper with the size larger than the RNA gel was soaked in 20 X SSC, and put on the surface of RNA. The gel was reversed by hand and put on the Whatman 3 MM paper wick. The air bubbles under the gel were removed. Next, the nylon membrane cut to the same size as the RNA gel was put on the gel carefully to prevent the occurrence of air bubbles. A stack of Whatman 3 MM filter papers (2.5 cm high) was placed on the membrane. A plastic plate (200 g weight) was put on top of stack and the transfer of RNA was allowed to proceed for 16-18 hr. Next, the stack Whatman 3 MM filter papers were removed. The RNA membrane was dried at 80°C for 3 hr, and then exposed to UV light for 3 min.

### 2.3.4 Hybridization of RNA gel blots with *Os4bglu12* gene specific probe

A gene-specific probe for Os4bglu12 was generated by amplification of 3'end region and 3'-UTR of *Os4bglu12* gene with 445-1 3'end forward primer (5'-ATGGAGCAAACGTGAAGGGAT-3') and 445-1 3'UTR reverse primer (5'-AACTGGATTACTTCCATCTC-3') using rice genomic DNA as the template. The amplification was performed with 30 cycles of 94°C, 45 sec, 45°C, 45 sec, and

72°C, 1 min, using Taq DNA polymerase. One microgram of *Os4bglu12* DNA probe was mixed with 45 μL sterile distilled water. The DNA probe was denatured by heating at 95$^{o}$C for 10 min and then immediately put on ice for 10 min. Next, five microliters of Redivue [$^{32}$P] dCTP radioisotope was added into a Rediprime II Random Labeling system tube (GE Healthcare, Buckinghamshire, UK.), then mixed well immediately and incubated at 37°C for 1 hr. To stop the random prime labeling reaction, five microliters of 0.5 M EDTA was added and the reaction mix was heated at 95°C for 10 min and cooled immediately on ice for 10 min. The reaction mixture was loaded onto spin column placed on 1.5 mL tube and centrifuged at 3000 x g for 2 min. The radioactivity of the labeled DNA probe in the flowthrough solution was measured. The total CPM (count per min) of the labeled DNA probe should be between one to two million CPM.

The RNA blots were inserted into a hybridization plastic bag filled with 20 mL of hybridization solution (Appendix C) and preincubated at 42°C for 2 hr. The RNA blots were hybridized with the α-$^{32}$P-labeled *Os4bglu12* gene-specific probe for 16 hr at 42°C in a flip-flop shaker. The blots were then washed once with 2 X SSC, 0.1% (w/v) SDS, for 30 min at 65°C and washed twice with 0.1 X SSC, 0.1% SDS for 15 min at 65°C. The membranes were wrapped with Saranwrap and exposed to a Fuji film imaging plate for 16 hr at room temperature. The positions of radioactive bands were visualized by using a Fuji Film BAS 1000 BioImaging Analyzer (Fuji Photo Film Co., Ltd, Tokyo, Japan).

## 2.4 Analysis of cell wall hydrolysis by rice endo-1,3-1,4- β-glucanase and Os4bglu12 β-glucosidase

### 2.4.1 Preparation of crude cell walls from rice seedling leaves

Ten-day-old rice seedling leaves were ground to a fine powder with mortar and pestle in liquid nitrogen. The fine powder was transferred into a glass beaker containing 10 volumes of hot 90% (v/v) ethanol warmed at 90°C in a water bath and stirred gently for 10 min to remove free mono- and oligo-saccharides from cell wall powder. The suspension of cell wall powder was cooled down to room temperature and transferred into a 50 mL Falcon tube and centrifuged at 7,000 x g for 20 min at room temperature. This hot ethanol wash was repeated for another 3 times and then the crude cell wall pellets were dried under vacuum and kept at -20°C until use.

### 2.4.2 Hydrolysis of crude cell walls by a recombinant rice endo-1,3;1,4-β-glucanase

A 50 mL plastic tube containing the reaction mixture consisting of 100 mg crude cell wall powder, 100 pg of the GST-fusion protein of rice endo-1,3;1,4-β-glucanase (see Appendix A), 10 mM sodium acetate (pH 5.2), in a total volume of 20 mL was incubated at 37°C for 6 hr. The reaction was stopped by boiling for 10 min. The reaction mixture was centrifuged at 7,000 x g for 20 min at 4°C. The supernatant was loaded on an AG501-X8D (Bio-Rad, Hercules, USA) ion exchange resin column (1 cm inner diameter, 4 cm in length) previously equilibrated with distilled water and the column was washed with 10 volumes of distilled water. The

unbound sugar fractions and the washed fractions were evaporated to dryness under vacuum. The concentrated fraction was dissolved in 200 μL distilled water and loaded on a silica gel 60 $F_{254}$ plate and developed twice in acetonitrile:water (3:1 by volume). To determine the positions of the cell wall products hydrolyzed by endo-1,3;1,4-β-glucanase, edge parts (5 mm in width) of both sides of the TLC plate were cut off and submerged in an orcinol-sulfuric acid solution containing 0.1% (w/v) orcinol and 3% (v/v) concentrated sulfric acid in ethanol. Then the separated edge parts of TLC plate were heated at 150°C for 10 min to visualize positions of sugar spots. Based on the positions of sugar spots of the edge parts, the silica gel layers on TLC plate regions which were determined to contain sugar spots were scratched off, the powders were packed into a small column (1.5 cm inner diameter, 9 cm in length) and sugars were eluted from a column with 10 volumes of 80% (v/v) ethanol and evaporated to dryness under vacuum.

### 2.4.3 Hydrolysis of purified cell wall oligosaccharides with a rice Os4bglu12 β-glucosidase

A reaction mixture consisting of 10 μL of each of purified cell wall hydrolysis product (from method 2.4.3), 1 μL (0.08 μg) of Os4bglu12 β-glucosidase and 2 μL of 50 mM sodium acetate (pH 5.2) was incubated at 37°C for 0, 10, 30 and 60 min. Each reaction was stopped by boiling for 10 min. The samples were loaded on a silica gel 60 $F_{254}$ plate (Merck) and developed twice in acetonitrile:water (3:1 by volume). Spots of sugars on TLC plates were visualized by soaking the plates in orcinol sulfuric acid reagent and baking at 150°C for 10 min.

## 2.5 Purification and determination of natural glycoside substrates from rice

### 2.5.1 Plant material

Rice seeds (*O. sativa* L. spp. *indica*) cultivar "KDML 105" were soaked in water overnight prior to sowing and germinated seeds were grown in the dark for 1 day and then transferred to a 12 hr dark/ 12 hr light cycle condition for an additional 6 days. Rice seedlings were separated into 3 parts, root, shoot and seed. Rice plants at flowering stage were separated into 2 parts, flower and leaf/stem. Separated rice tissues were stored at -80°C until natural substrate extraction.

### 2.5.2 Extraction of crude extracts from rice tissues

To prepare the crude extracts from rice tissues, approximately 500 g each of roots and shoots separated from 7-day-old rice seedlings, and leaf/stem parts of mature rice at flowering stage were ground in liquid nitrogen with a mortar and pestle to a fine powder. The powder was extracted by soaking in methanol in a ratio of 1 g rice tissue powder per 4 mL methanol and incubated overnight at room temperature. The rice tissue extracts were filtered through No.1 Whatman filter paper. Then, the chlorophylls were removed from the filtrate by partitioning with a mixture of acetone and hexane (1:2 by volume) in a separatory funnel. After partitioning, the methanol layer was transferred to a glass flask and evaporated to dryness by freeze dryer. This extract was denoted as the crude extract.

### 2.5.3 Separation of rice crude extract with Sephadex LH-20 chromatography

The rice crude extract was separated by Sephadex LH-20 chromatography. Dried crude extracts (0.8 g) were dissolved in 8 mL methanol and loaded onto a Sephadex LH20 column (3.5 cm x 100 cm) previously equilibrated with methanol. The compounds were eluted from the column with methanol at a flow rate of 1 mL/min and 5 mL fractions were collected. Fractions were either evaporated to dryness and dissolved in 0.5-1 mL methanol or concentrated to approximately 0.5-1 mL solution.

### 2.5.4 Identification of natural substrates of Os4bglu12 β-glucosidase by TLC

Thin layer chromatography (TLC) was used to further fractionate the compound fractions that were purified by Sephadex LH-20 chromatography (see method 2.5.3). Various solvent systems were applied to separate these compounds on the TLC plates. The solvents used in this study were chloroform, methanol, water, ethyl acetate, acetic acid and butanol. To identify the fractions that contain natural substrates of rice Os4bglu12 β-glucosidase, each fraction was incubated with recombinant rice Trx-Os4bglu12 β-glucosidases (from method 2.2). A control reaction mixture consisted of 10 μL fraction, 90 μL 50 mM sodium acetate (pH 5.0). The enzyme assay reaction mixture consisted of 10 μL (~0.8 μg) Trx-Os4bglu12 β-glucosidases, 10 μL fractions and 80 μL 50 mM sodium acetate (pH 5.0). The reactions were incubated at 37°C for 30-60 min and stopped by boiling for 5 min. The reaction mixture was evaporated to dryness by Speedvac evaporator and then dissolved with 10 μL methanol. The products in each reaction were separated on TLC

using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) as solvent. The positions of reaction products were visualized under UV light at 366 nm and/or stained with 10% sulfuric acid in methanol and baked at 120°C for 10 min. Natural glucoside substrates for Os4bglu12 β-glucosidases were identified by investigating the occurrences of new compound spots in the enzyme assay reaction compared to the control reaction.

### 2.5.5 Purification of natural substrates of rice Os4bglu12 β-glucosidase

In order to purify the natural substrates of Os4bglu12 enough for structural analysis. One thousand grams of rice tissues (including leaf/stem parts) were extracted with methanol as described in method 2.5.2, and approximately 8 g of crude extract was obtained after freeze drying. In each round, 0.8 g of crude extract was dissolved in 8 mL of methanol and loaded onto an LH-20 column following the method described in method 2.5.3. After investigating the presence of natural substrates of Os4bglu12 in the fractions by TLC, the fractions containing substrates were pooled. Fifty milligrams of a dried substrate fraction was separated on preparative TLC using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) as solvent. The positions of substrates were detected by visualizing spots under UV light and marked with pencil. Each silica layer band on a TLC plate was scraped off with a small spatula or a box knife and suspended in methanol. The suspension was stirred overnight at room temperature, and then centrifuged at 12,000 x g at room temperature for 10 min. The supernatant was transferred to new tube, while the precipitated silica gel was washed again with methanol and centrifuged at 12,000 x g. The supernatant was transferred to new tube and dried in a freeze dryer. Each fraction

was then assayed with Os4bglu12 β-glucosidase to see whether the compound it contained be hydrolyzed by this enzyme. The natural substrate fractions that were confirmed to contain compounds hydrolyzed by a rice Os4bglu12 β-glucosidases were loaded onto a Silica-60 (Si-60) column (15 cm x 1 cm), which was eluted with ethyl acetate:acetonitrile:methanol:water mixtures starting from low to high polarity as showed in Table 2.3 Thirty milliliters of each mobile phase was used for Si-60 chromatography.

The compounds were eluted from Silica-60 column and collected at a rate of 2 min/fraction and detected on TLC. The fractions containing purified substrates were pooled and evaporated to dryness with a Speedvac concentrator.

The natural substrates were further purified by reverse phase HPLC with a Mightysil ODS-column (RP-18 GP Aqua, 46 x 150 mm, 5 μm) (Kanto Kagaku, Tokyo, Japan). The elution systems that were tried to separate the compounds are listed in Table 2.4 The appropriate condition for separating substrates by HPLC in this study was isocratic elution with 40% methanol in water, at a flow rate of 0.8 mL/min performed at 40°C.

**Table 2.3** Ratios of solvents in mobile phases for Si-60 chromatography

| Solvent | Ethyl acetate | acetonitrile | methanol | water |
|---|---|---|---|---|
| 1 | 20 | 0 | 0 | 0 |
| 2 | 16 | 3 | 1 | 0 |
| 3 | 15 | 3 | 2 | 0 |
| 4 | 15 | 2 | 2 | 1 |
| 5 | 14 | 3 | 2 | 1 |
| 6 | 12 | 4 | 3 | 1 |
| 7 | 12 | 3 | 4 | 1 |
| 8 | 10 | 4 | 4 | 2 |
| 9 | 10 | 3 | 5 | 2 |
| 10 | 8 | 4 | 6 | 2 |
| 11 | 5 | 5 | 8 | 2 |
| 12 | 5 | 3 | 10 | 2 |
| 13 | 3 | 5 | 10 | 2 |
| 14 | 0 | 7 | 10 | 3 |
| 15 | 0 | 4 | 12 | 4 |
| 16 | 0 | 2 | 14 | 4 |
| 17 | 0 | 0 | 15 | 5 |
| 18 | 0 | 0 | 10 | 10 |

**Table 2.4** The elution systems for separating compounds by HPLC

| System | Condition |
|---|---|
| 1 | acetonitrile:water ; 0 to 3min (0:100), 5 min (50:50), 7 min (80:20), 13 min (85:15), 17 min (87:13), 21 min (90:10), 25 min (95:5), 28 to 30 min (100:0) |
| 2 | acetonitrile:water ; 0 to 3 min (0:100), 5 min (50:50), 7 min (60:40), 10 to 15 min (75:25), 18 min (80:20), 21 to 23 min (85:15), 25 min (90:10), 27 to 30 min (100:0) |
| 3 | acetonitrile:water ; 0 to 3 min (0:100), 5 min (30:70), 13 to 15 min (50:50), 23 min (80:20), 26 min (85:15), 30 min (95:5), 31 to 33 min (100:0) |
| 4 | methanol:water ; 0 to 3 min (0:100), 5 min (30:70), 10 min (45:55), 13 min (50:50), 40 min (90:10), 45 to 50 min (100:0) |
| 5 | methanol:0.1% Acetic acid in water ; 0 to 3 min (0:100), 7 min (45:55), 10 min (50:50), 25 min (55:45), 30 min (65:35), 33 min (90:10), 35 to 37 min (100:0) |
| 6 | methanol:water ; 0 to 3 min (0:100), 7 min (45:55), 12 to 20 min (51:49), 25 min (57:43), 29 min (65:35), 34 min (90:10), 35 to 40 min (100:0) |
| 7 | methanol:water ; 0 to 3 min (0:100), 5 min (45:55), 12 to 30 min (51:49), 35 min (57:43), 40 min (65:35), 43 min (100:0) |
| isocratic | 65%, 55% acetonitrile in water and<br>55%, 50%, 45% and 40% methanol in water |

### 2.5.6 Determination of natural glycoside substrate structure

Structures of purified natural substrates of Os4bglu12 β-glucosidase were analyzed by various spectoscopic technigues. LC-MS analysis was performed on Electrospray Ionization Mass Spectrometry (ESI-TOF-MS) JMS-T100CS (JEOL, Japan). Matrix-assisted Laser Desorption/ionization Mass Spectrometry (MALDI-TOF-MS) analysis of the isolated compounds were performed in the negative-ion mode with a Voyager-DE STR-H mass spectrometer (Applied Biosystems, Foster, CA, USA) operated at a -20 kV accelerating voltage, a pulse delay time of 145 ns and a grid voltage of 67% using a $C_{18}$ reverse-phase column. NMR spectra were recorded on a JOEL ECX-600 spectrometer ($^1$H-NMR at 600 MHz).

## 2.6 Hydrolysis of natural glycoside substrates by Os4bglu12 β-glucosidase

The natural glycoside substrates purified by above methods were subjected to assay with Os4bglu12 β-glucosidase. Control reaction mixture consisted of 10 μL of a fraction and 90 μL of 50 mM sodium acetate (pH 5.0). Assay reaction mixtures consisted of 10 μL (~0.8 μg) of Os4bglu12 β-glucosidase, 10 μL of a fraction and 80 μL of 50 mM sodium acetate (pH 5.0). The reactions were incubated at 37°C for 60 min, stopped by boiling for 5 min and loaded onto an AG501-X8D (Bio-Rad) ion exchange resin column (1 cm inner diameter, 8 cm in length) previously equilibrated with distilled water in order to remove salts. The unbound fraction that contains reaction products was eluted from the column with distilled water and concentrated to dryness by using a Speedvac concentrator. A concentrated sample was redissloved in

water and loaded on a silica gel 60 $F_{254}$ plate (Merck, USA) and developed once in acetonitrile:water (3:1 by volume). The aglycone spots were visualized under UV light, while sugar spots were detected by soaking a TLC plate in an orcinol sulfuric acid reagent followed by baking at 150°C for 10 min. Precipitates of aglycones of natural glycoside substrates found in Os4bglu12 β-glucosidase assay reaction was separated by centrifugation at 15,000 x g for 10 min at room temperature and also analyzed by TLC, as described above. In addition, the compound in assay reactions were subjected to separation by $C_{18}$ reverse phase column HPLC with isocratic elution with 55% methanol in water.

# CHAPTER III

# RESULTS

## 3.1 Recombinant protein expression of Os4bglu12 β-glucosidase

The recombinant Os4bglu12 β-glucosidase protein was produced in Origami B(DE3) *E.coli* transformed with pET32a+/DEST-*Os4bglu12* recombinant plasmid. The cDNA encoding the mature protein of AAA0201451 β-glucosidase (designated Os4bglu12) was previously cloned and inserted into pET32a+/DEST by Tassanee Onkoksoong (Figure 3.1). The construct was used to transform Origami B(DE3) *E. coli.* and recombinant thioredoxin-Os4bglu12 fusion protein (Trx-Os4bglu12) was expressed in soluble active form (Opassiri et al., 2006). Origami B(DE3) are redox-deficient *E. coli.* that allow disulfide bond formation in the cytoplasm, while the thioredoxin fusion tag can assist folding and catalyze disulfide bond formation under this condition (pET manual, Novagen, Madison, USA). Recombinant Trx-Os4bglu12 contains a $His_6$.tag, an Stag, and a cleavage site for enterokinase after the thioredoxin tag at the N-terminus. In this study, expression of recombinant Os4bglu12 protein was performed by inducing the cell cultures with 0.2 mM IPTG and the cell cultures were allowed to grow at 20°C for another 8 hr. Comparison of protein profiles by SDS-PAGE showed that induced cultures of Trx-Os4bglu12 had an intense band at approximately 69 kDa in total cell lysates and soluble fractions (Figure 3.2).

Purification of soluble Trx-Os4bglu12 fusion protein was done 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) with elution buffer containing 250 mM immidazole and a band corresponding to 69 kDa was observed in SDS-PAGE (Figure 3.3). The purified enzyme was found to hydrolyze *p*NPG with optimal activity at pH 5.0 at 37°C (Opassiri et al., 2006). This purified enzyme was subjected to use for identifying its natural substrates in rice.

## 3.2 Expression patterns of *Os4bglu12* gene in rice

Northern blot analysis was used to determine the transcript levels of *Os4bglu12* gene in rice. Specific probe was generated by amplifying the 3'end region of *Os4bglu12* gene, including a short 3'end coding region and part of the 3'UTR, with 445-1 3'end forward and 445-1 3'UTR reverse primers (see position of primers in Figure 3.1) and rice genomic DNA as the template. The 3'end fragment specific DNA probe for *Os4bglu12* (264 bp) (Figure 3.4) was $\alpha^{32}$P-labeled and RNA blots hybridized with the probe.

### 3.2.1 Expression patterns of *Os4bglu12* gene in rice tissues during germination and flowering stages

In 7-day-old rice seedlings, *Os4bglu12* transcripts were detected in high abundance in the shoot, and at low levels in the root and endosperm. In 6-week-old mature rice plants at flowering stage *Os4bglu12* mRNA was highly expressed in leaf sheaths and stems. The *Os4bglu12* transcripts were detected with moderate levels in node, flower, leaf blade and very low or no signal was seen in root (Figure 3.5).

```
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
445-13'end_f primer
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  *        445-13'UTR_r primer
ATATGTTTTGCGTGAATTTGGTACAAGAGATGGAAGTAATCCAGTTAAATAATAAATCCCTCTCTTACTGC
```

**Figure 3.1** The full-length cDNA sequence and deduced amino acid sequence of rice *Os4bglu12*. Underlined letters represent the regions of DNA sequence of PCR primers for cloning the mature protein encoding cDNA for recombinant protein production and 3'end DNA probe for northern blot analysis.

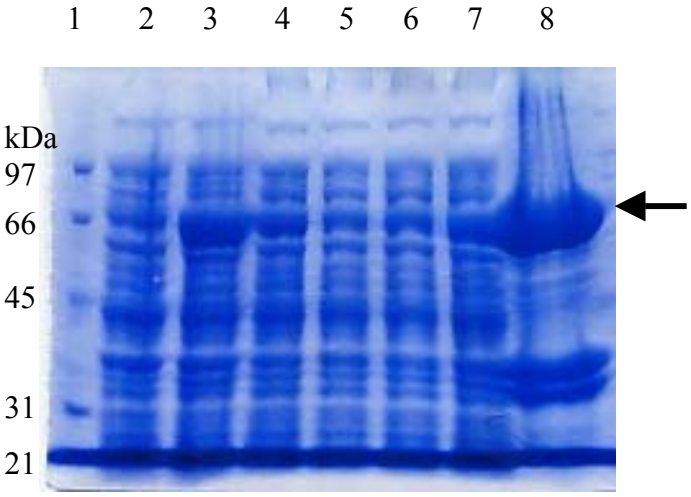



**Figure 3.2** SDS-PAGE profiles of recombinant Trx-Os4bglu12 fusion protein expressed in Origami B(DE3) *E. coli* transformed with pET32a+/DEST-*Os4bglu12*. The bacterial cell culture was incubated in the presence of 0.2 mM IPTG with shaking at 20°C for 8 hr. Lanes 1, standard marker (Bio-RAD); 2, total protein in *E. coli* cells containing pET32a+/DEST-*Os4bglu12* before induction with IPTG; 3, total protein in *E. coli* cells containing pET32a+/DEST-*Os4bglu12* induced by 0.2 mM IPTG at 20°C for 8 hr; 4 and 7, soluble fusion of Trx-Os4bglu12 protein; 5 and 6, flowthough fractions after passing crude enzyme through $Co^{2+}$ column; 8, insoluble protein fraction; The arrow points to the positions of Trx-Os4bglu12 protein monomer at 69 kDa.

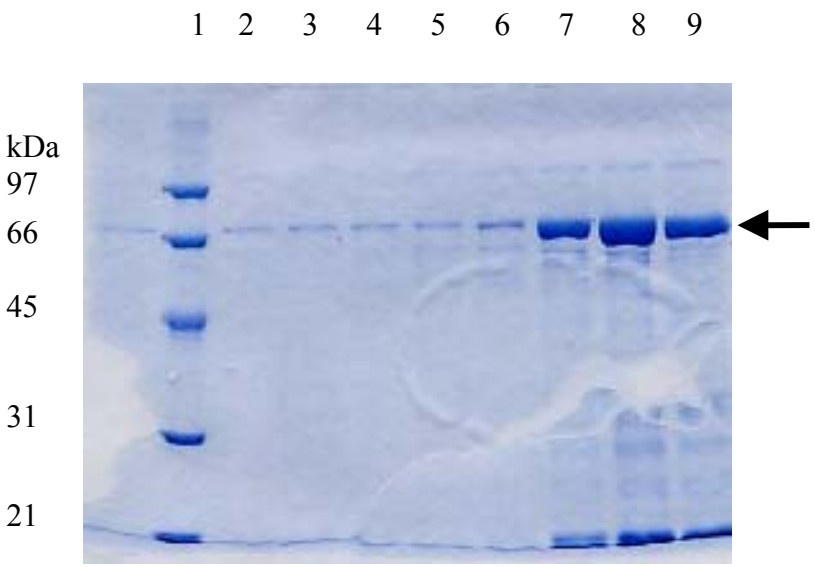



**Figure 3.3** SDS-PAGE profiles of recombinant Trx-Os4bglu12 fusion protein expressed in Origami B(DE3) *E. coli* after purifying with IMAC-$Co^{2+}$ chromatography. Lanes 1, standard marker (Bio-RAD); 2 to 9, purified protein in fractions 2 to 8, respectively. The arrow points to the positions of Trx-Os4bglu12 protein monomer at 69 kDa.

