CHARACTERIZATION OF β-GALACTOSIDASE FROM RICE (*Oryza sativa* L.)

Malika Chantarangsee

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การศึกษาสมบัติของเบตากาแลคโตซิเดสจากข้าว (*Oryza sativa* L.)

นางสาวมัลลิกา จันทรังษี

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(*Oryza sativa*, L.)

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

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ได้ทำการศึกษายีนที่ควบคุมการแสดงออกของเบตากาแลคโตซิเดส 2 ตัวคือ ยีน *OsBGal1* ซึ่งควบคุมการแสดงออกที่เมล็ดข้าวที่กำลังงอก และยีน *OsBGal 2* ควบคุมการแสดงออกที่ช่อดอก โดยพบว่ามีการแสดงออกในระดับสูงในรากและต้นอ่อนของเมล็ดข้าวที่กำลังงอก รวมทั้งในกาบใบของข้าวอายุ 15-30 วัน โดยพบว่ายีนทั้งสองมีการแสดงออกในระดับที่ต่ำในดอกและ immature seed และพบว่าเฉพาะ OsBGal2 มีการแสดงออกที่ mature seed โปรตีนเบตากาแลคโตซิเดสจากข้าวได้ถูกนำมาแสดงออกในรูปแบบของโปรตีนที่เชื่อมต่อกับไทโอรีดอกซินโดยใช้แบคทีเรียสายพันธุ์ OrigamiB (DE3) เพื่อที่จะผลิตเอนไซม์เบตากาแลคโตซิเดสที่มีกิจกรรมของเอนไซม์สูงซึ่งไม่พบในกลุ่มควบคุม ผลที่ได้จากการทำ western blot จากโปรตีนที่สกัดจากต้นข้าว ได้ข้อบ่งชี้ว่า โปรตีน OsBGal 1 แสดง immuno reactive ที่มีขนาดประมาณ 90 กิโลดาลตัน ส่วนโปรตีน OsBGal2 มีขนาดประมาณ 55 กิโลดาลตัน เมื่อเบตากาแลคโตซิเดสจากข้าวไอโซไซม์ที่ 1 (OsBGal1) ถูกผลิตขึ้นในสภาพที่เร่งปฏิกิริยาได้และถูกทำให้บริสุทธิ์ขึ้น พบว่าสามารถย่อยสลายพาราไนโตรฟีนอลเบตาดีกาแลคโตไซด์และยังเร่งปฏิกิริยาการนำกาแลคโตสไปเชื่อมต่อกับโมเลกุลของพาราไนโตรฟีนอลเบตาดีกาแลคโตไพราโนไซด์ และพาราไนโตรฟีนอลเบตาดีฟิวโคไพราโนไซด์แต่ไม่ย่อยพาราไนโตรฟีนอลตัวอื่น นอกจากกนี้ยังพบว่าสามารถย่อยสลายกาแลคโตโอลิโกแซคคาไรด์ที่มีพันธะ β-(1→3)-, β-(1→4)- และ β-(1→6)- ได้ผลผลิตเป็นน้ำตาลกาแลคโตส แต่ไม่ย่อยสลายน้ำตาลแลคโตสและสารที่มีโครงสร้างซับซ้อนมากขึ้น เช่น ไซโลกลูแคนโอลิโกแซคคาไรด์ เฮมิเซลลูโลซิคโพลีแซคคาไรด์ที่สกัดจากข้าวและเพคติน แต่กลับพบกาแลคโตสเกิดขึ้นเมื่อนำเอนไซม์ที่บริสุทธิ์ตัวดังกล่าวมาบ่มรวมกับสารสกัดที่ไม่ละลายในอัลกอฮอล์ที่สกัดจากรากและต้นอ่อนของข้าว

สาขาวิชาชีวเคมี ลายมือชื่อนักศึกษา_____

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MALLIKA CHANTARANGSEE: CHARACTERIZATION OF β-GALACTOSIDASES FROM RICE (*Oryza sativa* L.). THESIS ADVISOR: ASSOC. PROF. JAMES R. KETUDAT-CAIRNS, Ph.D. 157 PP.

β-GALACTOSIDASE/ RICE/ TRANSGLYCOSYLATION AND EXPRESSION

The genes encoding two isoforms of β-galactosidase expressed in germinating rice and panicle, designated *OsBGal1* and *OsBGal2*, were previously cloned and sequenced. The two genes were found to be relatively highly expressed in seedling roots and shoots and in leaf sheath in 15-30 day old plants. Both were also expressed at low levels in flowers and immature seeds, but only the *OsBGal2* transcript was found in mature seeds. The predicted mature proteins of the rice β-galactosidases were expressed as thioredoxin fusion proteins in *E. coli* strain OrigamiB (DE3) to produce β-galactosidase activities not found in control extracts. Western blot analysis indicated immunoreactive proteins of approx. 90 kDa for OsBGal1 protein and 55 kDa for OsBGal2 in extracts of these tissues. Purified OsBGal1 fusion protein hydrolyzed and transglycosylated *p*-nitrophenyl (*p*NP) β-D-galactopyranoside and *p*NP β-D-fucopyranoside. Other *p*NP-glycosides were not hydrolyzed. Galactose was released from β-(1→3)-, β-(1→4)- and β-(1→6)-linked di- and trisaccharides of galactose by this enzyme, but it did not hydrolyze larger [^{3}H]oligo-(1→4)-galactans (penta-, octa- and undecasaccharides), lactose, a galactosylated xyloglucan oligosaccharide (XLLG), total rice hemicellulosic polysaccharides or pectin. Galactose was also detected when the

purified OsBGal1 was incubated with rice root and rice coleoptile alcohol-insoluble residue.

School of Biochemistry

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LIST OF ABBREVIATIONS

AIR	Alcohol-insoluble residue
Ara	L-Arabinose
B:A:W	Butanol:acetic acid:water
B:Py:W	Butanol:pyridine:water
E:Py:A:W	Ethyl acetate:pyridine:acetic acid:water
EST	Express sequence tag
Fuc	L-Fucose
FW	Formular weight
g	Grams
g	Gravity
Gal	D-Galactose
GalA	D-Galacturonic acid
GH	glycosyl hydrolase
GH35	glycosyl hydrolase family 35
Glc	D-Glucose
l	Liter
LC/MS/MS	Liquid chromatography linked to tandem mass-Spectrometry
Man	D-Mannose
MLG	Mixed-linkage glucan

LIST OF ABBREVIATIONS (Continued)

MLG3-MLG6	Mixed-linkage glucan oligosaccharides
Mr	Molecular weight, relative
PC	Paper chromatography
OsBGal1	Rice β-glactosidase isozyme 1
OsBGal2	Rice β-glactosidase isozyme 2
PCR	Polymerase chain reaction
R_f	$\frac{\text{Distance moved by compound}}{\text{Distance moved by solvent front}}$
Rha	L-Rhamnose
Rib	D-Ribose
RT-PCR	Reverse transcription-polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
t	Time
TLC	Thin-layer chromatography
UTR	Untranslated region
UV	Ultraviolet
XLLG	Xyloglucan oligosaccharide
Xyl	D-Xylose
SDS	Sodium dodecyl sulphate

CHAPTER I

INTRODUCTION

1.1 Overview of β-galactosidase

β-galactosidase (EC 3.2.1.23) is an enzyme that catalyzes the hydrolysis of β-glycosidic bonds between nonreducing galactose residues and hydroxyl groups, such as in the cleavage of lactose to glucose and galactose (Figure 1.1). This enzyme has been found in numerous microorganisms, fungi, animals, and plants. It is a commercially important enzyme which has potential application in medicine, agriculture, biotechnology and food industry. In plants, β-galactosidase activity had been detected in almond, maize, tomato, asparagus, rice and root tip of various plants (Dey, 1986).

In microorganisms, the first purification of β-galactosidase from *Escherichia coli* was carried out by Cohn and Monod in 1961 who found that this enzyme can hydrolyze lactose and other β-galactosides to release monosaccharides (Wallenfels and Well., 1972). Moreover, the regulation and production of *LacZ* β-galactosidase in *E. coli* led scientists to understand more about gene regulation and made it an important model in the development of molecular biology. Many vectors in current use carry a short segment of *E. coli* DNA that contains the regulatory sequences (the lac promoter) and the coding information for the first 146 amino acid of β-galactosidase gene (*lacZ*) (Sambrook *et al*., 1989). The introduction of such vectors in

certain strains of *E. coli* containing the rest of the *lacZ* gene allows recombination to form a functional gene and production of *lacZ* β-galactosidase upon induction with lactose or the nonhydrolyzable lactose analogue IPTG. However, cloning of a DNA fragment into one of the cloning sites within this *lacZ* sequence will disrupt the protein coding sequence, resulting in a lack of β-galactosidase activity. Thus, cells containing recombinant clones appear white when grown on media containing X-Gal, while those with non-recombinant plasmid are blue due to the hydrolysis of X-Gal by β-galactosidase.

Moreover, for the application of β-galactosidase is of interest to food scientists for two main biotechnological uses: the removal of lactose from milk for lactose intolerant people and the production of galactooligosaccharides for use in probiotic food stuffs. β-galactosidase has been found in several strains of *Enterobacteriaceae,* and tests for fermentation of lactose play an important role in diagnostic bacteriology. Pluemsub (1998) noted that many microorganisms which have β-galactosidase activity have been shown to posses the capability to produce oligosaccharide sugars, which are widely used as food sweeteners.

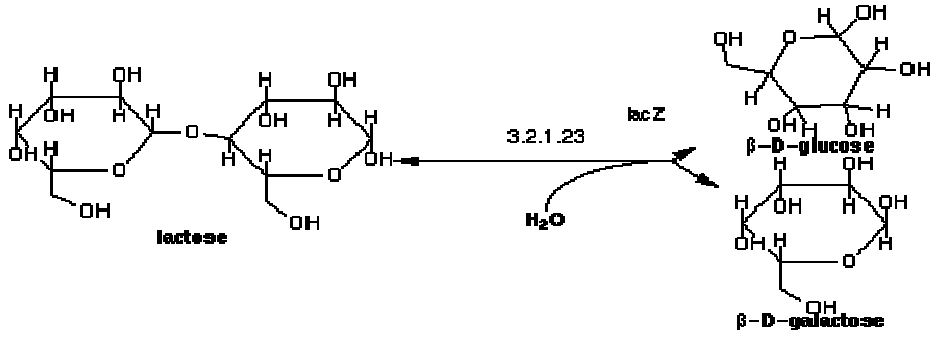


Figure 1.1 Enzymatic reaction of bacterial β-galactosidase from the Lac operon (Voet, D. and Voet, J., 1995).

The widespread occurrence of β-galactosidase in mammalian organs is related to the multiple physiological functions of the enzyme. In mammals, acid β-galactosidase is a lysosomal enzyme, which hydrolyzes the terminal β-D-galactoside residue from glycolipids and glycoproteins during the normal catabolic process of these tissue constituents (Nanba and Suzuki, 1990). Its deficiency has been shown to cause a number of diseases, such as GM_1 gangliosidosis, which is a disorder of sphingolipid degradation, galactosidosis, which is a disorder of glycoprotein degradation and Morquio B syndrome, which is a disorder of glycosaminoglycan degradation, as well as mucolipidosis II and III, which are caused by defects in transport of the precusor enzyme (Oshima *et al*., 1988). β-galactosidase has been reported in the intestine of various animals, such as dog, rabbit, calf, sheep, and goat, in the digestive juice of snails, and in the saliva and intestinal secretions of human, where it is essential for the breakdown of nutritional β-galactosides.

In plants, β-galactosidase activity has been found in the seeds of many species, including cucumber, pumpkin, Thai jute, mung bean and blackwood (Surarit *et al*., 1995), as well as in ripening fruit and senescing tissues (Goulao, 2007). β-galactosidase is widely distributed in nature, but the physiological role of this enzyme in plants is still not well characterized. β-galactosidase activity is often measured using artificial substrates, however numerous studies have shown that β-galactosidase catalyzes the hydrolysis of terminal galactosyl residues from carbohydrates, glycoproteins and glycolipids. Its activity may be related to the degradation of fruit cell walls, such as in ripening tomatoes and apples. β-galactosidase action has been proposed to release stored galactose as a source of energy for rapid growth, release

free Gal during normal metabolic recycling of galactolipids and cell wall components, and degrade cell wall components during senescence (Smith and Gross, 2000). In addition, it has been suggested that β-galactosidase is more specifically involved in the degradation of the core structure of arabinogalactan, which contains β-1,4-linked-D-galactopyranoside units.

Physiological and biochemical data show that Gal is the most dynamic sugar residue of the cell wall during tomato fruit development (Kim *et al*., 1991). In particular, these physiological studies showed that there was a significant net loss of galactosyl residues from the wall throughout fruit development and the rate of galactosyl residue loss increased during ripening. The presence of at least 7 isozymes of β-galactosidase in tomato fruit, suggests they may indeed play several roles including the release of galactosyl residues from cell wall pectins. (Smith and Gross, 2000).

Cell walls of monocotyledons are quite different from those of dicotyledons and have a low content of galactose containing pectic polysaccharides, so the role of β-galactosidase participating in the metabolism of cell walls in monocotyledons remains to be investigated (Jarvis *et al*., 1988). King and Davies (1995) suggested that β-galactosidase in asparagus spears functions mainly as a cell wall glycosidase which removed galactose units in an exo fashion from cell wall polysaccharide. Moreover, the proportion of galactose in spear cell wall decreases after harvesting and the released galactose residues may be used as a respiratory substrate for the rapidly deteriorating asparagus spear (O'Donoghue *et al.* , 1998).

1.2 Glycosidases

O-Glycoside hydrolases (3.2.1.-) are a widespread group of enzymes which hydrolyse α or β glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glycosyl hydrolases transfers the glycosyl bond to a water molecule; glycosyl transferases transfer this glycosyl bond to the -OH group of another glycosyl residue. Enzymes which catalyze the hydrolysis of glycosidic linkages are widely distributed in nature and include β-galactosidase, invertase, maltase, α-galactosidase, β-glucosidase, amylase etc. Glycosidases are also known as carbohydrolases. The existence of glycosidases has been known for more than 100 years and they were the very first biological catalyst investigated. Different glycosidases form different products and display a complete range of different bond specificity. For example, α-amylase hydrolyzes endo-α-(1-4) bonds in polysaccharide chains and produce linear α-(1-4) linked oligosaccharides with α-D-glucose residues on the reducing end of the oligosaccharide released from the nonreducing end (so-called retaining enzyme) (Boel *et al*., 1990), whereas a β-amylase also hydrolyzes α-(1-4) bonds, but cuts off only maltose from the non-reducing ends (exo) and forms a different anomeric configuration (β-D-glucose; a so-called inverting enzyme) (Mikami *et al*., 1992). Some enzymes prefer to use substrates of a certain chain length, for instance, cyclodextrin glycosyltransferases use predominantly linear and singly branched chains of amylopectin with a degree of polymerization of 13, 14 or 15 (Lawson *et al*., 1994).

1.3 Catalytic mechanism

The enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis that requires two critical residues: a proton donor and a nucleophile/base (Davies and Henrissat, 1995). This hydrolysis occurs via two major mechanisms giving rise to either an overall retention or an inversion of anomeric configuration (McCarter and Withers, 1994) In the proposed mechanism for inverting enzymes, the glycosidic oxygen is initially protonated by a general acid catalyst, followed by a nucleophilic attack on the C1 atom of the sugar by a water molecule, which is activated by extraction of proton by a carboxylate base (Figure 1.2a), leading to inversion of the anomeric conformation. This is known as a single displacement mechanism (Koshland, 1953), bond breaking and bond making both proceed in a single concrete step. The reaction rate depends on the concentrations of both nucleophile and substrate.

The retaining reactions proceed via a double-displacement mechanism (Vernon, 1967). The first step involves a similar protonation of the glycosidic oxygen by a general acid, as in the inverting mechanism, creating an intermediate, which is attacked by a water nucleophile assisted by the base form of the acid catalysis in the second step (Figure 1.2b, c). Each step inverts the configulation of the anomeric carbon. The two displacement steps therefore create an overall retention of the configuration. For retaining enzymes, the intermediate could either be an oxo-carbonium ion (Figure 1.2b) which is electrostatically stabilized by a carboxylate, or involves formation of a covalent bond (Figure 1.2c), in which one of the catalytic carboxylates is presumed to act as a nucleophile.

Transglycosylases (e.g. Cyclodextrin glycosyl transferases) employ a reaction mechanism similar to that described for retaining hydrolases. For these enzymes however, the second step of the reaction does not involve a water nucleophile, but the non-reducing end of a saccharide, possibly assisted by the base form of the acid catalyst (Sinnott, 1990).

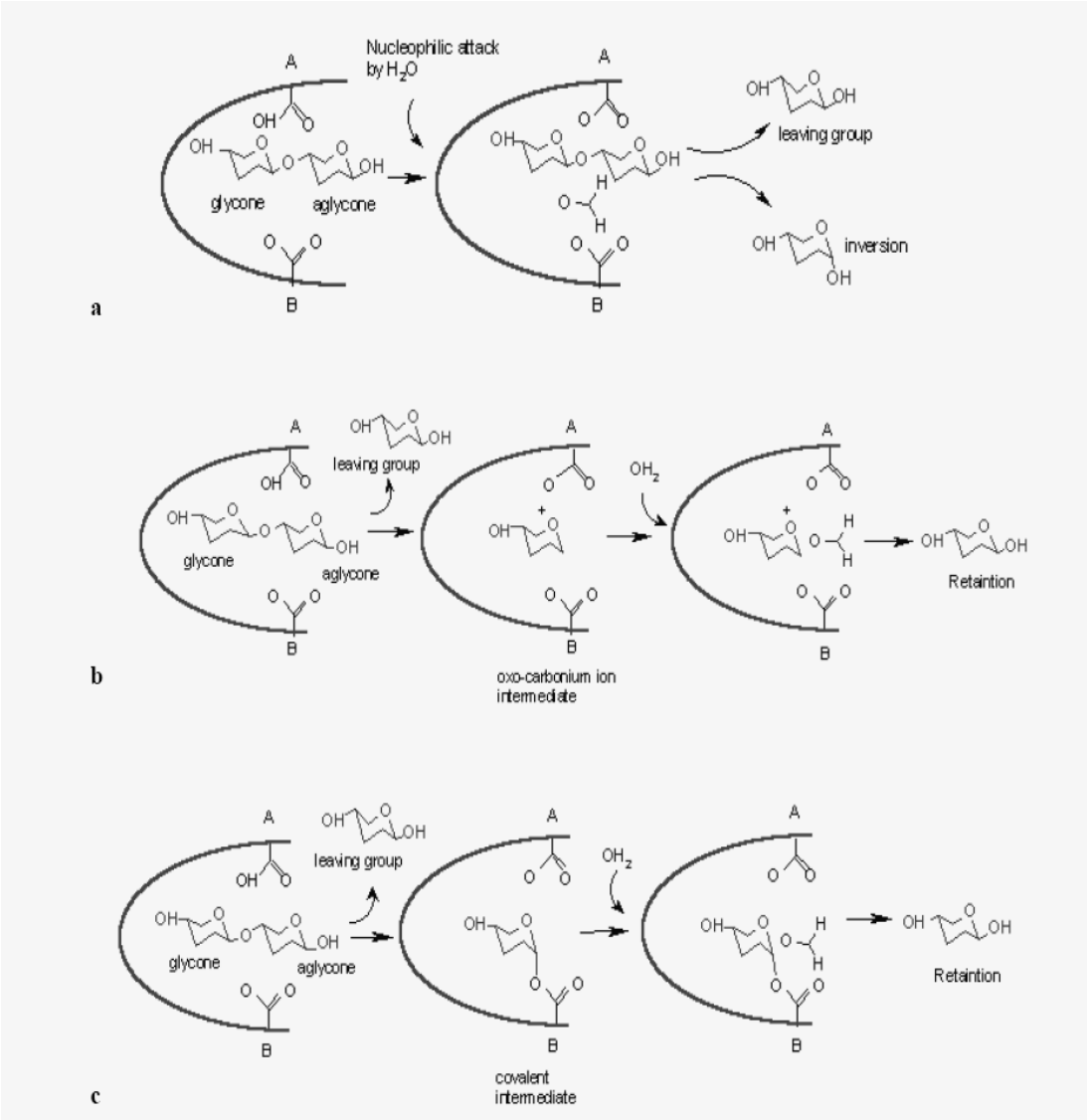


Figure 1.2 Catalytic mechanisms of glycosidases: inverting enzyme (a) and retaining enzyme acting through an exo-carbonium ion (b) or via a covalent intermediate (c).

1.4 Plant Glycosyl Hydrolases

Glycosyl hydrolases are a set of gene families that are of particular interest to investigate in plants. Plants have a wide variety of glycosides and polysaccharides, which carry-out many functions. These include cell wall polysaccharides, which form the bulk of the structure and biomass of plants, as well as starch and other forms of storage carbohydrates. Glycosides found in plants include glycolipid components of cellular membranes and pigments, as well as many reactive or bioactive species that are blocked from their activity by sugars for storage or to allow reactions at other positions on the molecule. The compounds with sugar blocking groups include phytohormones, which can be released to active forms by glycosyl hydrolases, lignin precursors, which can be transported to the area of lignification, then activated, and defense compounds, which can be activated by mixing of the glycosyl hydrolase with the glycoside upon fungal invasion or herbivore breakage of the cells. So, the glycosyl hydrolases may play important roles in plant growth, development, and adaptation. In addition, glycosyl hydrolases have great potential for biotechnological applications, such as in release of nutrients and flavor compounds in plant-derived foods and feeds, conversion of certain sugars, like lactose in milk products, to more desirable sugars, and in biomass conversion of cellulose and other polysaccharide waste to useful products, including fuels. Beta-glycosidases, including beta-glucosidases, beta-galactosidases and related enzymes are particularly interesting in this regard.

Glycosyl Hydrolases have been divided into families related by their amino acid sequences by Henrissat and colleagues (Henrissat, 1991; Henrissat and Bairoch, 1993, 1996). These classifications have identified over 100 such families at this point

in time, as documented in the CAZY (Carbohydrate Active Enzyme) website (http://www.cazy.org). The families can be grouped into clans related by their 3 dimensional structures. Clan A, for instance, is made up of enzymes with $(\beta/\alpha)_8$ barrel structures with carboxylic acids that act as catalytic acid/bases and nucleophiles on beta-strands 4 and 7, respectively. This clan includes families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 50, 51, 53, 59, 72, 79 and 86, which include enzymes with a wide variety of activities, despite in the similar structures.

1.5 β-Galactosidases

The enzymes responsible for hydrolyzing galactosidic linkages are termed as galactosidases. There are two types of galactosidases: α-galactosidases and β-galactosidases, depending on the configulation of the anomeric carbon atom of galactose in the substrate molecule on which they act (Figure 1.3).