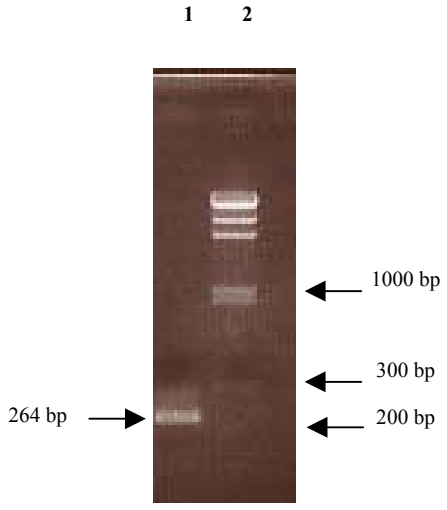



**Figure 3.4** 1% agarose gel electrophoresis of PCR product of 3'end region *Os4bglu12* DNA amplified with the 445-1 3'end forward and 445-1 3'UTR reverse primers using rice genomic DNA as template. Lane 1, PCR products of 3'end region of *Os4bglu12* gene and lane 2, DNA Marker.

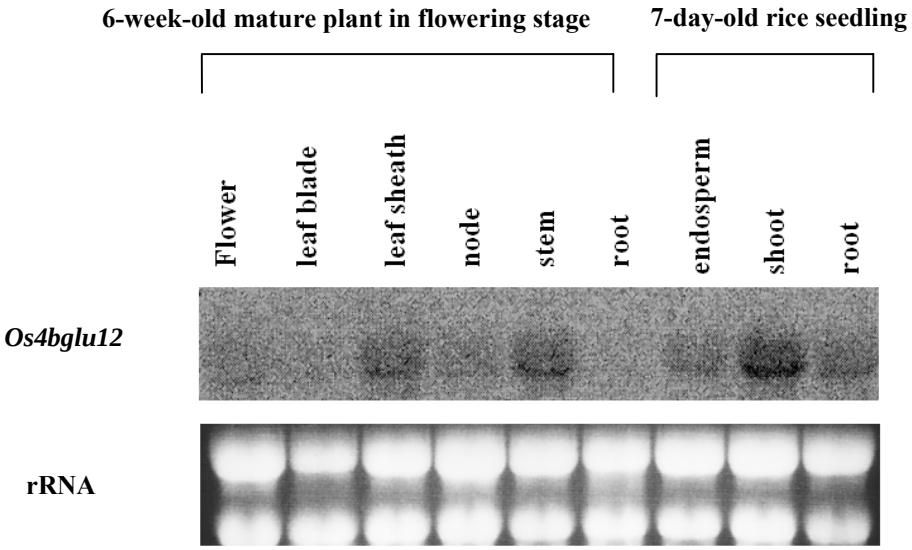



**Figure 3.5** Northern blot analysis of *Os4bglu12* expression levels in various parts of 7-day-old rice seedling and 6-week-old mature plant at flowering stage. RNA blot was hybridized with the α-$^{32}$P-labeled 264 bp 3'end fragment of *Os4bglu12* DNA probe. Thirty micrograms of total RNA was loaded in each lane on 1.2% agarose formaldehyde gel. The ethidium bromide-stained gel below the blot indicates the equal RNA loading before blotting.

The temporal expression pattern of *Os4bglu12* in seedling tissues during germination was examined using total RNA extracted from whole rice seedlings. The transcript of *Os4bglu12* was not detected in seeds before germination (day 0). The transcript could be detected at a moderate level on day 1 in germinated seeds. Then, the expression of *Os4bglu12* increased and reach the highest level on day 2, and the high transcript level could be detected upto day 4. After that, the expression level of this gene decreased gradually from day 6 (Figure 3.6).

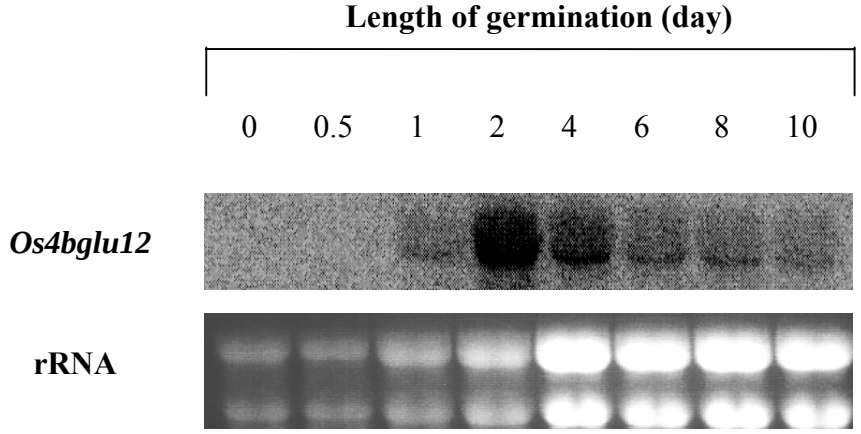



**Figure 3.6** Northern blot analysis of *Os4bglu12* expression in rice seedlings during germination and growth. Thirty micrograms of total RNA was loaded on 1.2% agarose formaldehyde gel. RNA blot was hybridized with the α-$^{32}$P-labeled 264 bp 3'end fragment of *Os4bglu12* cDNAs probe. The ethidium bromide-stained gel below the blot indicates the RNA loading before blotting.

### 3.2.2 Effects of environmental stresses and plant hormones on *Os4bglu12* gene expression in rice seedlings

Northern blot analysis was also used to determine the effects of environmental stresses and plant hormones on the expression of *Os4bglu12* gene in rice seedling during germination. The transcript levels of *Os4bglu12* were compared between 12-day-old rice seedlings that had been exposed to various conditions for 2 days to 12-day-old rice seedlings grown at 28°C (control). A significant increase in *Os4bglu12* mRNA levels was detected when rice seedlings were exposed to methyl jasmonate. The mRNA transcripts were detected at levels similar or slightly decreased compared to the control when the seedlings were exposed to 37°C,

drought, salt, mannitol and gibberellic acid. The transcript levels decreased when the seedlings were treated with ethylene and wounding, and decreased dramatically when plants were grown under cold (5°C) and flooding conditions, and in the presence of abscissic acid (Figure 3.7).

LeÓn et al. (2001) reported that plant hormones such as methyl jasmonate, ethylene and abscissic acid are involved in diverse plant responses to abiotic stresses especially in wounding stress. Therefore, a more detailed time-course study of *Os4bglu12* expression was performed in 10-day-old rice seedlings treated with methyl jasmonate, ethephon and wounding. The expression of *Os4bglu12* was induced as early as 5-10 hr after treatment with wounding and decreased gradually thereafter (Figure 3.8A). Similar gene expression patterns were seen in rice seedlings treated with methyl jasmonate and ethephon. *Os4bglu12* mRNA levels significantly increased within 10 hr after treating with methyl jasmonate, after that the mRNA levels decreased slowly (Figure 3.8B). Treatment with ethephon induced a rapid temporary increase in *Os4bglu12* mRNA levels within 10 hr, after which the mRNA levels decreased rapidly within 1 day and then decreased gradually (Figure 3.8C).

The association of the induced expression patterns of *Os4bglu12* gene in response to wounding, methyl jasmonate and ethephon indicated that these treatments rapidly induced the expression of *Os4bglu12* and a high level of expression maintained within 10 hr.

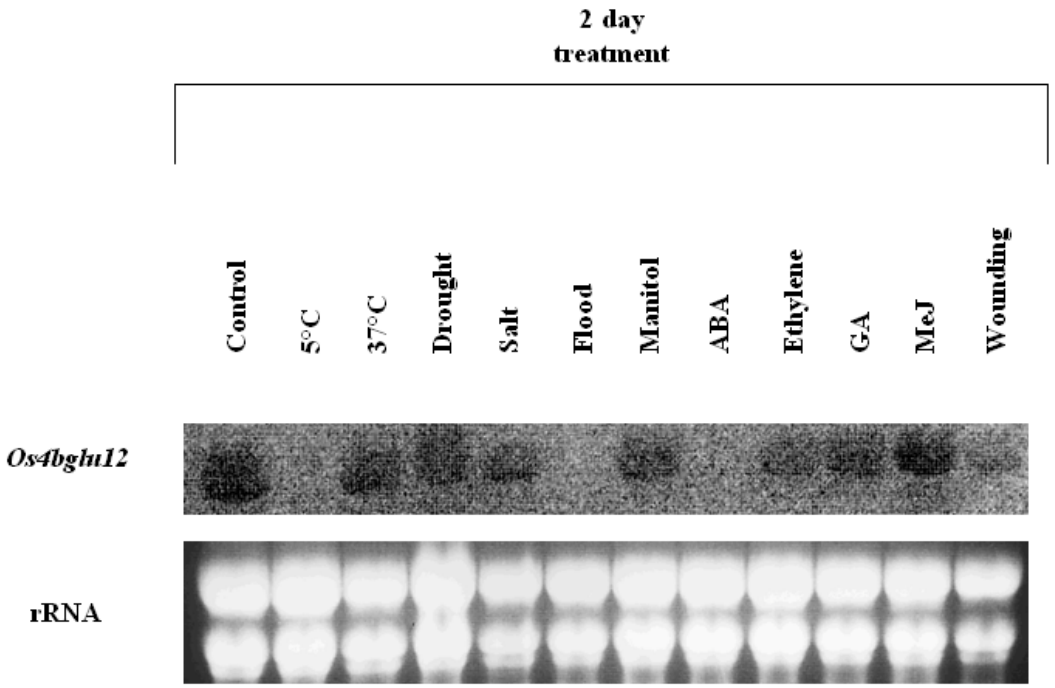



**Figure 3.7** Northern blot analysis of *Os4bglu12* expression in 10-day-old rice seedlings grown under with various abiotic stresses and plant hormones for an additional 2 days. Thirty micrograms of total RNA was loaded on a 1.2% agarose formaldehyde gel. The RNA blot was hybridized with the α-$^{32}$P-labeled 264 bp 3'end fragment of Os4bglu12 DNA probe. The ethidium bromide-stained gel below blots indicates the equal RNA loading before blotting. Control (grown at 28°C ), cold (grown at 5°C ), 37°C, drought (dehydrated until losing 50% of the fresh weight was lost), salt (irrigated with 0.3 M NaCl), flood (plant were submerged in distilled water), mannitol (irrigated with 0.5 M mannitol), wounding (aerial parts such as leaves and stems were crushed by plastic ruler at intervals of 1 cm). For plant hormone treatment, seedlings were both irrigated and sprayed with GA ($10^{-6}$ M gibberellic acid $A_3$), ABA ($10^{-3}$ M abscissic acid), ethylene ($10^{-6}$ M 2,4-dichlorophenoxyacetic acid), and MeJ ($10^{-6}$ M methyl jasmonate).

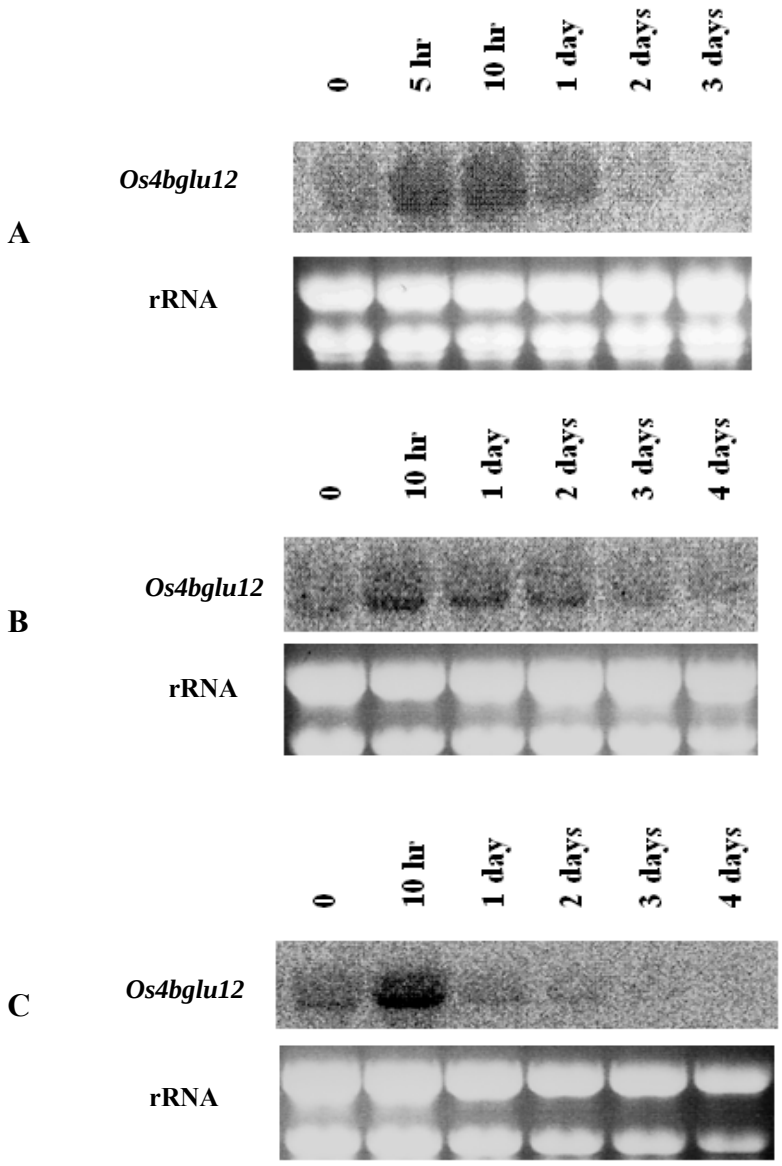



**Figure 3.8** RNA blot analysis of *Os4bglu12* expression in 10-day-old rice seedlings treated with A. wounding, B. methyl jasmonate and C. ethephon at different time-points. Thirty micrograms of total RNA were loaded on 1.2% agarose formaldehyde gel. RNA blot was hybridized with the α-$^{32}$P-labeled 264 bp 3'end fragment of Os4bglu12 DNA probe. The ethidium bromide-stained gel below blots showed the RNA loading before blotting.

## 3.3 Hydrolysis of rice cell wall by Os4bglu12 β-glucosidase

### 3.3.1 Hydrolysis of rice crude cell wall by recombinant rice Os4bglu12 β-glucosidase and endo-1,3;1,4-β-glucanase

In order to study the roles of Os4bglu12 in rice both under normal conditions and in response to stresses, a Trx-Os4bglu12 fusion protein expressed in *E.coli* (see section 3.1) was used to identify its natural substrates. It was previously found that this enzyme preferentially hydrolyzed β-(1,4)-linked oligosaccharides of 3-6 residues and laminaribiose (Opassiri et al., 2006). To demonstrate this more directly, the experiments were performed to assay the activitiy of Os4bglu12 on the hydrolysis of rice cell wall β-glucans. A fine powder of crude cell walls of rice seedlings were incubated with Os4bglu12 and aliquots were taken from the reaction mixture at indicated periods of time and analyzed by TLC (Figure 3.10). TLC analysis showed that Os4bglu12 could not hydrolyze rice cell wall polysaccharides directly, since almost no spots of mono- or oligosaccharides were detectable after incubation with Os4bglu12 (Figure 3.9A). It was previously found that the expression of rice endo-1,3;1,4-β-glucanase gene increased in rice seedling leaves in response to wounding, ethephon and methyl jasmonate (Takashi Akiyama, unpublished data). If Os4bglu12 and endo-1,3;1,4-β-glucanase can cooperate in cell wall hydrolysis, this might clarify the roles of both enzymes in response to these stresses. So, an experiment was set up to test if Os4bglu12 is able to hydrolyze oligosaccharides produced from the hydrolysis of rice cell walls by recombinant rice endo-1,3;1,4-β-glucanase. The hydrolysis of crude cell walls by a recombinant rice endo-1,3;1,4-β-glucanase resulted in final products composted of a three major oligosaccharides

(Figure 3.9B, lane 3). A mixture of rice endo-1,3;1,4-β-glucanase cell wall hydrolysis products was incubated with Os4bglu12 and aliquots were taken from the reaction mixture at different times and analyzed by TLC (Figure 3.10B). As seen on the TLC, Os4blu12 could hydrolyze one of the major oligosaccharide spots in hydrolysis products which migrates between spots of cellotetraose and cellotriose standards, more rapidly than other oligosaccharides. In addition, the glucose product released by Os4bglu12 trended to increase during incubation from 10 to 60 min (Figure 3.9B).

### 3.3.2 Substrate specificity of rice Os4bglu12 β-glucosidase toward purified rice cell wall oligosaccharides

As shown in the above results, the extensive hydrolysis of rice crude cell walls by a recombinant rice endo-1,3;1,4-β-glucanase generated three major rice cell wall hydrolysis products, which were denoted as Os1, Os2 and Os3, respectively (Figure 3.11 A-C). Os1 migrates between the spots of cellotetraose and cellotriose standards, Os2 migrates between the spots of cellotriose and cellobiose, and Os3 has $R_f$ value (0.53) equal to laminaribiose.

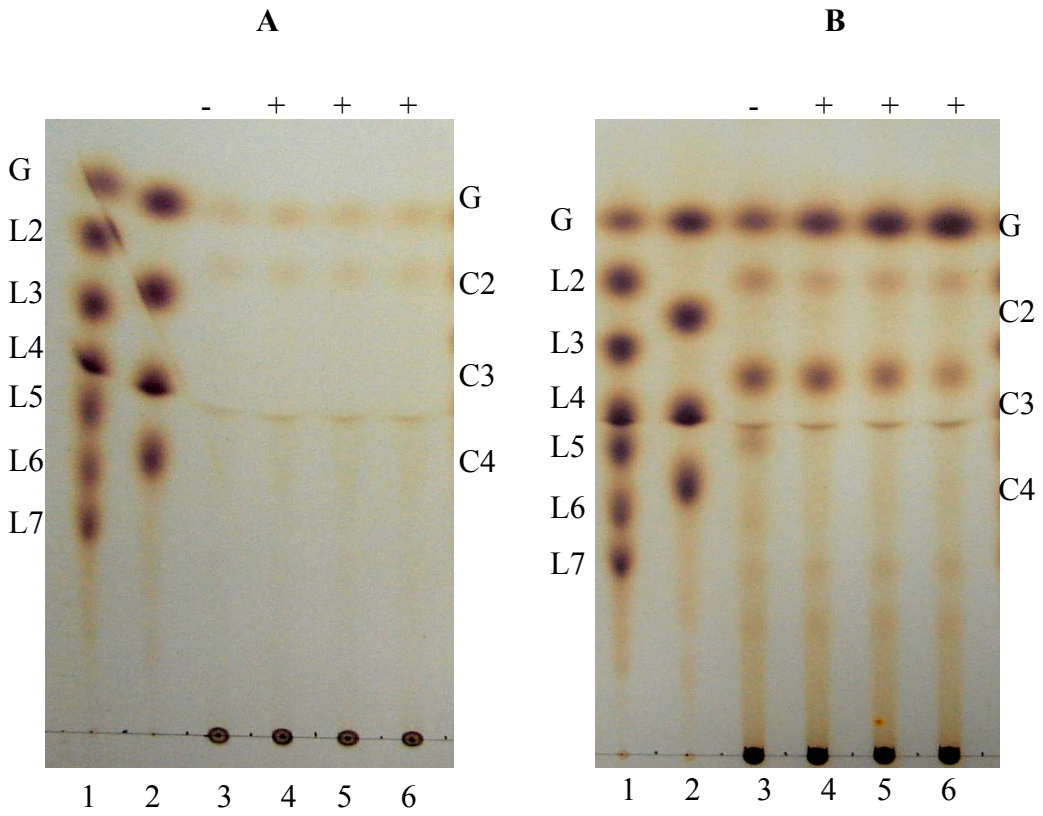



**Figure 3.9** TLC analysis of rice cell wall hydrolysis by rice endo-1,3;1,4-β-glucanase and Os4bglu12. **A.** Time course of reaction of crude cell wall incubated with Os4bglu12 and **B.** Time course of reaction by Os4bglu12 with which crude cell wall was preincubated with rice endo-1,3;1,4-β- glucanase. Lane 1, glucose (G) and laminari-oligosaccharide standard of degree of polymerization (DP) 2-7 (L2-L7); lane 2, cello-oligosaccharide standard of DP 2-4 (C2-C4), lane 3, rice cell wall extracts incubated without enzyme (-), lane 4-6, the samples incubated with Os4bglu12 (+) for 10, 30 and 60 min, respectively. The reaction mixtures composed of Os4bglu12 and crude cell walls in 50 mM sodium acetate buffer (pH5.0) were incubated at 37°C and the reactions were stopped by boiling. Separation of products released by the enzymes was carried out by TLC on a silica 60 plate using acetonitrile:water (3:1 by volume) as developing solvent and spots of sugars were visualized by heating at 150° C for 5 min after spraying with orcinol-sulfuric acid reagent.

In order to determine the substrate preference of Os4bglu12 against these three compounds. The products of cell wall hydrolysis by endo-1,3;1,4-β-glucanase were separated on TLC using acetonitrile:water (3:1 by volume) as developing solvent. Os1, Os2 and Os3 were purified by scratching out the silica layers containing each compound from the TLC and further purified by ion-exchage chromatography. Each product was indicated with Os4bglu12 and aliquots were sampled at indicated time intervals (10, 30 and 60 min) and analyzed by TLC (Figure 3.10A-C). TLC analysis of Os1 hydrolysis products indicated that Os4bglu12 hydrolyzed Os1 oligosaccharide more rapidly than Os2 and Os3 and Os1 was cleaved to give glucose, Os2 and Os3 as product (Figure 3.10A). TLC analysis of Os2 hydrolysis products revealed that Os4bglu12 hydrolyzed Os2 slowly to give Os3 and glucose as products (Figure 3.10B). Os4bglu12 hydrolyzed Os3 to give glucose as the end product. Based on these results, it was likely that Os4bglu12 hydrolyzed Os1 to release Os2 and glucose and then further hydrolyzed Os2 to generate Os3 and glucose (Figure 3.10C). Finally, the enzyme hydrolyzed Os3 to release glucose residues.

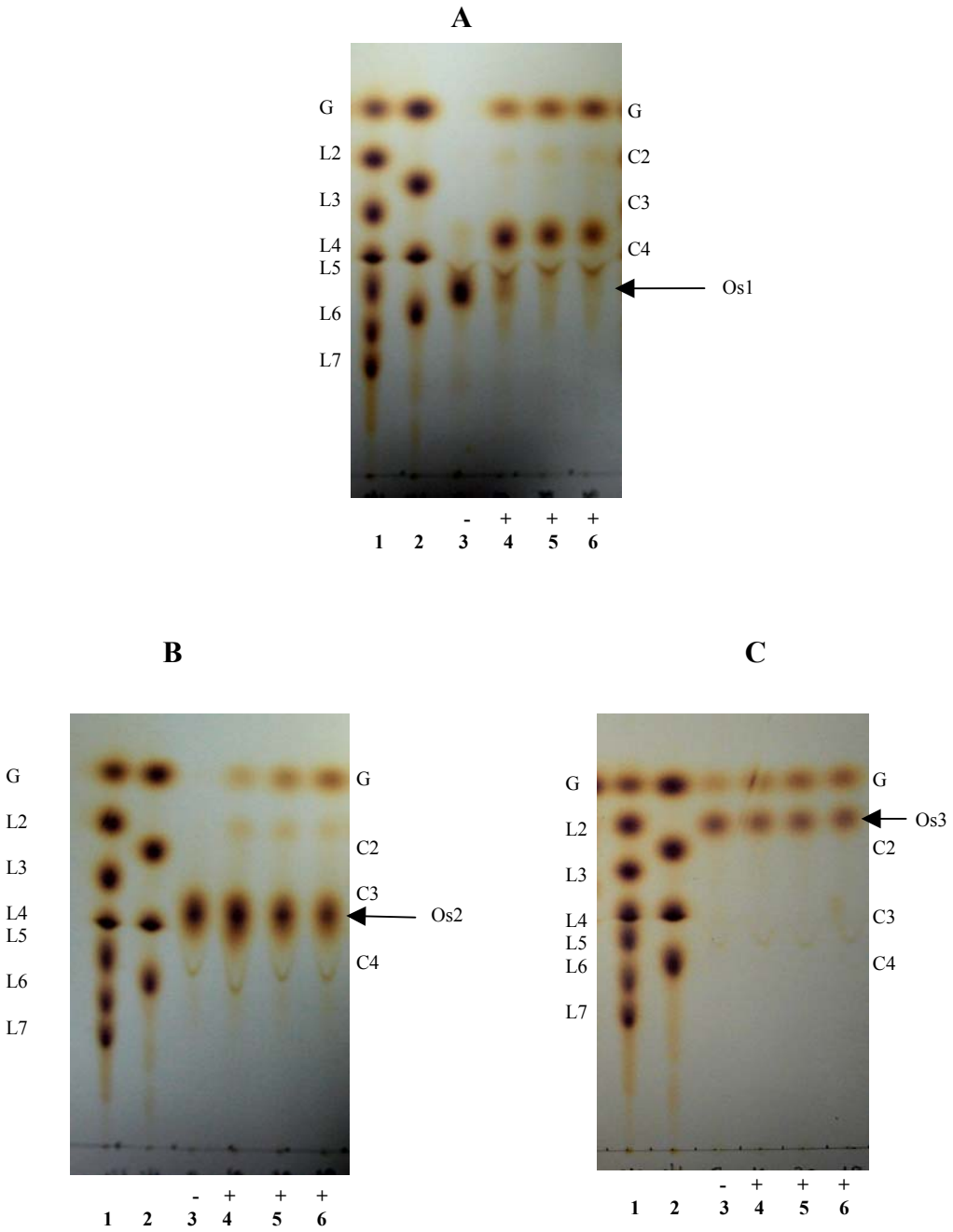



**Figure 3.10** Hydrolysis of rice endo-1,3;1,4-β-glucanase cell wall hydrolysis products by Os4bglu12 β-glucosidase. The cell wall hydrolysis products derived from the hydrolysis of rice cell wall by endo-1,3;1,4- β-glucanase were separated on TLC using acetinitrile:water (3:1 by volume) as developing solvent. Then, the Os1, Os2, and Os3 were purified by scratching out the compound from the silica layers, further

purified by ion-exchange chromatography and subjected to hydrolyzed by Os4bglu12. A. Os1, B. Os2 and C. Os3 incubated with Os4bglu12. Lane 1, glucose (G) and laminari-oligosaccharide standard of DP 2-7 (L2-L7); lane 2, cello-oligosaccharide standard of DP 2-4 (C2-C4); lane 3, control (-) without Os4bglu12, lane 4-6, the sample incubated with Os4bglu12 (+) in 10, 30 and 60 min, respectively. Reaction mixture composed of Os4bglu12 and crude cell walls in 50 mM sodium acetate buffer (pH5.0) were incubated at 37°C and the reactions were stopped by boiling. Separation of products released by the enzymes was carried out by TLC on a silica 60 plate using acetonitrile:water (3:1 by volume) as developing solvent and spots of sugars were visualized by heating at 150°C for 5 min after spraying with orcinol-sulfuric acid reagent.