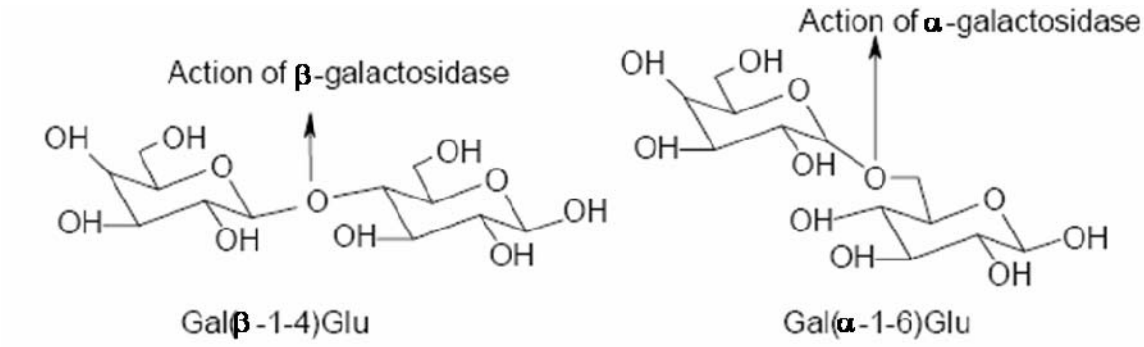


Figure 1.3 Configuration of the anomeric carbon atom of galactose in the substrate molecule

β-D-Galactosidases (EC 3.2.1.23), which hydrolyze the glycosidic bond between a nonreducing galactose residue and another sugar or alcohol, have been described in many different organisms, including microorganisms, plants, and animals. In these organisms, β-galactosidases are involved in breakdown of carbohydrates, glycolipids and glycoproteins containing galactose. Aside from their natural functions, β-galactosidases have several important applications, including use as a marker in genetic engineering and hydrolysis of lactose and production of probiotics in the dairy and food industries [Vasiljevic and Jelen, 2003].

To date, the β-galactosidases that have been described at the molecular level fall into 4 related glycosyl hydrolase (GH) families: 1, 2, 35, and 42, with the enzymes that have been characterized in plants being in family 35 [Henrissat, 1991]. GH family 35, like other families including β-galactosidases, falls into GH Clan A, in which the catalytic domain is a $(\beta/\alpha)_8$ barrel, which catalyzes hydrolysis of the glycosidic bond by a retaining mechanism [Jenkins *et al*., 1995; Henrissat *et al*., 1995]. In this clan, the catalytic acid/base and nucleophiles are found at the carboxyl ends of β-strands 4 and 7, respectively. The catalytic nucleophiles have been defined for the GH family 35 β-galactosidases from *Xanthamonas manihotas* and *Bacillus circulans* by labeling with a 2-deoxy-2-fluoro-galactoside [Blanchard *et al*., 2001]. The acid/base and nucleophile could also be putatively identified from the crystal structure of the *Penicillium* sp. β-galactosidase [Rojas *et al*., 2004].

The functions of β-galactosidases are various. In *Escherichia coli*, the lacZ β-galactosidase allows the bacteria to hydrolyze lactose for use as an energy source, and other bacterial and fungal β-galactosidases may have similar roles. In mammals,

lactase–phlorizin hydrolase (LPH) plays a similar role in allowing digestion of lactose in milk, while lysosomal β-galactosidases are involved in the degradation of glycolipids, glycoproteins and proteoglycans [Callahan, 1999; van der Spoel *et al*., 2000]. The importance of these enzymes in underscored by the fact that their deficiencies cause human metabolic disorders, lactose intolerance for LPH and G_{M1}-gangliosidosis and Morquio B syndrome for lysosomal β-galactosidase. In plants, the activity of β-galactosidases has mainly been described in the processes of growth, development, senescence and fruit ripening, where they are typically thought to act on cell wall carbohydrates [Konno and Kato, 1992; Ross *et al*., 1994; Carey *et al*., 1995; Li *et al*., 2001]. Though animals and microorganisms have only a few β-galactosidases, mRNA for at least 7 family 35 glycosyl hydrolase genes likely to be β-galactosidases have been detected in ripening tomato [Smith and Gross, 2000]. Knock-down of one of these genes by antisense RNA has significant effects on tomato ripening and particularly the cell wall [Smith *et al*., 2002]. Thus, it appears that at least some of these enzymes are involved in cell wall remodeling, though the large number expressed in the fruit suggests they may play a variety of roles.

Most of the studies on plaṇt β-galactosidases have been done on dicotyledons, in which the cell wall contains a relatively large amount of pectic polysaccharides compared to gramineous monocotyledons. It was suggested that β-galactosidases from several plants are involved in the removal of galactose from pectic and hemicellulosic polysaccharides and glycoproteins of cell walls [Kotake *et al*., 2005]. The increase in β-galactosidase in asparagus during harvest-induced senescence would support such a role in a non-gramineous monocot [O'Donoghue *et al*., 1998],

which has cell wall structure more similar to dicots than gramineous monocots. Relatively little work has been done on gramineous monocots, in which the role in the cell wall is less obvious.

Among grains, β-galactosidases have been described in barley and rice. Giannakouros *et al*. [1991] were able to separate 4 isozymes of β-galactosidase from germinating barley. Recently, a β-galactosidase purified from barley was shown to be a heterodimer with 35 and 45 kDa subunits, which appeared to have originated from the same precursor protein [Triantafillidou and Georgatsos, 2001].

1.6 Rice β-galactosidase

Rice (*Oryza sativa* L.) is one of the world's most important crops. Knowledge of rice molecular genetic and physiology are important to develop the molecular crop improvement techniques (Gurdev and Gary, 1991). Moreover, rice is the primary monocot genome model, with a complete genome sequence available. Therefore, studies in rice allow the use of genome sequence data, as well as cDNA tools, like expressed sequence tags (ESTs) and full length cDNA clones, to aid the investigation.

Germination and seedling development of rice start when seed dormancy has been broken with adequate water and the seed is exposed to a temperature ranging from about 10-40°C. The physiological definition of germination is usually the time when the radical or coleoptile (embryo shoot) emerges from the ruptured seed coat. The rice grain is similar to the other cereal grains in its mechanism of starch, protein, and lipid biosynthesis during grain ripening. Many metabolic and structural changes have been in observed. Many enzymes, including phosphatases, proteases, esterases,

lipases, peroxidases, catalase, and glycosidases, such as β-glucosidase, α- and β-galactosidase, increase by the fourth day of germination and have peak activities by the fifth to seventh day (Palmiano and Juliano, 1973). The site of production of many of these enzyme during seed germination is in the aleurone layer.

Konno and Tsumuki (1993) found that rice β-galactosidase that was purified by DEAE ion exchange, Sephacryl S200 gel filtration and affinity chromatography using on *p*-aminophenyl-β-D-thiogalactopyranoside-linked Sepharose 4B gave a single protein band with a molecular mass of 42 kDa, similar to the size found for β-galactosidase from radish seeds (45 kDa, Sekimato *et al*., 1989). The enzyme from rice shoot could hydrolyze larchwood arabinogalactan in an exo-fashion similar to its action on *pNP*-β-D-galactopyranoside, which was used as substrate in the standard assay. In addition, they found that the enzyme exhibited hydrolytic activity toward lactose and had a small amount of activity toward polysaccharides containing β-D-glucopyranosyl linkages, such as laminarin and lichenan. An arabinose and galactose rich, CDTA-extracted fraction from rice cell walls also served as a substrate from which galactose and glucose were released. Recently, Kaneko and Kobayashi [2003] isolated a β-galactosidase from the medium of rice suspension cells, and showed it to contain 40 and 47 kDa subunits. This protein was also found to release galactose from cell-wall-derived polysaccharides, consistent with a role of rice β-galactosidases in cell wall remodeling.

To date, rice β-galactosidases have not been well studied, X-Gal staining of germinating rice (Figure 1.4) shows the strong localization to the embryo and aleurone layer, and later to the shoot and root tip, (Chantarangsee, 2001) suggesting

β-galactosidases may play a role in carbohydrate mobilization and growth. Moreover, two full-length cDNA clones, OsBGal1, derived from the cDNA library screening, and the related clone, OsBGal2 (E1405) provided by the MAFF clone bank were sequenced. Sequence comparison suggested that the amino acid sequence of the OsBGal1 clone contains a galactose-binding lectin domain at the C-terminus that might have a function in binding to the cell wall, while the OsBGal2 clone lacked this domain. For OsBgal2, preliminary EST analysis indicated expression in leaves, including leaves under certain stresses, such as drought and rice blast fungus (*Magnaporthe grisea)* infection (Chantarangsee, 2001). Thus, β-galactosidases may have an unexpected role in response to biotic and abiotic stress. However, the precise role of the β-galactosidase in rice plant can only be speculated. To evaluate the role of the gene in rice, expression of the protein in *E. coli* or yeast expression system to produce enough enzymes to characterize the β-galactosidase activity was done here. Further study of the protein purified from rice was begun to evaluate posttranscriptional modification, such as glycosylation and proteolytic processing, along with localization in the cells or tissues.

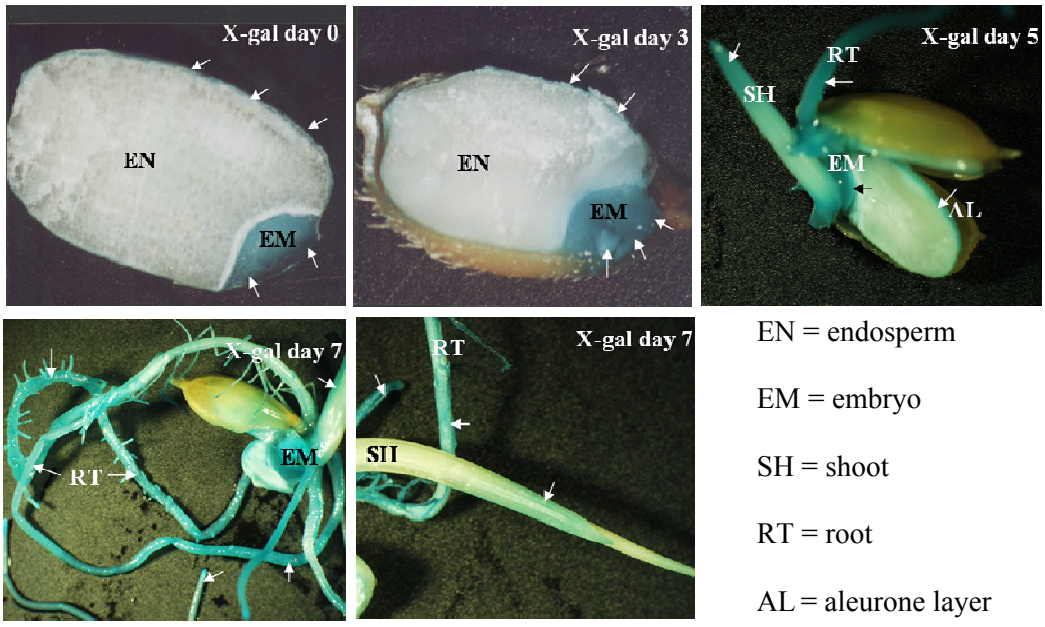


Figure 1.4 Localisation of β-galactosidase activity in rice seeds and seedlings 0-7 days after soaking. The seed was cut in half before application of the staining solution (X-Gal). The arrows point to staining tissues. The abbreviations are: AL, aleurone; EM, embryo; RT, root; SH, shoot

In order to begin to identify the rice β-galactosidase genes, the sequences of two β-galactosidases mRNA from rice were determined by cDNA library screening and database searching. For sequence determination of the first rice β-galactosidase cDNA (*OsBGal1*), a partial cDNA sequence was previously identified in Prof. Dr. Tatsuhito Fujimura's laboratory by cDNA library screening. A 400 bp cDNA fragment was amplified from rice seedling RNA by RT-PCR with degenerate primers based on conserved protein sequences found in GH family 35. This product was used to probe an immature rice seed cDNA library, resulting in the cloning of a 2,529 bp partial cDNA, the uninterrupted reading frame (URF) of which appeared to be

missing the start codon. The sequence was used to search the Monsanto rice genome database, and was found to match the OSM198 contig (found in Genbank accession no. **AC104428**, rice chromosome 3). A primer was designed based on the sequence just 5′ of the apparent start codon of the gene in this contig and used with primers from the 5′ end of the cDNA library clone to amplify the 5′ region by RT-PCR to verify the 5′ sequence of the mRNA. The complete cDNA sequence is in Genbank under accession no. **AF508799**. The longest open reading frame (ORF) in this sequence is 2,529 nucleotides and encodes an 843 amino acid protein. A second, closely related gene was identified by a BLAST search of dbEST, and the clone for the EST (E1405) was sequenced completely. The sequence was found to match a genomic sequence in Genbank Accession **AP004729**, which maps to chromosome 6, along with several full-length cDNA clones in Unigene cluster OS6322, including **AK102756**, **AK059741**, and **AK105619** (Kikushi et al., 2003). This sequence contained a 2,151 bp ORF, encoding a 715 amino acid protein. The open reading frames (ORFs) of two β-galactosidase clones shared high amino acid sequence identity with each other and other published plant β-galactosidases. Though some gaps were seen over the length of the alignment of the two proteins, the primary reason for the greater length of OsBGal1 was a C-terminal extension, which is homologous to the galactose-binding lectin from sea urchin. This extension has been noted previously in several GH family 35 members, and has been speculated to act as a carbohydrate binding domain (Trainotti *et al*., 2001; Triantafillidou and Georgatsos, 2001). Both proteins were predicted to contain N-terminal signal sequences by SignalP, which predicted the first 25 residues of OsBGal1 and the first 20 residues of OsBGal2 would be cleaved from the protein during transport into the endoplasmic

reticulum (ER). The remaining 816 residues of OsBGal1 are predicted to form an 89.7 kD protein with a pI around 5.9, while the 697 residue mature OsBGal2 protein is predicted to have a Mw of 76.6 kD and a pI of 7.7. OsBGal1 contained two typical NXS/T *N*-glycosylation sites, while OsBGal2 contained only an NPT site, which is unlikely to be glycosylated.

1.7 Cell wall structure and metabolism

An early model of the plant primary cell wall is that the cellulose fibrils, which were coated with hemicellulose and the matrix composed of pectic polysaccharides and proteins. Cellulose consists of linear chains of β-(1,4)-linked residues, which are aggregated together with hydrogen bonds to form fibrils. Hemicellulose is composed of a variety of polymers, including xyloglucan, glucomannan, and galactoglucomannans. Pectic polysaccharides are also composed of a variety monosaccharides, the neutral pectic polysaccharides being arabinan, galactan or arabinogalactans (Fig 1.5). It is thought that these polymers are linked together in the three dimensional structure of the cell wall by a variety of covalent and noncovalent bonds. Cellulose fibrils are held together by hydrogen bonds and similar bonds account for the interaction of cellulose with hemicellulose. However, it has been postulated that, in addition to simply coating the cellulose fibril, hemicellulose molecules may cross-link the cellulose fibrils. Seymour et al. (1993) proposed releasing or relaxation of such linkages could be a major cause of cell wall loosening and softening.

Brett and Waldron (1996) noted that the enzymes that are located in the cell wall include peroxidase, cellulase, pectinase and a number of exoglucosidases, which

have been reported to be involved in cell wall turnover, including β-glucosidase, β-xyloidases, α-galactosidases, and β-galactosidases. Much attention have been given to xyloglucan endotransglycosylase (XET), which is found in a range of walls and may be involved in insertion of xyloglucan into the wall and possibly in control of wall extensibility. One possible natural substrate of β-galactosidase may be the polymers containing β-galactosidase in plant cell walls, such as arabinogalactan, a polymer of arabinose and galactose associated with the rigidity of the wall. In fact, in several cases, β-galactosidases from ripening fruit have been shown to attack native galactan polymer. These enzymes remove terminal β-galactosidase residues, so they can be considered as true β-galactosidases and could well be implicated in wall degradation.

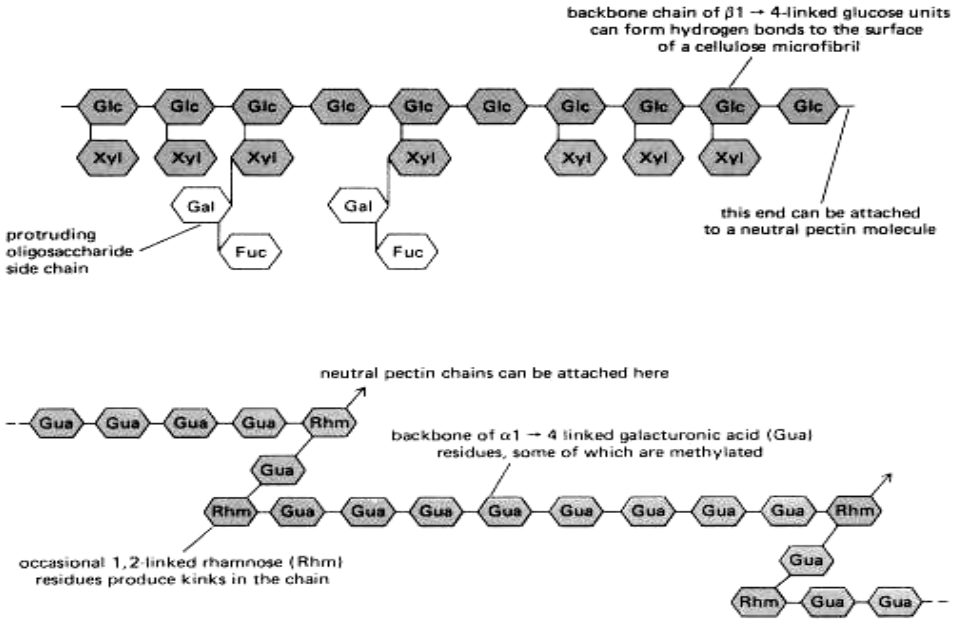


Figure 1.5 A hemicellulose molecule (A) and an acidic pectin molecule (B) from the cell wall of a higher plant (Albert, B. *et al.*, 1989).

1.8 The rice genome

Rice (*Oryza sativa* L.) is a model plant for the grasses which is the first crop plant to be sequenced. Sasaki and Burr (2002) noted that rice has the smallest genome of the major cereals, which also has dense genetic maps and relative ease of genetic transformation. The International Rice Genome Sequencing Project (IRGSP) was formally established in 1998 to obtain a complete sequence of the rice genome by pooling the resources of sequencing groups in ten nations. The IRGSP adopted the clone-by-clone shotgun sequencing strategy so that each sequence cloned can be associated with a specific position on the genetic map, and it follows a policy of immediate release of the sequence data to the public domain. In 2004, IRGSP released a high quality map-based draft sequence while 3 completely sequenced chromosomes were previously published: rice chromosome 1 (Sasaki *et al*., 2000), rice chromosome 3 (Feng *et al*., 2002) and rice chromosome 10 in 2003. The genome of the japonica subspecies of rice was sequenced and assembled by whole-genome shotgun sequencing by Goff *et al*., (2002). The assembled sequence covers 93% of the 420-megabase genome. Gene predictions on the assembled sequence suggest that the genome contains 32,000 to 50,000 genes. Homologues of 98% of the known maize, wheat, and barley proteins are found in rice.

Yu *et al*., (2002) produced a draft sequence of the rice genome for the most widely cultivated subspecies in China, *Oryza sativa* L. ssp. indica, by whole-genome shotgun sequencing. The genome was 466 Mb in size, with an estimated 46,022 to 55,615 genes. Functional coverage in the assembled sequences was 92.0%. About 42.2% of the genome was in exact 20-nucleotide oligomer repeats, and most of the transposons were in the intergenic regions between genes. Although 80.6% of

predicted *Arabidopsis thaliana* genes had a homolog in rice, only 49.4% of predicted rice genes had a homolog in *A. thaliana.*

The completion of the rice genome sequencing project has provided an opportunity to examine the gene structure for all members of GH family 35 and other gene families within a representative model mocot. The high-quality and map-based sequence of the entire genome is now available in public databases, the International Rice Genome Sequencing Project (2005) provided evidence finished quality sequence that cover 95% of the 389 Mb genome, including all of euchromatin and two complete centromeres. This genome sequences data will help to improve the nutrition quality and production of rice.

1.9 Objectives

The objectives of this thesis include

1. To determine the number and sequences of β-galactosidases in rice and the sequences of their protein products.
2. To determine the expression patterns of some β-galactosidase genes in different rice organs and developmental stages.
3. To express a β-galactosidase protein in recombinant systems in order to characterize the activity of the specific isozyme in terms of substrate specificity and other important parameters.
4. To produce antibodies specific for two isozymes in order to characterize the location of the protein in the plant and identify the size of the natural protein by western blotting.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Indica rice (*Oryza sativa* L., cv. KDML 105) seeds were purchased from the Department of Agriculture, Nakhon Ratchasima and were germinated for 10 days at room temperature under sterile conditions on tissue paper moistened with sterile distilled water.

2.1.2 Chemicals

Acrylamide, bis-N,N'-acrylamide, ammonium persulfate, N,N', N'',N'''-tetramethylethylenediamine (TEMED), lysozyme, sodium dedocyl sulfate (SDS) and Triton X-100 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Bromophenol blue, chloroform, copper sulfate, disodiumethylenediamine tetraacetate (EDTA), ethanol, glacial acetic acid, glycerol, glycine, hydrochloric acid, methanol, piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES), potassium chloride, potassium hydroxide, sodium dihydrogen phosphate, and sodium hydrogen phosphate were purchased from Carlo ERBA (Rodano, Milano, Italy). Calcium chloride, chloramphenical, Coomassie brilliant blue R-250, imidazole, diethyl pyrocarbonate, ethidium bromide, magnesium chloride, phenyl methylsulfonylfluoride (PMSF), and sodium citrate were purchased from Fluka (Steinheim, Swizerland). Bacto tryptone,

yeast extract, and Bacto agar were purchased from DIFCO (Grayson, IL, USA). Ampicillin was purchased from Merck (Damstadt, Germany). *Taq* DNA polymerase, *Pfu* DNA polymerase, agarose (molecular grade), deoxyribonucleotides (dATP, dCTP, dTTP, dGTP), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were purchased from Promega (Madison, WI, USA). Restriction endonucleases *Nco*I, *Xho*I, *Pst*I, *Eco*RI and *Eco*RV were purchased from New England Biolabs (Beverly, MA, USA). Other chemicals and molecular reagents used but not listed here were purchased from a variety of suppliers.

2.1.3 PCR primers

Oligonucleotide primers for cDNA amplification were designed from rice β-galactosidase cDNA sequence and ordered from Geneset/ Proligo (Singapore) and the BioServices Unit (BSU) of the National Science and Technology Development Agency (Bangkok, Thailand).

2.2 General methods

2.2.1 RNA extraction

Isolation of total RNA from rice was done using TRIZOL Reagent (GIBCO-BRL, Grand Island, NY) developed by Chomczynski and Sacchi (1987). One hundred milligrams of germinated rice seeds were pulvarized by grinding with a mortar and pestle in liquid N_2 transferred to a new sterile microtube containing 1 ml of Trizol reagent and mixed. The homogenized sample was incubated for 5 min at room temperature, followed by addition of 0.2 ml of chloroform. The mixture was mixed vigorously by inversion for 15 sec, incubated at room temperature for 2-3 min,

and then centrifuged at 12,000 x g for 1 min at 4°C. The colorless upper aqueous phase containing RNA was transferred into a fresh tube. The RNA from the aqueous phase was precipitated by mixing with 0.5 ml of isopropanol, left at room temperature for 10 min, and pelleted by centrifugation at 12,000 x g for 10 min at 4°C. The pellet of RNA was washed with 75% (v/v) ethanol (in DEPC–treated water) and dried briefly at 37°C. Finally the dried RNA pellet was resuspended in 20 ml of DEPC-treated water. The RNA solution was kept at -70°C.