## 3.4 Identification of natural glycoside substrates of Os4bglu12 β-glucosidase in rice

To identify other possible natural substrates of Os4bglu12, rice seedlings and tissues of rice plant at flowering stage were extracted with methanol, the extracts fractionated by LH-20 column chromatography and fractions tested for hydrolysis by recombinant Os4bglu12.

### 3.4.1 Identification of natural glycoside substrates of Os4bglu12 β-glucosidase from rice seedling roots.

The methanol crude extract of rice seedling roots was chromatographed on a Sephadex LH-20 column to separate compounds into different fractions by using methanol as eluent solvent. Many solvent systems were applied to separate the compounds in the fractions on TLC (Appendix B). The solvent system suited for separating the compounds from rice seedling roots was n-butanol:chloroform:methanol:water (3:8:7:2 by volume). Each fraction was chromatographed on the silica gel 60 $F_{254}$ TLC plate and the spot of compounds visualized under UV light. The identification of natural substrate of Os4bglu12 is based on an assumption that the spots of the fraction containing natural glycoside substrate after digesting with Os4bglu12 should disappear or be reduced as observed on the TLC. The occurence of differences in TLC spot patterns between the rice extracts incubated with and without Os4bglu12 revealed that, the LH-20 fractions 30 to 36 might contain the possible substrates of Os4bglu12 (Figure 3.11). The spots found in the reaction without enzyme disappeared in the reaction containing Os4bglu12. It was also found that new spots were present in the reaction containing Os4bglu12. The fractions 30-36 might contain the same compound because their spots have the same $R_f$ value as observed on TLC.

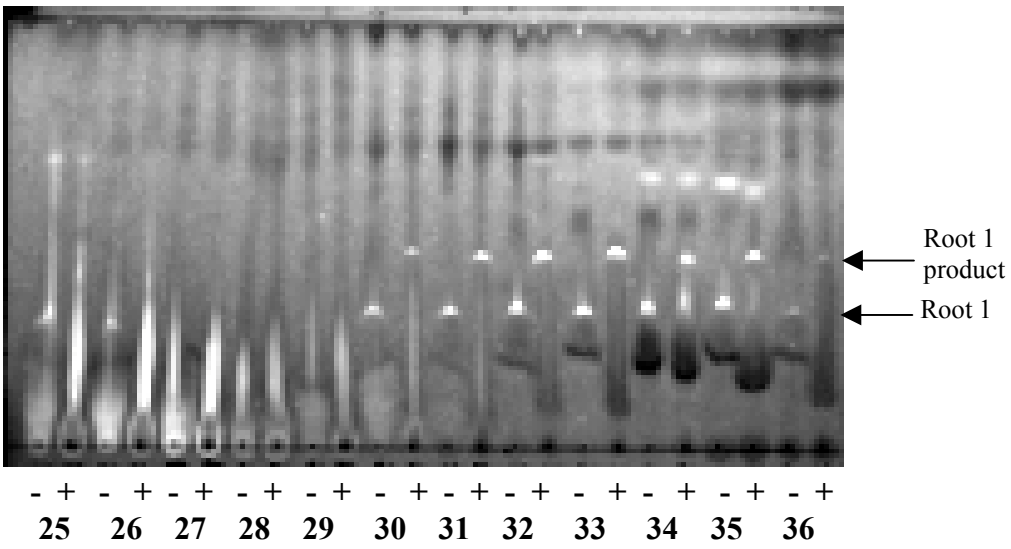



**Figure 3.11** TLC analysis of rice seedling root compounds separated by Sephadex LH-20 column chromatography. The compounds in each fraction were incubated with (+) and without (-) Os4bglu12. One hundred microliters reaction mixtures containing 10 μL of the Os4bglu12 (0.8 μg), a 10 μL aliquot of fraction in 50 mM sodium acetate buffer (pH5.0) was incubated at 37°C for 30 min. The compounds in the assay reactions were detected by TLC on a silica gel 60 $F_{254}$ plate using n-butanol:chloroform:methanol:water (3:8:7:10 by volume) as the developing solvent. The spots of compounds were visualized by exposing to UV light. The numbers under the TLC plate represent the fraction number. Root 1 presents the spot of the possible natural substrate of Os4bglu12, and Root 1 product presents the spot of possible product after digesting the root compound with Os4bglu12.

### 3.4.2 Identification of natural glycoside substrates of Os4bglu12 β-glucosidase from rice flower

The methanol crude extract from flower of rice at flowering stage was chromatographed on a Sephadex LH-20 column to separate compounds into different fractions by using methanol as the eluent. Each fraction were tested with Os4bglu12 and fraction components were detected by silica gel 60 $F_{254}$ TLC under UV light. The occurence of differences in TLC spot patterns between the rice extracts incubated with and without Os4bglu12 revealed that, the fractions 96-102 might contain the substrates of Os4bglu12 (Figure 3.12). The spots were found in the reaction without enzyme and disappeared in the reaction containing Os4bglu12. The solvent system suited for separating the compounds from flower parts of rice was n-butanol :methanol:acetic acid:water (15:1:2:2 by volume).

### 3.4.3 Identification of natural glycoside substrates of Os4bglu12 β-glucosidase from leaf/stem parts of rice at flowering stage

The methanol extract of leaf/stem parts of rice at flowering stage was purified by Sephadex LH-20 column to separate compounds into different fractions by using methanol as eluent solvent. Many solvent systems were applied to separate the compounds in leaf/stem parts of rice by TLC (Appendix B). As shown in Figure 3.13, the occurrence of differences in TLC spot patterns between the rice extracts treated with and without Os4bglu12 revealed that the pooled fractions 71 (68-71), 79 (72-79), 85 (80-85), 100 (86-100), 107 (101-107) and 114 (108-114) might contain natural substrates of Os4bglu12. The solvent system that was used to separate the compounds in these fractions is ethyl acetate:methanol:acetic acid:water (15:1:2:2 by

volume). It was observed that the migration of white spots in pooled fractions 71, 79, 85, 100, 107 was slightly different. Spots of Os4bglu12 hydrolysis products in the assay reaction of these fractions tended to shift down compared to the reaction without enzyme. These fractions might contain different compounds.

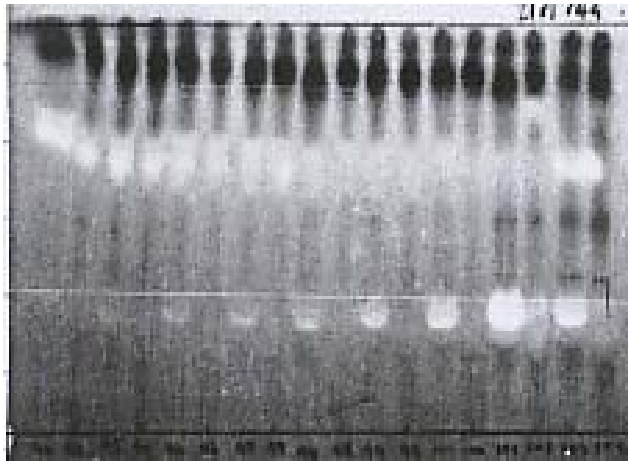



**Figure 3.12** TLC analysis of Sephadex LH-20 column chromatography fractions of rice flower extracts for compounds modified by Os4bglu12. The compounds in each fraction were incubated with (+) and without (-) Os4bglu12. One hundred microliters reaction mixtures containing 10 μL of Os4bglu12 (0.8 μg) and a 10 μL of an aliquot of fraction in 50 mM sodium acetate buffer (pH5.0) were incubated at 37°C for 30 min. The compounds in the assay reactions were detected by TLC on a silica gel 60 $F_{254}$ plate using n-butanol:methanol:acetic acid:water (15:1:2:2 by volume) as developing solvent. The spots of compounds were visualized by exposing to UV light. The numbers under the TLC plate represent the fraction numbers.

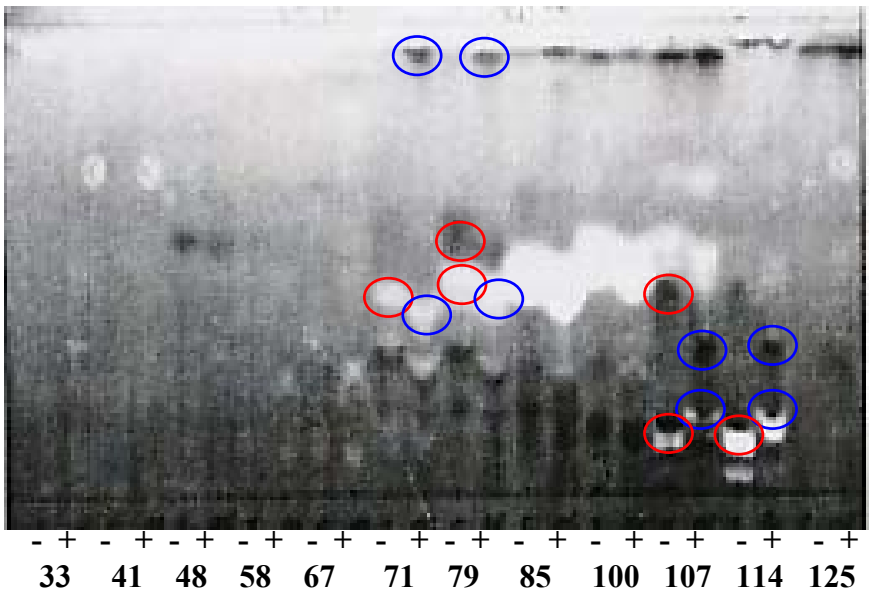



**Figure 3.13** TLC analysis of the compounds in leaf/stem of rice at fowering stage. The compounds in rice tissues were fractionated by Sephadex LH-20 chromatography. Each fraction was incubated with (+) and without (-) Os4bglu12. One hundred microliters reaction mixtures containing 10 µL of the Os4bglu12 (0.8 µg) and a 10 µL of an aliquot of compound in 50 mM sodium acetate buffer (pH 5.0) were incubated at 37°C for 30 min. The compounds in assay reactions were detected by TLC on a silica 60 $F_{254}$ plate using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) as developing solvent. The spots of compounds were visualized by exposing to UV light. The number under the TLC plate represent the number of pooled fractions.

## 3.5 Purification of natural glycoside substrates of Os4bglu12 β-glucosidase from leaf/stem parts of rice at flowering stage

### 3.5.1 Purification of natural glycoside substrates of Os4bglu12 β-glucosidase from leaf/stem of rice at flowering stage by LH-20 chromatography, preparative TLC and si-60 chromatography

In order to determine the mass and structure of these compounds, the large batch purification of compounds was carried out. This study was focused on only pooled fractions 71 and 79. Rice crude extract (0.8 g) was loaded on to LH-20 column and fractionated using methanol as eluent. The pooled fractions 71 and 79 included in 12 fractions (from a total 125 fractions) in each round. The above step was repeated for an additional 10 times to obtained enough of the compounds for mass and structural analysis. The compounds in pooled fractions 71 and 79 were separated by preparative TLC (Silica gel 60 $F_{254}$) using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) as the solvent. It was seen that there were many bands on the TLC plate visualized under UV light (Figure 3.14). So, each silica layer band was scratched off the TLC plates and extracted in methanol. The supernate containing compounds was dried and part was incubated with Os4bglu12. Ten microliters of the enzyme (~0.8 μg) was incubated with 10 μL of purified compounds in 80 μL of 50 mM sodium acetate buffer (pH 5.0) at 37°C for 30 min. Os4bglu12 could hydrolyze the compounds separated from layers 1, 2, 3 and 4, as observed by the disappearance of the spots of these compounds after digesting with Os4bglu12 on TLC (Figure 3.15). Only the dark spot compounds found in layer 1 was further separated by Si-60 chromatography using ethyl acetate:acetonitrile:methanol:water

mixture as eluent solvent by varying the volume ratio of each solvent from low to high polarity. Si-60 chromatography could separate the dark spot compounds (called Cl/sD) from the white fluorescent spot compounds (called Cl/sW) as observed by TLC (Figure 3.16).

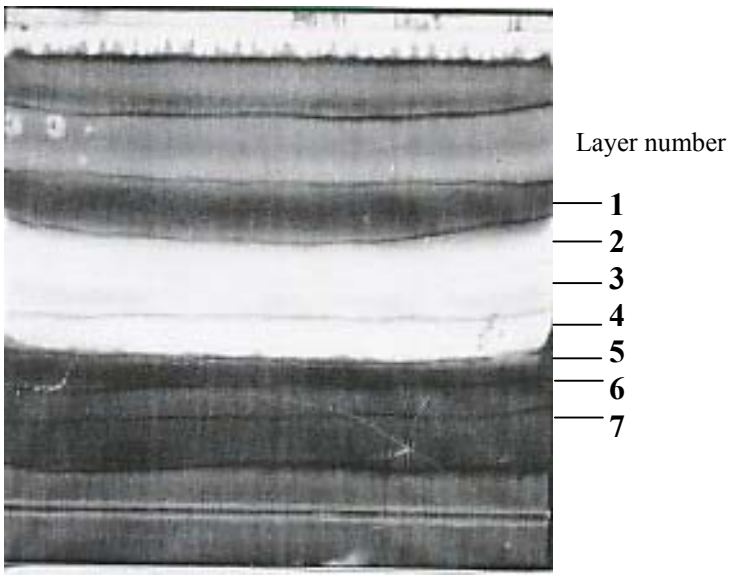



**Figure 3.14** Separation of compounds in pooled fractions 71 and 79 by preparative TLC (Silica gel 60 $F_{254}$). Ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) was used as the developing solvent.

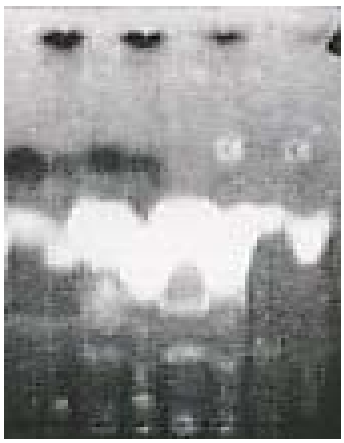



**Figure 3.15** Hydrolysis of compounds in layer 1 to 4 separated from preparative TLC by Os4bglu12. The compounds separated from each TLC layer were incubated with (+) and without (-) Os4bglu12. The number under the TLC plate represent layer number. One hundred microliters reaction mixture containing 10 μL of the Os4bglu12 (0.8 μg), 10 μL of an aliquot of a band extract in 50 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The compounds in assay reactions were detected by TLC on a silica 60 $F_{254}$ plate using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by volume) as developing solvent. The spots of compounds were visualized by exposing to UV light.

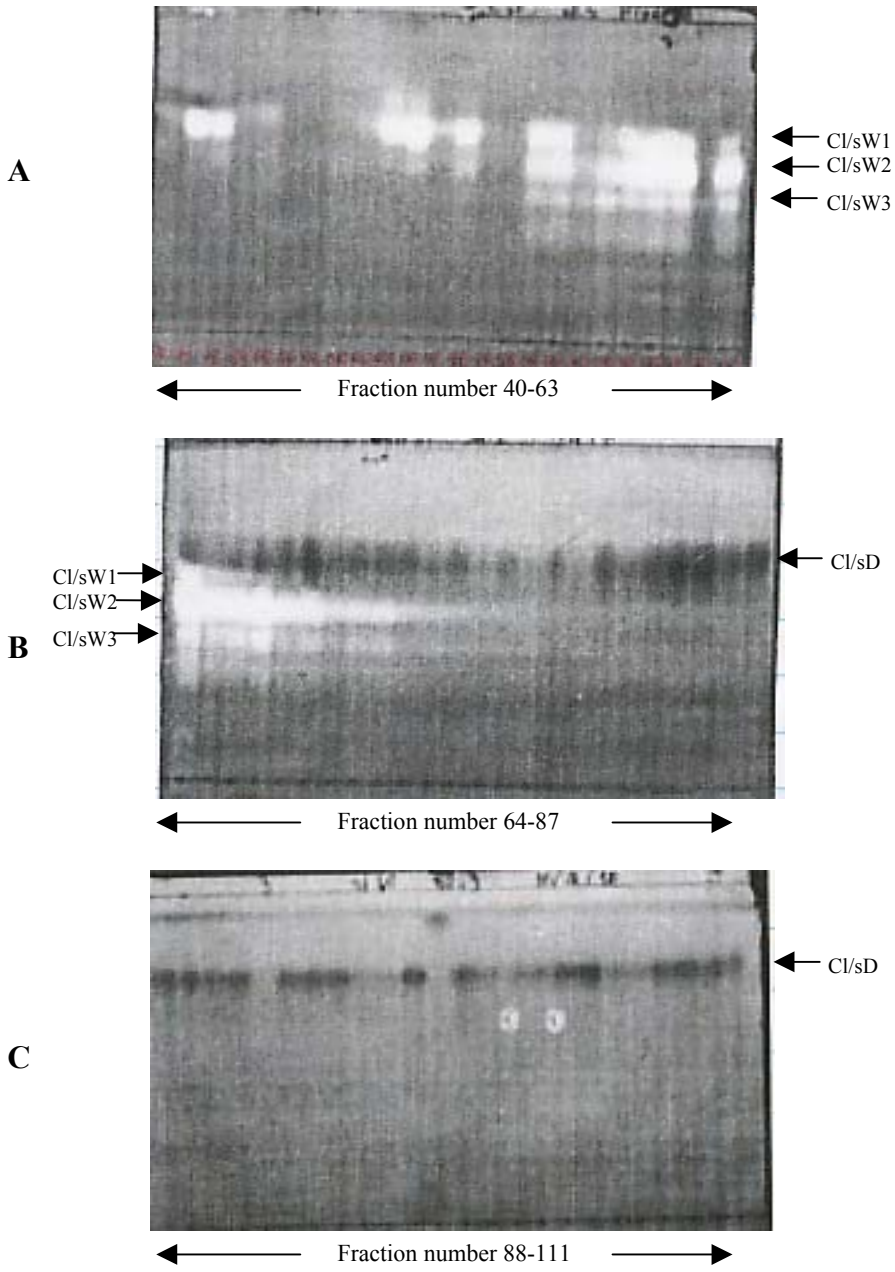



**Figure 3.16** Purification of compounds in layer 1 that were previously separated by preparative TLC using Si-60 chromatography. The compounds in each fraction were detected on TLC using ethyl acetate:methanol:acetic acid:water (15:1:2:2 by vlolume) as developing solvent and the spots of compounds detected by UV light. The numbers under the TLC plate represent fraction numbers. Cl/sD marks dark spot compounds, Cl/sW marks white fluorescent spot compounds.

### 3.5.2 Purification of natural glycoside substrates of Os4bglu12 β-glucosidase by HPLC

To obtain the natural glycoside substrates of Os4bglu12 that have purity high enough for determining its mass and structure, the dark spot compounds, Cl/sD, were further separated by HPLC with a Mightysil ODS reverse phase ($C_{18}$) column (46 x 150 mm). The elution solvent system applied for separating the compounds in the Cl/sD fraction was 40% (v/v) methanol in water, which was used in isocratic elution. Cl/sD could be separated into at least three peaks at retention times 7.60, 9.15 and 11.93 min and the name of these peaks were denoted as Cl/sD1, Cl/sD2 and Cl/sD3, respectively (Figure 3.17). The UV wavelengths at which these compound could be detected by absorbence were 254 and 330 nm. This suggests that Cl/sD contains at least three different components. On the other hand, Cl/sW was eluted as a broad peak, suggesting that Cl/sW may consist of multiple components or the elution condition was not appropriate (Figure 3.18). Therefore, only purified Cl/sD fractions were taken to determine mass their and structure by ESI-TOF-MS, MALDI-TOF-MS and $^{1}$H-NMR.

In summary, the starting material needed to separate enough of the Cl/sD compounds for mass and $^{1}$H-NMR analysis was 1000 g fresh weight of leaf/stem tissues, which corresponded to approximately 8 g of crude extract. After purifying the compounds by LH-20 chromatography only 0.12 g of pooled fractions 71 and 79 was obtained. After separating the compound in these fractions by preparative TLC, only 18.4 mg of compounds in layer 1 could be obtained. Approximately eighteen milligram of compounds separated from layer 1 were purified by Si-60 chromatography, and approximately 1.3 mg of Cl/sD and 0.7 mg of Cl/sW could be

obtained. Only Cl/sD was further purified by HPLC ($C_{18}$ reverse phase column) and approximately 0.3 mg of Cl/sD1, 0.2 mg of Cl/sD2 and 0.4 mg of Cl/sD3 could be collected. Approximately 0.2-0.3 mg of each compounds was used in the mass analysis and NMR analysis.

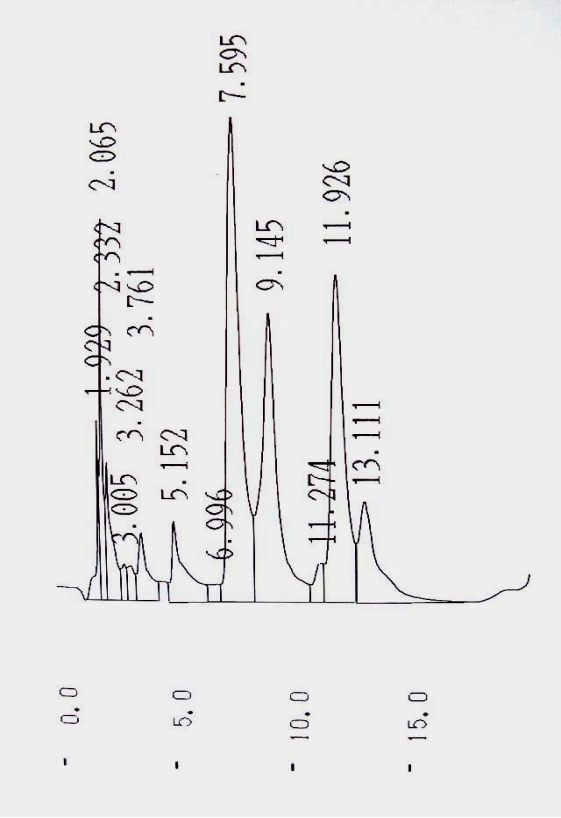



**Figure 3.17** HPLC chromatogram of Cl/sD separated on a $C_{18}$ reverse phase column, visualized at 330 nm. The isocratic solvent elution system used to separate the compounds in Cl/sD was 40% (v/v) methanol in water. The purified compounds were collected from 3 peaks at retention times 7.60, 9.15 and 11.93 min, which were denoted as Cl/s1D1, Cl/s1D2 and Cl/s1D3, respectively.

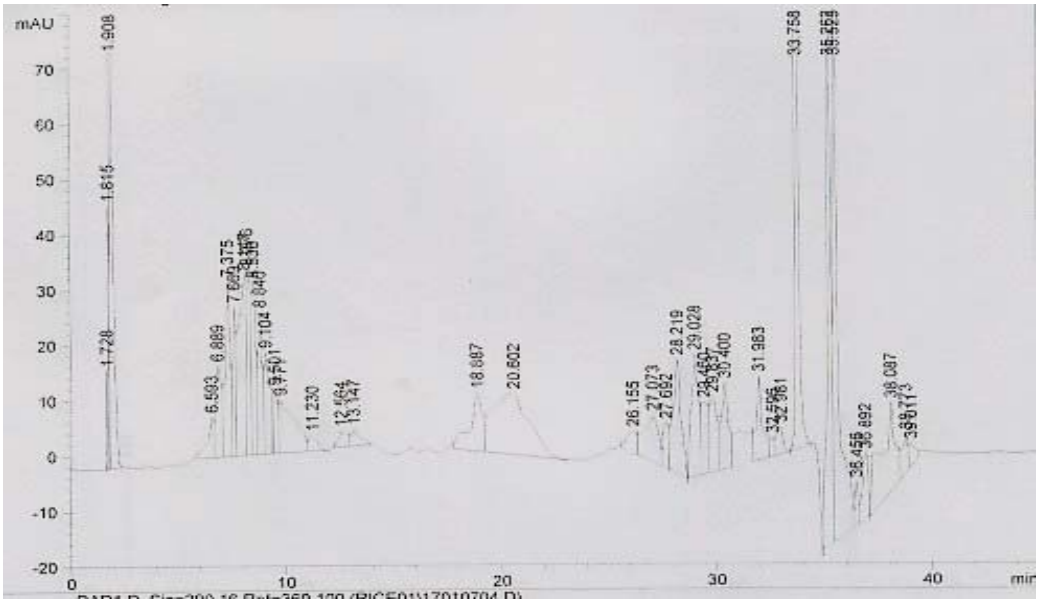



**Figure 3.18** HPLC chromatogram of Cl/sW purified by C18 reverse phase column, visualized at 330 nm. The solvent system used to separate the compounds was the step gradient of acetonitrile:water; 0 to 3min (0:100), 5 min (50:50), 7 min (80:20), 13 min (85:15), 17 min (87:13), 21min (90:10), 25 min (95:5), 28 to 30 min (100:0).

## 3.6 Hydrolysis of natural glycoside substrates by Os4bglu12 β-glucosidase

### 3.6.1 Analysis of the glycone portions of Cl/sD and Cl/sW

To determine the sugar moiety of natural substrates of Os4bglu12, Cl/sD and CL/sW were incubated with Os4bglu12 and sugar spots were analyzed by TLC. TLC analysis showed that sugar spot released from Cl/sW by Os4bglu12 migrated slightly faster than that of L-fucose, and apparently much faster than that of D-glucose (Figure 3.19).

Analysis of sugar moieties of Cl/sD1, Cl/sD2 and Cl/sD3 on the TLC revealed that the spots of sugar released from these compounds by Os4bglu12 had a similar mobility as that of D-glucose (Figure 3.20).

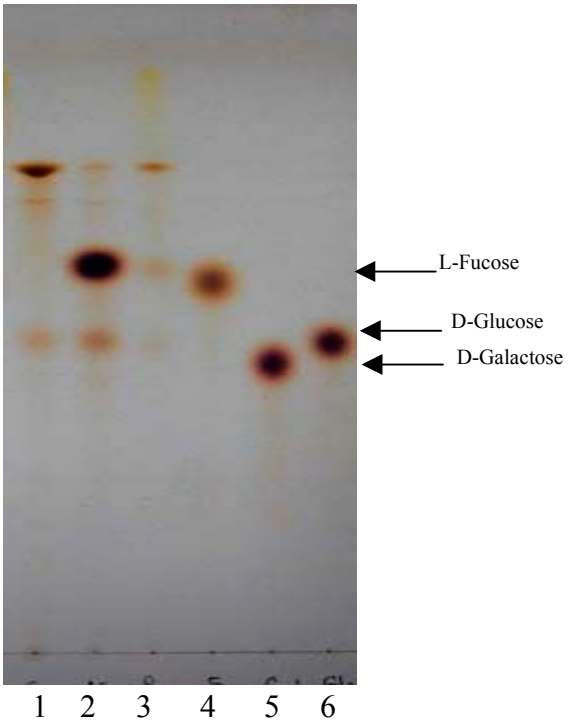



**Figure 3.19** Hydrolysis of Cl/sW glycoside by Os4bglu12 detected by TLC. Lane 1 Cl/sW glycoside; lane 2, Cl/sW glycoside incubated with Os4bglu12; lane 3, precipitates of Cl/sW products after digesting with Os4bglu12; lane 4 fucose; lane 5, glucose; lane 6, galactose standard. The reaction containing 10 µL of the Os4bglu12 (0.8 µg) and 10 µL of an aliquot of compound in 50 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The products released by the enzyme were separated on TLC using acetonitrile:water (3:1 by volume) as solvent system and spots were visualized by staining with orcinol-sulfuric acid reagent and heating at 150°C for 5 min.

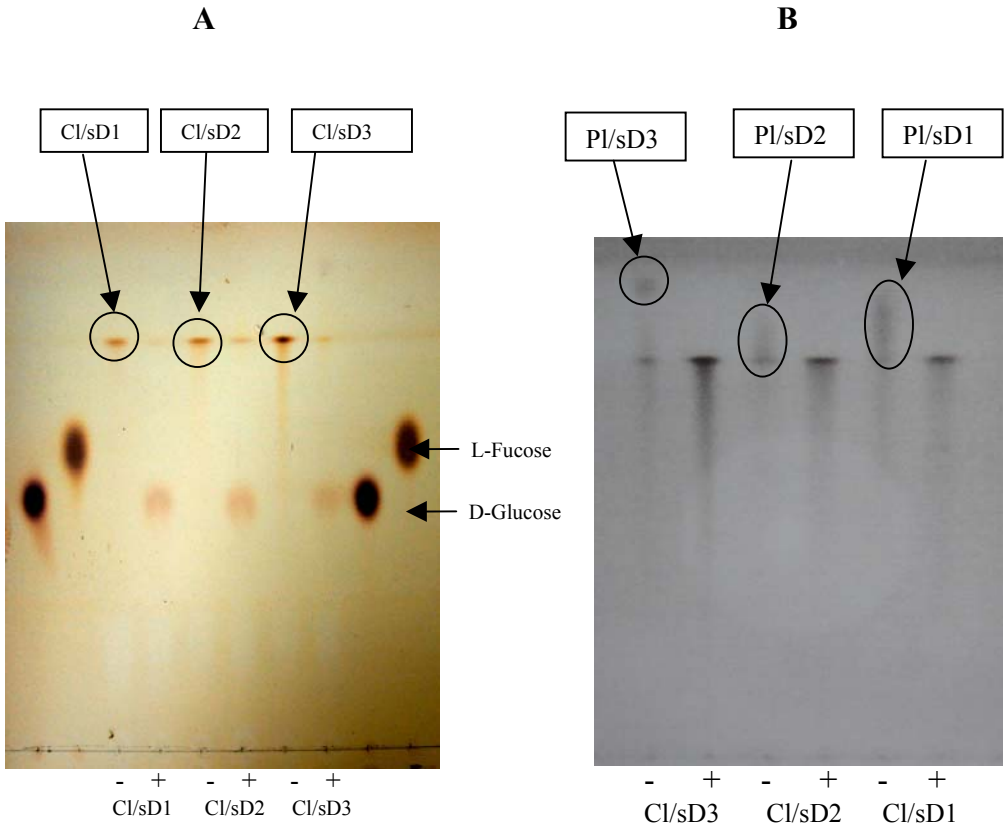



**Figure 3.20** Hydrolysis of Cl/sD1, Cl/sD2 and Cl/sD3 glycosides by Os4bglu12. The compounds separated by HPLC were incubated with (+) and without (-) Os4bglu12. One hundred microliters reaction mixture containing 10 µL of the Os4bglu12 (0.8 µg) and 10 µL of compound in 50 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The presence of compounds in assay reactions were detected by TLC using acetonitrile:water (3:1 by volume) as developing solvent. The spots on A. were visualized by heating at 150°C for 5 min after staining with orcinol-sulfuric acid reagent and B. were detected with UV light. The products released from the hydrolysis of Cl/sD1, Cl/sD2 and Cl/sD3 by Os4bglu12 were denoted as Pl/sD1, Pl/sD2, and Pl/sD3, respectively.

### 3.6.2 Analysis of aglycone portions of Cl/sD glycosides

To analyze the aglycone portions of Cl/sD glycosides after digesting with Os4bglu12, the Cl/sD1, Cl/sD2, and Cl/sD3 glycosides were incubated with (+) and without (-) Os4bglu12 and analyzed on TLC. TLC analysis showed the disappearance of the spots of Cl/sD1, Cl/sD2, and Cl/sD3 in the reactions containing Os4bglu12. There are three new spots which are the hydrolysis products of Cl/sD1, Cl/sD2, and Cl/sD3 released by the enzymes called, Pl/sD1, Pl/sD2, and Pl/sD3, respectively, as observed on the TLC (Figure 3.20B).

The assay reactions containing Os4bglu12 were separated by HPLC with a Mightysil ODS reverse phase (C18) column (46 x 150 mm). The solvent system used to separate the compounds in each assay reaction was 55% (v/v) methanol in water isocratic elution. It was found that the retention time of Pl/sD1 and Pl/sD2 was observed at 7.33 and 6.29 min, respectively (Figure 3.21 A and B). There were 2 peaks of products released from Cl/sD3 by Os4bglu12 as shown on HPLC chromatogram at the retention time 5.11 and 7.12 min (Figure 3.21 C).

It is interesting to note that significant amount of precipitates was detected in hydrolysis products of Cl/sD after digesting with Os4bglu12. The precipitates is soluble in methanol but not much soluble in distilled water. A spot of precipitates migrated much faster than that of an intact Cl/sD without digestion with Os4bglu12 and the spot was weakly stained with an orcinol-sulfuric acid reagent which was used to visualize spots of sugar (Figure 3.22). These data indicate that the precipitated materials are likely to be an aglycone of Cl/sD which was released from Cl/sD glycoside by hydrolysis with Os4bglu12.

On the other hand, hydrolysis products of Cl/sW with Os4bglu12 showed much less precipitates than that of Cl/sD. This indicates that the aglycone of Cl/sW seems to be more hydrophilic than that of Cl/sD. Another interesting point to be noted is that the sugar released by Os4bglu12 from Cl/sD before separating by HPLC had similar mobility to L-Fucose (Figure 3.22).

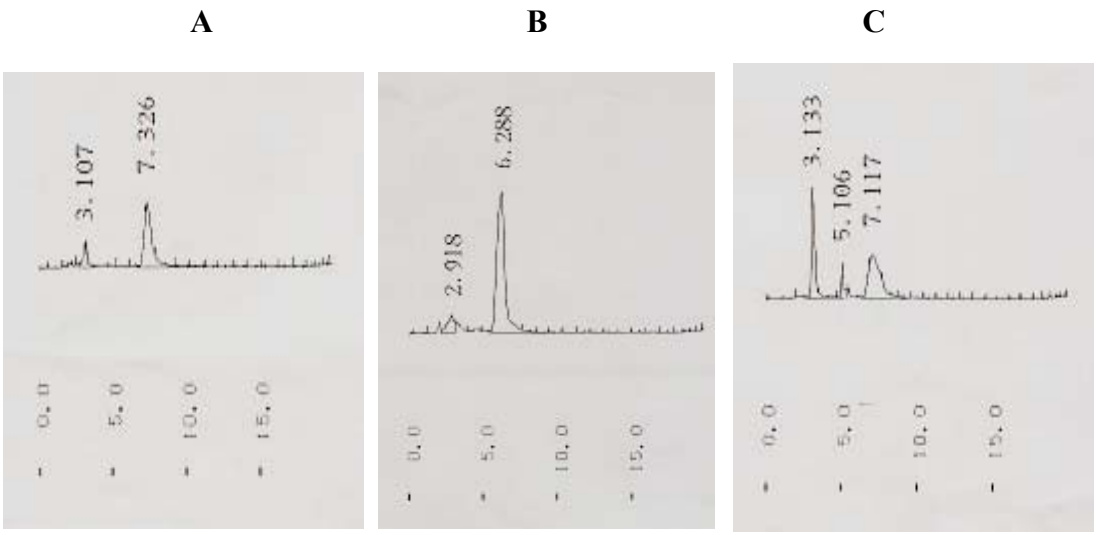



**Figure 3.21** HPLC elution profiles of the hydrolysis products released from A. Cl/sD1, B. Cl/sD2, and C. Cl/sD3 by Os4bglu12. The reaction containing 10 µL of the Os4bglu12 (0.8 µg) and 10 µL of compound in 50 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The compounds in assay reactions were separated by $C_{18}$ reverse phase chomatography using 55% methanol in water as an isocratic elution solvent.

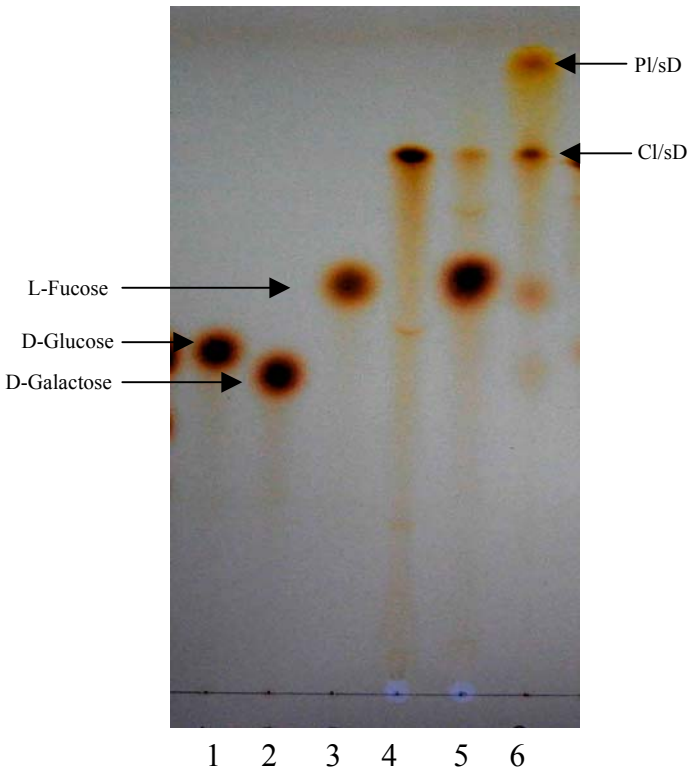



**Figure 3.22** Hydrolysis of Cl/sD glycoside by Os4bglu12 detected by TLC. The reaction containing 10 µL of the Os4bglu12 (0.8 µg) and 10 µL of compound in 50 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The products released by the enzyme were separated on TLC using acetonitrile:water (3:1 by volume) as the solvent system and spots were visualized by staining with orcinol-sulfuric acid reagent and heating at 150°C for 5 min. Lane 1, glucose; lane 2, galactose; lane 3, fucose; lane 4, Cl/sD glycoside; lane 5, Cl/sD glycoside incubated with Os4bglu12; lane 6, precipitates of Cl/sD products after digesting with Os4bglu12. Pl/sD represents the product released from Cl/sD by the enzyme.

## 3.7 Mass and structural analysis of Cl/sD compounds.

Mass and structures of the Cl/sD1, Cl/sD2 and Cl/sD3 compounds were analyzed by ESI-TOF-MS, MALDI-TOF-MS and $^{1}$H-NMR.

### 3.7.1 Cl/sD1 compound

In mass spectral analysis, the ESI-TOF-MS spectrum of Cl/sD1 exhibited positive ions at m/z 493 ($[M+H]^+$), 515 ($[M+Na]^+$), 1007 ($[2M+Na]^+$) (Figure 3.23); and a negative ion at m/z 491 ($[M-H]^-$) (Figure 3.24). The MALDI-TOF-MS spectrum of Cl/sD1 found an ion at 493 ($[M+H]^+$) (Figure 3.25). Based on these results, a molecular weight of a Cl/sD1 compound was estimated to be 492 corresponding to molecular formula $C_{23}H_{24}O_{12}$.

In NMR analysis, only $^{1}$H-NMR of Cl/sD1 could be analyzed because of a limited amount of purified compounds obtained. The $^{1}$H-NMR spectrum of Cl/sD1 was shown in Figure 3.26. The signals at lower magnetic field, 6.4-8.6 ppm, were observed in the spectrum of Cl/sD1 and these signals indicated an aromatic group. The signal of an anomer proton was visualized around 5.07 ppm. Therefore, it could suggest that Cl/sD1 is likely a glycoside because it contains one sugar group and an aglycone part that contains an aromatic group. In order to search the known compounds that probably have the same molecular weight and aromatic group like Cl/sD1 glycoside, the molecular formula $C_{23}H_{24}O_{12}$ was searched against SciFinder Scholar (American Chemical Society). The formula matched 104 structures. Among these compound structures, tricin-O-β-D-glucosides were previously reported to be presented in rice (Adjei-Afriyie et al., 2000; Chung et al., 2005; Cai et al., 2005) (Figure 3.27).