2.2.2 Determination of quantity and purity of RNA

The concentration of the RNA solution was determined by measuring the UV absorbance at 260 nm of a 1 µl aliquot, diluted to 1 ml in distilled water. The A_{260}/ A_{280} was measured with a Lambda Bio20 UV/VIS Spectrometer (Perkin Elmer, Shelton CT). Normally, a A_{260}/A_{280} ratio of approximately 1.8-2.0 indicates good purity of RNA. The RNA content was calculated using the A_{260} value of single strand RNA according to following equation:

$$\text{Total RNA} = (A_{260} \times \text{dilution factor} \times 40\ \mu g/ml)$$

One absorbance unit was assumed to be equivalent to 40 µg/ ml RNA (Sambrook *et al*., 1989)

2.2.3 Reverse transcription of RNA to cDNA

One microliter of 50 mM Oligo $(dT)_{20}$ primer and RNA (5 µg) were combined and the volume adjusted to 10 µl with DEPC-treated water in 0.5 ml tube. The tube was incubated at 65°C for 15 min and then placed on ice immediately to denature the secondary structure which may impede full-length cDNA synthesis. In the meantime,

5 X cDNA synthesis buffer was mixed in a microtube on ice, which was composed of 4 μl 5 X cDNA synthesis buffer, 1 μl 0.1 M DTT, 1 μl *RNase*OUT (40 units/μl), 1 μl DEPC-treated water, 1 μl 10 mM dNTP mix, and 1 μl Thermoscript reverse transcriptase (15 units). The master reaction was pipeted into the RNA-primer tube on ice which was then transferred to a GeneAmp® System 9700 thermocycler (PE Applied Biosystems, Foster City, CA) preheated to 55°C and was incubated for 60 min. The reaction was terminated by heating the tube at 80°C for 5 min and then placed on ice. One microliter of *RNase* H (40 units) was added and the reaction was incubated at 37°C for 20 min to digest the complementary RNA which was basepaired with the first strand cDNA. *RNase* H digestionnimprove the RT-PCR signal of many targets and is required for the efficient and consistent amplification of long RT-PCR templates. The first strand cDNA was kept at -30°C ready for use in PCR reaction.

2.2.4 DNA analysis by agarose gel electrophoresis

The amplified PCR products were analyzed on 1% agarose gel electrophoresis in 1 X TAE buffer (0.04 M Tris-HCl, pH 8.0, 0.04 M acetate, 0.001 M EDTA), as described by Sambrook et al. (1989). The agarose gel (molecular grade) was prepared in a Pharmacia GNA-100 Gel Electrophoresis Apparatus (Pharmacia Biotech, San Francisco, CA). DNA samples were mixed 5:1 with 6 X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) and applied to the gel wells. Electrophoresis was performed at a constant 95 V for 40-50 min. After electrophoresis, the gel was stained in 0.1 mg/ml ethidium bromide solution for 5-10 min, and destained with distilled water for 20 min. The DNA bands in the gel were

visualized by UV light transillumination with a Fluor-S^{TM} MultiImager (Bio-RAD Laboratories, Richmond, CA). The size of DNA fragments were estimated by comparison with 100 bp and 1 kb EZ DNA markers (Bio-RAD).

2.2.5 Purification of PCR products from agarose gels

The DNA bands in agarose gels were extracted with the QIAQuick gel purification kit (QIAGEN, Hilden, Germany) by the recommended protocol. The PCR products from a 100 μl reaction were separated by electrophoesis on 1% TAE agarose gels. The DNA bands were excised, placed in clean 1.5 ml microtubes, and washed once with distilled water. Three volumes of QG buffer were added to 1 volume of gel and the tube was incubated at 50°C for 10 min with vortexing every 2-3 min. One gel volume of isopropanol was added to the sample and mixed. The sample was applied to the QIAQuick column and centrifuged for 1 min at 13,000 x g, and the flow-through was discarded. Seven hundred fifty microliters of PE buffer was added to the column, which was then allowed to stand for 2-5 min and centrifuged at 13,000 x g for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min. The column was placed in a clean 1.5 ml microtube, and the DNA eluted from the column with 50 μl EB buffer (10 mM Tris-HCl, pH 8.5) by centrifugation at 13,000 x g for 1 min and the DNA stored at -30°C.

2.2.6 Quantification of DNA yield

Five microliters of DNA solution was mixed with 995 μl distilled water, the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) measured and the A_{260}/A_{280} ratio calculated with a UV/VIS Lamda Bio20 spectrophotometer (Perkin Elmer).

Normally, an A_{260}/A_{280} ratio of approximately 1.6-1.8 indicates good purity of DNA. The DNA content was calculated using the A_{260} value of double strand DNA with the following equation:

$$\mu g/ml \text{ of DNA} = (A_{260} \text{ x dilution factor x } 50\ \mu g/ml)$$

$$= (A_{260} \text{ x } 200 \text{ x } 50\ \mu g/ml)$$

One A_{260} unit equals 50 μg of double strand DNA/ml (Sambrook *et al*., 1989).

2.2.7 Vector preparation for ligation reaction

pBlueScript II SK(+) (2.966 kb) (Stratagene, La Jolla, CA) was isolated from the culture of DH5α cells with the QIA miniprep kit, as described in method 2.2.11. The plasmid was digested with the restriction enzyme *Eco*R V (Promega, Madison, WI) or *Sma* I (GIBCO, BRL, Invitrogen Corp.) to obtain blunt-ended vectors. The digestion reaction mix for the restriction enzyme *Eco*R V included 10 μg pBluescript II SK(+) plasmid, 2 μl 10X Buffer D, 0.2 ml BSA, 10 units of *EcoR* V and sterile distilled water to bring the volume up to 20 μl. The digestion reaction mix for the restriction enzyme *Sma* I included 10 μg pBlueScript II SK(+) plasmid, 2 μl 10 X React 4 buffer, 0.2 μl 100 X BSA, 10 units of *Sma* I, and sterile distilled water to bring the volume to 20 μl. The reaction mix was incubated at 37°C for 3-4 h. To remove the uncut plasmid, the digest reaction products were separated on a 0.8% agarose gel and purified by QiaQuick gel extraction kit, as previously described in method 2.2.5.

2.2.8 Ligation of DNA fragments into vector

Blunt-end ligation of purified PCR products into pBlueScript II SK(+) vector was done with T4 DNA ligase (GIBCO-BRL, Invitrogen) according to the supplier's directions. The amount of vector and purified PCR product (ng) was calculated according to the following equation:

$$\text{Amount of PCR product (ng)} = \text{(amount of vector, ng)} \times \frac{\text{size of PCR product, kb}}{\text{Size of vector, kb}} \times \text{(molar ratio of PCR product:vector)}$$

For good ligation efficiency, the amount of vector is about 60-100 ng, and the molar ratio between PCR product insert and vector is 3:1 to 5:1 for blunt end ligation. Just prior to use, the 5 X DNA ligase reaction buffer was thawed at room temperature and mixed vigorously to dissolve any precipitated material. The reaction mix consisted of 4 μl 5 X ligase reaction buffer, 60-100 ng purified pBluescript II SK(+) vector digested with *EcoR* V or *Sma* I, 60-120 ng purified PCR product, 1 unit T4 DNA ligase, and sterile distilled water to bring the volume up to 20 μl. The reaction mix was incubated at 14°C for 16-24 h. This reaction mix was used directly to transform the *E. coli* cloning host strain DH5α. It is noted that T4 DNA ligase is unstable on ice for long period, so it was returned to -20°C within 5-10 min after taking it out.

2.2.9 Transformation and selection

Transfer of ligation reactions or plasmid into *E. coli* DH5α was done as described by Sambrook et al. (1989). A 50 μl aliquot of frozen competent cells was thawed on ice for 5 min. The plasmid (10-100 ng) or 5 μl ligation reaction mix was added to thawed competent cells, mixed gently by swirling the tube and incubated on

ice for 30 min. The cells were transformed by heat shock at 42°C for 60 s and immediately chilled on ice for 2 min. The transformed cells were grown in 450 μl of SOC medium or LB broth (Appendix C) by shaking at 200 rpm for 45 min. For blue-white colony selection of recombinant pBlueScript II SK(+) vector, 200 μl of cell culture was spread on an LB plate containing 100 μg/ml of ampicillin which was pre-spread with 25 μl of 50 mg/ml X-Gal and 50 μl of 0.1 M IPTG (Appendix C). For antibiotic selection of recombinant pET clones, 200 μl of cell culture was spread on LB plate containing appropriate antibiotics for each vector and host system. After spreading, the plate was sat 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 h). Among the clones produced the blue colonies had no insert (self-ligated pBlueScript plasmid).

2.2.10 Plasmid isolation by boiling miniprep

Preparation of recombinant plasmid for preliminary analysis was done using the boiling miniprep method (Sambrook et al., 1989). Normally, the size of recombinant plasmid is larger than nonrecombinant plasmid. Single colonies were picked with sterile toothpicks, restreaked on LB plates containing appropriate antibiotics, and grown at 37°C overnight. The fresh overnight colonies were inoculated in 5 ml LB broth containing appropriate antibiotics and grown overnight with 200 rpm shaking at 37°C. The cell cultures were pelleted in microtubes by centrifugation at 3,000 x g for 3 min. The cell pellets were resuspended in 350 μl STET buffer (Appendix C) and mixed with 20 μl freshly prepared 10 mg/ml

lysozyme. The suspensions were boiled at 100°C for 40 s to break the cells. The broken cells were precipitated by centrifugation at 12,000 x g for 10 min at room temperature, and the cell pellets were removed with sterile toothpicks and discarded. To precipitate the plasmids in the supernatant, 400 µl isopropanol was added and the tubes were incubated at room temperature for 5 min, then centrifuged at 12,000 x g for 5 min at 4°C to pellet the plasmids. The pellets were resuspended in 50 µl 10 mM Tris-HCl, pH 8.0, containing 0.1 mg/ml RNase A, and incubated to degrade the RNA at 37°C for 30 min. The circular sizes of purified plasmids were compared to plasmid without insert by electrophoresis on 1% agarose gels. The plasmids containing insert were stored at -30°C.

2.2.11 Restriction enzyme digestion for plasmid analysis

To determine the size of DNA inserts in the pBlueScript II SK(+) plasmid, restriction endonucleases whose sites are present in the flanking regions of *Eco*R V (*Eco*R I and *Xho* I) and *Sma* I (*Bam*H I and *Xho* I) were used to cut the recombinant plasmids. The reaction mix was prepared including 3-5 µg plasmids, 2 µl 10 X Buffer D, 0.2 µl 100 X BSA, 5 units of each restriction enzyme and sterile distilled water to 20 µl. The reaction was incubated at 37°C overnight. One percent agarose gel electrophoresis was carried out to determine the DNA insert size.

2.2.12 QIAGEN plasmid miniprep

The QIA prep spin miniprep plasmid extraction kit (QIAGEN) was used to purify recombinant plasmid for sequencing using the recommended protocol. A recombinant colony was inoculated in 5 ml LB broth with appropriate antibiotics and

the cells were grown by shaking at 200 rpm (12-16 h) at 37°C. The cultured cells were pelleted by centrifugation at 3,000 x g for 3 min at room temperature. The cell pellet were resuspended completely in 250 µl P1 buffer containing RNase A. Two hundred fifty microliters of P2 buffer was added to the resuspended cells, and mixed by inverting the tube gently (to avoid genomic DNA shearing) 4-6 times until the solution became viscous and slightly clear. Three hundred fifty microliters of N3 buffer was added and mixed immediately, to avoid localized precipitation, by inverting the tube gently 4-6 times. The tube was centrifuged at 13,000 x g for 10 min to compact the white pellet. The supernatant was applied to a QIA prep column by pipetting, centrifuged at 13,000 x g for 1 min, and the flow-through was discarded. The column was washed by applying 0.75 ml PE buffer and centrifuging it at 13,000 x g for 1 min. The flow-through was discarded, and the column was centrifuged for an additional 1 min. The column was placed in a new 1.5 ml microtube and 25 µl distilled water was added to the center of column. The column was allowed to stand for 1 min, and centrifuged at 13,000 x g for 1 min to elute the plasmid DNA. The DNA was stored at -30°C.

2.2.13 DNA sequencing

Purified DNA samples were sequenced in cycle-sequencing using the ABI PRISM dye labeled terminator kit (Big Dye, PE Applied Biosystems, Foster City, CA) with the recommended protocol. The reaction mix was composed of 4 µl Terminator Ready Reaction Mix, 2 µl purified plasmid DNA (100 µg/µl), 1 ml 3.2 pmol/µl primer and 3 µl distilled water. Amplification was done using the Gene Amp PCR system 9700 (PE Applied Biosystems). The program was conducted by rapidly

increasing the temperature to 96°C, followed by 25 cycles of 96°C for 20 s, 50°C for 10 s and 72°C for 4 min. The amplified products were purified by ethanol precipitation as describe in the kit manual. The purified DNA pellet was dissolved in 20 µl TSR solution, heated at 100°C for 2 min, quickly cooled on ice for 3 min and loaded onto an ABI 310 DNA automated sequencer.

2.2.14 Analysis of DNA sequences

The data obtained from DNA sequencing was interpreted and converted to single letter code in text file format by the Chromas 1.56 program (Technelysium Pty. Ltd). The DNA sequences were also corrected by manual inspection of the chromatogram. The sequences were analyzed using the computer analysis programs in the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/). The sequence was translated to deduced amino acid sequences by the 6 frame translation program and antisense sequences converted to sense strand sequences with the reverse complement program in the BCM Sequence Utilities. The sequences were confirmed to correspond to β-galactosidase by a local alignment search of the Genebank database with the BLAST program (Altschul *et al*, 1997) at the National Center for Biotechnological Information (NCBI, http://www.ncbi.nlm.nih.gov). Comparison of the DNA fragment sequences was done with the ClustalX implementation of CluistalW (Jeanmougin *et al*., 1998; Thompson *et al*., 1994) or BLAST2 at NCBI. The cDNA sequences of *OsBGal1* and *OsBGal2* were searched against the dbEST database using BLASTn to identify matching ESTs in rice, and these ESTs were used to search the Rice Genome Project database (http://rgp.dna.affrc.go.jp) mapped EST markers to identify the chromosomal locations of *OsBGal1* and *OsBGal2*.

Protein sequence alignments were done with the ClustalX implementation of ClustalW. Protein analyses were done at the Expasy proteomics server (http://www.expasy.org/), signal sequences were predicted by SignalP (Nielson et al., 1997) and cellular locations were predicted by PSORT (Nakai and Horton, 1999).

2.2.15 DNA probe labeling

cDNA probe was isotopicly labeled with [α-^{32}P]dCTP using Ready-To-Go DNA labeling Beads (Amersham Pharmacia Biotech). Thirty nanograms of DNA were adjusted to a volume of 48 μl. The DNA solution was denature by heating at 100°C for 5 min, then put on ice for 5 min to fix the denatured form of DNA, then centrifuged briefly. Forty-eight microliters of denature DNA and 2 μl [α-^{32}P]dCTP (300 Ci/mmol) were added into the microtube containing the DNA labelling beads, and gently mixed by pipetting up and down 4-5 times. The reaction were removed by gel filtration on Sephadex G-50 (packed in a 1 ml plastic sylinge column) with centrifugation at 3,4000 X g for 4 min. Before hybridization, labelled DNA was denatured by heating at 100°C for 5 min, then placed on ice immediately.

2.3 Rice β-galactosidase gene identification and analysis

2.3.1 Database searching and sequence analysis

All family 35 β-Galactosidase genes in the rice genome were identified by a tBLASTn search of the National Center for Biotechnology Information (NCBI) Genbank nr, Monsanto rice genome, and Syngenta Torrey Mesa Research Institute (TMRI) rice genome databases for japonica rice and the Beijing Genomics Institute

(BGI) database, for indica rice (Altschul et al. , 1997; Bary et al. , 2001; Goff et al. , 2002; Yu et al. , 2002, ; Kikushi et al. , 2003) . The number and sequences of rice glycosyl hydrolase family 35 β-galactosidases were predicted from the NCBI, TMRI, and BGI databases. Expressed sequence tags (ESTs) corresponding to GH family 35 genes were identified with tBLASTn (to find all rice ESTs) and BLASTn (to identify ESTs from a specific gene) searches of the NCBI dbEST database. When available, gene assignments were compared with the Unigene cluster containing the ESTs. Identified genes were retrieved and translated with the 6-frame translation facility of the Baylor College of Medicine (BCM) search launcher (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html), and the translations compared to the BLAST results to identify coding regions. The splice sites were identified as "GT" and "(C)AG" which maintained the proper reading frame and gave the best sequence alignment with known proteins. The predictions were checked with full-length cDNA and EST sequences, when possible, and also compared to gene predictions in the database. Putative signal peptides were analyzed based on the SignalP program (Nielsen, 1997), and putative N-glycosylation sites were identified manually or with the software at the Expasy web page (http://www.Expasy.org). Conserved domains of the protein were analyzed using NCBI Conserved Domain Search (http://www.ncbi.nlm.nuh.gov/).

Protein sequences were aligned with the Clustalx 1.83 implementation of Clustalw (Jeanmougin *et al*, 1998; Thompson *et al*, 1994) using the default parameters. The alignment was edited with the Genedoc program (http://www.psc.edu/biomed/genedoc).

2.4 Expression pattern of rice β-galactosidase genes by northern blot analysis

2.4.1 Northern hybridization

Total RNA was subjected to electrophoresis on a 1.2 % (w/v) formaldehyde agarose gel and transferred to a Hybond-N + nylon membrane (Amersham) using 20 X SSC Sambrook et al., 1989). The gene-specific primers used to amplified the probes were designed from the 3'UTR of each *β*-galactosidase gene,

Table 2.1 Primers used for amplification of 3'UTR probes for OsBGal1 and OsBGal2

Primer name	Primer sequence
MOSM1983pf8	5'-CAAAGCACACAGAAAGCGATG-3'
MOSM1983pr9	5'-TGCTCACTGCACAATCAACG-3'
ME14053pf1	5'-AGGAAAGTGGGGGCGTATAG-3'
ME14053pr12	5'-TCCCATTTACAACTCAACGTTGC-3'

The probe was amplified using rice cDNA as template. The PCR reaction mix included 1 ml first-strand cDNA, 2.5 μl 10 X HotStar Taq buffer, 2 μl 2.5 mM dNTP mix, 1 μl 10 mM MOSM1983pf8 primer, 1 μl 10 μM MOSM1983pr primer for *OsBGal1* probe and 1 μl 10 mM ME1405pf1 primer, 1 μl 10 mM ME1405pr12 primer for *OsBGal2* probe, 0.625 unit HotStar Tag DNA polymerase (QIAGEN), and sterile distilled water to bring the volume to 25 μl. The amplification was done using 35 cycles of 94°C 30 s, 48°C 30 s, and 72°C 1 min. The amplified DNA products were electrophoresis on 1% agarose gel. The DNA was cut and purified as described in method 2.2.5 and isotopicly labelled with [α-^{32}P]dCTP as described in method 2.2.15.

The blots were hybridized with the *OsBGal1* and *OsBGal2* radiolabeled gene specific probe for 16 h at 65°C. The blots were then washed once in 0.1% SDS, 2 X SSC for 20 min at 65°C and twice in 0.1 % SDS, 1 X SSC for 20 min at 65°C, and exposed to x-ray film. To ensure equal loading of RNA in individual lanes (20 μg $lane^{-1}$), gels were stained with ethidium bromide for comparison of the intensity of rRNA bands prior to blotting.

2.5 Recombinant protein expression in *Escherichia coli*

2.5.1 Amplification of the cDNA to insert in expression vectors

The cDNAs encoding the predicted mature protein products of the *OsBgal1* and *OsBgal2* genes were cloned into a pET vector and expressed in *E. coli*. The primers specific for Os*Bgal1* and *OsBgal2* (Table 2) were designed to amplify the cDNA coding the mature proteins and introduce restriction enzyme sites complementary to the cloning sites of the expression vector to flank the cDNA, allowing inframe translation of the proteins.

An *OsBGal1*, cDNA encoding the mature protein product was cloned into pET32a(+) (Novagen, Madison, WI). The *OsBgal1* cDNA prepared for cloning into pET32a(+) was amplified with indica rice (*Oryza sativa* L., cv. KDML 105) cDNA as a template and MOSMex-F and MOSMex-R primers (Table 2) to introduce *Nco*I and *Xho*I sites at the 5' and 3' ends, respectively.

The PCR reaction mix included 1 μl indica rice cDNA, 2.5 μl of 10 X *Pfu* DNA polymerase buffer (Promega), 2 μl of 2.5 mM dNTP, 1 μl each of 10 mM MOSMex-F and MOSMex-R primer, 0.75 unit *Pfu* DNA polymerase (Promega) and sterile distilled water to give the final volume of 25 μl. The PCR was performed by

incubating for 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 4 min, and a final extension of 10 min at 72°C after the last cycle.

The *OsBgal2* cDNA prepared for cloning into pET32a(+) was PCR amplified using the E1405 clone plasmid as template with the ME1405ex-F and ME1405ex-R primers to introduce *Nco*I and *Xho*I sites at the 5' and 3' ends, respectively.

The PCR reaction mix included 1 μl cDNA, 2.5 μl of 10 X *Pfu* DNA polymerase buffer (Promega), 2 μl of 2.5 mM dNTP, 1 μl each of 10 mM ME1405ex-F, 1 μl of 10 mM ME1405ex-R, 0.75 unit *Pfu* DNA polymerase (Promega) and sterile distilled water to give the final volume of 25 μl. The PCR was performed by incubating for 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 4 min, and the final extension of 10 min at 72°C after the last cycle.

The PCR products were evaluated to confirm they were the correct size on 1% agarose gel electrophoresis in 1 X TAE buffer (0.04 Tris-HCl pH 8.0, 0.04 M acetic acid, 0.001 M EDTA, pH 8.0) as described by Sambrook *et al.* (1989). The agarose gel (molecular grade) was prepared using a GNA-100 Pharmacia gel electrophoresis Apparatus (Pharmacia Biotech, San Francisco, CA). DNA samples were prepared by mixing 5:1 with 6 X loading dye (0.25% bromophenol blue, 0.25 xylene cyanol FF and 30% glycerol) and applied to the gel wells. Electrophoresis was performed at a constant at 100 V for 40-50 min. After electrophoresis, the gel was stained with 0.1 μg/ml ethidium bromide solution for 5-10 min and destained in distilled water for 20 min. The band in the gel was visualized by UV light transillumination, with Fluor - S^{TM} MultiImager (Bio-RAD Laboratories, CA). The size of DNA were estimated by

comparing with 1 kb ladders (New England Biolabs, Beverly, MA), or 100 bp DNA marker (Bio-RAD, Richmond, CA).