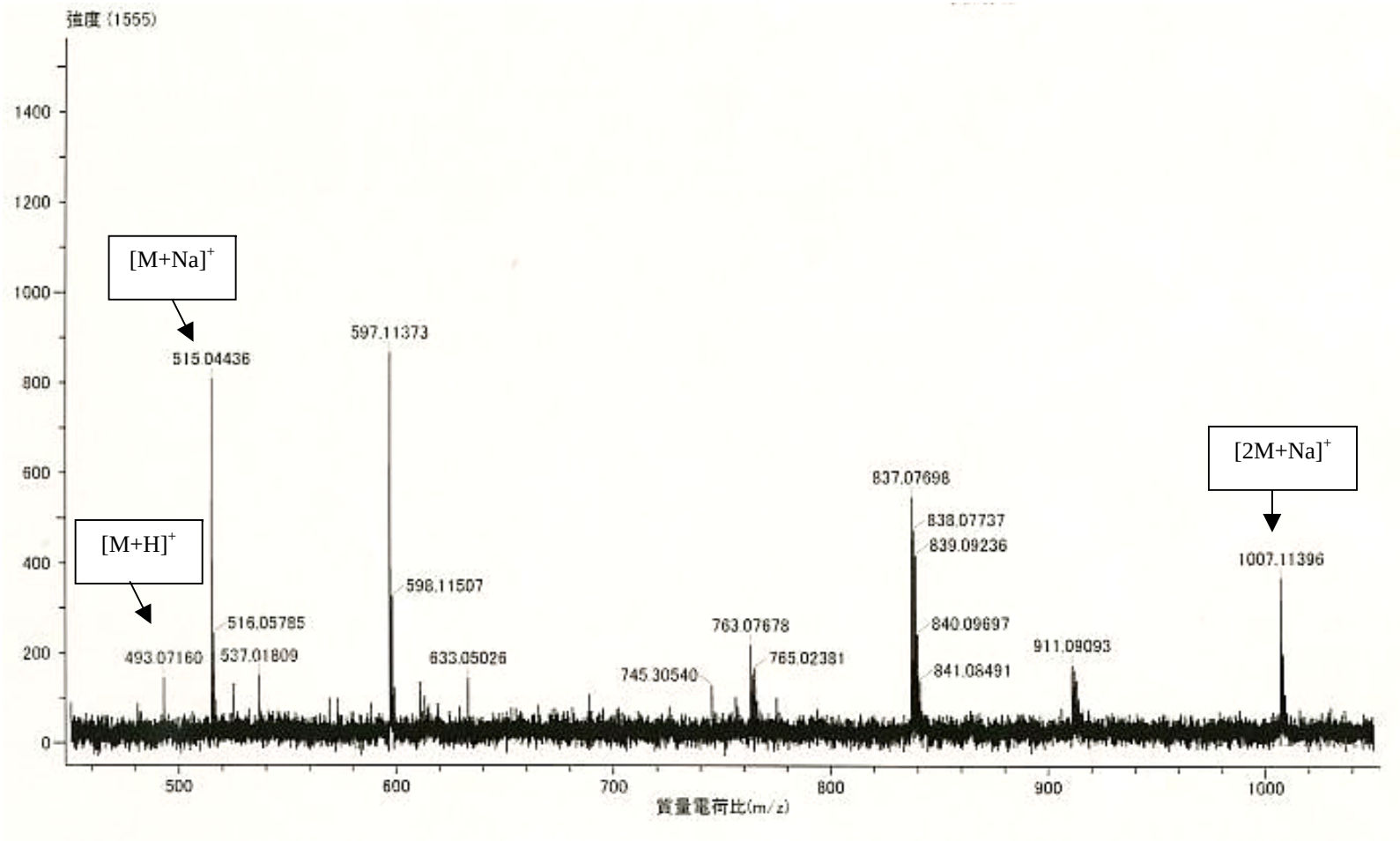



**Figure 3.23** ESI-TOF-MS mass spectrum of the Cl/sD1 compound**.**

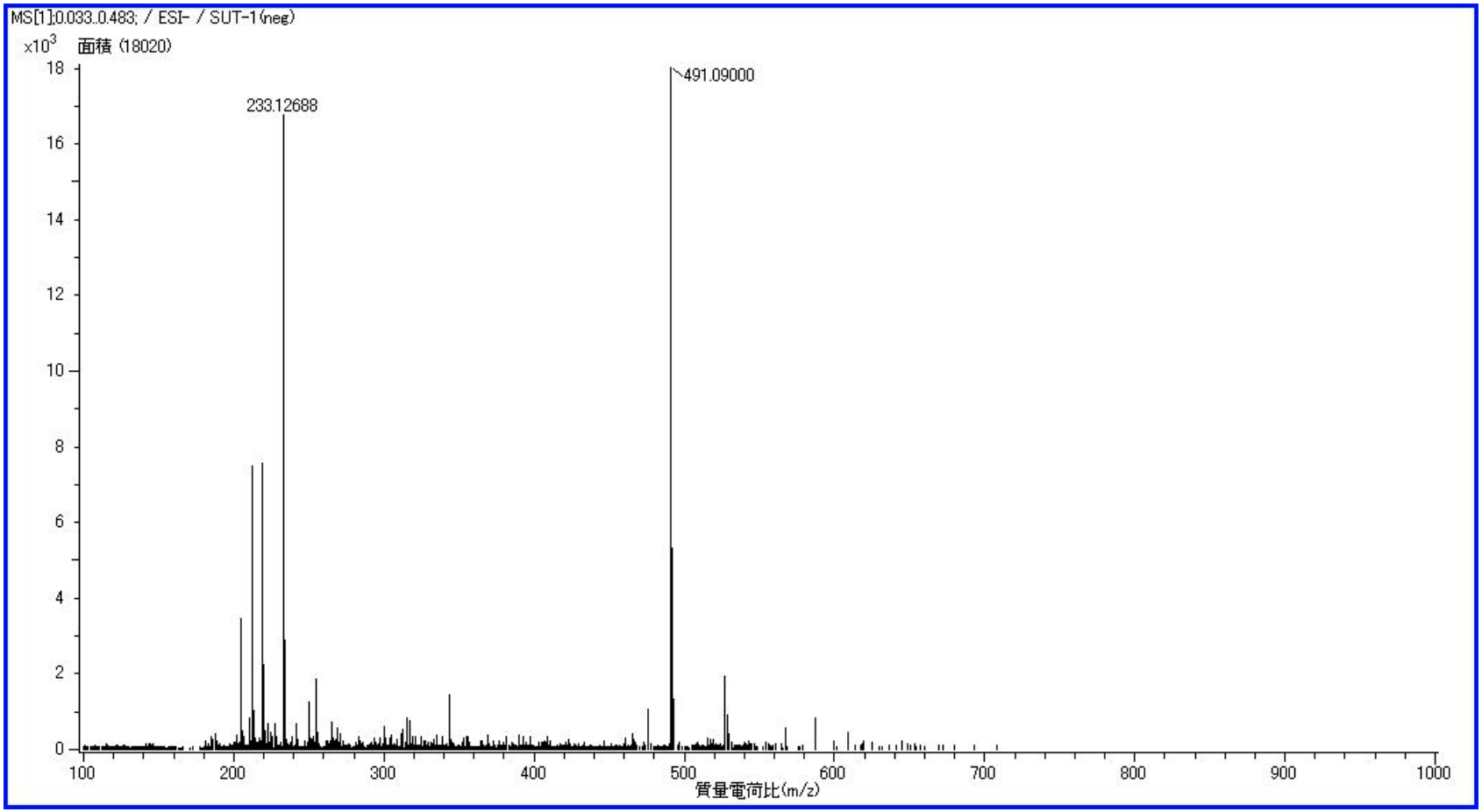



**Figure 3.24** ESI-TOF-MS mass spectrum of the Cl/sD1 compound in negative mode**.**

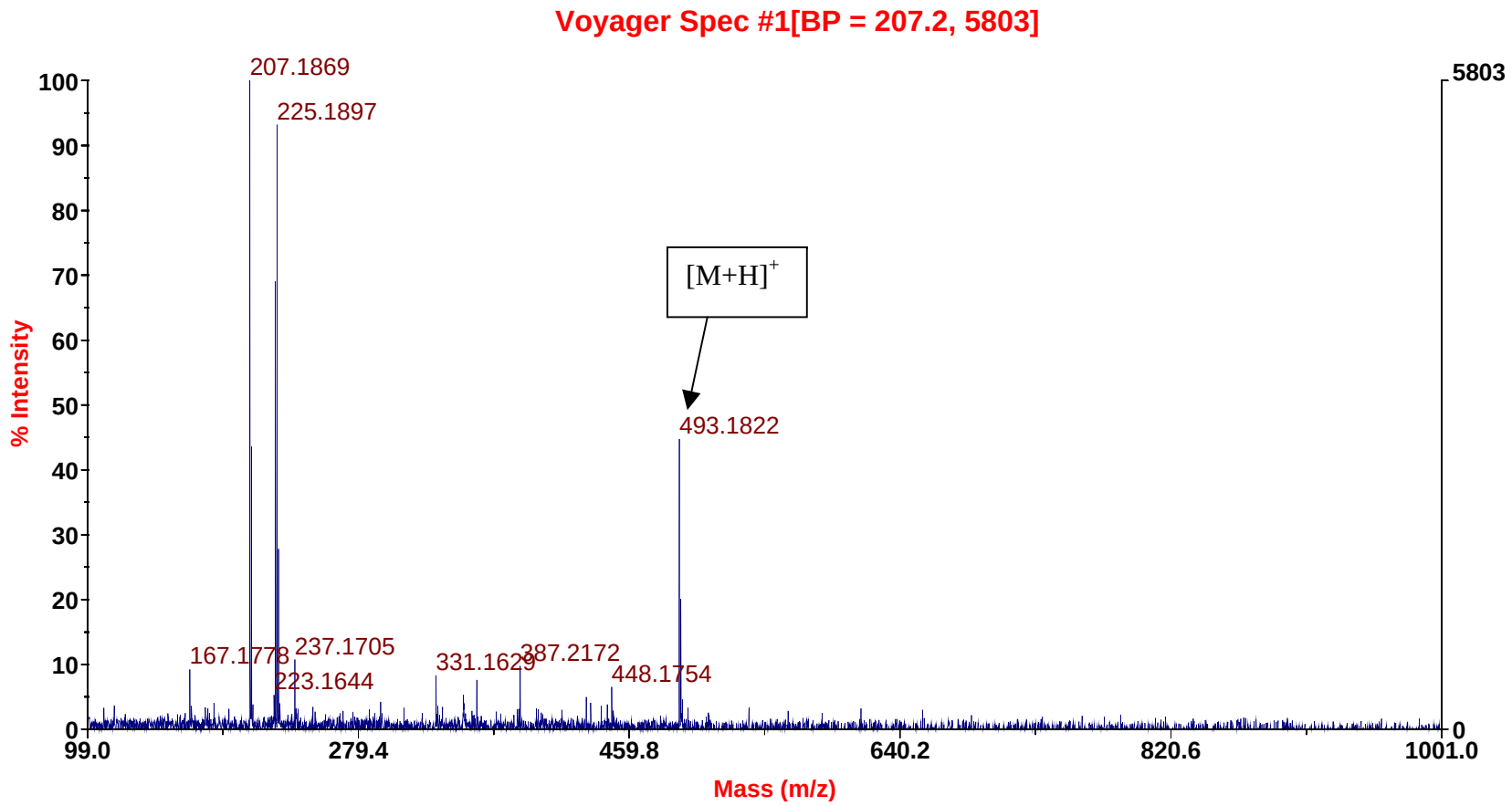



**Figure 3.25** MALDI-TOF-MS mass spectrum of the Cl/sD1 compound in positive mode**.**

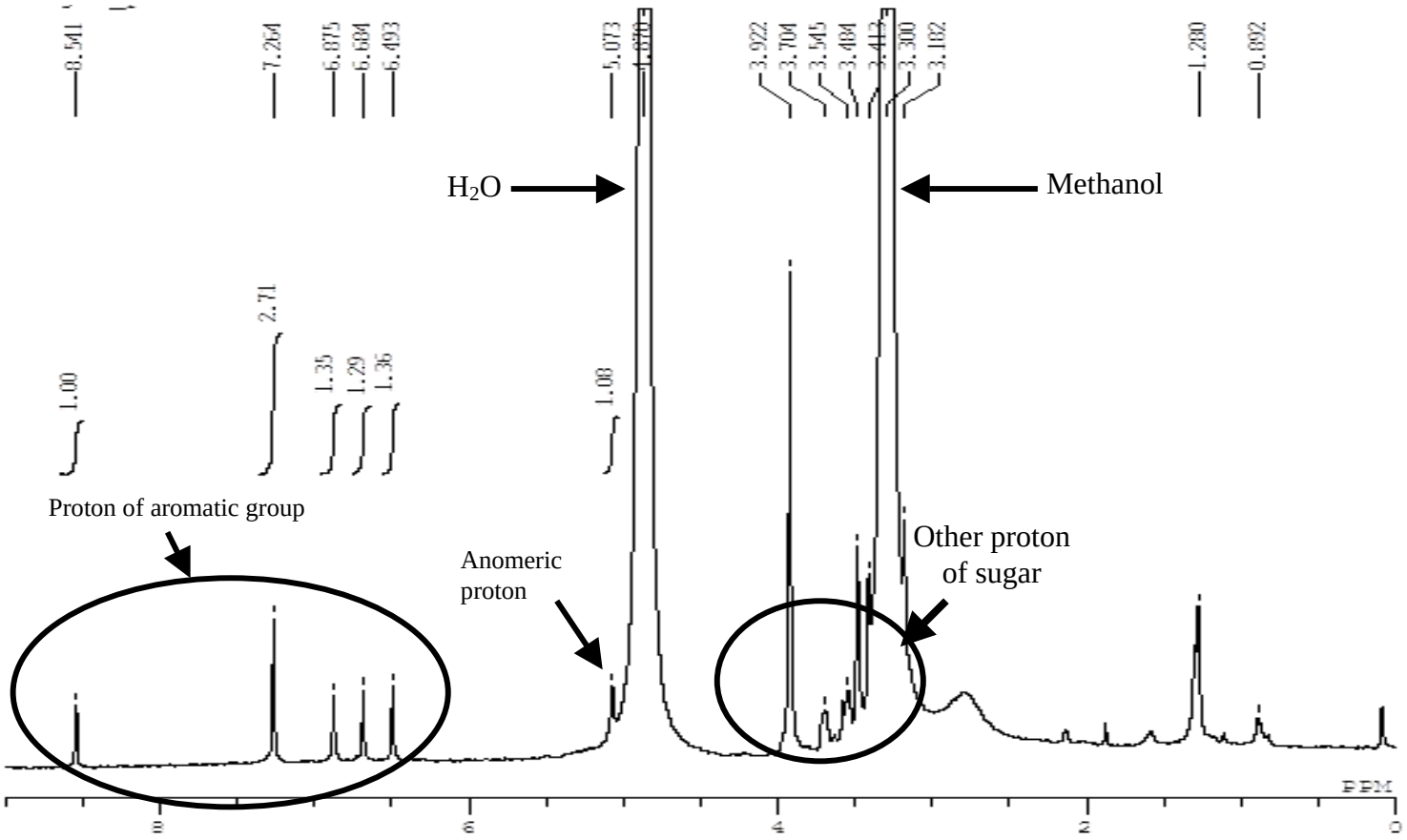
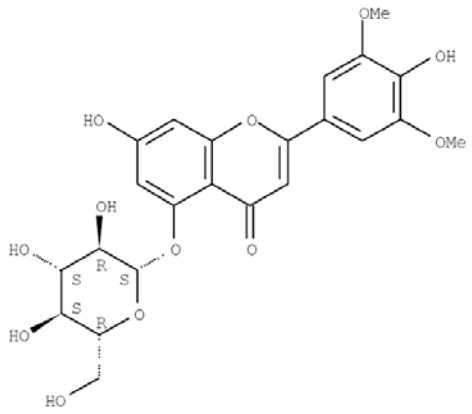
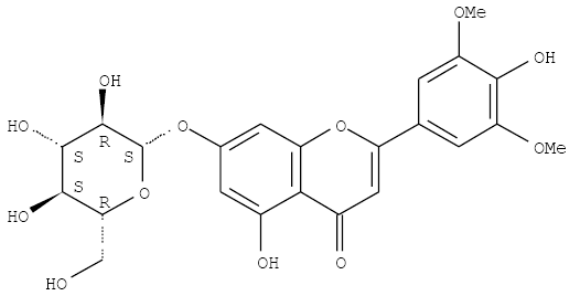



**Figure 3.26** $^{1}$H-NMR spectrum of the Cl/sD1 compounds.

**A**



**B**



**C**

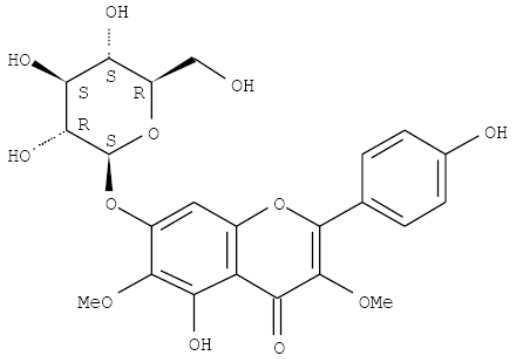


**Figure 3.27** The structure of A. Tricin 5-O-β-D-glucoside**,** B. Tricin-7-β-D-glucopyranoside**,** C. Trihydroxy-3,6-dimethoxyflavone 7-O-β-D-glucoside**.**

### 3.7.2 Cl/sD2 and Cl/sD3 compounds

Both ESI-TOF-MS and MALDI-TOF-MS spectra of Cl/sD2 exhibited the same positive ions found at m/z 679 ($[M+H]^+$) and 701 ($[M+Na]^+$) (Figure 3.28 and Figure 3.29). Normally, ESI-TOF-MS can detect the signals of both $[M+H]^+$ and $[M+Na]^+$ ions, but MALDI-TOF-MS usually detect only $[M+H]^+$ ion. So, it has been impossible to estimate molecular weight of Cl/sD2.

In the $^1$H-NMR spectrum of Cl/sD2, the of the anomeric protons were visualized at 4.9 and 5.07 ppm. This result indicated that there is more than one molecule of monosaccharides present in Cl/sD2. In addition, the signals of aromatic groups were observed at 6.4-8.6 ppm. (Figure 3.30). These results might also imply that there are more than one compound in the Cl/sD2 fraction.

Interpretation of mass spectrum of Cl/sD3 compounds could not be done. Since, the Cl/sD3 sample was not ionized well, so a clear signal was not detected (Figure 3.31). In addition, many signals were observed in the range of anomeric protons in the Cl/sD3 $^1$H-NMR spectrum. Like Cl/sD1 and Cl/sD2, the signals indicated aromatic groups were also visualized in this sample (Figure 3.32). Based on these results, it is likely that there is more than one compound in Cl/sD3 sample.

In summary, the $^1$H-NMR analysis revealed that aromatic groups are present in all three fractions. In addition, all three fractions contain sugar in their structures.