To purify the PCR product from agarose gels, the DNA bands were extracted with the QIAQuick gel purification kit (QIAGEN) by the vendor's recommended protocol. The PCR products from a 100 μl reaction mix were separated on 1% TAE agarose gels. The DNA bands were excised and placed in a clean 1.5 ml microtube, and the gel was washed once with distilled water. Three volumes of QG buffer were added to 1 volume of gel and the tube was incubated at 50°C for 110 min with vortexing every 2-3 min. One gel volume of isopropanol was added to the sample and mixed. The sample was applied to the QIAQuick column and centrifuged for 1 min at 12,000 x g, and the flow-through was discarded. Seven hundred fifty microliters of PE buffer was added to the column, which was then allowed to stand for 2-5 min and centrifuged at 12,000 x g for 1 min. The column was placed in a clean 1.5 ml microtube, and the DNA was eluted from the column with 50 μl EB buffer (10 mM Tris-HCl pH 8.5) by centrifugation at 12,000 x g for 1 min and stored at -30°C.

Table 2.2 Primers used for amplification of *OsBGal1* and *OsBGal2* cDNA

Primer name	Primer sequences
MOSMex-F	5'-TCCCCATGGCGGTGACGTACGACAAG-3'
MOSMex-R	5'-GAACTCGAGCTATGCAGCGGTAGAGCA-3'
MESTex-F	5'-CCACCATGGCGGTCACCTACGACCAC-3'
MESTex-R	5'- TCCCTCGAGACATGTCGTCCTCGTCATC-3'

2.5.2 Construction of recombinant plasmids

The PCR products were cloned into the *Eco*R V site of pBluescript II SK(+) and transformed into *E. coli* DH5α. The resultant recombinant plasmids were purified and digested with restriction enzymes, as described in method 2.2.10. The gel purified inserts for *OsBGal1* were cloned into the *Nco* I and *Xho* I sites of pET32a(+) (Novagen). The ligation reaction mix was transformed into DH5α *E. coli* and spread on LB agar plates containing 50 μg/ml ampicillin.

To identify the recombinant pET plasmids. The transformant colonies were randomly selected and grown in LB broth with 50 μg/ml ampicillin and incubated in a shaking incubator overnight at 37°C. Plasmids were isolated with the QIA Prep Spin Miniprep kit (QIAGEN) and checked by digestion with *Nco* I and *Xho* I restriction enzymes and by sequencing.

2.5.3 Transformation of recombinant plasmid into expression host cells

The recombinant pET32a(+)-*OsBgal1* and pET32a(+)-Os*Bgal2* plasmidswere transformed into OrigamiB (DE3) *E. coli* (Novagen). Competent cells of Origami B (DE3) were prepared as described in Appendix C. Plasmids (10-100 ng) were used to transform a 80 μl aliquot of the frozen competent cells, as described in method 2.2.8, and spread on LB agar plates containing 50 μg/ml ampicillin, 15 μg/ml kanamycin, and 12.5 μg/ml tetracycline.

2.5.4 Recombinant protein expression in *Escherichia coli* and extraction of protein from induced cells

To express the proteins in *E. coli*, the selected clones were grown at 37°C overnight in LB broth containing 50 μg/ml ampicillin, 15 μg/ml kanamycin, and 12.5 μg/ml tetracycline. The cultures were inoculated into LB broth containing the same antibiotics and the cultures were grown in a shaking incubator at 37°C. After the optical density at a wavelength of 600 nm of the cultures reached 0.5-0.6, IPTG was added to a final concentration of 0.4 mM and the cultures were shaken at 200 rpm at 18 °C for an additional 16 h. Induced cultures were transferred to 50 ml centrifuges, which were then chilled on ice for 10 min. The cells were harvested by centrifugation at 5000 rpm at 4°C for 15 min and the cell pellet was kept at -70°C for 30 min or longer until used for protein extraction.

To determine the protein expression level, the total protein profiles of host cell containing control-empty plasmids and recombinant plasmids were compared. For this, the cell pellet from 1 ml liquid culture was collected in a microtube and resuspended in 40 μl of 2X SDS sample buffer (125 mM Tris, pH 6.8, 15% glycerol, 1% SDS, 5.6% β-mercaptoethanol, 0.05% bromophenol blue). The suspension was boiled for 5 min and 8-12 μl were loaded onto an 12% SDS polyacrylamide gel (SDS-PAGE) as described in Appendix C.

To extract the protein from induced recombinant cells, the bacterial pellet was thawed and suspended in 5 ml per gram cell freshly prepared extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, 200 μg/ml lysozyme, 1% Triton-X100, 1 mM phenylmethylsulfonylfluoride (PMSF), 40 μg/ml DNase I), and incubated at room temperature for 30 min. The cell suspensions were sonicated on ice 3 times at 50 W output for 10 s, with 1 min cooling in between, with an Ultrasonic

Processor GE 100 probe sonicator (Treadlitei, Woodstock, MA). The soluble protein fraction was recovered by centrifugation at 12,000 x g at 4°C for 10 min.

2.6 Characterization of crude OsBGal1 and OsBGal2

2.6.1 Preparation of alcohol-insoluble residue (AIR) and alcohol soluble residue from maize and rice

Two grams dried maize tissues were homogenized in 200 ml of 70% ethanol. Alternatively, 20 g fresh weight of rice root and rice coleoptile were liquidized in 140 ml ice-cold absolute ethanol plus 40 ml H_2O to give a final solvent composition of 200 ml 70% ethanol (the tissue was assumed to contain about 20 ml H_2O). The tissue was homogenized by 1-min bursts at full power in a Polytron liquidizer with cooling to 0°C between each burst. For complete extraction of low-M_r cell components, the suspension was stirred at room temperature with a magnetic stirrer several hours. The AIR was collected on a filter (e.g. a fine nylon gauze), and washed with several 100 ml volumes of 70% ethanol. To monitor progress of removal of low-M_r compounds, the A_{280} of a sample of each filtrate was measured and the final residue was resuspended in more 70% ethanol, and stored in a tightly-stoppered bottle at about 4°C. For prepation pf alcohol-soluble residue from rice, 20 g fresh weight, 140 ml ice-cold absolute ethanol and 40 ml H_2O were added to give a final solvent composition of 200 ml 70% ethanol (the tissue was assumed to contain about 20 ml H_2O). The tissue was homogenized by 1-min bursts at full power in a Polytron liquidizer with cooling to 0°C between each burst. For complete extraction of low-M_r cell components, the suspension was stirred at room temperature with a magnetic

stirrer bar several hours. The alcohol insoluble material was removed by centrifugation at 12,000 rpm for 5 min and the supernatant (alcohol soluble fraction) was collected and stored in a tightly-stoppered bottle at about 4°C.

2.6.2 Thin layer chromatography (TLC)

The products of OsBGal1 and OsBGal2 hydrolysis of *p*NPGal were also detected by thin-layer chromatography (TLC). The assay mixtures (110 μl) containing 5 mM *p*NPGal, 50 mM NaOAc, pH 5.0, and 100 μg crude extracts of OsBGal1 and OsBGal2 were incubated for 5 min to 16 h at 37°C. Four microliters of reaction mixture were spotted on a 0.25-mm F_{254} silica-coated aluminium plate (Merck, Darmstadt, Germany) and chromatographed vertically with EtOAc-pyridine-HOAc-H_2O (6:3:1:1) as solvent, followed by detection of *p*NP groups under 365-nm UV light, then treatment of the plates with staining solution (0.5% thymol, 5% H_2SO_4, 95% ethanol) and baking at 110°C for 5 min for visualization of sugar moieties.

2.6.3 Hydrolysis of XLLG-SR, rice coleoptile AIR and rice root AIR by crude OsBGal1 and OsBGal2

Xyloglucan oligosaccharides (XGOs) were a mixture containing principally XLLG (nonasaccharide) XXLG (octasaccharides) and XXXG (heptasaccharides) (see Fry et al., 1993). The XGO mixture was converted to XGO-SRs (XLLG-SR, XXLG-SR, XXXG-SR, all of which act as fluorescent acceptor substrates for XETs) by the method of Fry (1997). The XGO-SRs at 6.5 mM (sulphorodamine basis) were dissolved in 25 mM MES buffer, pH 5.5. Crude extracts of BGs were incubated in the XGOs-SR for 0-27 h and analyzed by TLC. For analysis of remaining low M_r XGO-

SRs after incubation in the presence of BGs for 0-27 h, the reaction mixture was resolved by thin layer chromatography (TLC) on silica gel in butanol/acetic acid/ water (2:1:1 [v/v/v]). The plate was illuminated with green light and the fluorescent spots from the sulforodamine conjugates were photographed through an orange filter.

2.6.4 Hydrolysis of oligosaccharide and polysaccharides substrates

Galactose released from β-(1→3), (1→4), and (1→6)-linked di- and trisaccharides of galactose and from cell wall polysaccharides was detected semi-quantitatively by TLC. Larch-wood arabinogalactan, galactan (prepared from gum arabic), apple pectin, citrus pectic acid, birchwood xylan, oat xylan and lactose were purchased from Sigma (St. Louis, MO, USA) for these tests. Galactosylated xyloglucan oligosaccharides (XLLG and XXLG) were conjugated to sulphorhodamine (XGO-SRs) by the method of Fry [34]. Lupin (1→4)-galactan was subjected to partial acid hydrolysis, and the oligosaccharides were purified by paper chromatography; the penta-, octa- and undeca-saccharides were eluted, radiolabelled with NaB^3H_4 (Hetherington and Fry, 1993) and re-purified by paper chromatography. Alcohol-insoluble residues and total extractable hemicelluloses were obtained from maize cell-suspension cultures and rice seedlings (root and coleoptile) as described by Fry (2000). Solutions or suspensions containing 0.5% (w/v) polysaccharide substrates or 5 mM galacto-oligosaccharides were incubated with 10 μl of crude extracts of OsBGal1-expressing, OsBGal2-expressing or control plasmid containing *E. coli*, or purified OsBGal1, in 50 mM sodium acetate, pH 4.0, in a final volume of 110 μl for 16 h or 3 days. For the controls, equal amounts of substrates were incubated under the same conditions but in the absence of enzyme. TLC was routinely on silica-gel

60 F_{254} (Merck, Darmstadt, Germany) in 7:1:2 (v/v/v) 1-propanol: ethanol: water with detection by charring after spraying with 20% (v/v) H_2SO_4 in methanol. For analysis of remaining XGO-SRs after incubation in the presence of OsBGal1 and OsBGal2 for 0-27 h, the reaction mixture was resolved by TLC on silica gel in 2:1:1 (v/v/v) butanol:acetic acid:water. The plate was viewed under 254-nm UV light. [^{3}H]Oligogalactans and their products were separated by TLC in the same solvent and detected by fluorography and scintillation-counting.

2.6.5 Hydrolysis of galactose containing polysaccharide by crude extract of rice OsBGal1 and rice OsBGal2.

In order to examine the action of rice OsBGal1 and rice OsBGal2 on natural substrates, aliquots of the possible galactose containing polysaccharide substrates (shown in Table 2.3) were treated with crude extract of rice OsBGal1 and OsBGal2. Enzyme activity was measuring using reaction mixtures (50 μl) consisting of 100 μg crude enzyme, 0.1% (w/v) polysaccharides or 5 mg of alcohol insoluble residue (AIR) extracted from maize, rice root and rice coleoptile in 50 mM sodium acetate buffer, pH 4.0. After incubation at 37°C for 0-24 h, mono- and oligosaccharides in enzymatic hydrolysates were separated on TLC. In all cases, (0.5% v/v) chlorobutanol was added to the incubation mixture in order to avoid bacterial contamination (chlorobutanol did not affect β-galactosidase activity *in vivo*). For the controls, equal amounts of crude cell extract from induced *E. coli* containing the empty vector were treated with those substrates under the same conditions.

Table 2.3 Possible galactose-containing substrates and suitable solvent systems used for TLC

Substrate	Solvent system used for TLC
Xyloglucan oligosaccharide	B/A/W (2:1:1)
AIR from maize, rice root, rice coleoptile	EtOAc/Py/A/W (6:3:1:1)
Alcohol extracted from maize, rice root, rice coleoptile	B/A/W (2:1:1)
Arabinogalactan from birchwood	B/A/W (2:1:1)
Pectin from apple	B/A/W (2:1:1)
Pectin from citrus	B/A/W (2:1:1)
Hemicellulose B from barley, maize, grass	B/A/W (2:1:1
Polygalacturonic acid	B/A/W (2:1:1)
Gum Arabic	B/A/W (2:1:1)
Lactose	B/A/W (2:1:1)
Galactobioside	B/A/W (2:1:1)
Galactan	B/A/W (2:1:1)
Tran-zeatin riboside	B/A/W (2:1:1)
β-(1→ 4) -[^{3}H]Gal_{5}-ol	B/A/W (2:1:1)
β-(1→ 4) -[^{3}H]Gal_{8}-ol	B/A/W (2:1:1)
β-(1→ 4) -[^{3}H]Gal_{11+}-ol	B/A/W (2:1:1)
2'Fucosyl- [^{3}H] lactitol	B/A/W (2:1:1

B = butanol, A = acitic acid, W = distilled water, EtOAc = ethyl acetate, and Py = pyridine.

2.7 Purification and characterization of recombinant OsBGal1 expressed in *E. coli*

2.7.1 Purification of recombinant protein by Ni-NTA superflow

Purification of BGal1 was done with Ni-NTA superflow (QIAGEN) at 4°C. Ten milliliters of the soluble extract of bacteria expressing BGal1 protein from pET32a(+) was added to a 1 ml Ni-NTA superflow column (QIAGEN) equilibrated with 8 ml (8 volumes) of equilibration buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl). The column was washed with 4 ml (4 volumes) of equilibration buffer. The recombinant protein was eluted with 4 ml (4 volumes) of elution buffer (10 mM L-histidine in equilibration buffer). The 0.5 ml fractions with β-galactosidase activity were pooled, and concentrated 5 fold and exchanged with 50 mM sodium phosphate, pH 7.0, in a 10 kDa cutoff centrifugal ultrafiltration membrane.

2.7.2 Purification of recombinant protein by ion-exchange chromatography (DEAE)

The sample was applied to a DEAE anion-exchange column (5 ml) previously equilibrated with low salt buffer (50 mM sodium phosphate, pH 7.0). The column was washed with 2 volumes of the same buffer and eluted with a linear gradient of 0-1 M NaCl in 50 mM sodium phosphate, pH 7.0, in a total volume of 50 ml, followed by 20 ml of 1 M NaCl at a flow rate of 1 ml min^{-1}. Fractions of 1 ml each were collected. Active fractions were pooled and concentrated in a 10 kDa Mw cut-off centrifugal ultrafiltration membrane (Amicon).

2.7.3 Protein determination

Protein concentration of the crude extracts of *E. coli* were estimated by the method of Lowry (Lowry *et al*., 1951) using bovine serum albumin (BSA) as standard (15-105 μg BSA). The dilution of protein sample in 1 ml was mixed with 1 ml alkaline copper solution (0.05% $CuSO_4$, 0.1% potassium-sodium tartate, 5% Na_2CO_3 and 0.4M NaOH). The reaction was left to stand for 10 min at room temperature and 0.5 ml diluted Folin reagent (2:1 in water) was then added. The mixture was vigorously mixed by vortexing and allowed to stand at 28°C for 30 min. The absorbance at 750 nm was measured with a Jenway 6405 UV/VIS Spectrophotometer (Essex, UK).

Purified protein concentration was determined by Bio-RAD Protein Assay kit (Bio-RAD) using BSA as the standard (2-10 μg). Appropriate dilutions of protein samples were prepared in 0.2 ml volumes and mixed with 0.8 ml Bio-RAD Protein Assay solution. The reaction was allowed to proceed for 10 min and the absorbance at 595 nm was measured using a Jenway 6405 UV/VIS Spectrophotometer (Essex, UK).

2.7.4 Analysis of protein by LC/MS/MS

The purified OsBGal1 protein was analysed for size and purity by SDS-PAGE according to the standard method (Laemmli, 1970) on a 10% polyacrylamide gel. The bands in the purified protein lane were excised from the gel and digested with trypsin. The peptide fragments were analyzed by high-performance 2-Dimensional Ion Trap Mass spectrometer (LC/MS/MS) at the Bio-Technology Service Unit of the

National Center for Genetic Engineering and Biotechnology, Thailand, to identify peptides from OsBGal1 beta-galactosidase and its thioredoxin tag.

2.8 Biochemical characterization

2.8.1 β-galactosidase assays and kinetics study

The activity of β-galactosidase was determined in a reaction mixture (110 μl) consisting of the enzyme, 5 mM *p*-nitrophenol β-D-galactoside, and 50 mM NaOAc, pH 4.0. After incubation at 37°C for 10 min, the reaction was terminated by addition of 0.2 M Na_2CO_3 (100 μl) and monitored at 405 nm. One unit of enzyme activity liberates 1 μmol of *p*-nitrophenol (*p*NP) per minute.

The kinetic parameters, K_m and V_{max} of OsBGal1 were calculated according to the method of Lineweaver and Burk with the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). About 0.56 μg of purified OsBGal1 from the DEAE column was assayed using various concentrations of *p*NPGal (0.1, 0.15, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mM) in 50 mM NaOAc buffer, pH 4.0 in a final volume of 110 μl. The reaction was allowed to continue for 10 min at 37°C before being stopped with 2 volumes of 0.2 M Na_2CO_3. Three replicates each of substrate concentrations were used. Protein assays were performed colorimetrically with the Bio-RAD Protein Assay (Bio-RAD) using BSA as standard.

2.8.2 pH and temperature profile of activity

The pH optima of OsBGal1 and OsBGal2 were determined by assaying β-galactosidase activity over a pH range of 2.5-9.0, using 50 mM citrate phosphate

buffer (pH 2.5-6.0), 50 mM sodium phosphate buffer (pH 6.0-8.0) and 50 mM Tris-HCl buffer (pH 7.0-9.0) at 0.5 pH unit intervals. The activity at a given pH was measured by mixing the enzyme solution with 5 mM final concentration of *pNP*-β-galactoside in the same buffer. The reactions were allowed to proceed for 10 min at 37°C and the amount of product released determined as described previously (2.7.1).

2.8.3 Effect of metal ions and possible inhibitors on enzyme activity

Inhibition studies were done by incubating the enzyme for 30 min in the presence of 10 mM metal ions and possible inhibitors: $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $NiCl_2$, $CoCl_2$, $HgCl_2$, $FeCl_3$, EDTA, δ-gluconolactone, and galactonic acid δ-lactone. Galactose and lactose were similarly tested at 5 mM. The standard 5 mM *p*NP-Gal reactions were then done in the presence of the metal ions or other compounds. For selected compounds, 1 mM and 100 mM concentrations were also tested.

2.9 Production of OsBGal1 and OsBGal2 antibodies

2.9.1 Peptide synthesis as antigen

In order to determine the location of the β-galactosidases in rice plants and to detect of the expression of recombinant rice BGal protein in *E. coli*, antibodies against OsBGal1 and OsBGal2 were produced. Antibodies against peptides designed from the translated cDNA were produced in New Zealand white rabbits

Polyclonal antibodies were produced in rabbits with specific peptides derived from the catalytic domains of OsBgal1 (N'-CEGKEFGAAGKAYIN-C') and OsBgal2 (N'-CGESVMGSGAKSRIPD-C'). The peptides were conjugated to the pre-activated

Imject Supercarrier BSA or mcKLH, (PIERCE, Rockford, IL) as described by the manufacturer's instruction, and the conjugates were purified by Dextran gel filtration column chromatography. The conjugates were used to inject rabbits after mixing 1:1 with Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for two subsequent booster injections. Innoculations were done by intermuscular (thigh) and intradermal injections with these preparations. The rabbit's serum was collected once before the first injection (pre-immune bleed) and 10 days after each antigen injection. The rabbit's serum titer was tested by indirected ELISA.

2.9.2 Determination of titer from rabbit's serum by indirect ELISA

OsBGal1 and OsBGal2 synthetic peptide were dissolved to 5 mg/ml in phosphate buffered saline (PBS), pH 7.4. One hundred microliters of one peptide solution were added to each well of a 96-well plate, while 100 μl of 100 μg/ml BSA was added to negative control wells. The plate was then wrapped and kept at 4°C overnight. After that the coated plate was washed 3 times with PBS, and 200 μl of 4% skim milk were added to each well and left at room temperature for 1 h. Then, the blocked plate was washed 3 times with PBS, and 100 μl of diluted rabbit anti-OsBGal1 or OsBGal2 serum in 4% skim milk powder in PBS with 0.05% Tween 20 were added to each well. The plates were incubated at room temperature for 2 h, followed by washing 3 times with PBS. The plates were then reacted with a 1:5000 dilution of HRP-conjugated goat anti-rabbit antibody in PBS (100 μl/well) at room temperature for 1 h, followed by washing 3 times with PBS. The antigen-antibody reaction was visualized by adding 100 μl of 3, 3', 5, 5'-tetramethylbenzidine (0.1 mg

TMB in 100 μl DMSO with 9.9 μl of sodium citrate, pH 6.5, and 5 μl of 30% H_2O_2) in to each well.

2.9.3 Purification of OsBGal1 and OsBGal2 antibodies on peptide-linked resins

The OsBGal1 and OsBGal2 antibodies were affinity purified on immobilized OsBGal1 and OsBGal2 peptide resins, respectively. The resins were produced by linkage of 4.6 mg of the peptide to 1 g of cyanogen bromide-activated resin (Sigma), according to the supplier's directions. Anti-OsBGal1 or Anti-OsBGal2 antibody diluted in PBS (3 ml) was loaded onto the affinity column, which was then washed with 20 mL PBS, followed by 20 mL of Tris buffer (50 mM Tris-HCl, 0.1% Triton X-100, 0.5 M NaCl), pH 8.0, 20 ml of Tris buffer, pH 9.0, and finally 20 ml of sodium phosphate buffer (50 mM sodium phosphate, pH 6.3, 0.1% Triton X-100, 0.5 M NaCl). The specific antibody was eluted with 20 ml of glycine buffer, pH 2.5 (50 mM glycine-HCl, 0.1% Triton X-100, 0.15 M NaCl) and collected into four volumes of 1 M Tris-HCl, pH 9.0. The antibody was desalted on a prepacked Sephadex^{TM} G-25 M column (Amersham Biosciences) using PBS as the desalting buffer.

2.10 Immunolocalization of OsBGal1 and OsBGal2 in rice

2.10.1 Western blot analysis

For the immunoblot of total protein from rice plant, total protein were isolated from root and shoot of 7-day rice seedlings and also from root, stem and leaf of 1-month rice. Typically, 0.5 g of fresh tissue was ground in liquid nitrogen in a motar and resuspended with 2 ml of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 400 mM

sucrose, 1 mM PMSF, 100 μM soybean trypsin inhibitor and 0.1% Triton X-100. After centrifugation (12,000 x g, 4 °C, 15 min), the supernatant was collected and the protein concentration was determined by the method of Bradford (1976).

Twenty-five micrograms of total protein was separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences) on a semi dry gel blotting system (Bio-Rad) in 1X transferring buffer (10% MeOH in Tris-glycine buffer, pH 8.3) at a constant 400 mA for 2 h. The blot was blocked with 4% BSA in PBS (20 mM phosphate buffer, 150 mM NaCl), pH 7.4, with 0.05% (v/v) Tween 20 (PBST) overnight, then incubated with affinity purified OsBGal1 or OsBGal2 antibody at 1:2,500 dilution in blocking buffer for 1 h. Duplicate gels for the two western blots were run in parallel. After incubation with antiserum, the membrane was washed 3 times with PBST, followed by incubation in a 1:5,000 dilution of goat anti-rabbit antibody coupled to horseradish peroxidase (HRP, Sigma) in PBST for 1 h. The membrane was washed 3 times with PBST with 15 min per wash. The blot was developed with ECL western blotting detection reagents (Amersham Biosciences), and the blot was exposed on x-ray film.

For immunoblot of protein samples from extracts of *E. coli* induced to express OsBGal1 or control plasmids and the final purified protein from DEAE ion exchange chromatography were mixed with one-fourth volume of the 4X SDS-gel sample buffer. The samples were boiled at 100°C for 5 min, and then the denatured proteins were fractionated by 10% SDS-PAGE by the method of Laemmli (1970). The gels were then blotted and OsBGal1 detected with OsBGal1 antibody, as described for protein from rice extracts. Crude extracts from cells expressing OsBGal2 and the

control plasmid were also analysed by western blot as described above, but with OsBGal2 antiserum as the primary antibody.

2.10.2 Localization of OsBGal1 and OsBGal2 in rice seedlings at 0-7 days

For immunohistochemical localization, japonica rice (*Oryza sativa* L., cv. nipponbare), prepared as described for X-Gal staining, were incubated in 70% PBS at room temperature, followed by 0.1 M glycine (pH 7.0), and 0.1% Triton X-100 for 20 min each. The seeds were blocked for nonspecific binding with 4% BSA in PBS buffer for 30 min, then incubated with OsBGal1 or OsBGal2 antiserum at 1:2000 dilution for 1 h. After incubation in antiserum, the seeds were washed 2 times in PBST, then incubated in a 1:5000 dilution of goat anti-rabbit antibody coupled to horseradish peroxidase (Sigma) in PBST for 1 h. The rice seeds were washed 2 times in PBST with 5 min per wash, then stained with an aminoethyl carbazole substrate kit (ZYMED Laboratories) for 3 min and the reaction was stopped by soaking the seed in distilled water. The staining was photographed under a Nikon SMZ-U stereomicroscope (Nikon, Tokyo, Japan) at 10X magnification.

2.10.3 Localization of OsBGal1 and OsBGal2 in 5-day rice root sections by immunoperoxidase staining

Rice root (5 days) were used for all microscopic studies here. Tissues were fixed by perfusion with Bouin's solution for overnight. Tissues were then dehydrated and embedded in paraffin for sectioning. The tissue sections (7-μm thick) were deparaffinized and rehydrated through decreasing concentrations of ethanol (Abs. EtOH→ 95%→ 80%→ 70%). The sections were treated with 1% lithium carbonate in

70% ethanol to quench endogenous peroxidase and to remove residual picric acid. The sections were then treated with 300 mM glycine to neutralize residual formaldehyde. To block non-specific binding, the sections were incubated for 15 min at room temperature with 4% BSA in PBS. Separate sections from each tissue were then incubated for 2 h at room temperature with purified anti-OsBGal1 IgG and anti-OsBGal2 IgG. Following successive washing with 0.1% Tween 20 in PBS (PBST), the sections were incubated with HRP-conjugated secondary antibody and the enzymatic reaction was developed with *NOVA Red*^{TM} substrate. The concentrations of chemicals and conditions for this treatment were described by the manufacturer. The red color product of the peroxidase reaction signified β-galactosidase localization. Sections treated with preimmune rabbit serum IgG or PBS were used as negative controls. Finally, the sections were viewed under a light microscope.

CHAPTER III

RESULTS

3.1 Identification of rice β-Galactosidase genes

Rice genes homologous to glycosyl hydrolase family 35 β-galactosidases have been identified from the Torrey Mesa Research Institute (TMRI), GenBank at NCBI and Beijing Genome Institute (BGI) rice genome databases. The retrieved gene sequences were searched against the dbEST expressed sequence tag database to identify tissues of expression of each gene in rice. Fifteen β-galactosidase genes (including *OsBGal1* and *OsBGal2*), were identified, as listed in Table 3.1. The rice β-galactosidase genes were mapped to chromosomes 1, 2, 3, 5, 6, 8, 9 and 10 (Table 3.1).

Analysis of rice genomic and cDNA sequences in the public databases identified the gene structures of the 15 β-galactosidase genes belonging to glycosyl hydrolase family 35 (Figure 3.1). A phylogenetic tree of these genes based on their amino acid sequences appeared to be divided in to 5 main branches (Figure 3.2), with the gene structures within each branch being similar. For instance, OsBGal12, OsBGal14, and OsBGal15 were found in one branch and have the same splice sites with 17 exons of almost the same size, but introns of different length.

Table 3.1 Summary of rice gene identified as homologous to glycosyl hydrolase family 35 β-galactosidases from the Torrey Mesa Research Institute (TMRI), GenBank at NCBI, and the Beijing Genome Institute (BGI) databases.

Gene number	**Database Gene Accession**	**Chromosome location (locus)**	**cDNA clones Unigene cluster & number of ESTs**	**Expression tissues of ESTs and cDNA**
OsBGal 1	AC104428.2, AC107225.2, AAAA01002951, CL030879.63	Os03g0165400	AF508799, AK102192, AK060645, Unigene OS13559: 105 ESTs	stem, shoot, leaf, immature seed, root, callus, panicle at flowering, flower
OsBGal 2	AP004729, AAAA01003471, CLO06101.54.66, CLO16983	Os06g057360	AK102756, AK059741, AK105619, Unigene OS6322: 203 ESTs	Callus, etiolated shoot, panicle at flowering, flower, leaf, root
OsBGal 3	AP002908, AP003546, AAAA01012445, AAAA01022298, CLC21503	Os01g0580200	AK103045, Unigene Os18562 43 ESTs	Callus, green shoot, immature leaf, panicle at flowering,
OsBGal 4	AP003922, AP003297, NT_079952, CLO04124.242	Os01g0875500	AK102715, AK101399, Unigene Os.9892 107 ESTs	Flower, callus, roots, panicle at flowering
OsBGal 5	AP003445, AP003447, AAAA01000473, CL024037.17, CL004328.202.182 , CL024778.114.123	Os01g0533400	AK119447 Unigene Os.41043: 3 ESTs	Stem
OsBGal 6	AP008211.1 AC135419, AC135429, CLB8049.3	Chrom 5 20854058-20845697	Unigene Os.82841: 1 EST	Unknown
OsBGal 7	AP004996, AAAA01011567, AAAA01017443, CL037070.87	Os02g0219300	AK061043, AK119269, AK059059, AK059794, Unigene Os.14358: 57 ESTs	Shoot, anther, stem, panicle at flowering, root, callus
OsBGal 8	AC134234, AAAA01001024, HTC149654-	Os03g0255100	AK061989, AK067479, Unigene	shoot, leaf, Endosperm; Immature Seed;

	A01.R.5.1		Os.22360 252 ESTs	panicle at flowering, flower, mixed, stem, root, callus, seed
OsBGal 9	AC119291	Os05g0539400	AK068572, Unigene Os.14570: 65 ESTs	Shoot, leaf, panicle at flowering, stem, immature seed, callus, whole plant, callus, seed, root
OsBGal 10	AP003912, AY459336, AAAA01005991, CL034244.24	Os08g0549200	AK069066, AK070916, Unigene Os.18310: 136 ESTs,	Flower, panicle at flowering, stem, 800 per million transcripts in flower library (34/43,008)
OsBGal 11	AP006149, AAAA01013958, CL009772.405	Os09g0539200	AK119414, Unigene Os.49945	seed
OsBGal 12	AC091749.8, AC131374, AE017076, AAAA010005757, CL027104.6, CL010261.145.10	Os10g0330600	AK119350 Unigene Os.46702: 3 ESTs	flower
OsBGal 13	AL935071, BX664709, CLO04181.73.15, CLO07520.141	Os12g0429200	AK065546, Unigene Os.52193: 36 ESTs	shoot, stem, leaf, flower, inflorescence, callus
OsBGal 14	AP008216.1 AC090498, NT080065	Chrom 10 9501961-9496993	AK242716 AK242960 Os.22528: 65 ESTs	Flower, immature seed, panicle, 3500 transcripts per million in flower library (37 of 13,353)
OsBGal 15	AP008212 AP004733, CL032501.65	Chrom 6 25034594-25026949	None	Unknown

In this table, Genbank nr database entries (mainly RGP japonica rice), Beijing Genomics Institute (BGI) scaffold entries (starting with AAAA) and Syngenta Torrey Mesa Genomics Institute (TMRI, japonica rice) database contigs (starting with CL or HTC) are listed in column 2. Expression tissues are taken from source data on EST and full-length cDNA entries found matching these genes.

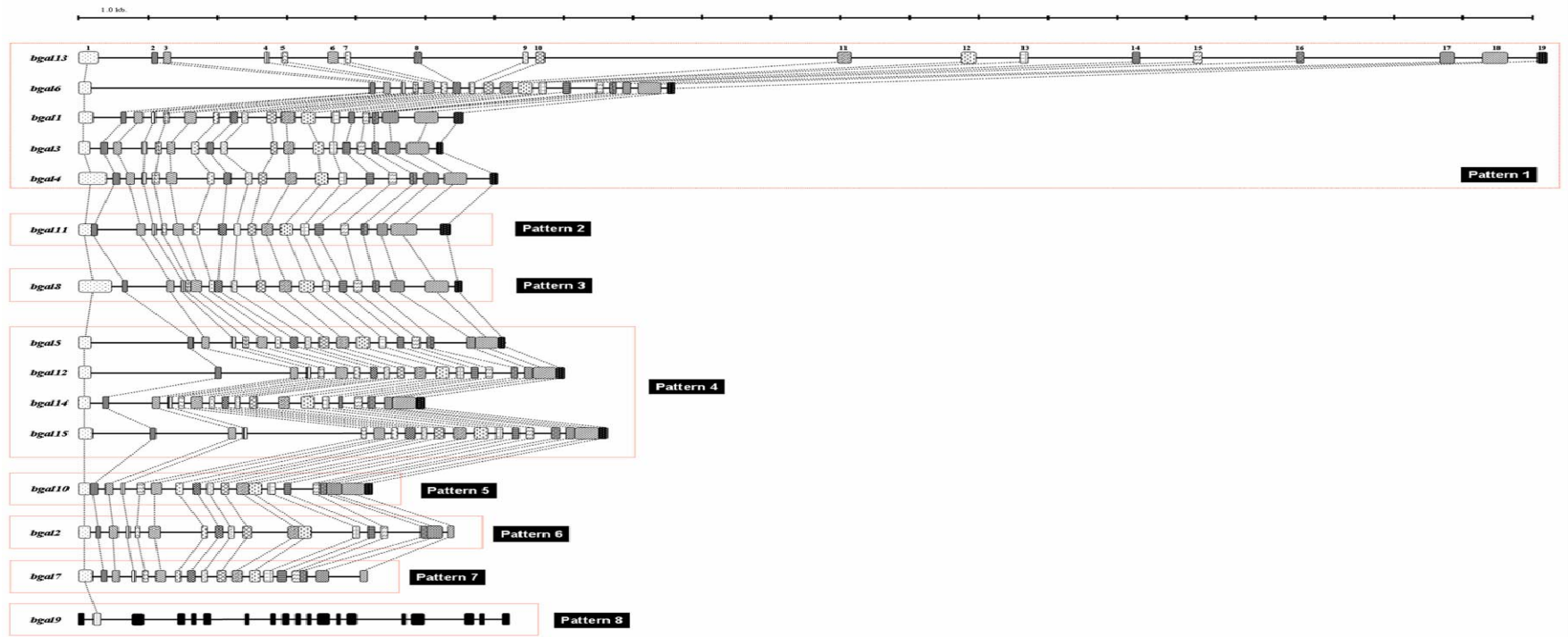


Figure 3.1 Comparison of the structures of putative rice β-galactosidase genes from GH35 family, which showed eight different splicing patterns. Most of the positions of splicing along the genes showed highly conserved intron phasing, but some genes appeared to have undergone intron loss. OsBGal19, however, had an unrelated gene structure, with only a few similar splice sites (pattern 8).

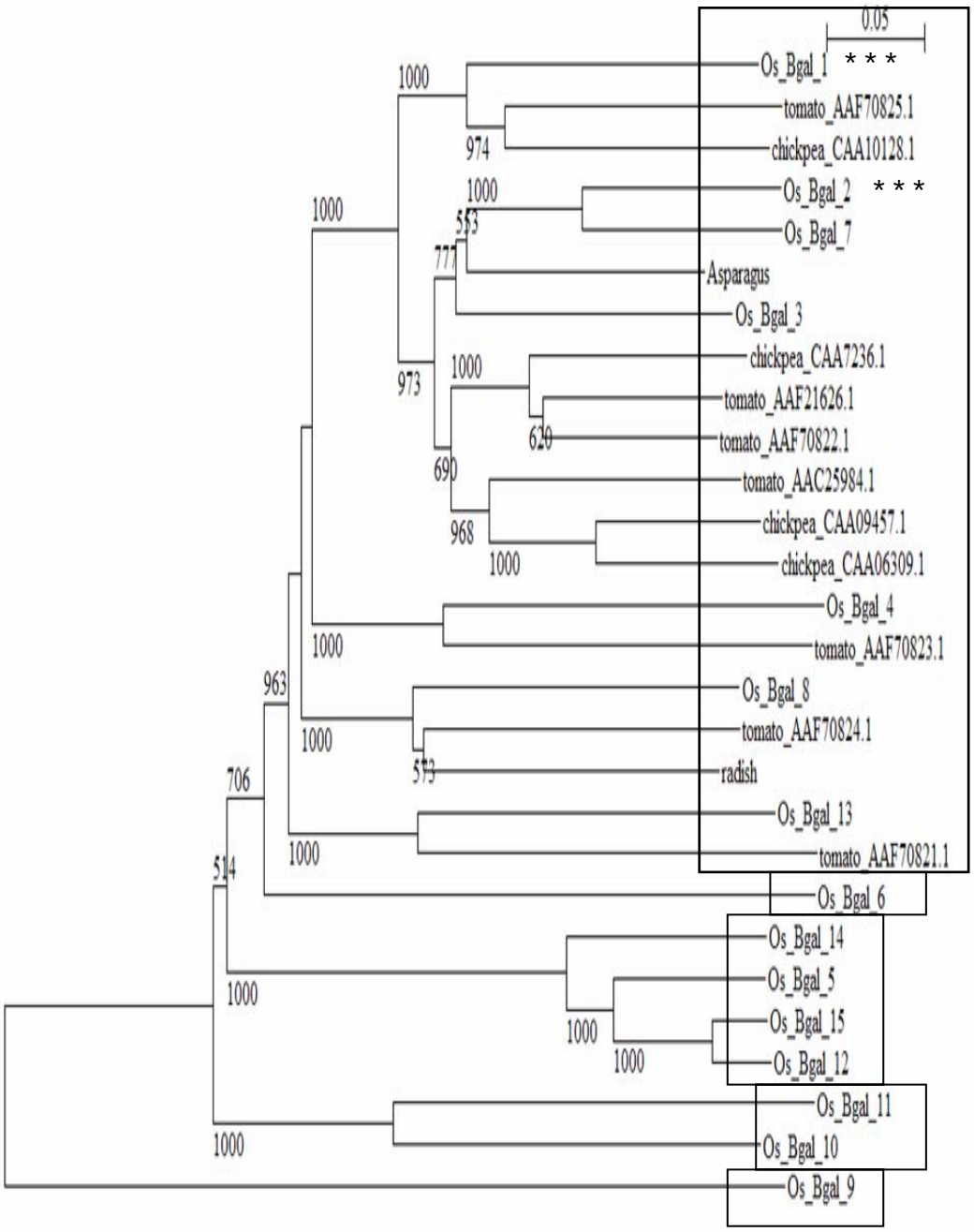

0.05
Os_Bgal_1 * * *
tomato_AAF70825.1
chickpea_CAA10128.1
Os_Bgal_2 * * *
Os_Bgal_7
Asparagus
Os_Bgal_3
chickpea_CAA7236.1
tomato_AAF21626.1
tomato_AAF70822.1
tomato_AAC25984.1
chickpea_CAA09457.1
chickpea_CAA06309.1
Os_Bgal_4
tomato_AAF70823.1
Os_Bgal_8
tomato_AAF70824.1
radish
Os_Bgal_13
tomato_AAF70821.1
Os_Bgal_6
Os_Bgal_14
Os_Bgal_5
Os_Bgal_15
Os_Bgal_12
Os_Bgal_11
Os_Bgal_10
Os_Bgal_9
1000
974
1000
553
771
1000
973
1000
620
690
968
1000
1000
963
1000
573
706
1000
514
1000
1000
1000
1000

Figure 3.2 Phylogenetic tree showing relationship of OsBGal1 and OsBGal2 to other plant β-galactosidases. The two cloned beta-galactosidases were compared at the protein level to other GH family 35 genes, as well as the genes characterized from chickpea, radish and tomato to show their relationship to each other and the other members of this family. The Genbank accession numbers are given in the names for the chickpea (Esteban et al., 2005) and tomato (Smith and Gross, 2000), while "Asparagus" refers to Genbank accession number CAA54525.1 (King and Davies, 1995) and "radish" to accession number BAD20774.2 (Kotake et al., 2005). The rice genes are derived from the following loci or accessions: OsBGal 1, Os03g0165400; OsBGal 2, Os06g057360; OsBGal 3, Os01g058200; OsBGal 4, Os1g0875500; OsBGal 5, Os01g0533400; OsBGal 6, accession AP008211.1 (chromosome 5); OsBGal 7, Os02g0219300; OsBGal 8, Os03g0255100 (Kaneko and Kobayashi, 2003); OsBGal 9, Os05g0539400; OsBGal 10, Os08g0549200; OsBGal 11, Os09g0539200; OsBGal 12, Os10g0330600; OsBGal 13, Os12g0429200; OsBGal 14, AP008216 (chromosome 10); OsBGal 15, AP008212 (chromosome 6). Proteins with C-terminal galactose-binding lectin domains are indicated by stars. Despite OsBGal 1 having a C-terminal lectin-like domain and OsBGal 2 lacking this domain, the two cluster relatively closely together, along with OsBGal 7 (lacking the domain), OsBGal 3 (with the domain) and family 35 enzymes from chickpea and tomato, some of which contain the C-terminal domain and some of which do not.

3.2 Expression pattern of rice β-Galactosidase genes.

Preliminary information on gene expression was obtained by BLASTn evaluation of the NCBI dbEST database (Table 3.1). To determine the endogenous expression pattern of the *OsBGal1* and *OsBGal2* β-galactosidase genes in the rice plant, probes were designed from the 3'UTR and amplified by PCR from cDNA or from rice genomic DNA (Figure 3.3). The *OsBGal1* and *OsBGal2* probes were then used to identify mRNA in tissues of 7-day-old seedlings and 6-week-old mature plants by northern blot analysis.

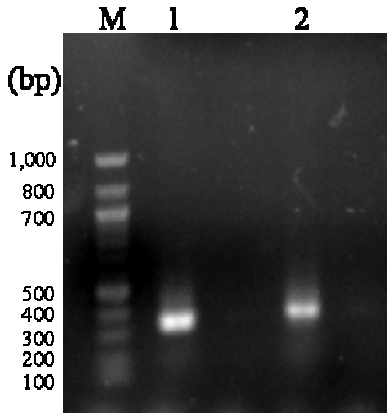


Figure 3.3 1% agarose gel of 3'UTR probes for expression analysis. The primers used for amplification were designed to amplified 250-300 bp probes from the 3'UTR and C-terminal encoding region.

M: EZ load 100 bp PCR Molecular Ruler

Lane 1: *OsBGal1* 3'UTR PCR product

Lane 2: *OsBGal2* 3'UTR PCR product

RNA gel blot analysis was used to determine transcript levels in tissues of 7-day-old seedlings and 6-week-old mature plants, relative to 28S and 18S rRNA bands, which acted as the loading control (Figure 3.4). The effect of environmental conditions on β-galactosidase gene expression in rice seedlings was also evaluated (Figure 3.5). However, the northern blots of the mRNAs of the two genes gave high background despite the use of specific 3' UTR probes, with multiple bands, including large, diffuse bands. Therefore, semiquantitative RT-PCR was used to probe the *OsBGal1* and *OsBGal2* expression pattern

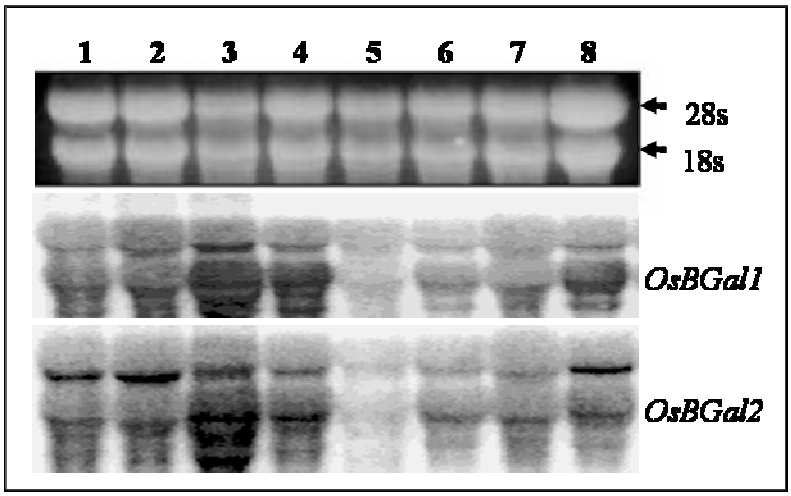


Figure 3.4 Northern blot analysis of *OSBGal1* and *OsBGal2* transcript levels in various tissues from mature plant and 7-d-old seedlings. Twenty micrograms of total RNA from the tissues loaded in each lane. The ethidium bromide-stained gel (top) shows the loading of ribosomal RNA was nearly equal in all lanes. The RNA blots were hybridized with α^{32}P-labeled 3'UTR cDNA probes of *OsBGal1* and *OsBGal2*. Lane 1, leaf blade; lane 2, leaf sheath; lane 3, root; lane 4, stem; and lane 5, node from 6-week-old rice plant, and lane 6, shoot; lane 7, endosperm; and lane 8, root from 7-d-old seedlings. A high background was seen with a similar pattern for both genes, so the expression patterns are unclear.