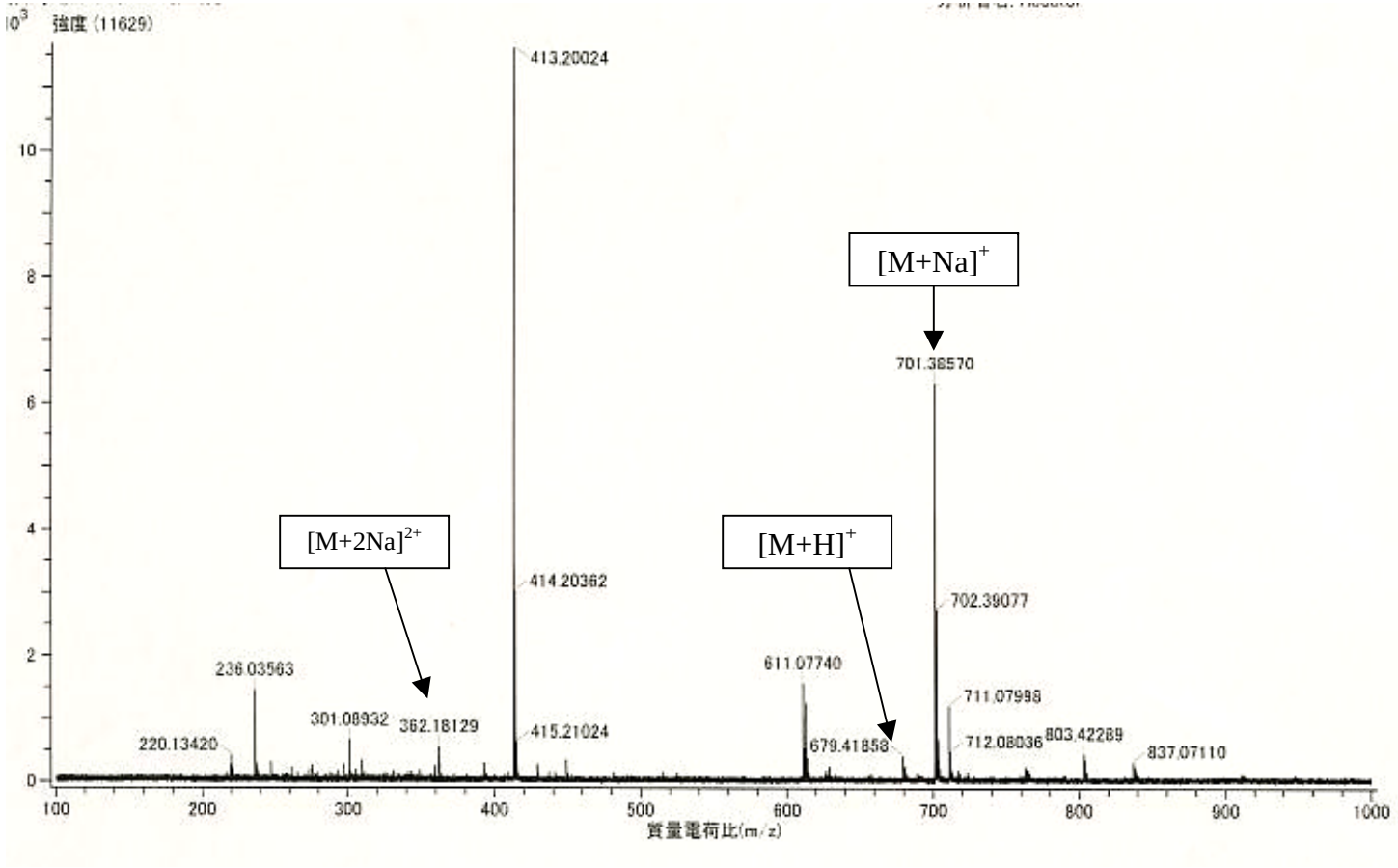



**Figure 3.28** ESI-TOF-MS mass spectrum of the Cl/sD2 compound in positive mode.

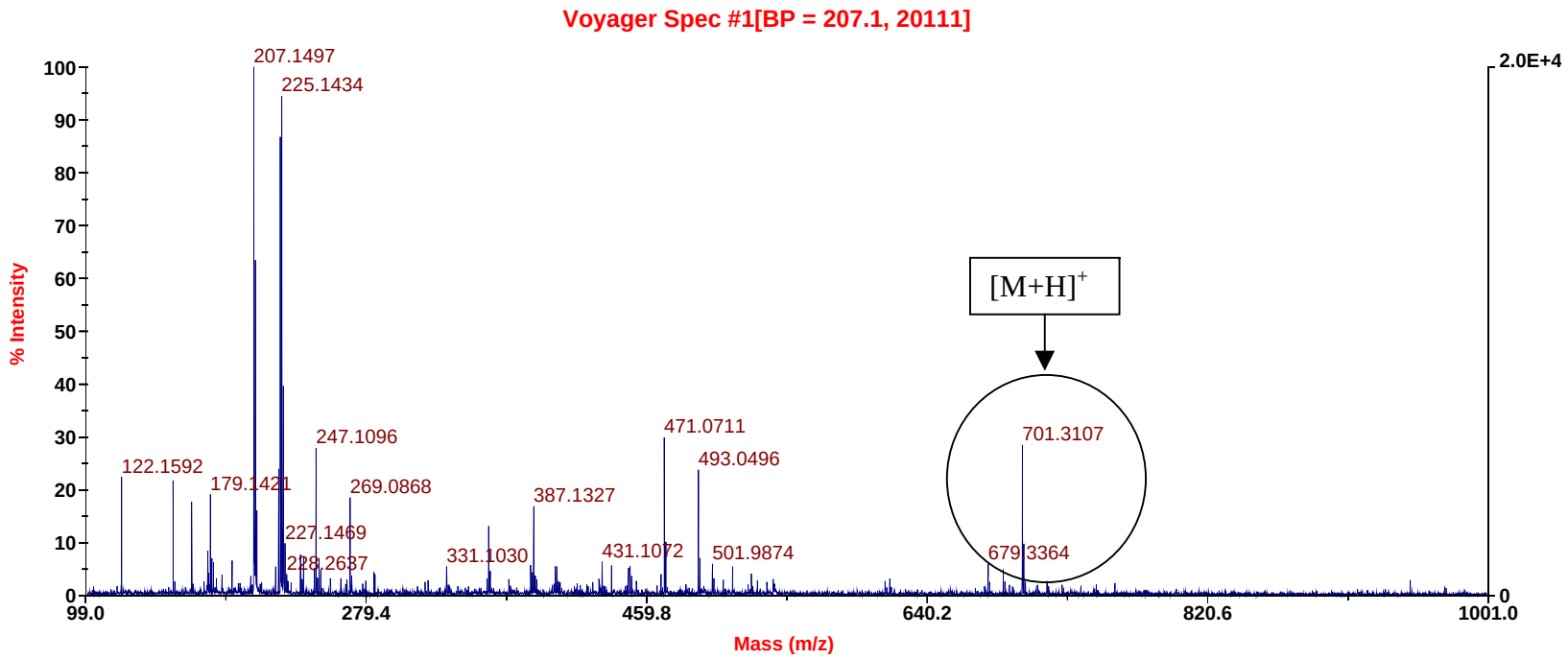



**Figure 3.29** MALDI-TOF-MS mass spectrum of the Cl/sD2 compound.

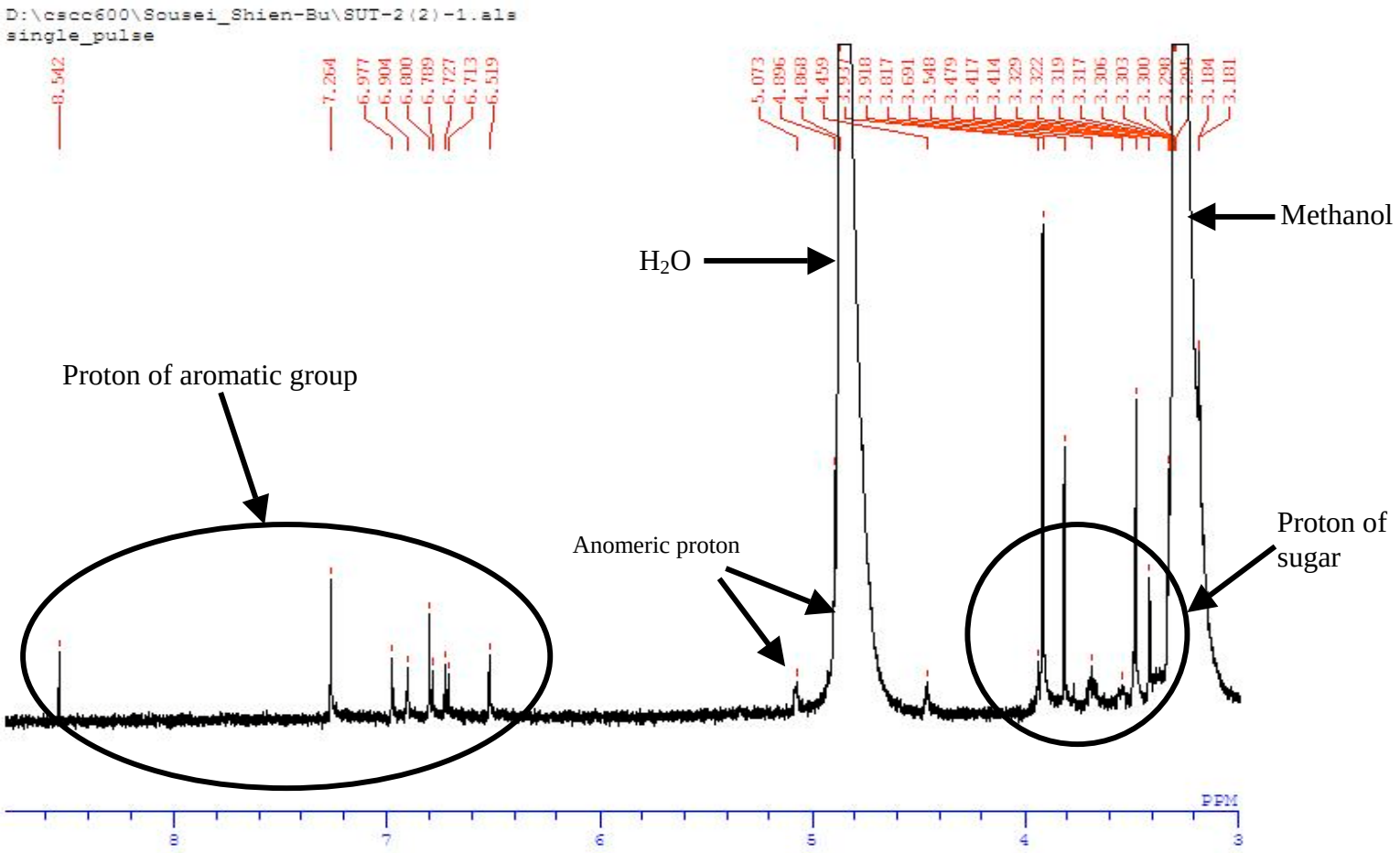



**Figure 3.30** $^{1}$H-NMR spectrum of the Cl/sD2 compounds.

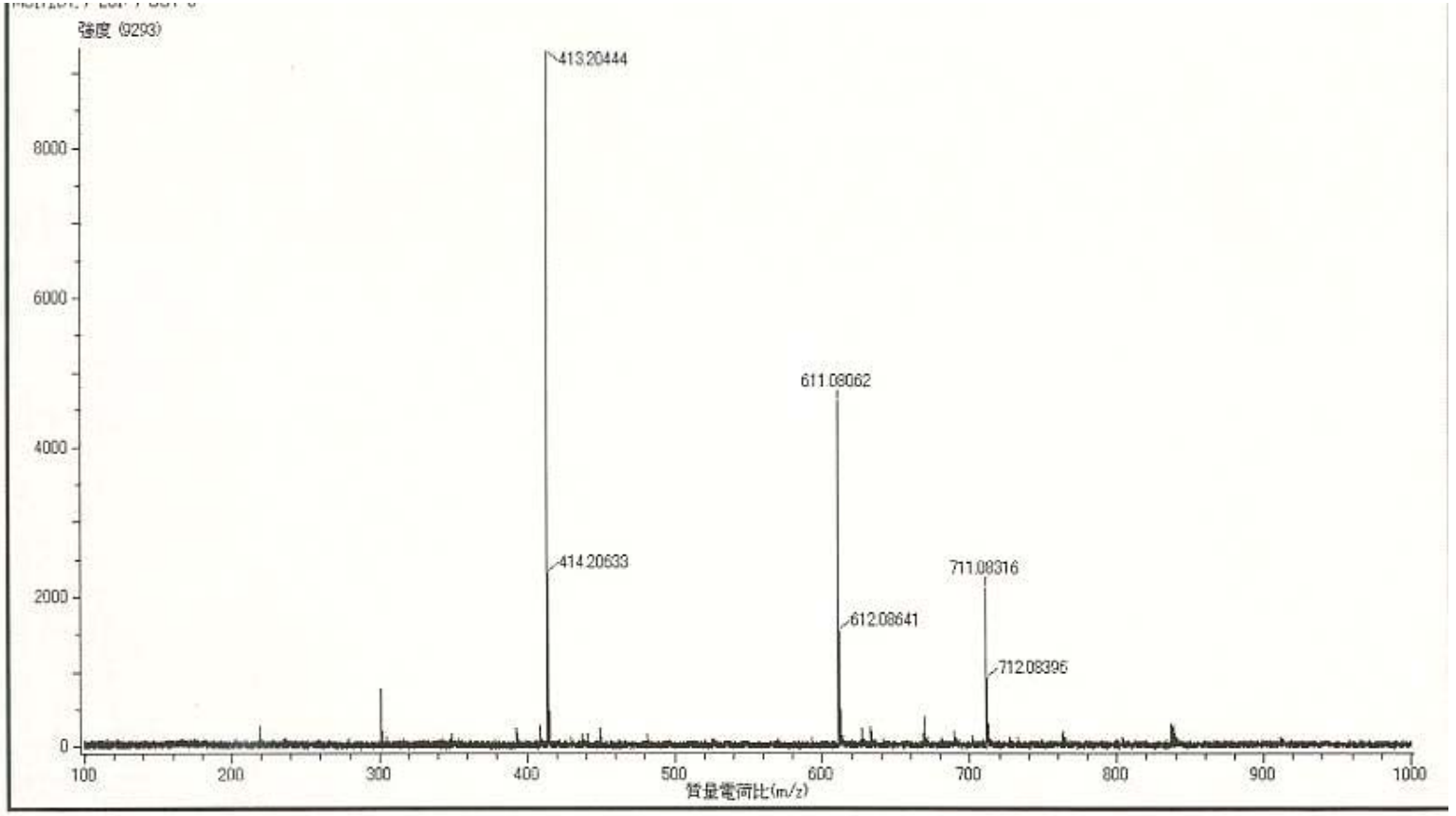



**Figure 3.31** ESI-TOF-MS mass spectrum of the Cl/sD3 compound in positive ion mode.

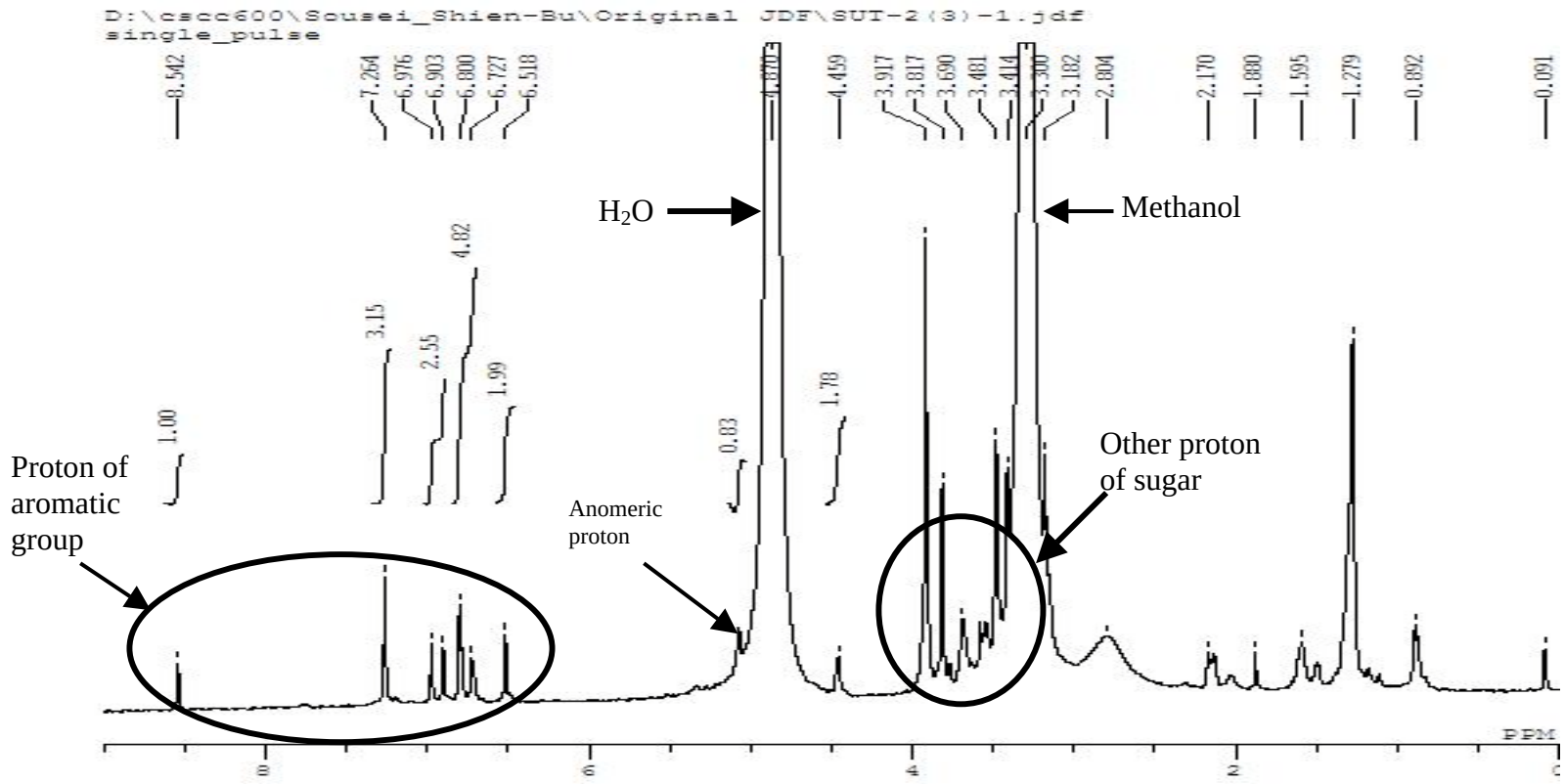



**Figure 3.32** $^{1}$H-NMR spectrum of the Cl/sD3 compounds.

# CHAPTER IV

# DISCUSSION

The full length cDNA of glycosyl hydrolase family 1 β-glucosidase, Os4bglu12 was previously isolated by RT-PCR from rice seedlings (Opaasiri et al., 2006). In this study, it was shown that *Os4bglu12* gene is highly expressed in shoots during germination and in leaf sheaths and stems of 6-week-old mature rice plants. The mRNA levels of *Os4bglu12* in 10-day-old rice seedlings significantly increased in response to methyl jasmonate, ethephon and wounding stress. The function of Os4bglu12 in hydrolyzing natural substrates in rice seedlings and leaf/stem parts of adult plants were characterized.

## 4.1 Expression of *Os4bglu12* gene in rice

It was shown that *Os4bglu12* gene is highly expressed in shoots, but expressed at low levels in other germinating seed parts. In 6-week-old mature rice plants, the high abundance of *Os4bglu12* mRNA was detected in leaf sheaths and stems, while the expression of this gene was at moderate or low levels in other plant parts.

Similar expression patterns of β-glucosidase genes were observed in rice *bglu1* and *bglu2* β-glucosidase genes. Northern blot analysis showed that *bglu1* and *bglu2* are highly expressed in seedling shoots, but in adult plants *bglu1* is highly expressed in flowers, while *bglu2* is in node (Opassiri et al., 2003). Other grass β-

glucosidase studied so far were present in seedling including maize, sorghum, rye and oat except for barley enzymes seen in maturing grains and seedling (Esen, 1992; Cicek and Esen, 1998; Sue et al., 2000; Nisius, 1988; Hrmova et al., 1998; Leah et al., 1995).

In addition, the temporal expression of *Os4bglu12* in seedlings results in the increased mRNA levels on day 1, which reached a maximum on day 2 and decreased by day 4 and then decreased rapidly on day 6 until day 10. In contrast, the mRNA levels of rice *bglu1* and *bglu2* genes increased on day 4, and maintained a similar high level of expression from day 4 to day 10, and decreased on day 12 and 14 (Opassiri et al., 2003). The occurrence of the mRNAs of these β-glucosidase genes in rice seedlings on different time frames during development implies that different rice β-glucosidase isozymes may play different roles during growth and development. *Os4bglu12* might act in the activation of phytohormone or cell wall recycling which is necessary for the prenetration of shoot and root primodia through the seeds in germination. After this stage, rice *bglu1* and *bglu2* might play the roles in rapid cell expansion and cell division during the elongation of rice seedling shoots and roots. Mundy and Fincher (1986) reported that barley β-glucosidase and endoglucanase are first synthesized during germination in aleurone and scutellum in response to gibberellic acid. In addition, Leah et al. (1995) proposed that barley BGQ60 β-glucosidase, the enzyme that hydrolyze β-linked glucose oligosaccharides, might be involved in the reorganization and alteration of specific β-linked polysaccharides during the extensive cell expansion which occurs during seed development (Carpita and Gibeaut, 1993). In addition, the high expression of *Os4bglu12, bglu1* and *bglu2* in different tissues of mature rice plants might indicate functional differences between

different β-glucosidase isozymes. However, these enzymes might have functional redundancy associated with growth and development in adult plants.

Opassiri et al. (2006) reported there are ESTs for 33 rice glycosyl hydrolase family 1 (GH 1) β-glucosidase gene from source libraries including callus, seedling (shoot and root), immature and mature plant parts (stem, leaf, root, flower, and immature seeds). The source libraries that have ESTs for *Os4bglu12* include callus, seedling shoot and root, spikelet before heading and whole plant at tillering and trefoil stages. The occurence of the ESTs/cDNAs of β-glucosidase genes in tissues may correlate with growth and development. As mentioned by Xu et al. (2004), the members of a given subfamily of GH 1 family may have the same biochemical functions and may be expressed in different cell or tissues. However, the multiple isozymes of rice β-glucosidase may also represent functional redundancy and be expressed in the same tissues (Opassiri et al., 2006).

## 4.2 Expression of *Os4bglu12* gene in response to environmental stresses and phytohormones

Some environmental conditions have an effect on the transcript levels of *Os4bglu12* in rice after 10-day-old seedlings were grown under various conditions for an additional 2 days. Northern blot analysis showed that the *Os4bglu12* mRNA levels significantly increased in rice seedlings in response to methyl jasmonate. Other stresses, including heat, drought, salt, osmotic stresses and gibberellic acid treatments did not affect the expression of this gene. The transcript levels decreased when plants were exposed to ethylene, abscissic acid, wounding, cold, and flood stresses. However, the two days response may be different from more immediate responses.

So, the time frame must be considered. It was previously found that the transcripts of the EST contig BE607353 and BG101702, whose sequence are homologous to rice *bglu1* and *Os4bglu12*, respectively, increased in response to high salinity stress in salt-tolerant rice (var Pokkali), but not in the salt-sensitive cultivar IR29 (Kawasaki et al., 2001). The increase in the transcript of *Os4bglu12* in response to methyl jasmonate and salt stress suggests that this enzyme might play a role in cooperation with other components in the signaling cascade associated with stress response or the response itself. Opassiri (2003) reported that *bglu1* and *bglu2* mRNA levels decreased when 6-day-old seedlings were exposed to drought stress for 2 days, while cold, osmotic, salt and flooding stresses, ethylene and ABA have little or no effects on *bglu1* transcript levels. Opassiri et al. (2006) also reported the presence of rice β-glucosidase ESTs from stressed plant tissue libraries, such as salt, drought, cold, heat stresses and fungal infection. However, the high or low abundance of ESTs for some rice genes might not reflect the relative expression levels in particular tissues or growth conditions because there may be bias in the selection of plant parts and developmental stages for preparation of EST cDNAs. (Opassiri et al., 2006; Xu et al., 2004).

## 4.3 Effect of methyl jasmonate, ethephon and wounding on *Os4bglu12* gene expression

Methyl jasmonate, ethephon, systemin and ABA have been implicated in putative wounding and other stress signaling in higher plants (LeÓn et al., 2001). This evidence led to a more detailed time course study of *Os4bglu12* gene expression in 10-day-old rice seedlings exposed to methyl jasmonate, ethephon and wounding

treatments. Methyl jasmonate and ethephon have been reported to be involved in wound signaling in several plant species (McColud and Baldwin, 1997; Dahl and Baldwin, 2004). Accumulation of these hormones is known to increase transiently after wounding or herbivore attack (Ziegler et al., 2001). Ethylene concentration has been reported to increase strongly when *Manduca sexta* is feeding on tobacco plants (Kahl et al., 2000). The increasing concentrations of jasmonic acid , methyl jasmonate and ethylene in wound-induced plants were reported in tobacco and tomato (Dahl and Baldwin, 2004; ÓDonnell et al., 1996; Xu et al., 1994). When wounded or herbivore attacked, plants respond quickly with a complex sequential action of different signals leading to defense gene expression (Wasternack et al., 2006). The jasmonic acid-mediated wound signal transduction pathway requires the activation of wound-induced protein kinase which is a defense-related mitogen activated protein kinase (MAPK) (Seo et al., 1995). Rojo et al. (2003) proposed the antagonistic interactions of jasmonic acid and ethylene on defense gene expression, resulting in resistance of *Arabidopsis thaliana* to herbivore. There are many strategies that plants use in defense, including 1) enhancement protective compounds for injury healing or resistance against pathogen attack, such as phenylpropanoid synthesis and production of antimicrobial substances (ie, proteinase inhibitors); 2) formation and emission of volatiles which attract insect predators; and 3) cross-linking of cell walls to restore damaged tissues and protect them from subsequent pathogen or insect attack (Dixon and Paiva, 1995; Wasternack et al., 2006; Bradley et al., 1992).

Northen blot analysis revealed that the expression of *Os4bglu12* was upregulated in a similar manner in response to wounding, methyl jasmonate and ethephon treatments, and the mRNA levels reached to maximum within 5 to 10 hr

after the beginning of the treatments and then decreased by the end of 3 or 4 days treatments. 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 observed from subtractive hybridization cDNA library screening. According to above studies, an increase in the mRNA levels of *Os4bglu12* gene induced by mechanical wounding and herbivore attack may be mediated by the methyl jasmonate and ethylene signal transduction pathway. Os4bglu12 might be involved in the cell wall cross-linking process to restore damaged tissues or releasing a volatile compound from its glycoside. However, it is difficult to confirm about the functional significance of *Os4bglu12* gene stimulation caused by wounding or other stresses. So, isolation of natural substrate(s) from rice and determination of the conditions under which each substrate is hydrolyzed were studied.

## 4.4 Natural substrates of Os4bglu12 and its possible roles in rice

Since the high transcripts of *Os4bglu12* gene were detected in rice both under normal and stress conditions, identification of the natural substrates of this enzyme was performed to indicate its roles in rice plants.

### 4.4.1 Hydrolysis of rice cell wall by Os4bglu12

It was previously reported that rice endo-1,3;1,4-β-glucanase genes were induced considerably by wounding and fungus infection in rice seedling leaves (Simmons et al., 1992; Nishizawa et al., 2003). Endo-1,3;1,4-β-glucanases specifically hydrolyze 1,3;1,4-β-glucans which are constituents of grass cell walls.

The northern blot analysis showing the induction of mRNA levels of both endo-1,3;1,4-β-glucanase (Akiyama T., unpublished data) and *Os4bglu12* β-

glucosidase gene in rice seedling following wounding, methyl jasmonate and ethephon treatments supports the idea that these enzymes may play cooperative roles in hydrolysis of 1,3;1,4-β-glucans of cell walls. In agreement with this hypothesis, 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. The hydrolysis of rice cell wall powder by recombinant endo-1,3;1,4-β-glucanase resulted in 3 major products composed of two oligosaccharides, namely Os1 and Os2, and disaccharide, namely Os3. Os1 migrated between cellotriose and cellotetraose on TLC and it is likely to be an oligosaccharide with DP of 4. Os2 showed a mobility in between cellobiose and cellotriose on TLC, so it is likely to be an oligosaccharide with DP of 3. Os3 is thought to be laminaribiose (β-1,3-linkage) because it has an $R_f$ value egual to this disaccharide. In general, endo-1,3;1,4-β-glucanase hydrolyze (1-4)-β-glucosidic linkages where these linkages are adjacent to a (1-3)-β-D-glucosyl residue and usually release two major hydrolysis products, (1-3),(1-4)-β-D-tri- and tetrasaccharides ($G4G3G_{red}$ and $G4G4G3G_{red}$, where G represents a β-glucosyl residue, 3 and 4 are (1-3) and (1-4) linkages, respectively; and "red" indicates reducing terminus). However, these enzymes also release higher oligosaccharides of up to 10 or more (1-4)-β-D-glucosyl residues with a single reducing terminal (1-3)-β-D-glucose (Woodward et al., 1983; Wood et al., 1994). If the mode of action of recombinant rice endo-1,3;1,4-β-glucanase is similar to the other enzymes in the same group, structures of Os1 and Os2 oligosaccharides are most likely to be $G4G4G3G_{red}$ and $G4G3G_{red}$, respectively.

The time coursed analysis of the hydrolysis of Os1, Os2 and Os3 by Os4bglu12 revealed that the enzyme hydrolyzed Os1 to produce Os2 and free glucose. The Os2 was further cleaved by the enzyme to produce Os3 disaccharide and

free glucose. Finally, the Os3 disaccharide was cleaved by the enzyme to release glucose residues. The mode of action of Os4bglu12 in the hydrolysis of rice cell wall is some what similar to barley BGQ60 β-glucosidase. The barley BGQ60 enzyme hydrolyzed the oligosaccharide products, G4G3G$_{red}$ and G4G4G3G$_{red}$, released from barley-1,3-1,4-β-glucans by barley endo-1,3;1,4-β-glucanase (Leah et al., 1995). In addition, the study from Hrmova and Fincher (2001) indicated that an oligosaccharide G4G4G4G3G$_{red}$ has a very similar shape to cellopentaose G4G4G4G4G$_{red}$ particularly at the nonreducing end. It has been shown that Os4bglu12 preferentially hydrolyzed cello-oligosaccharide (Opassiri et al., 2006) and its ability to hydrolyze laminaribiose, suggests that (1,3);(1,4)-β-D-oligosaccharides could be completely depolymerized to glucose by Os4bglu12. Since Os4bglu12 could not hydrolyze laminari-oligasaccharides with DP higher than 2 (Opassiri et al., 2006), 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 termini of (1,3), (1,4)-oligosacchrides.

According to the data derived from by this study and Opassiri et al. (2006), it would indicate that rice Os4bglu12 β-glucosidase may be involved in the complete depolymerization to release glucose of at least three major cell wall polysaccharides present in rice tissues, including the (1,4)-β-glucan (cellulose), (1,3) (1,4)-β-glucan and (1,3)-β-glucan. So, these would suggest the role for Os4bglu12 in regeneration of cell walls (this includes damaged cell wall caused by wounding) by futher hydrolyzing the oligosaccharides released from the cell walls by endoglucanases, thereby promoting reconstruction of cell wall architecture. The reorganization and alteration of specific β-linked polysaccharides involves selective

recycling of monosaccharides derived from β-linked polysaccharide to other polymers. In grasses, glucose is selectively cleaved from β-glucans and may re-enter sugar-nucleotide interconversion pathways contributing to the synthesis of new β-linked polysaccharides either as β-glucans or other types of polymers (Gibeaut and Carpita, 1991; Carpita and Gibeaut, 1993). This implies that the presence of the transcripts of *Os4bglu12* gene in rapid growing tissues of rice seedlings, mature rice plants and the wound-preferential expression might be associated with the cell wall recycling process, as described above.

Recently, there have been some reports found that not only photosynthesis and breakdown of sucrose and starch, the hydrolysis of cell wall polysaccharides also likely generates sugar signals (Rolland et al., 2006). Several cell wall glycosyl hydrolases are upregulated under stress conditions such as dark, sugar depletion, senescence and pathogen infection (Contento et al., 2004; Fujiki et al., 2001; Lee et al., 2004; Rolland et al., 2006). Therefore, some roles in sugar sensing for Os4bglu12 also can not be ruled out, and the enzymes may play more than one role.

### 4.4.2 Natural glycoside substrates of Os4bglu12

To study other possible natural substrates of Os4bglu12, rice seedlings and tissues from rice plant at flowering stage were extracted with methanol and the fractions separated by LH-20 chromatography were tested for hydrolysis by recombinant Os4bglu12 β-glucosidase. The occurence of differences in TLC spot patterns between the rice extracts treated with and without recombinant Os4bglu12 revealed that Os4bglu12 could hydrolyze at least two, one and seven compounds

present in 7-day-old seedlings, flower and leaf/stem portions of rice at flowering stage, respectively.

In this study, however, only three fractions from leaf/stem parts of adult plants, denoted Cl/sD1, Cl/sD2 and Cl/sD3, could be purified. The $^{1}$H-NMR analysis indicated the presence of aromatic group spectra in spectra of all fractions. The presence of the spectra of anomer protons indicated that Cl/sD1 and Cl/sD2 contain one and two molecules of monosaccharide, respectively. Cl/sD3, however, contains more than one sugar containing compounds.

The mass of Cl/sD1 glycoside could be estimated to be 492 from the ESI-TOF-MS and MALDI-TOF-MS data, corresponding to the molecular formula $C_{23}H_{24}O_{12}$. The molecular formula $C_{23}H_{24}O_{12}$ matched 104 known compounds found by SciFinder Scholar. Three structures of tricin-O-β-D-glucosides, one major group of flavonoid glucosides found in rice, were also included in the output of this analysis. Tricin-O-β-D-glucosides were found in many of rice tissues, including rice hulls, bran, leaves and stems (Adjei-Afriyie et al., 2000; Chung et al., 2005; Cai et al., 2005). Adjei-Afriyie et al. (2000) reported that these compounds have probing stimulant activity for brown planthopper. Based on the above results, it could be speculated that Cl/sD1 might have the structure grouped into tricin-O-β-D-glucosides. If this hypothesis is true, Os4bglu12 might act in releasing of active flavonols for defense against herbivore attack or pathogenic microorganisms infection after wounding. Consistent with this hypothesis, the transcript levels of the EST contig BPHiw028 homologous to the *Os4bglu12* gene was induced in response to brown planthopper attack (Wang et al., 2005). In addition, our study also supported that the transcripts of *Os4bglu12* gene was upregulated in response to wounding.

Accumulation of many glycosides in rice grown under environmental stresses and in transgenic rice have been reported. Marino et al. (2005) found that indole-3-acetic acid glucoside are accumulated in tissues of tryptophan-overproducing transgenic rice and Chern et al. (2005) also found the accumulation of salicylic glucoside in rice overproducing NH1, a key regulator of salicylic acid which mediates systematic acquired resistance, in transgenic rice. Makham et al. (1998) reported increased levels of flavone glucosides in UV-tolerant rice after exposing to high UV-B levels. As suggested by Opassiri et al. (2006), high amounts of some metabolic compounds under the above conditions are corrected by converting them to glucoside-conjugated forms. This process might help, not only in the storage of these compounds, but also in the transport of soluble forms of the compounds between different plant tissues. However, it still neccessary to shown whether or not these compounds are later reactivated by β-glucosidases (Opassiri et al., 2006).

For our work, it is still neccessary to clarify the structure of Cl/sD glycosides and show whether or not these compounds are accumulated in response to wounding or pathogen and herbivore attack. Though Os4bglu12 is though to function in hydrolysis of oligosaccharides released from the cell walls, it might be possible that this enzyme plays more than one role. Recently barley β-glucosidase, which is thought to help in hydrolysis of cell wall oligosaccharides during germination has been found to hydrolyze cyanogenic glycosides from barley leaves (Nielsen et al., 2005). This gives support to the possibility of one enzyme playing roles in both the cell wall hydrolysis and defense.

# CHAPTER V

# CONCLUSION

The *Os4bglu12* cDNA encoding mature proteins was previously cloned and inserted into pET32a+/DEST, and successfully expressed as soluble protein in Origami B (DE3) (Opassiri et al., 2006). In this study, the transcript level of *Os4bglu12* in rice was determined by northern blot analysis using a specific DNA probe generated by amplification of 3'end region of *Os4bglu12,* including a short 3'end coding region and part of the 3'UTR. In 7-day-old rice seedlings *Os4bglu12* transcripts were detected in high abundance in the shoot. In 6-week-old mature rice plants at flowering stage *Os4bglu12* mRNA is highly expressed in leaf sheaths and stems. The expression of *Os4bglu12* in seedling tissues during germination could be detected on day 1, then increased to reach the highest level on about day 2 and decreased gradually from day 6. A significant increase in *Os4bglu12* mRNA levels was detected in 12-day-old-rice seedlings treated with methyl jasmonate for 2 days and detected at levels similar or slightly decreased compared to the control when the seedlings were treated with 37°C, drought, salt, mannitol and gibberellic acid. The transcript levels decreased when the seedlings were treated with ethylene, abscissic acid, wounding, cold, and flooding stresses.