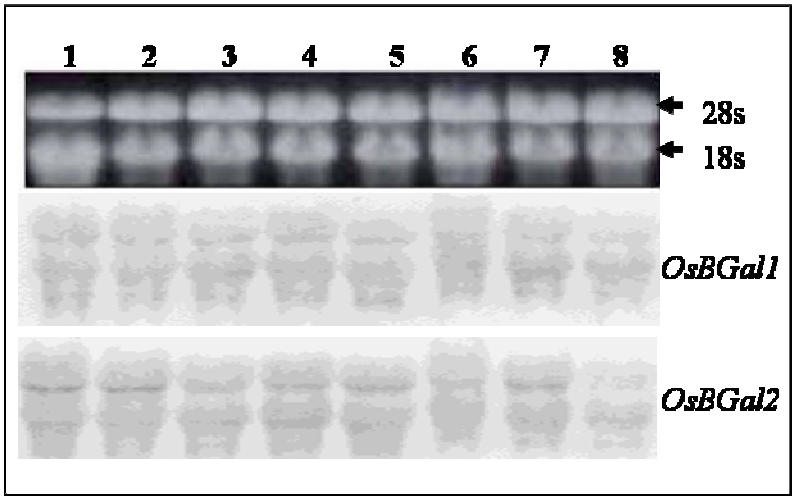


Figure 3.5 Northern blot analysis of *OsBGal1* and *OsBGal2* transcript levels in 7-d-old seedlings grown an additional 2 days under various stress conditions. Twenty micrograms of total RNA from the seedlings were loaded in each lane. The ethidium bromide-stained gel serves as a loading control (top). The RNA blot was hybridized with α^{32}P-labeled 3'UTR cDNA probes of *OsBGal1* and *OsBGal2*. Lane 1, control; lane 2, cold stress at 5°C; lane 3, cold stress at 12°C; lane 4, heat stress at 40°C; lane 5, drought for 2 days; lane 6, 0.3 M NaCl; lane 7, flooding for 2 days; lane 8, 0.5 M manitol for 2 days. Signals were weak and diffuse, so the specific signal is unclear.

3.3 Recombinant protein expression of rice OsBGal1 and OsBGal2

In this study, the *OsBGgal1* cDNA was amplified using rice seedling cDNA as the template. To obtain the full-length mature protein coding sequence of the *OsBGal1* cDNA, the first stand cDNA was synthesized from mRNA extracted from 3-4 days old rice seedling as the template (from Opassiri, 2003). The first strand cDNA was then used as the template in a PCR reaction with the specific sense primer MOSMex-F and antisense primer MOSMex-R, derived from the *OsBGal1* cDNA library clone AF508799

sequence to amplify a cDNA encoding the predicted mature protein of *OsBGal1* (Figure 3.6).

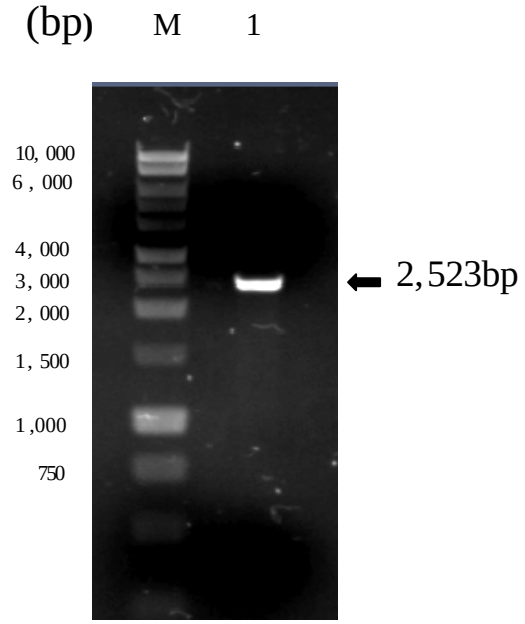


Figure 3.6 1% agarose gel electrophoresis of PCR product of full mature protein encoding *OsBGal1* cDNA amplified with the MOSMex-F and MOSex-R primers. Lane M, Hi-low DNA marker; lane 1, PCR product of full expression *OsBGal1* cDNA.

This PCR product was gel purified and cloned into pBlueScript II SK (+) vector. The PCR product was ligated with *EcoR* V digested pBluescript II SK (+) vector and transformed. The recombinant plasmid was selected and digested with *Xho* I and *Nco* I enzymes, as shown in Figure 3.7. The gel purified digested *OsBGal1* cDNA was cloned

into the pET32a(+) expression vector to produce recombinant protein for functional analysis.

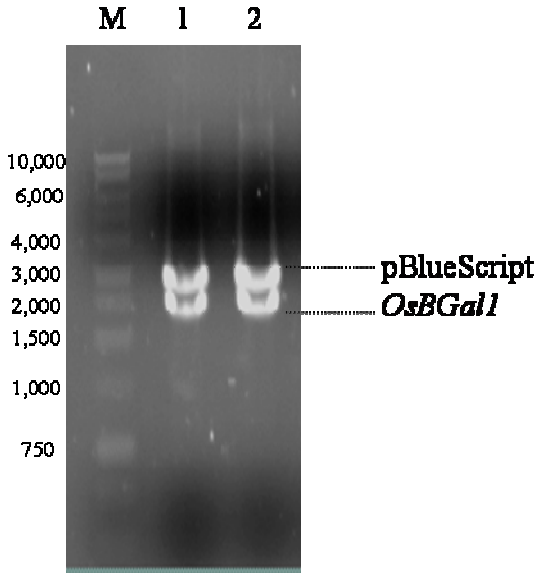


Figure 3.7 1% agarose gel of *OsBGal1* cDNA digested from pBluescript II SK (+) by *Xho* I and *Nco* I. Lane M, Hi-low DNA marker; lanes 1 and 2, *OsBGal1* cDNA digested by *Xho* I and *Nco* I.

To prepare for OsBGal2 protein expression, the cDNA fragment encoding the predicted mature β-galactosidase protein was generated by PCR with primers which inserted an *Nco* I restriction site at the 5' end of the fragment and an *Xho* I restriction site at the 3' end (Figure 3.8). The PCR product was cloned into the *Eco*R V site of

pBluescriptKS(+/-). After the sequence was confirmed by automated sequencing, the insert was cut out with *Nco* I and *Xho* I (with a partial *Nco* I digest for *OsBGal2*, which has an internal *Nco* I site, Figure 3.9), gel purified and cloned into the pET32a expression vector.

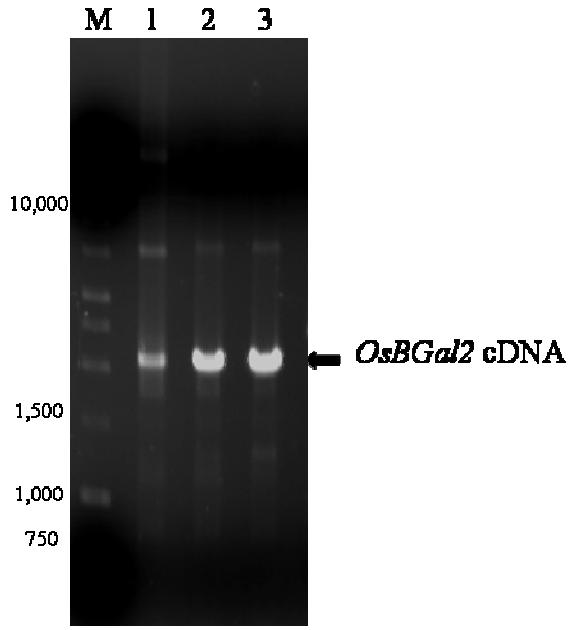


Figure 3.8 1% agarose gel electrophoresis of PCR product of a cDNA encoding the predicted mature OsBGal2 protein amplified with the MESTex-F and MESTex-R primers. Lane M, Hi-low DNA marker; lanes 1, 2 and 3, PCR products of OsBGal2 with 1/10, 1/100 and 1/500 dilution of the cDNA template, respectively.

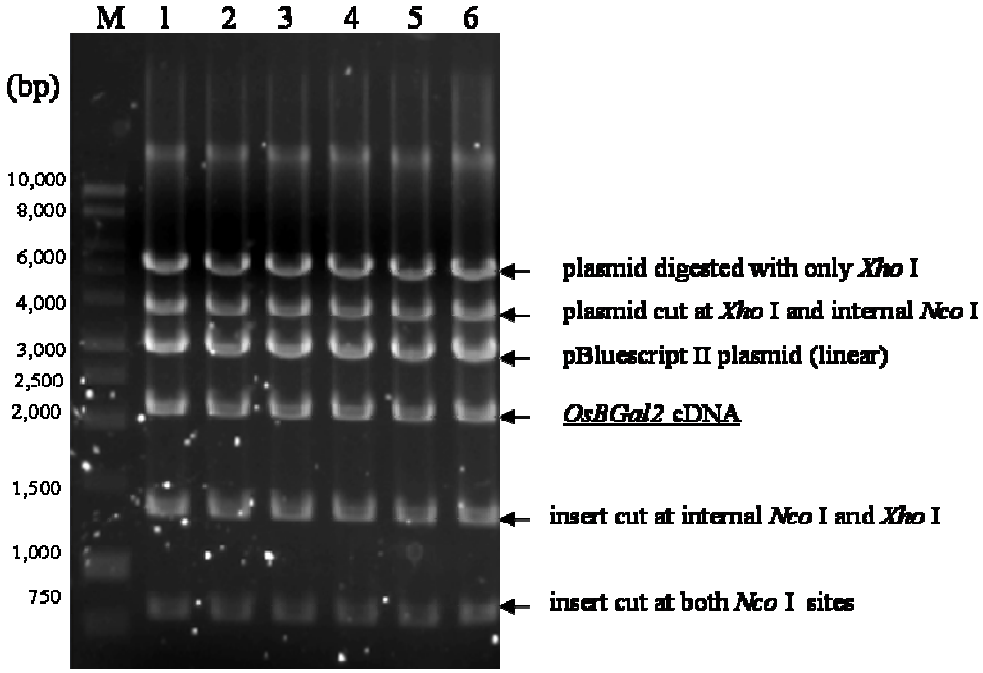


Figure 3.9 1% agarose gel of *OsBGal2* cDNA digested from pBluescript II SK (+) by *Xho* I followed by a partial digest with *Nco* I. The arrows indicate the plasmid-derived bands with the label for the correct size band of OsBGal2 cDNA underlined. Lane M, Hi-low DNA marker; lanes 2-7, *OsBGal2* cDNA digested from pBluescript II SK (+) by *Xho* I and *Nco* I.

When the OrigamiB (DE3) cells containing the pET32-OsBGal1 plasmid were induced with IPTG at 20°C, the expressed thioredoxin-OsBGal1 fusion protein was detected at 97 kDa on SDS-PAGE. This band was absent from the profiles of induced cultures containing pET32a(+) plasmid without an OsBGal1 cDNA insert (negative control). However, most of the expressed protein was localized to inclusion bodies, though some active soluble protein was also produced. The SDS-PAGE profiles of total cells and the insoluble fraction including inclusion bodies are shown in Figure 3.10

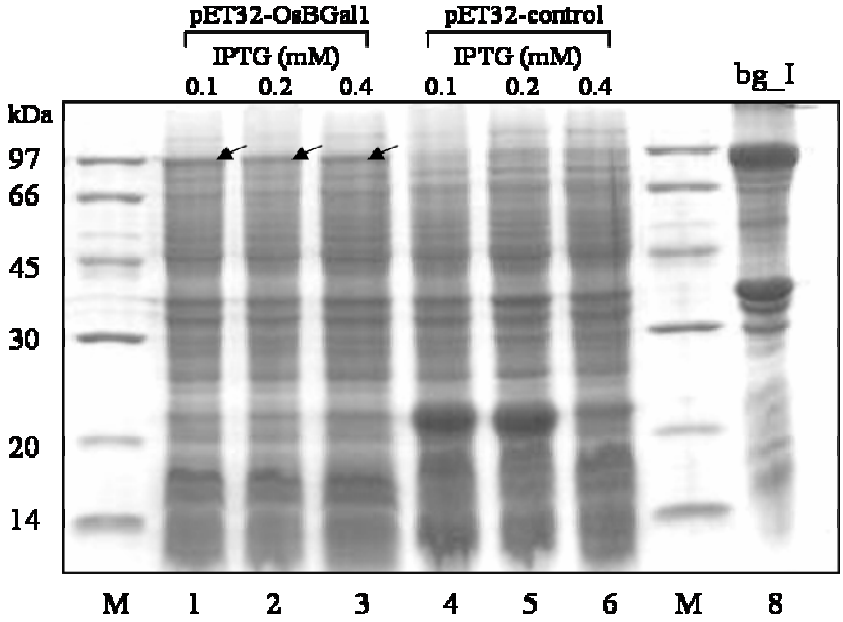


Figure 3.10 10% SDS-PAGE of protein expression in OrigamiB (DE3) *E. coli* transformed with the recombinant plasmid pET32-OsBGal1 and the pET32a (+) negative control. Lane M, molecular weight markers; lanes 1, 2 and 3, total protein extracts of cells containing the recombinant plasmid pET32-OsBGal1 after incubation in the presence of 0.1, 0.2, and 0.4 mM IPTG, respectively, at 20°C for 14 h. Lanes 4, 5 and 6, total protein extracts of cells containing pET32a(+) negative control after incubation in the presence of 0.1, 0.2, and 0.4 mM IPTG, respectively, at 20°C for 14 h. Lane 8, insoluble crude extract from *E. coli* with the pET32-OsBGal1 construct.

To check for the β-galactosidase activity of the recombinant fusion proteins, *p*NP-β-D-galactopyranoside hydrolysis assays were done with the soluble crude protein extract and compared to those from cells containing pET32a(+) control (Table 3.2). OsBGal1 and OsBGal2 were produced in active form and the soluble extracts from cells containing recombinant plasmids hydrolyzed *p*NP-β-D-galactopyranoside at high levels compared to extracts from cells with empty plasmid.

Table 3.2 Activity in crude extracts of recombinant OrigamiB (DE3) *E. coli* expressing OsBGal1 and OsBGal2 fusion proteins.

	Specific Activity (nmol $min^{-1}mg^{-1}$)[a]		
Substrate	**control pET32a(+)**	**OsBGal1- pET32a(+)**	**OsBGal2- pET32a(+)**
*p*NP-β-D-Galactopyranoside	1.5 ± 0.05	12.1 ± 4.30	83.0 ± 1.10
*o*NP-β-D-Galactopyranoside	2.0 ± 0.05	5.8 ± 0.05	4.0 ± 0.05
*p*NP-β-D-Glucopyranoside	nd	nd	nd

[a]Assays were done with 5 mM substrate at 37°C for 20 min at pH 5.0, and are expressed as nitrophenol produced per min per mg of total protein in the extract.

nd means not detectable

OsBGal2 protein expressed in OrigamiB (DE3) *E. coli* transformed with pET32a(+) seemed to be active, as the soluble cell extract could hydrolyze *p*NPGal at a high level compared to the control, though the OsBGal2 fusion protein appeared to be mainly in the insoluble cytoplasmic fraction (inclusion bodies). When the total protein fraction was checked on SDS-PAGE, the OsBGal2 fusion protein was detected at about 90 kDa (the expected size). This band was absent from the profiles of induced cultures containing pET32a (+) plasmid without an OsBGal2 cDNA insert (negative control). The top two of these bands reacted with anti-OsBGal2 catalytic domain peptide antibodies on a western blot as shown in Figure 3.11.

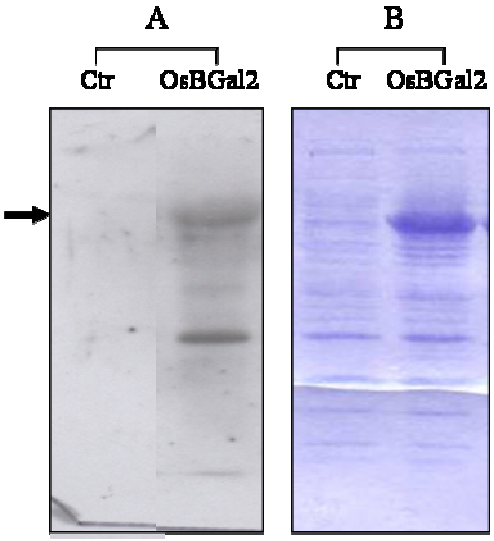


Figure 3.11 Immunoblot (A) and 10% SDS-PAGE gel (B) of total cell protein of *E. coli* strain OrigamiB (DE3) induced to express protein from pET32a-OsBGal2. Five micrograms of proteins were loaded in each lane. Samples are the total cell fractions from *E. coli* with control pET32a (Ctr) and expression construct (OsBGal2). Detection on the blot in A was with affinity-purified antibodies against an OsBGal2 catalytic domain peptide and the gel in B was with coomassie brilliant blue.

3.4 Characterization of crude OsBGal1, OsBGal2 and OsBGal13

The crude rice β-galactosidase from *E. coli* extracts was tested for activity against various substrates. Beside OsBGal1 and OsBGal2, we had cloned another rice β-galactosidases cDNA as previously shown in Table 3.1. AK065546 cDNA clone (OsBGal13) was amplified and cloned into pET32 expression vector and expressed in *E. coli* by Onkoksoong and Ketudat-Cairns (unpublished data) and the crude protein extracted from OsBGal13 was also used to test for the natural substrate with some galactose-containing polysaccharides.

Crude protein extracts of OsBGal1 and OsBGal2 were tested for hydrolysis of various galactose containing substrates. The crude enzymes catalyzed the hydrolysis of *p*NPGal, as detected by the formation of free *p*NP (Figure 3.12 A) and free galactose (Figure 3.12 B). Extended incubation gave lower R_f (i.e. larger) *p*NP-containing products, indicating that OsBGal1 and OsBGal2 could transglycosylate the *p*NPGal:

$$\text{Gal-}p\text{NP} + \text{Gal-}p\text{NP} \rightarrow \text{Gal-Gal-}p\text{NP} + p\text{NP}$$

in addition to hydrolyzing it:

$$\text{Gal-}p\text{NP} + H_2O \rightarrow \text{Gal} + p\text{NP}$$

Besides galactose, small amounts of ribose were formed (Figure 3.12 B), probably by hydrolysis of ribonucleotides co-extracted with the OsBGal1 and OsBGal2, ribose formation was not dependent on the addition of *p*NPGal. In addition to *p*NPGal, cell wall polysaccharide model substrates were also examined (Table 3.3).

Table 3.3 Substrates which were not and were cleaved by crude OsBGal1 and OsBGal2

Substrate	OsBGal1	OsBGal2	Time
*p*NP-β-D-galactoside	+	+	1h
*p*NP-β-D-glucoside	-	-	1 h
Maize AIR	-	-	24 h
Rice coleoptile AIR	-	-	24 h
Rice root AIR	-	-	24 h
Rice coleoptile alcohol extract	-	-	24 h
Rice root alcohol extract	-	-	24 h
Pectin	-	-	24 h
Galactan	-	-	24 h
Gum Arabic	-	-	24 h
Hemicellulose (barley, grass, maize)	-	-	24 h
Lactose	-	+	24 h
Adenosine	-	-	24 h
Uridine	-	-	24 h
Acetyl galactoside	-	-	24 h
Polygalacturonic acid	-	-	24 h
[^{3}H] oligo-(1→4)-galactans	-	-	24 h
XGO-SR_s	-	-	24 h
Tran zeatin riboside	-	-	24 h

Note that only crude extract from OsBGal2 could hydrolyze lactose which could be detected on TLC. (- means no hydrolytic product was found and + means the hydrolytic product was found on TLC).

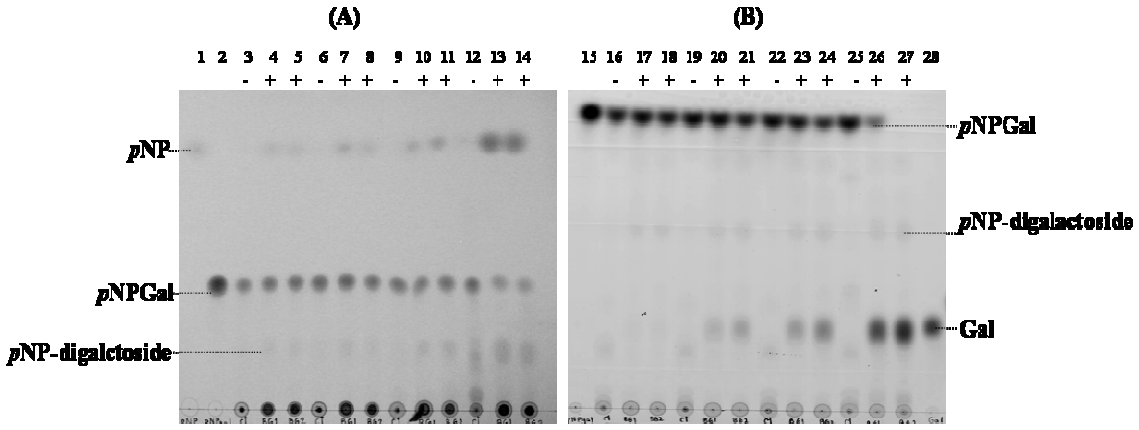


Figure 3.12 Transglycosylation of *p*NPG by crude extracts from OsBGal1 and OsBGal2 incubated with 5 mM *p*NPG in 50 mM NaOAc, pH 5.0, at 37°C for 5 min to 24 h. (A) the reactions were spotted onto an F_{254} Silica plate and developed with EtOAc/Pyridine/HOAC/H_2O (6:3:1:1) as solvent and detection with 365 UV light, while (B) was developed with Butanol/ Acetic/ Water (2:1:1) and detected with thymol/ H_2SO_4 staining. Samples were incubated crude enzyme extracts with (+) OsBGal1 (lanes 4, 7, 10, 13, 17, 20, 23 and 26) and OsBGal2 (lanes 5, 8, 11, 14, 18, 21, 24 and 27) and extracts of control pET32a containing cells (lanes 3, 6, 9, 12, 16,19, 22 and 25). Purified OsBGal1 gave similar transglycosylation. Lane 1, pNP standard; lanes 2 and 15, pNP-β-D-galactoside standard; lane 28, galactose standard. Reactions in lanes 3-5 and 6-18 were incubated for 5 min, those in lanes 6-8 and 9-21 for 1 h, those in lanes 9-11 and 22-24 for 4 h, and those in lanes 12-14 and 25-27 for 24 h.

A preliminary search for natural substrates of OsBGal1 and OsBGal2 in maize alcohol insoluble residue showed no hydrolysis to galactose, and ribose was the sole detectable product (Figure 3.13). When 0.5 mg of maize AIR was incubated with crude extract for 0-36 h at room temp, the unexpected product of free ribose was detected on TLC after staining with thymol/H_2SO_4. When the same reaction mixture was spotted on paper chromatography by using ribose as the internal marker, free ribose was also found as the product (data not shown).