Furthermore, a more detailed time course study of *Os4bglu12* expression revealed that the expression of Os4bglu12 in 10-day-old rice seedlings was induced as early as 5-10 hr after wounding and thereafter decreased gradually, similar gene

expression patterns were seen in rice seedlings treated with methyl jasmonate and ethylene.

A recombinant rice Os4bglu12 could not hydrolyze rice cell wall polysaccharides directly but it could hydrolyze oligosaccharide hydrolysis products released from rice cell walls by recombinant rice endo-1,3;1,4-β-glucanase. Three major rice cell wall hydrolysis products were denoted as Os1, Os2 and Os3, respectively. It is likely that Os4bglu12 hydrolyzed Os1 to produce Os2 and glucose, then the enzyme further hydrolyzed Os2 to Os1 and glucose. Finally, Os3 was cleaved by the enzyme to release glucose residues.

To identify other possible natural substrates of this enzyme, rice seedlings and tissues from rice plants at flowering stage were extracted with methanol, the extracts fractionated by LH-20 column chromatography and fractions tested for hydrolysis with Os4bglu12 by TLC. Os4bglu12 could hydrolyze the compounds extracted from 7-day-old rice seedling roots and flower and leaf/stem parts of rice at flowering stage. The substrates of Os4bglu12 found in leaf/stem parts of mature rice plants, namely Cl/sD were purified using various chromatographic techniques, including LH-20 chromatography, silica-gel TLC, Si-60 chromatography, and HPLC with a $C_{18}$ reverse phase chromatography. The solvent system used for separating compounds in Cl/sD by HPLC was 40% (v/v) methanol in water which was used in isocratic elution. Cl/sD could be separated into at least three peaks at retention times of 7.60, 9.15 and 11.93 min, the names of which were denoted as Cl/sD1, Cl/sD2 and Cl/sD3, respectively. On TLC, the sugar spots released from these compounds by Os4bglu12 had a similar mobility to that of D-glucose. The structure of these natural substrates were studied by mass spectroscopy and NMR spectroscopy. $^{1}$H-NMR analysis

indicated the presence of aromatic group and anomeric protons of sugar molecules in these compounds. Only the molecular weight of the Cl/sD1 could be estimated to be 492, corresponding to molecular formula $C_{23}H_{24}O_{12}$.

In conclusion, the fundamental knowledge of rice Os4bglu12 β-glucosidase, including temporal and spatial expression, the induction of the gene transcript in response to stresses, and the function of the enzyme in hydrolyzing natural substrates were characterized. This work is a good start toward determining the roles of this glycosyl hydrolase family 1 β-glucosidase in rice.

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# APPENDICES

# APPENDIX A

# CLONING OF *Os4bglu12* β-GLUCOSIDASE AND RICE ENDO-1,3-1,4-β-GLUCANASE

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

A full coding cDNA of *Os4bglu12* rice 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). A forward primer 445-1full (5'-TGTCCATGGCGGCAGCAG-3'), and the reverse primer 445-1_3'UTRr (5'-AACTGGATTACTTCCATCTC-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 TGGCCTAC AATAGCGCCGGCGAG-3’) and 445-1stop (5’-ATCATTTCAGGAG GAACTTCTTG-3’) primers and *Pfu* DNA polymerase to introduce a direction 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 directions (Invitrogen). The cDNA insert in the pENTR-D/TOPO vectors was subcloned into the pET32a+/DEST Gateway expression vector by LR Clonase recombination following the recommended protocols (Invitrogen) and thoroughly sequenced.

## 2. Expression and purification of recombinant rice endo-1,3;1,4-β-glucanase for preparation of cell wall oligosaccharides

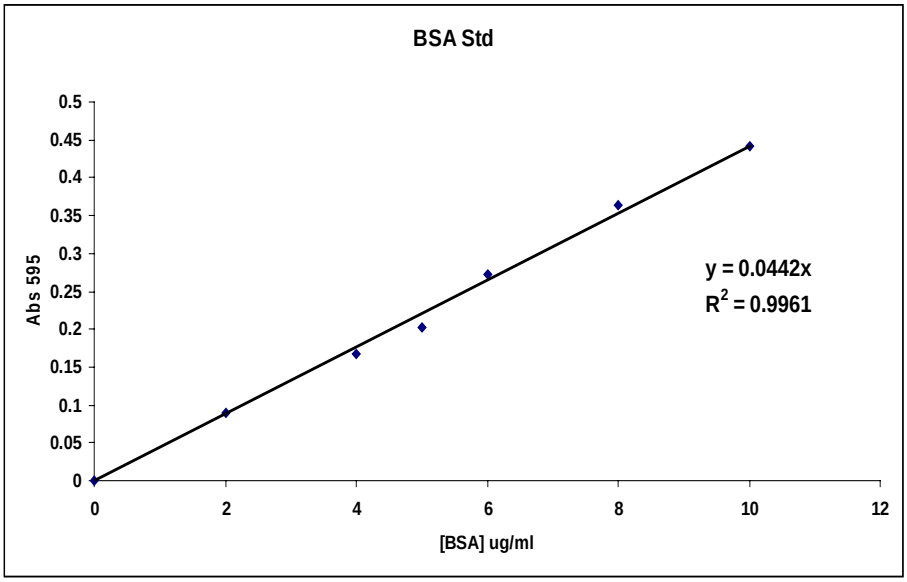
Expression of recombinant rice endo-1,3;1,4-β-glucanase protein was made by GST (glutathione S- transferase) gene fusion system (GE Healthcare, Buckinghamshire, UK) by Dr. Takashi Akiyama. The coding region for a rice endo-1,3;1,4-β-glucanase mature protein contig was amplified by a pair of primers: endo-1,3;1,4-β-glucanase forward primer (5’GATGGTGGATCCGAGGCGGAGGCG3’) containing *Bam*HI site and endo-1,3;1,4-β-glucanase reverse primer (5’ATGCGTGA ATTCGTACGGAATGCA3’) containing *Eco*RI site. PCR amplification was done by using the first stand cDNA synthesized from mRNA of rice seedlings (*O. sativa* L.cv.Yukihikari) as a template. The purified DNA products were digested with *Bam*HI and *Eco*RI restriction enzymes and ligated into the *Bam*HI and *Eco*RI sites of

pGEX4T-3 (GE Healthcare) to produce the pGST-endo-1,3;1,4-β-glucanase recombinant plasmid. The pGST-endo-1,3;1,4-β-glucanase recombinant plasmid was transformed into *E. coli* strain DH5α cells. The selected clone was grown at 37°C until the optical density at 600 nm reached 0.6 and induced with 0.1 mM IPTG. After 5 hr incubation at 37°C, the cells were harvested by centrifugation and sonicated in phosphate buffered saline solution (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) on ice. The recombinant GST-endo-1,3;1,4-β-glucanase fusion protein was purified on a glutathione-Sepharose 4B column (1.5 cm X 4 cm, GE Healthcare, USA.) as described by manufacturer. Protein concentration was determined by the method of Bradford (1976) with the Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as a standard (see method 2.2.9). Protein samples were separated by SDS-PAGE (LaemmLi 1970) and stained with Coomassie Brilliant Blue R250 (see method 2.2.9) to confirm the purity of the recombinant protein.

# APPENDIX B

# STANDARD CURVE, TLC AND HPLC CHROMATOGRAMS OF RICE COMPOUNDS SEPARATED BY VARIOUS SOLVENT SYSTEMS

## 1. Standard curve of BSA by Bio-RAD protein assay.

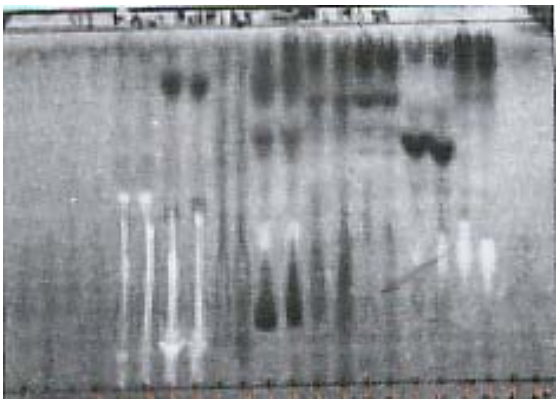


## 2. TLC chromatogram of rice extracts separated by various solvent systems

The crude extracts of rice seedling root and leaf/stem parts of rice at flowering stage were fractionated by LH-20 chromatography and fractions tested for hydrolysis by Os4bglu12 β-glucosidase. The assay reaction mixture (+) consisting of 10 μL fraction, Os4bglu12 β-glucosidase (~ 0.8 μg), 50 mM sodium acetate pH 5.0, in a total volume of 100 μL was incubated at 37°C for 30-60 min and stopped by boiling for 5 min. The control reaction (-) mixture consisted of 10 μL fraction, 90 μL 50 mM sodium acetate pH 5.0. The compounds in each reaction were separated on TLC using various solvent systems.

### 2.1 Rice seedling roots

**2.1.1** n-Butanol:methanol:water (60:15:25)



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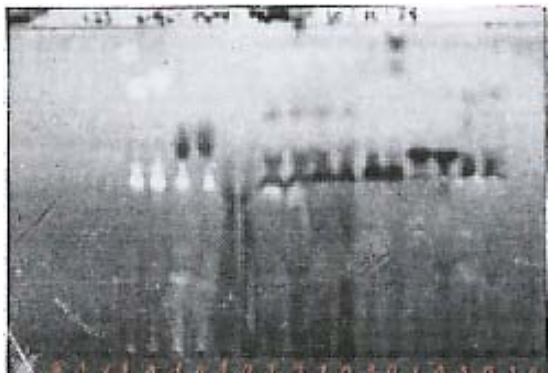
3 12 19 24 28 34 39 43 52 57 66

**2.1.2** Ethyl acetate:methanol:acetic acid:water (75:5:10:10)



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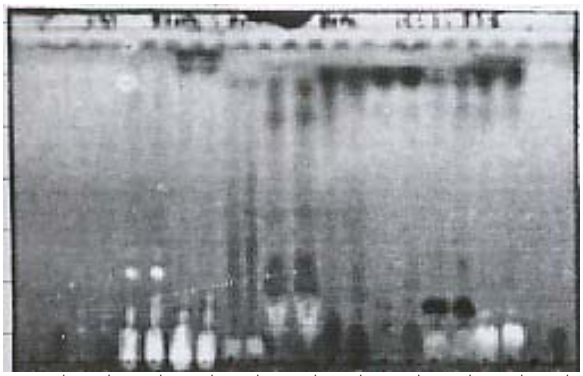
**2.1.3** Ethyl acetate:methanol:water (58:30:12)



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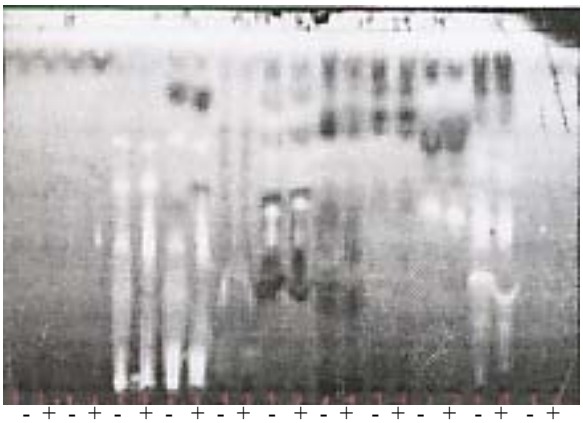
**2.1.4** n-Butanol:methanol:water (60:25:15)



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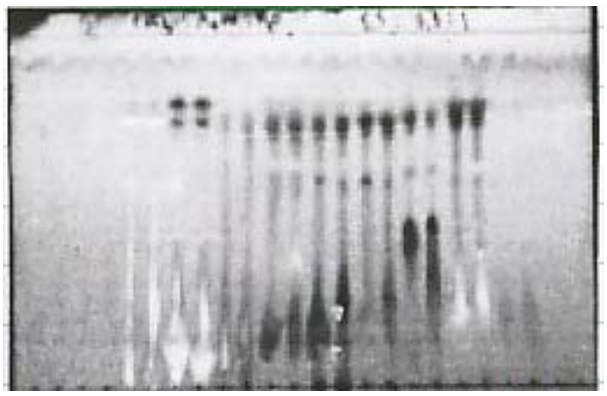
**2.1.5** Chloroform:methanol:water (55:35:10)



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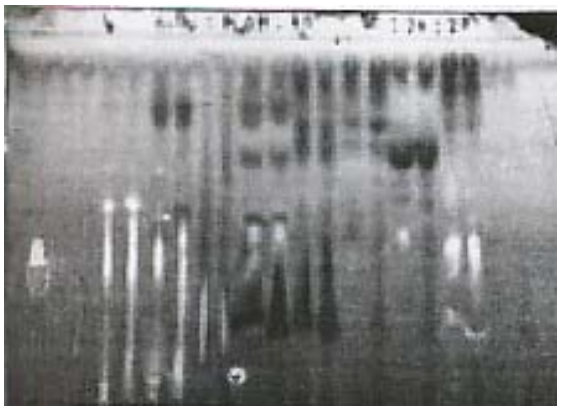
3 12 19 24 28 34 39 43 52 57 66

**2.1.6** n-Butanol:methanol:water (60:20:20)



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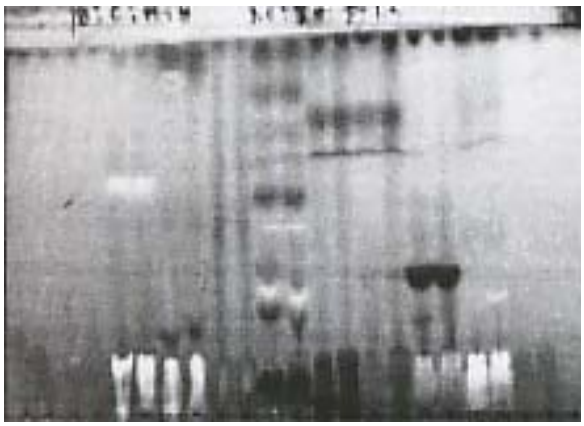
3 12 19 24 28 34 39 43 52 57 66

**2.1.7** n-Butanol:chloroform:methanol:water (35:30:20:15)



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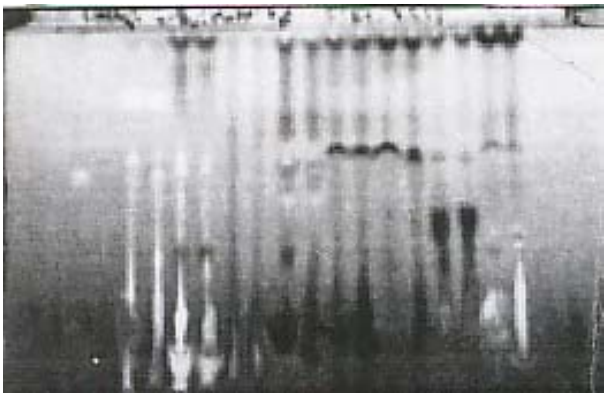
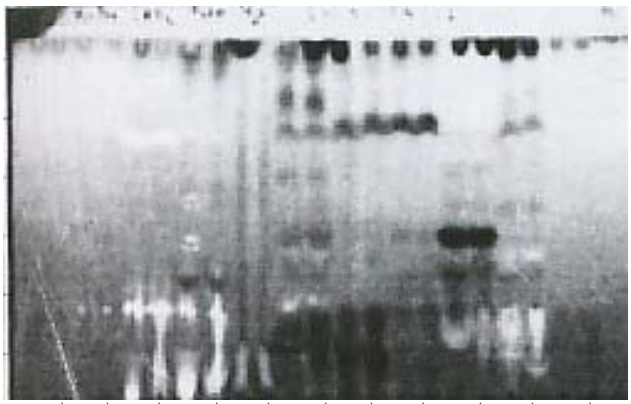
3 12 19 24 28 34 39 43 52 57 66

**2.1.8** n-Butanol:chloroform:methanol:water (45:10:30:15)

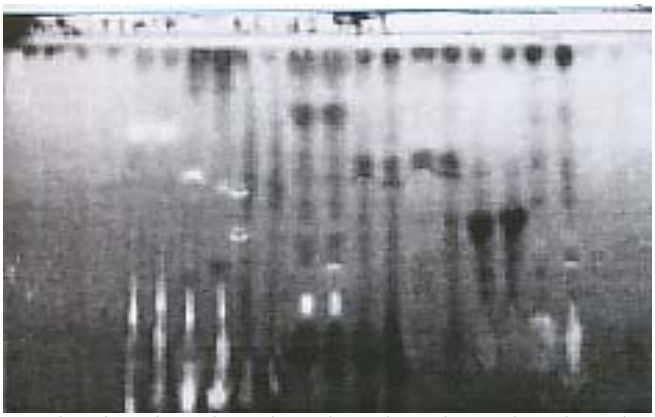

**2.1.9** n-Butanol:chloroform:methanol:water (5:50:35:10)




**2.1.10** n-Butanol:chloroform:methanol:water (25:35:28:12)



**2.1.11** n-Butanol:chloroform:methanol:water (15:42:33:10)

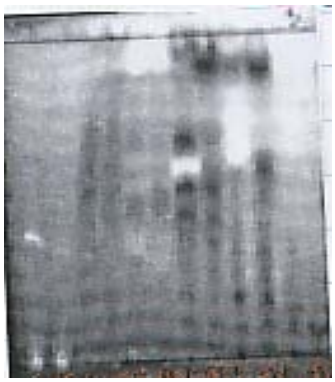



## 2.2 Leaf/stem parts of rice at flowering stage

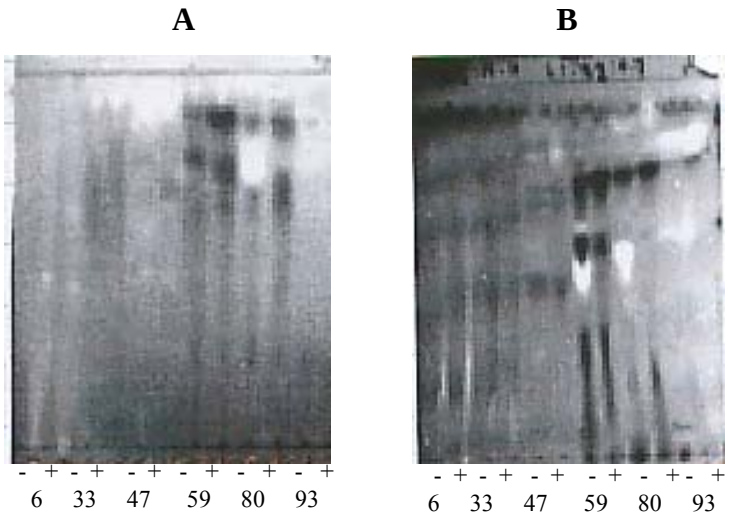
**2.2.1** A. Dichloromethane:methanol:water (60:32:8)

and B. n-butanol:methanol:acetic acid:water (75:5:10:10)

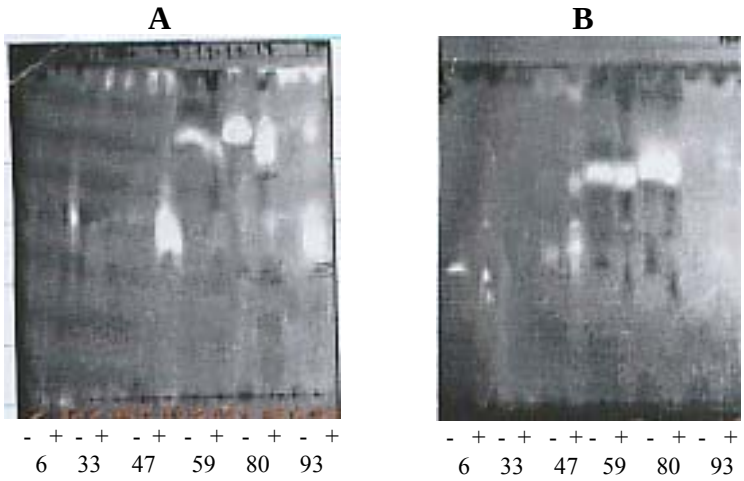
**A** **B**

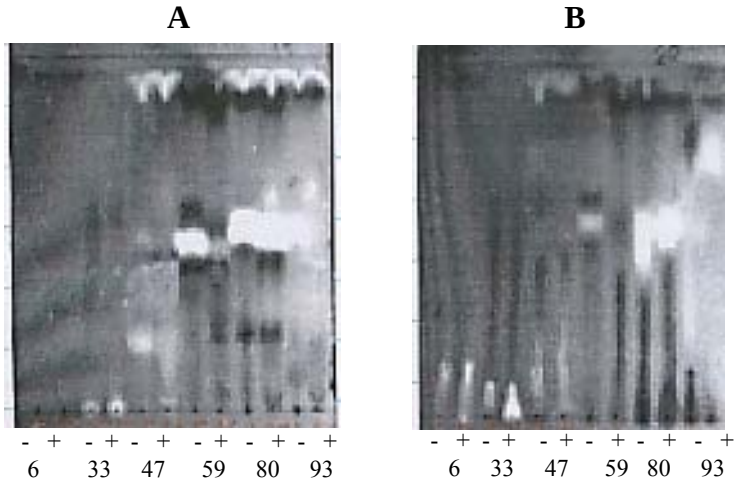
**2.2.2** A. Iso-propanol:methanol:acetic acid:water (75:5:10:10) and B. chloroform:methanol:water (61:32:7)



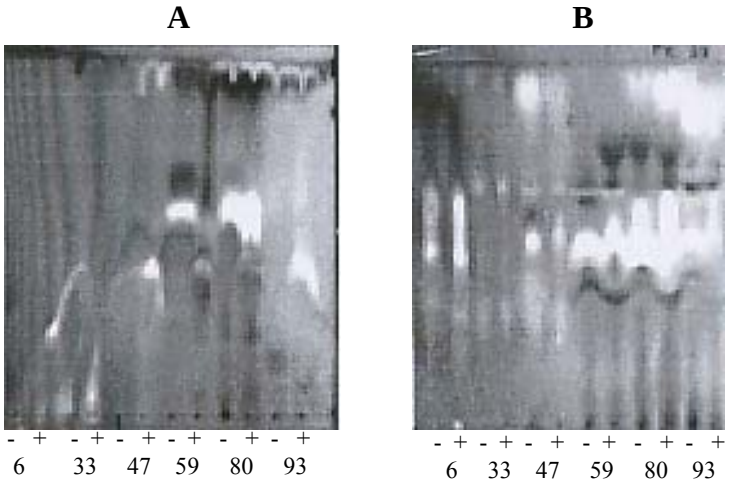

**2.2.3** A. Ethyl acetate:methanol:acetic acid:water (57:13:17:13) and B. chloroform:acetic acid:water (60:15:25)



**2.2.4** A. Ethyl acetate:methanol:acetic acid:water (67:8:10:16) and B. chloroform:n-butanol:water (57:13:17:13)

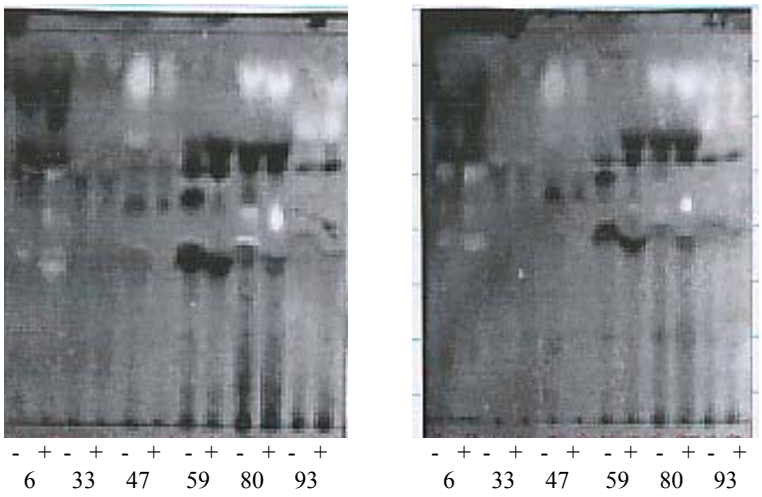



**2.2.5** A. Ethyl acetate:methanol:acetic acid:water (57:13:17:13) and B. chloroform:acetic acid:water (60:15:25)



**2.2.6** A. Ethyl acetate:methanol:acetic acid:water (57:13:17:13) and B. chloroform:acetic acid:water (60:15:25)

**A** **B**

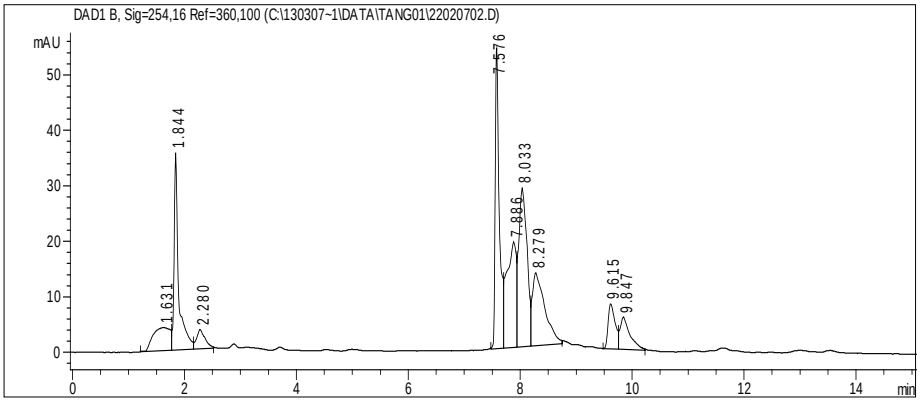



**2.2.7** Ethyl acetate:methanol:acetic acid:water (57:13:17:13)

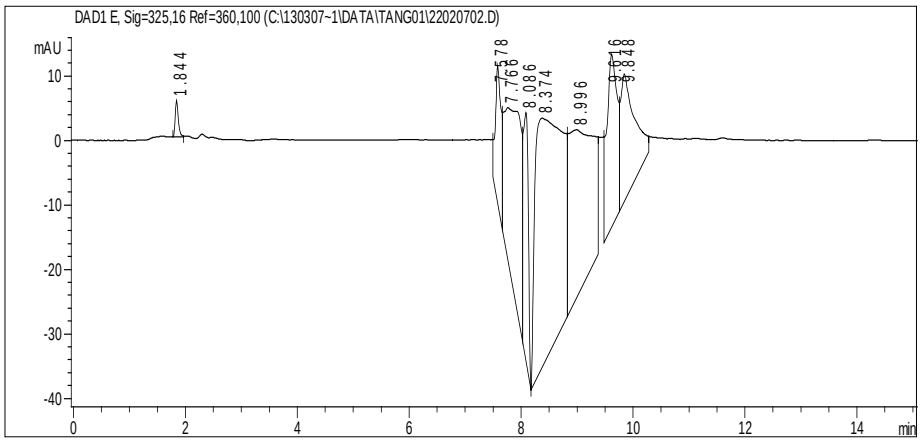
## 3. HPLC Chromatograms of the Cl/sD compounds isolated from leaf/stem of rice at flowering stage

**3.1** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of acetonitrile:water, (0 to 3 min (0:100), 5 min (30:70), 13 to 15 min (50:50), 23 min (80:20), 26 min (85:15), 30 min (95:5), 31 to 33 min (100:0)) at a flow rate of 0.8 mL/min.