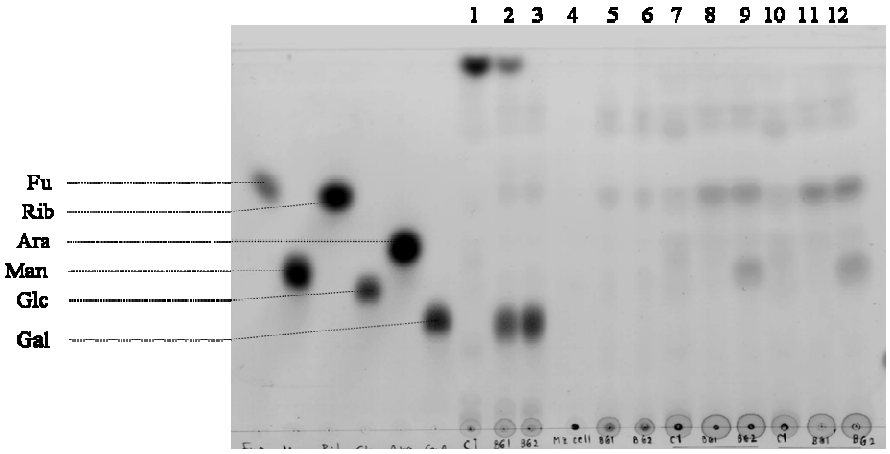


Figure 3.13 Hydrolysis of maize AIR with OsBGal1 and OsBGal2, assayed by TLC. One milligram of maize cell AIR was incubated with crude extracts of OsBGal1, OsBGal2 and control *E. coli* cells. Samples were incubated for 0-36 h, and silica TLC was performed with EtOAc/Pyridine/HOAC/H_2O (6:3:1:1) as solvent and the plate was stained with thymol/H_2SO_4. Lanes 1, 2 and 3, soluble crude extracts from control pET32a, OsBGal1 and OsBGal2 incubated with 5 mM *p*NPGal for 24 h; lane 4, maize cell AIR control, lanes 5 and 6, soluble crude extracts from OsBGal1 and OsBGal2; lanes 7, 8 and 9, soluble crude extract from control pET32a, OsBGal1 and OsBGal2, respectively, incubated with maize cell AIR for 24 h; lanes 10, 11 and 12, soluble crude extracts from control pET32a, OsBGal1 and OsBGal2, respectively, incubated with maize cell AIR for 36 h.

To understand about the ribose formation, uridine and adenosine were used to test for hydrolysis by the crude protein extracts and the hydrolysis product detected on TLC plate. To confirm the β-galactosidase activity and see if the substrates compete, 5 mM of *p*NPGal was also co-incubated in the reaction mixture of 0.5 mg of uridine (Figure 3.14) and 0.5 mg of adenosine (Figure 3.15). After 24 h of incubation of those substrates with crude enzymes and control protein (the extract of negative control cells without inserted gene), ribose was detected on the TLC plate, while only the crude OsBGal1, OsBGal2, and OsBGal13 coincubated with *p*NPGal yielded galactose as the product.

In order to determine the natural substrates of the enzymes in the rice plant, extracts of rice plants were tested for compounds that were hydrolyzed by the crude extracts of the enzymes from *E. coli*. OsBGal1, OsBGal2, and OsBGal13 have been tested, but these 3 isozymes were not seen to hydrolyze components of the alcohol insoluble extract. No substrate could be clearly identified for these crude extracts of OsBGals from *E. coli* cells. Figure 3.16 shows the initial results from screening of rice coleoptile and rice root alcohol insoluble residue on TLC.

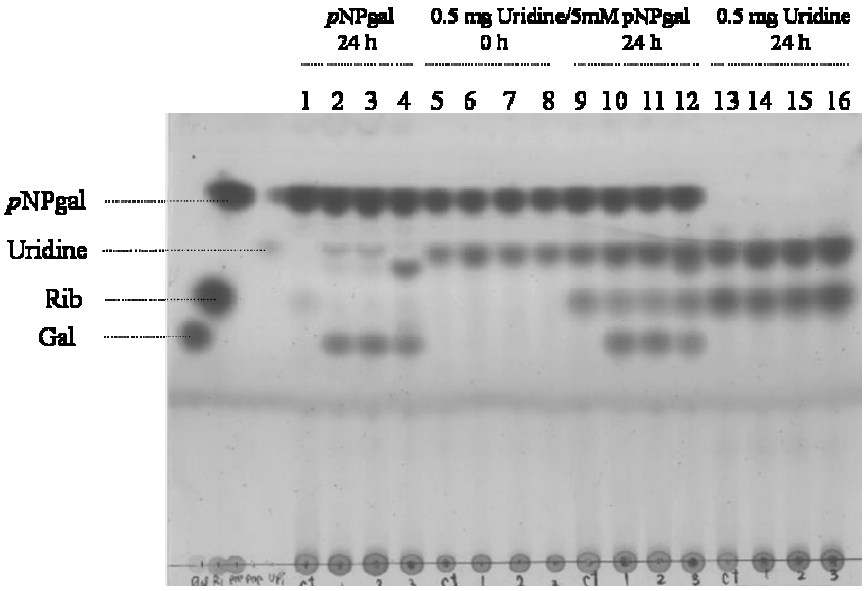


Figure 3.14 TLC of OsBGal1, OsBGal2 and OsBGal13 hydrolysis of uridine and *p*NP-Gal. 0.5 mg of uridine was incubated with crude extracts of cells that expressed OsBGal1, OsBGal2 and control plasmid. Samples were incubated for 0 or 24 h and spotted on a silica gel TLC that was developed with EtOAc/Pyridine/HOAc/H_2O (6:3:1:1) and stained with thymol/H_2SO_4**.** Lanes 1, 2, 3 and 4, soluble crude extracts from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with 5 mM *p*NPGal for 24 h; lanes 5, 6, 7 and 8, soluble crude extracts from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with 5 mM *p*NPGal and 0.5 mg uridine for 0 h; lanes 9, 10, 11 and 12, soluble crude extract from control pET32a, OsBGAl1, OsBGal2 and OsBGal13, respectively, incubated with 5 mM *p*NPGal for 24 h; lanes 13, 14, 15 and 16, soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with 0.5 mg uridine for 24 h.

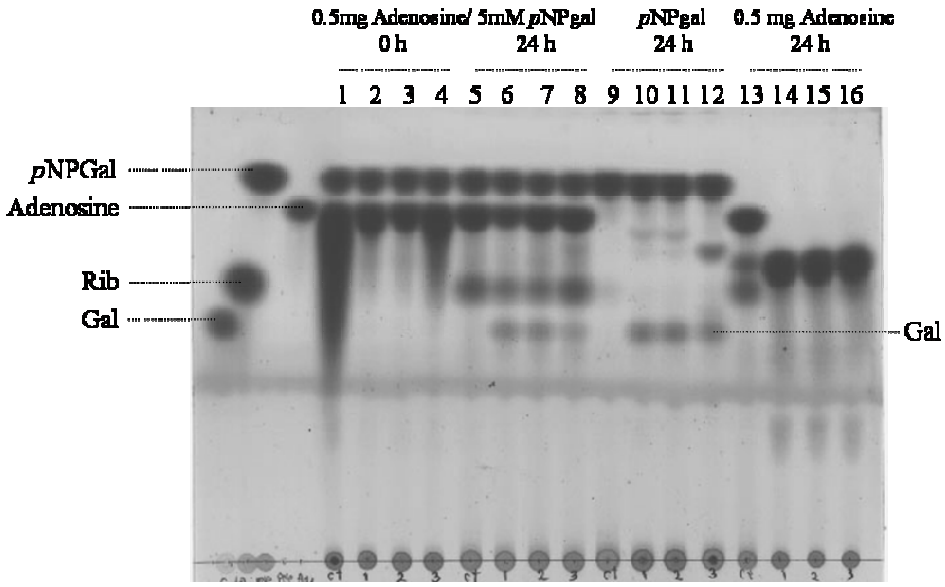


Figure 3.15 TLC of OsBGal1 and OsBGal2 hydrolysis of adenosine and *p*NPGal. 5 mM *p*NPGal and/ or 0.5 mg of adenosine was incubated with crude extract of cells that expressed OsBGal1, OsBGal2 and control *E. coli* cells. Samples were incubated for 0-24 h and the silica gel TLC was developed with EtOAc/Pyridine/HOAC/H_2O (6:3:1:1) and stained with thymol/H_2SO_4**.** Lanes 1, 2, 3 and 4, soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13 incubated with 5mM *p*NPGal and 0.5 mg adenosine for 0 h; lanes 5, 6, 7 and 8, soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13 incubated with 5 mM *p*NPGal and 0.5 mg uridine for 24 h; lanes 9, 10, 11 and 12, soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with 5 mM *p*NPGal for 24 h; lanes 13, 14, 15 and 16, soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with 0.5 mg adenosine for 24 h.

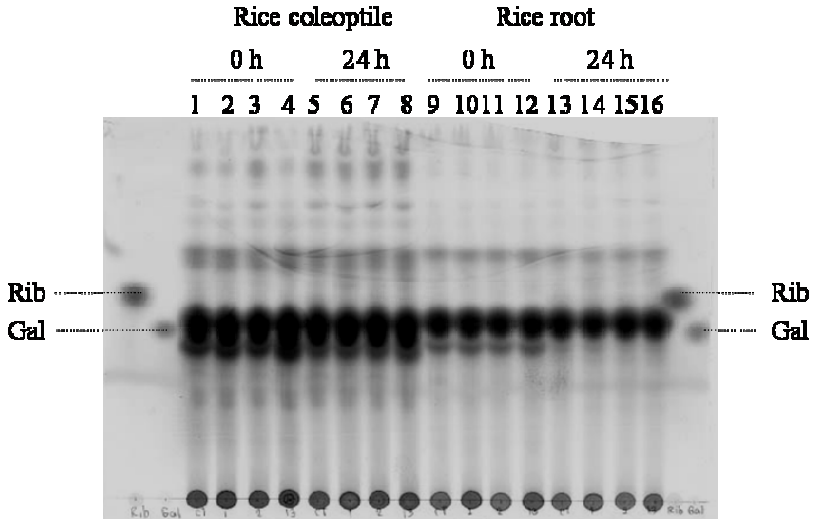


Figure 3.16 Rice alcohol insoluble residue hydrolysis by OsBGal1, OsBGal2 and OsBGal13 assayed by TLC. One milligram of rice coleoptile and rice root AIR was incubated with crude extracts of crude OsBGal1, OsBGal2 , OsBgal13, and control *E. coli* cell. Samples were incubated for 0 or 24 h and a TLC was run on a silica-gel plate in EtOAc/Pyridine/HOAC/H_2O (6:3:1:1) and stained with thymol/H_2SO_4**.** Lanes 1, 2, 3 and 4, 0.5 mg rice coleoptile AIR incubated with soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, for 0 h**;** lanes 5, 6, 7 and 8, 0.5 mg rice coleoptile AIR incubated with soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, for 24 h**;** lanes 9, 10, 11 and 12, 0.5 mg rice root AIR incubated with soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13 for 0 h**;** lanes 13, 14, 15 and 16, 0.5 mg rice root AIR incubated

with soluble crude extract from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated for 24 h.

When the crude extracts from control *E. coli* cells without a recombinant gene inserted (negative control), OsBGal1, OsBGal2, and OsBGal13 were tested for hydrolysis of lactose for 0-24 h. The paper chromatogram indicated the hydrolysis products of lactose only occurred with crude OsBGal2, while the other crude extracts from *E. coli* cells could not hydrolyse lactose (Figure 3.17), suggesting that β-galactosidase produced from crude OsBGal2 *E. coli* extract could hydrolyze lactose to give glucose and galactose as the products.

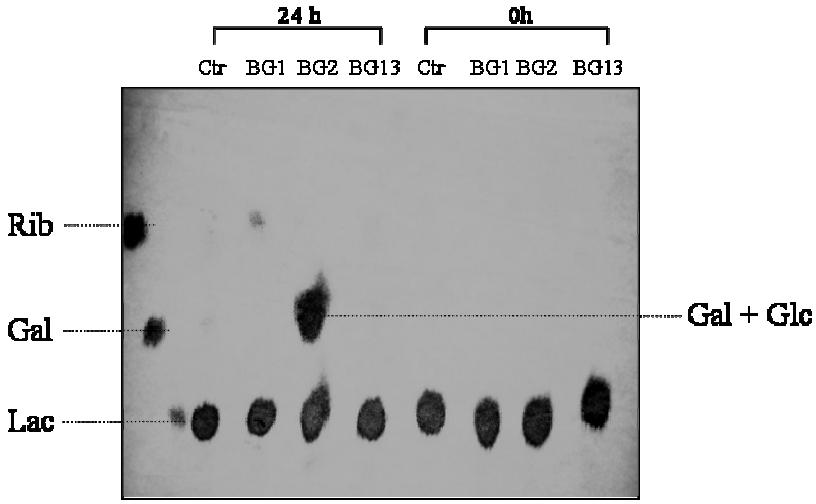


Figure 3.17 Paper chromatogram showing the action on hydrolysis of lactose by OsBGal2. 0.5 mg of lactose was incubated with crude extracts of control *E. coli* cells and those producing OsBGal1 (BG1), OsBGal2 (BG2) and OsBGal13 (BG13). Samples were incubated for 0 or 24 h and paper chromatography was run with EtOAc/Pyridine/HOAC/H_2O (6:3:1:1) solvent and stained with $AgNO_3$.

Xyloglucan oligosaccharides (XGOs), a mixture containing principally XLLG (nonasaccharide), XXLG (octasaccharide), and XXXG (heptasaccharide) was used to test for hydrolysis by crude extracts from *E. coli* cells induced to produce OsBGal1, OsBGal2, OsBGal13 or a control thioredoxin tag. The XGO mixture was converted to XGO-SRs (XLLG-SR XXLG-SR XXXG-SR, all of which act as fluorescent acceptor substrates for XETs) as previously described by Fry (1997). Figure 3.18 shows that the TLC profiles of XGO-SRs after 1-27 h incubation were similar to that of the starting material, which indicated that no hydrolysis of the fluorescently labelled substrate had occurred during the assay. So, the recombinant rice β-galactosidases had no apparent activity on these xyloglucan oligosaccharides.

Tritiated oligo-(1→4)-galactans, and 2'fucosyl lactitol, which have terminal non-reducing β-D-galactopyranosyl residues, were not substrates. As shown in Figure 3.26, 10 kBq of each compound were tested for hydrolysis of crude extract from *E. coli*. The TLC profiles of β-(1→ 4)-[^{3}H]Gal$_5$-ol, β-(1→ 4)-[^{3}H]Gal$_8$-ol, β-(1→ 4)-[^{3}H]Gal$_{11+}$-ol and 2'fucosyl-[^{3}H]lactitol after 24 h incubation were similar to those of the starting materials (Figure 3.18), which indicated that no hydrolysis of the [^{3}H] labelled substrate had occurred during the assay.

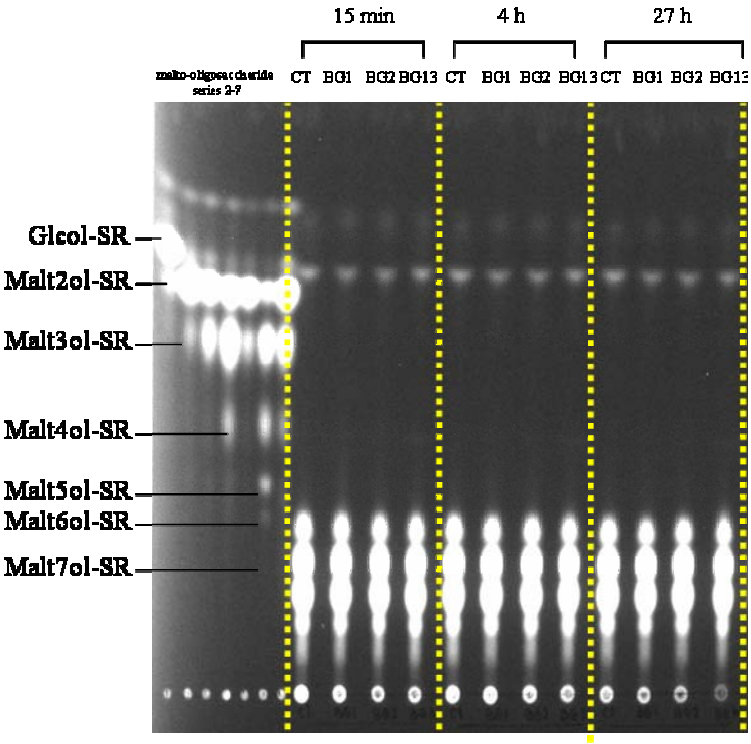


Figure 3.18 Thin- layer chromatographic (TLC) profiles of XGO-SRs incubated with OsBGal crude extracts after 15 min-27 h. The XGO-SRs at 6.5 mM were dissolved in 25 mM MES buffer, pH 5.5. Crude extracts of OsBGal1 (BG1), OsBGal2 (BG2), and OsBGal13 (BG13) were incubated in the XGOs-SR for 0-27 h and analyzed by silica gel TLC in the solvent of BuOH/HOAc/H_2O (2:1:1) and detected under UV light. The profiles were similar to that of the starting material, which indicates that no hydrolysis of the fluorescently labelled substrate occurred during the assay.

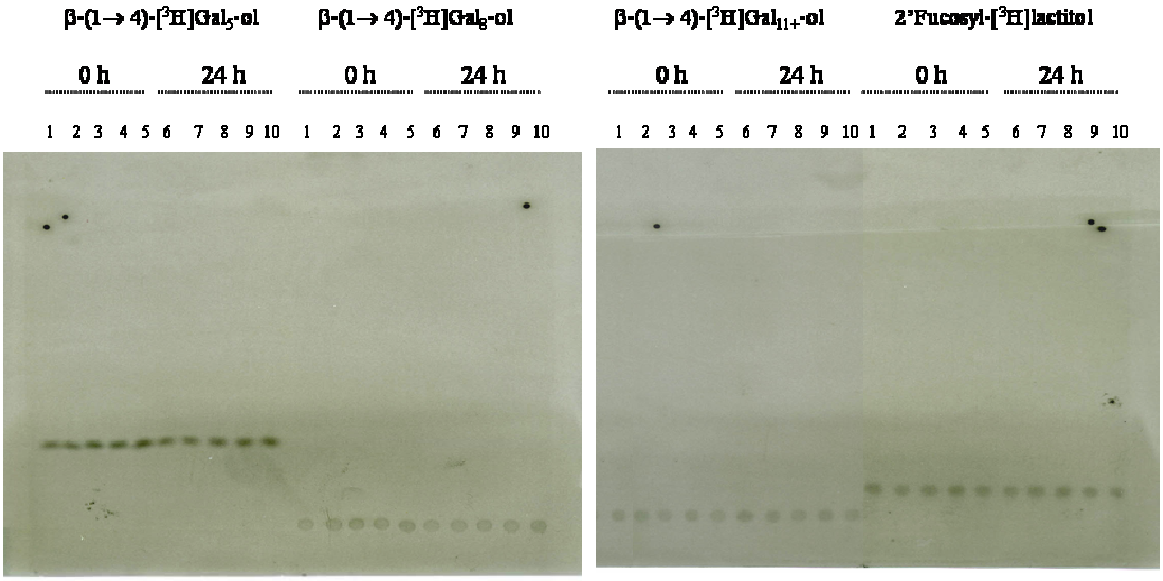


Figure 3.19 Thin-layer chromatographic (TLC) profile of β-(1→ 4)-[^{3}H]Gal$_5$-ol, β-(1→ 4)-[^{3}H]Gal$_8$-ol, β-(1→ 4)-[^{3}H]Gal$_{11+}$-ol and 2'Fucosyl-[^{3}H]lactitol TLC in the solvent of BuOH/HOAc/H_2O (2:1:1) before and after 24 h digestion. Lane 1, [^{3}H]oligo-(1→4)-galactan control**;** lanes 2, 3, 4 and 5, soluble crude extracts from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with [^{3}H]oligo-(1→4)-galactans (penta-, octa- and undecasaccharides) and 2'Fucosyl-[^{3}H] lacitol for 0 h; lane 6, [^{3}H]oligo-(1→4)-galactans (penta-, octa- and undecasaccharides) control, lanes 7, 8, 9 and 10, soluble crude extracts from control pET32a, OsBGal1, OsBGal2 and OsBGal13, respectively, incubated with [^{3}H]oligo-(1→4)-galactans and 2'fucosyl -[^{3}H] lacitol for 24 h.

3.5 Purification and characterization of OsBGal1

3.5.1 Purification of OsBGal1

The OsBGal1 thioredoxin fusion protein was purified by Ni-NTA agarose affinity chromatography and DEAE anion-exchange chromatography (Figure 3.20). The enzyme was purified 22.8 fold over the crude extract with 4.9% yield of total activity (Table 3.5). Approximately 0.12 mg of purified protein could be obtained per 500 mL of bacterial culture. The recombinant protein was eluted from the column and two major bands corresponding to 97 and 66 kDa were observed in SDS-PAGE (Figure 3.21) and were detected by western blots with the antibodies raised against an OsBGal1 catalytic domain peptide. Additional bands of smaller molecular weight were also present, but they did not react with the antibody (Figure 3.21).

Table 3.4 Purification of OsBGal1 expressed in *E. coli* strain [OrigamiB (DE3)]

Purification step	Total protein mg	Total Activity[a] units	Specific Activity units mg protein^{-1}	Yield %	Purification fold
Crude extract	61.80	2.75×10^{-2}	4.95×10^{-4}	100	-
Ni-NTA column	2.99	1.03×10^{-2}	3.44×10^{-3}	37.5	6.95
DEAE column	0.12	1.36×10^{-3}	1.13×10^{-2}	4.94	22.8

[a]Activity determined with 5 mM pNP-β-D-galactoside (*p*NPGal) as the substrate; 1 unit catalyses the hydrolysis of 1 µmol *p*NPGal per min at 37°C for 10 min .

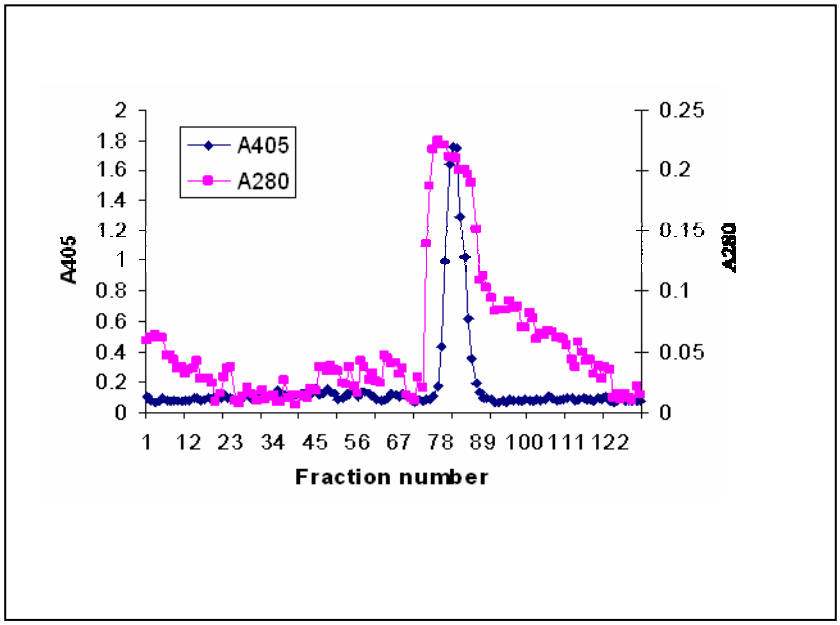


Figure 3.20 The chromatogram of purification of expressed OsBGal1 protein by ion-exchange chromatography. The blue line shows the absorbance of protein at 280 nm and the red line shows the activity in terms of absorbance at 405 nm of *p*NP released from 5 mM *p*NP-β-D-galactopyranoside as substrate. The reaction was performed by incubating 5 µl of each sample fraction with 5 mM *p*NPGal in 110 µl total reaction at 37°C for 10 min and stopping the reaction with 80 µl of 0.2 M Na_2CO_3.