**A**

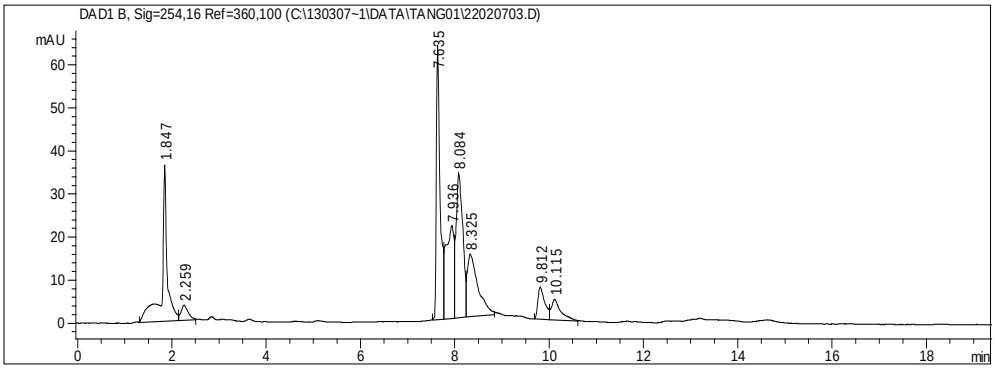



**B**

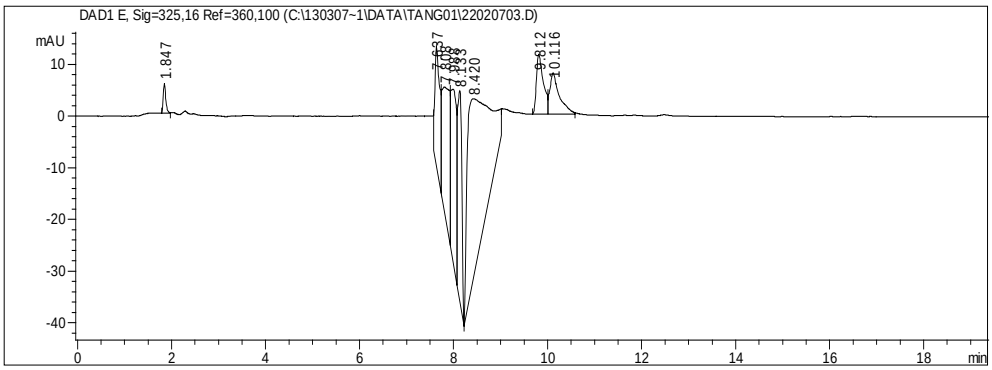


**3.2** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of acetonitrile:water (0 to 3 min (0:100), 5 min (20:80), 13 to 15 min (40:60), 18 min (50:50), 23 min (80:20), 26 min (85:15), 29 min (87:13), 30 min (95:5), 31 to 33 min (100:0)) at a flow rate of 0.8 mL/min.

**A**

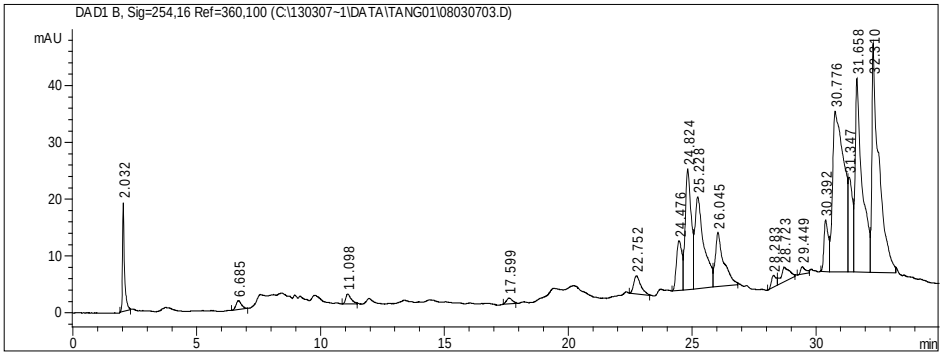



**B**

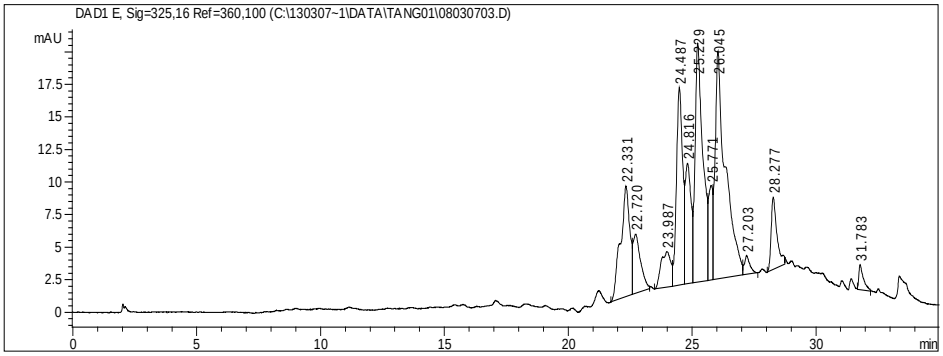


**3.3** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of methanol:water, (0 to 3 min (100), 5 min (20:80), 10 min (30:70), 18 min (40:60), 25 min (60:40), 28 min (85:15), 30 to 35 min (100:0)) at a flow rate of 0.8 mL/min.

**A**

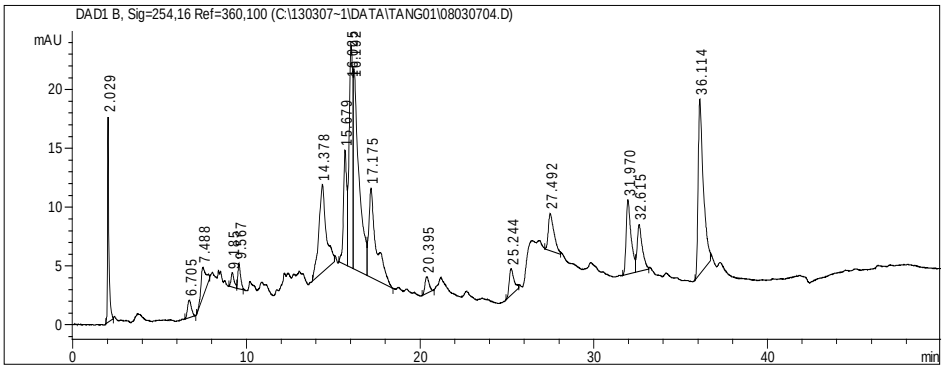



**B**

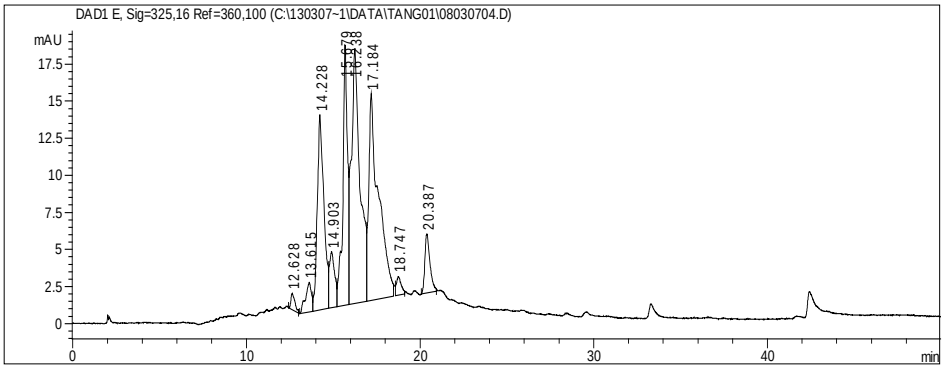


**3.4** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of methanol:water, (0 to 3 min (0:100), 5 min (30:70), 10 min (45:55), 13 min (50:50), 40 min (90:10), 45 to 50 min (100:0)) at a flow rate of 0.8 mL/min.

**A**

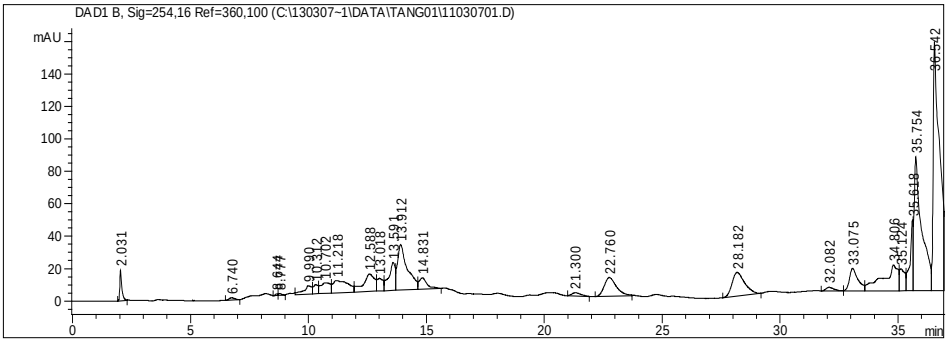



**B**

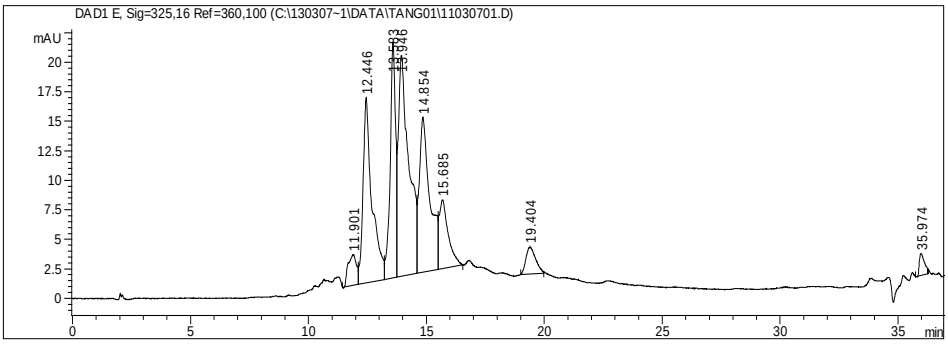


**3.5** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of methanol:0.1% Acetic acid in water, (0 to 3 min (0:100), 7 min (45:55), 10 min (50:50), 25 min (55:45), 30 min (65:35), 33 min (90:10), 35 to 37 min (100:0)) at a flow rate of 0.8 mL/min.

**A**

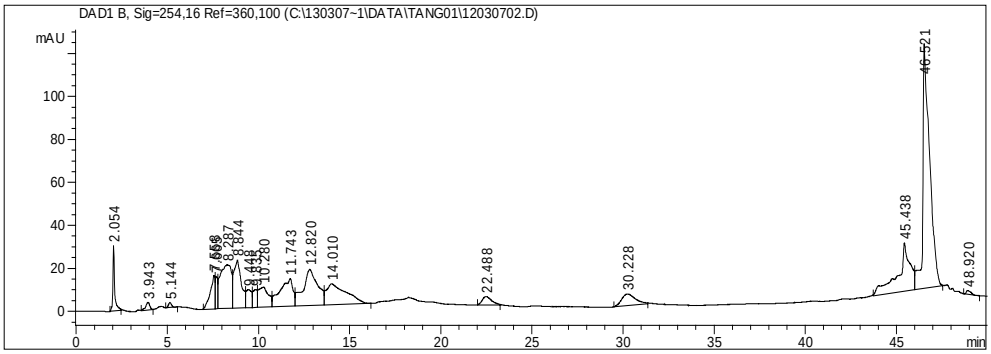



**B**

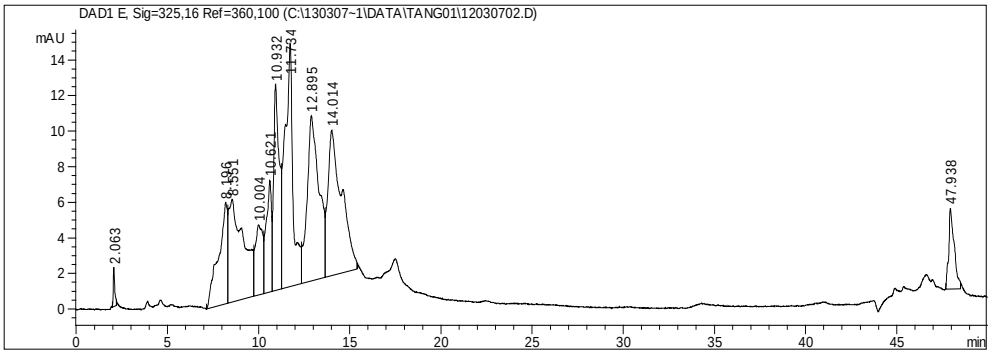


**3.6** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at (A) 254 nm and (B) 325 nm. The solvent systems used to separate the compounds was step gradient of methanol:water, 0 to 3 min (0:100), 5 min (45:55), 12 to 30 min (51:49), 35 min (57:43), 40 min (65:35), 43 min (100:0) at a flow rate of 0.8 mL/min.

**A**

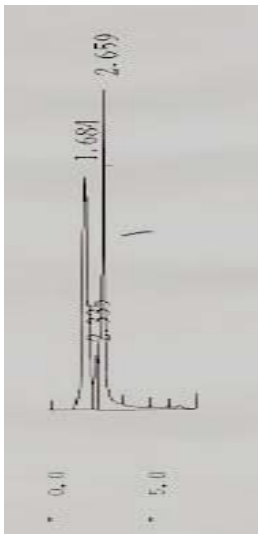



**B**

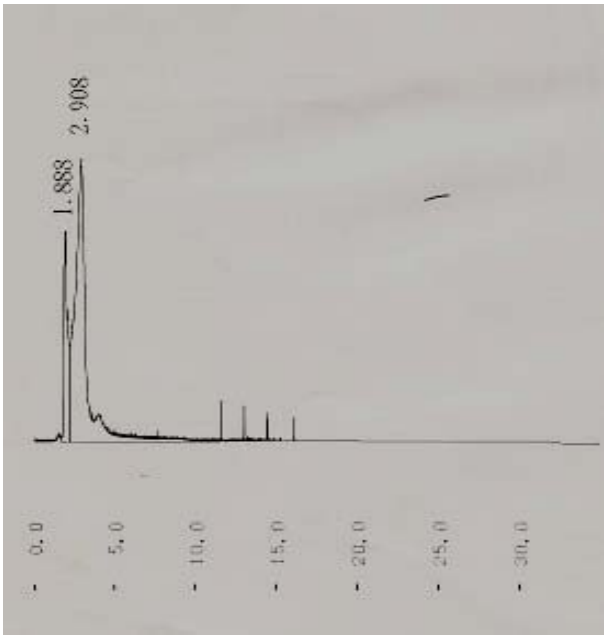


**3.7** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at 330 nm. The solvent systems used to separate the compounds was isocratic of 65% acetonitrile in water at a flow rate of 0.8 mL/min.

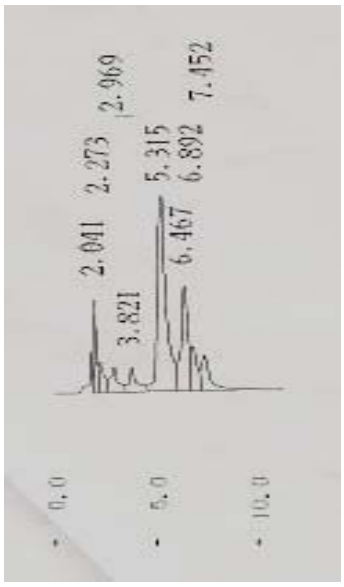

**3.8** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at 330 nm. The solvent systems used to separate the compounds was isocratic of 55% acetonitrile in water at a flow rate of 0.8 mL/min.



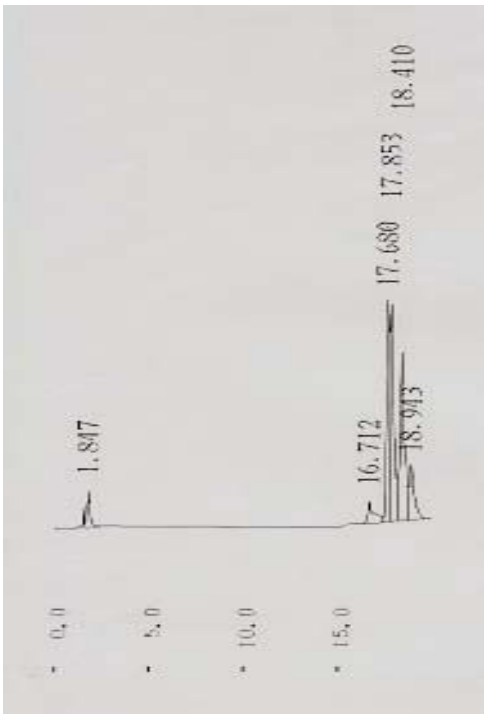
**3.9** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at 330 nm. The solvent systems used to separate the compounds was isocratic of 55% methanol in water at a flow rate of 0.8 mL/min.




**3.10** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at 330 nm. The solvent systems used to separate the compounds was isocratic of 45% methanol in water at a flow rate of 0.8 mL/min.



**3.11** HPLC chromatogram of Cl/sD separated by $C_{18}$ reverse phase column, visualized at 330 nm. The solvent systems used to separate the compounds was step gradient of methanol:water, (0 to 12 min ( 35:65), 12 to 20 min (50:50)) at a flow rate of 0.8 mL/min.



# APPENDIX C

# 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 μ g/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 tetraaacetate .$2H_2O$) 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. Reagents for total RNA isolation

### 4.1 1 M Tris-HCl pH 8.0 (100 mL)

Dissolve 12.11 g of Tris Base in 80 mL distilled water, then adjust pH with HCl to pH 8.0 and volume to 100 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min and store at room temperature.

### 4.2 10 M LiCl (100 mL)

Dissolve 42.39 g LiCl in distilled water to 100 mL final volume. Sterilize by autoclaving at 121°C for 20 min and store at room temperature.

### 4.3 RNA extraction buffer (1 L)

To prepare solution mix 100 mL of 1 M Tris-HCl pH 8.0, 10 mL of 10 M LiCl, 20 mL of 0.5 M EDTA, then adjust volume to 1 L final volume and add 10 g of SDS (sodium dodecyl sulfate). Store at room temperature.

### 4.4 3 M Sodium acetate pH 5.2 (100 mL)

Dissolve 24.6 g sodium acetate in 80 mL distilled water, adjust pH with gacial acetic acid to pH 5.2 and the volume to 100 mL. Sterilize by autoclaving at 121°C for 20 min and store at room temperature.

### 4.5 TE saturated phenol (pH 9.0)

Mix 300 mL TE (pH 8.0), 300 mL melted phenol at 50°C, and 4.5 g 8-hydroxyquinoline, then incubate at 4°C overnight and remove supernatant. Store at 4°C.

## 5. Reagents for northern blot analysis

### 5.1 10 X MOPS buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA) (1 L)

Dissolve 41.7 g MOPS free acid, 6.8 g sodium acetate, and 3.7 g EDTA in 700 mL sterile DEPC treated water, then adjust pH 7.0 with NaOH and the volume to 1 L with DEPC treated water. Store at 4°C.

### 5.2 1 X MOPS buffer (running buffer) (1 L)

Mix 100 mL of 10 X MOPS buffer, 20 mL 37% (=12.3 M) formaldehyde and 880 mL DEPC treated water and store at 4°C.

### 5.3 1.5% Formaldehyde agarose gel (100 mL)

Dissolve 1.5 g agarose in 100 mL 1 X MOPS buffer and heat in microwave.

### 5.4 RNA premix (500 µl)

Mix 62.5 µl 10 X MOPS, 109 µl 37% formaldehyde and 312.5 µl formamide and mix with RNA solution in ratio 1 : 1 (v/v).

**5.5 5 X RNA loading buffer (10 mL)**

Mix 16 µl saturated bromophenol blue, 80 µl of 0.5 M EDTA (pH8.0), 720 µl 37% formaldehyde, 3084 µl formalmide, 4 mL 10 X MOPS buffer, and 2 mL of 100% glycerol and then add DEPC treated water to final volume 10 mL (Stability 3 months at 4°C.

**5.6 20 X SSC (3 M NaCl, 0.3 M sodium citrate) (1 L)**

Dissolve 175.3 g NaCl, 88.2 g sodium citrate in 800 mL distilled water and adjust pH to 7.0 and the volume to 1 L. Sterilize by autoclaving at 121°C for 20 min and store at room temperature.

**5.7 Hybridization buffer (1 mM EDTA, 0.5 M phosphate buffer, 7% SDS, 1% BSA) (250 mL)**

Mix 0.5 mL of 0.5 M EDTA (pH 8.0), 85.5 mL of 1 M $Na_2HPO_4$, 39.5 mL of 1 M $NaH_2PO_4$, and 17.5 g SDS and adjust the volume to 250 mL with distilled water. Sterilize by autoclaving at 121°C for 20 min, then store at room temperature and sit to cool down to 60°C and add 2.5 g BSA. Store at room temperature.

## 6. Solutions for protein

**6.1 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.

**6.2 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.

**6.3 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.

**6.4 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.

**6.5 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.

**6.6 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.

### 6.7 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.

### 6.8 10% (w/v) Ammonium persulfate (1 mL)

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

## 7. Buffers and reagents for enzyme assay

### 7.1 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.

### 7.2 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.

### 7.3 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.

### 7.4 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.

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

### 8.1 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.

### 8.2 10% Triton X-100 (100 mL)

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

### 8.3 100 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.

### 8.4 10 mg/mL Lysozyme (1 mL)

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

**8.5 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.

**8.6 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.

**8.7 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.

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