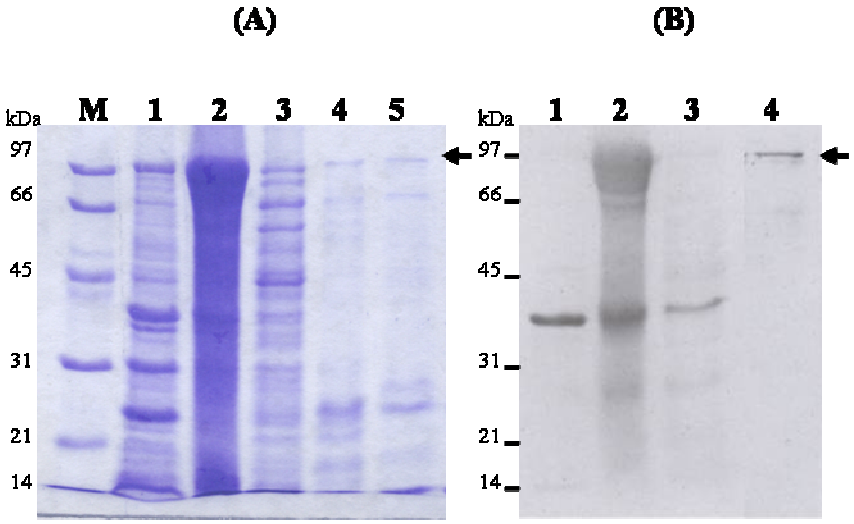


Figure 3.21 10% SDS-PAGE gel (A) and immunoblot (B) of pET32a-riceBGal1 expressed in *E. coli* strain OrigamiB (DE3) at different purification steps. Five micrograms of protein were loaded in lane 1. A. Coomassie brilliant blue stained gel. Lane M, molecular weight marker; lane 1, soluble crude extract from *E. coli* with control pET32a; lane 2, insoluble crude fraction from *E. coli* with the OsBGal1 expression construct; lane 3, soluble crude extract from *E. coli* with OsBGal1 expression construct; lane 4, OsBGal1 purified on a Ni-NTA column; lane 5, OsBGal1 purified by NiNTA and DEAE column chromatography. B. Immunoblot with affinity-purified antibodies against an OsBGal1 catalytic domain peptide. Lane 1, total cell extract of control cells after expression from empty vector; lane 2, total cell extract of cells expressing OsBGal1; lane 3, soluble crude extract from *E. coli* with BGal1 expression construct; lane 4, purified OsBGal1 protein after IMAC and DEAE chromatography.

3.5.2 Identification of OsBGal1 protein by LC/MS/MS

The purified OsBGal1 was resolved on 10% SDS-PAGE, and the 97 kDa (A), 66 kDa (B), and 29 kDa bands (C) (Figure 3.22) were excised and subjected to in-gel digestion with trypsin and mass spectrometry of the resulting peptides confirmed that the 97 kDa and 66 kDa bands contained both OsBGal1 and thioredoxin peptide, while the prominent non-immunoreactive band at approximately 29 kDa appeared to be a degradation product containing mainly thioredoxin tag. (The mass spectrum data, peptides and protein match details were shown in Appendix A.

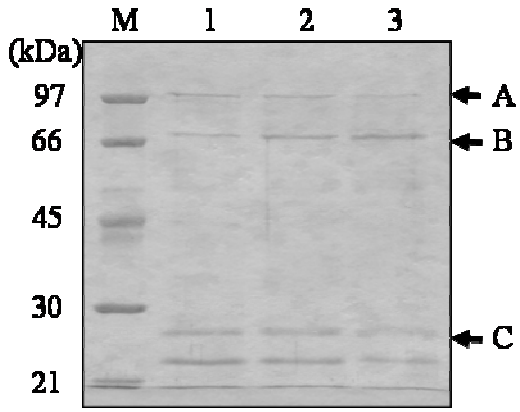


Figure 3.22 10% SDS-PAGE of pET32a-OsBGal1 expressed in OrigamiB (DE3) *E .coli* after anion-exchange chromatography. Lane M, Molecular weight marker; lanes 1, 2 and 3, OsBGal1 after purification by IMAC and DEAE columns. Arrows point to three protein bands that contained OsBGal1 and thioredoxin peptides, as confirmed by LC/MS/MS.

Eight peptides in the amino acid sequence of OsBGal1 from Chantarangsee, 2001 (Figure 3.23) were identified by LC/MS/MS of the 97 kDa band. For the 66 kDa band only four peptides gave the complete match with OsBGal1 (Figure. 3.23).

Figure 3.23 Peptides identified in tryptic digests of the 97 kDa and 66 kDa bands of the OsBGal1 protein by LC/MS/MS**.** The nine identified peptides (P1-P9) that gave complete matches with the OsBGal1 β-galactosidase sequence are presented in bold and underlined within the context of the predicted mature sequence of OsBGal1. (A) indicates sequences of peptides of the 97 kDa band, (B) indicates sequences of peptides of the 66 kDa band, while (A&B) indicates the sequences of peptides seen in both 97 and 66 kDa bands.

3.5.3 Substrate specificity

Purified OsBGal1 protein was tested for hydrolysis of a number of artificial substrates and cell wall-derived polysaccharides by incubating it with the substrates for 24 h and separating the products on silica gel TLC plates, followed by detection by staining (Table 3.5). Purified OsBGal1 protein could hydrolyse *p*NP-β-D-fucoside (= 6-deoxy-D-galactoside) in addition to *p*NPGal, but no other *p*NP-glycosides tested were hydrolysed. Moreover, the OsBGal1 enzyme released significant amounts of galactose from β-(1→3), β-(1→4)- and β-(1→6)-β-linked galactose di and trisaccharides with β-(1→3) galactobiose and galactotriose appearing to be hydrolysed the fastest (Figure 3.24). The enzyme was unable to hydrolyze lactose, unlike *lacZ* β-galactosidase from *E. coli* and many plant β-galactosidases.

Table 3.5 Substrates tested for hydrolysis by purified OsBGal1. Galactose and fucose release has detected by TLC. Release is indicated by the "+", while the other remaining substrates did not reveal any product on TLC, as indicated as the "-" .

Substrate	OsBGal1	Time
*p*NP-β-D-galactosideside	+	30 min
*p*NP-β-D-fucoside	+	30 min
*p*NP-β-D-glucoside	-	30 min
*p*NP-β-D-xyloside	-	30 min
*p*NP-β-D-arabinoside	-	30 min
*p*NP-β-D-mannoside	-	30 min
Maize AIR	-	24 h
β-(1→3)-galactobiose	+	24 h
β-(1→3)-galactotriose	+	24 h
β-(1→4)-galactobiose	+	24 h
β-(1→4)-galactotriose	+	24 h
β-(1→6)-galactobiose	+	24 h
β-(1→6)-galactotriose	+	24 h
Rice coleoptile AIR	+	24 h
Rice root AIR	+	24 h
Rice coleoptile alcohol extract	-	24 h
Rice root alcohol extract	-	24 h
Pectin	-	24 h
Galactan	-	24 h
Arabinogalactan	+	24 h
Gum Arabic	-	24 h
Lactose	-	24 h
Adenosine	-	24 h
Uridine	-	24 h
Polygalacturonic acid	-	24 h
Tran zeatin riboside	-	24 h

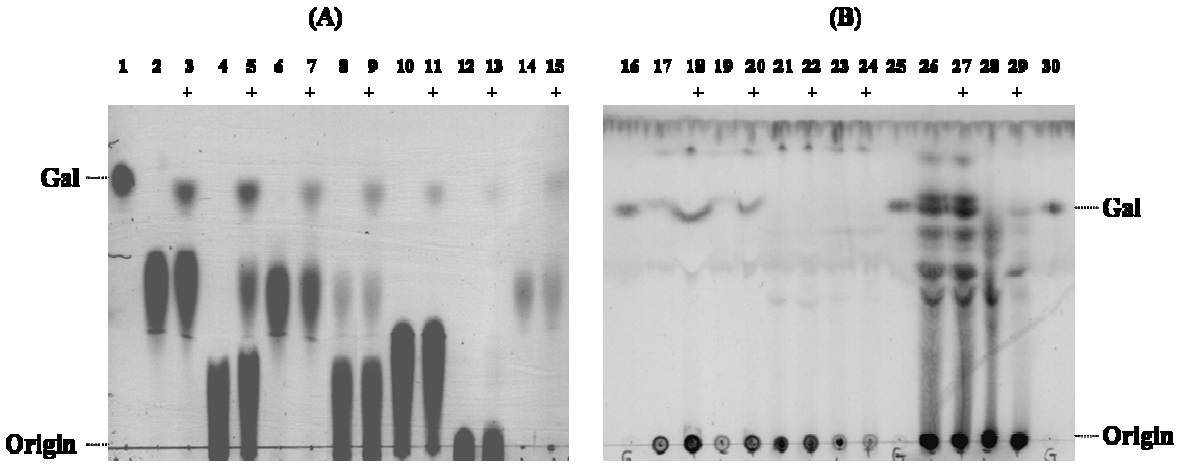
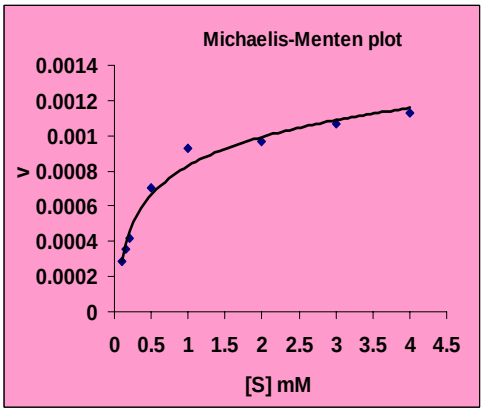


Figure 3.24 Substrate specificity of rice BGal1 assayed by TLC. 5 mM galactooligosaccharides (A) and 0.5% galactose-containing polysaccharides (B) were incubated with purified OsBGal1. Samples were incubated for 16 h and spotted on silica gel TLC plates in BuOH/HOAc/H_2O (2:1:1) and the products were detected by staining with 20% (v/v) H_2SO_4 in methanol. Samples were incubated with (+) or without (-) enzyme. Three times as much enzyme was used for polysaccharide substrates. Lanes 1, 16, 25 and 30, galactose standard (Gal); lanes 2 and 3, β-(1→3)-galactobiose; lanes 4 and 5, β-(1→3)-galactotriose; lanes 6 and 7, β-(1→4)-galactobiose; lanes 8 and 9, β-(1→4)-galactotriose; lanes 10 and 11, β-(1→6)-galactobiose; lanes 12 and 13, β-(1→6)-galactotriose; lanes 14 and 15, β-(1→4)-galactobiose; lanes 17 and 18, rice root alcohol-insoluble residue; lanes 19 and 20, rice coleoptile alcohol-insoluble residue; lanes 21 and 22, rice root hemicellulose; lanes 23 and 24, rice coleoptile hemicellulose; lanes 26 and 27, galactan; and lanes 28 and 29, larchwood arabinogalactan. Apparent release of galactose was seen for all galactose-oligosaccharides, rice root and coleoptile alcohol-insoluble residues, and larchwood arabinogalactan, while the possible release from galactan was unclear due to a high background in the substrate.

3.5.4 Study of kinetic parameters of OsBGal1

Kinetic analysis of the purified OsBGal1 with *p*NPGal as substrate under the standard assay conditions gave an apparent K_m of 0.32 mM and V_{max} of 2.1 μmole/min/mg.

(A)



(B)

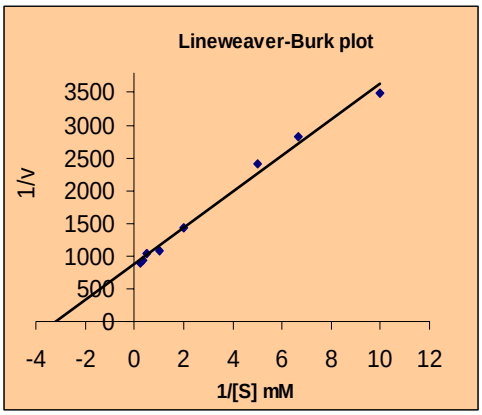


Figure 3.25 Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) for hydrolysis of *p*NPGal substrate by purified OsBGal1. The assays were carried out at different concentrations at 37°C for 10 mins. The velocity, V, is expressed as nmole/min/mg.

3.5.5 pH and temperature profile of OsBGal1 activity

The pH dependence of OsBGal1 activity was determined with *p*NPGal as substrate (Figure 3.26), and the optimum pH was 3.0-3.5, but the enzyme completely lost activity at pH 2.5 and lost nearly 50% of its activity by pH 5.0 on the basic end, as shown in Figure 3.26. The optimum temperature range for β-galactosidase activity was 50-60°C in a 10 min assay. Above 60°C, the activity decreased rapidly, likely because of thermal inactivation of the enzyme.

(A) **(B)**

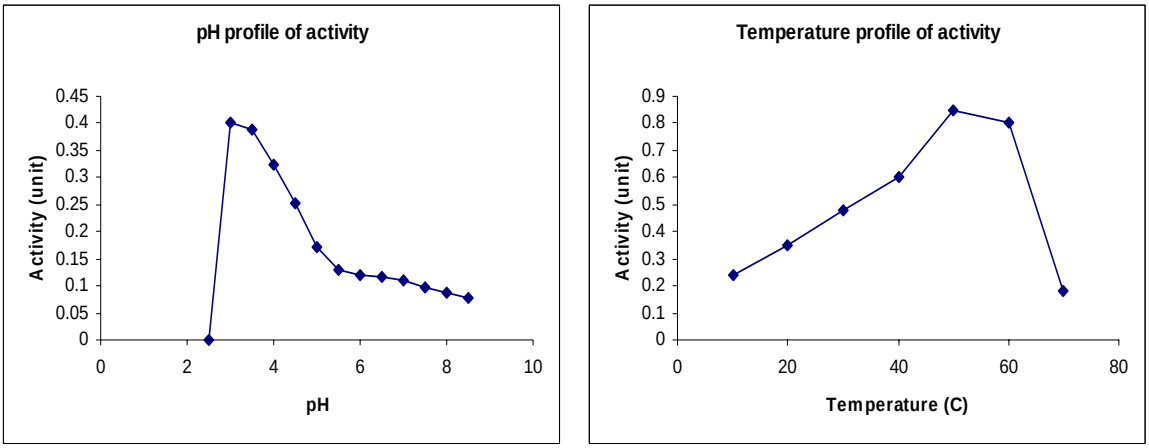


Figure 3.26 The pH and temperature profiles for OsBGal1 β-galactosidase activity. The activity was assayed over a pH range 2.5-8.5 (A) and over a temperature range of 15-70 °C (B) with 5 mM *p*NPGal for 10 min.

3.5.6 Inhibition studies

The effects of various sugars (each at 10 mM final concentration) on the hydrolysis of *p*NPGal by OsBGal1 under standard assay conditions was studied (Table 3.6). When tested for the effect of different sugars, only slight inhibition of enzyme activity was observed with galactose and lactose.

Table 3.6 Effects of various carbohydrates (5 mM final concentration) on the rate of *p*NPGal hydrolysis by purified OsBGal1 using standard assay conditions (5 mM final concentration of *p*NPGal).

Carbohydrate	**Relative activity**
None	100
D-Glucose	100
D-Galactose	90
Maltose	100
Lactose	85
D-Fructose	100
D-Xylose	100
L-Arabinose	100
Cellobiose	100

3.5.7 Effect of different cations on the activity of purified rice OsBGal1

The effect on β-galactosidase activity of various cations, each added to a final concentration of 10 mM to the standard assay, is shown in Table 3.7. The divalent cations Cu^{2+} Hg^{2+} and Fe^{3+} and to a lesser extent Mg^{2+} inactivated OsBGal1 β-galactosidase activity, with complete inactivation by Hg^{2+} and Fe^{3+} at lower concentration (1 mM), while most of the divalent cations tested did not greatly affect the enzyme activity when added to a final concentration of 10 mM. EDTA led to a 40% increase in activity at 10 mM, suggesting some metal ion in the enzyme preparation which is sequestered by the EDTA might be inhibiting the enzyme. Delta-galactonolactone, IPTG and δ-gluconolactone caused 62%, 38% and 40% inhibition, respectively, at 10 mM (Table 3.7).

Table 3.7 Effect of different cations and other potential inhibitors on the activity of purified OsBGal1

Cation	**Relative activity**				
	250 mM	**200 mM**	**100 mM**	**10mM**	**1 mM**
None	100	100	100	100	100
$MgCl_2$	-	-	-	73.4	-
$CaCl_2$	-	-	-	86.2	-
$MnCl_2$	-	-	-	119	-
$ZnSO_4$	-	-	-	85.9	-
$CuSO_4$	-	-	-	0	-
$NiSO_4$	-	-	-	116	-
$CoCl_2$	-	-	-	82.6	-
$HgCl_2$	-	-	-	0	0
$FeCl_3$	-	-	-	0	0
EDTA	-	-	69.5	143	-
IPTG	-	21.8	23.3	61.9	75.2
Imidazole	35.6	-	85.6	114	-
Histidine	103.5	107	123	125	-
δ-gluconolactone	-	16.8	21.8	60.2	65.2
δ-galactonolactone	-	-	16.5	37.8	58.1

Enzyme activity was determined under standard assay conditions using *p*NPGal as the substrate with the respective compound added to the enzyme for 30 min before the assay and added to the assay to give the stated final concentrations.

3.6 Immunoocalization of OsBGal1 and OsBGal2 in rice

3.6.1 Production of OsBGal1 and OsBGal2 antibodies

To determine the localization of protein β-galctosidases in rice and to identify bands of the OsBGal1 and OsBGal2 recombinant protein expressed in *E. coli*, polyclonal antibodies against synthetic peptides derived from OsBGal1 (N'-CEGKEFGAAGKAYIN-C') and OsBGal2 (N'-CGESVMGSGAKSRIPD-C') sequence were produced in New Zealand white rabbits. Rabbit serum was collected before and after the antigen injection and each rabbit's serum titer was tested by indirect ELISA. From the titer determination shown in Figure 3.27, anti-OsBGal1 polyclonal antibody from the third and the fourth bleeds of rabbit M1 was more suitable compared with the other anti-sera which had been taken from the preimmune serum and the first immune bleeding. At the same titer, anti-OsBGal1 from the third and the fourth bleeds gave higher absorbance compared to other bleeds. The second and fifth bleed also appeared useful, but the sixth bleed gave a weaker response. Therefore, the anti-OsBGal1 from rabbit M1 gave a titer of about 1:100 to 1:500, so a comparable dilution was used as a starting point for detection of the protein on western blot analysis and immunolocalization in rice. For anti-OsBGal2 polyclonal antibody, rabbits's serum was also collected before and after the antigen injection from three rabbits, M4, M5, and M6, and the rabbits' serum titer was tested by indirect ELISA. As shown in the Figure 3.28, the anti-OsBGal2 taken from rabbits M4 (A) and M5 (B) gave the highest absorbance at a dilution of 1:500. On the other hand, the anti-OsBGal2 serum from rabbit M6 gave the highest absorbance of about 3.8 at a titer of 1:50 (Figure 3.28 C) and the absorbance decreased at a dilution of 1:100 and dropped to almost the same absorbance as the preimmune at a dilution of 1:2000. So, the OSBGal1 antiserum from rabbit M1 from the third and the fourth

immune bleeds and OsBGal2 antisera of rabbits M4 and M5 from the second bleeds were collected and use to purify specific antibodies.

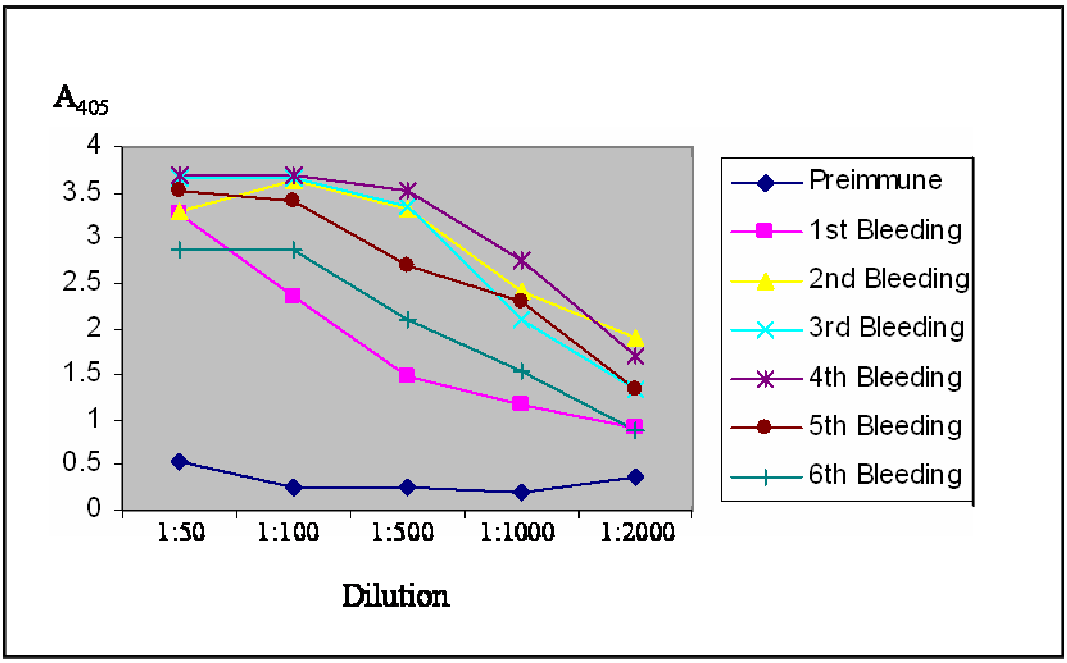


Figure 3.27 Titer determination of polyclonal anti-OsBGal1 antibody from rabbit M1. 5 mg/ml OsBGal1 synthetic peptide was used to coat the well of a 96-well plate, and 100 µl of each dilution (1:50, 1:100, 1:500, 1:1000 and 1:2000) of pre-immune serum (—◆—), anti-OsBGal1 serum from the first bleeding (—■—), the second bleeding (—▲—), the third bleeding (—×—), the fourth bleeding (—✳—) , the fifth bleeding (—●—) and the sixth bleeding (—+—) were added to wells. The plates were incubated at room temperature for 2 h, followed by washing 3 times with PBS and reacted with a 1:5000 dilution of HRP-conjugated goat anti-rabbit antibody in PBS (100 µl/well) at room temperature for 1 h. After washing 3 times with PBS buffer. The antigen-antibody reaction was visualized by adding 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (0.1 mg TMB in 100 µl DMSO with 9.9 µl of sodium citrate, pH 6.5, and 5 µl of 30% H_2O_2) in to each well, then measuring absorbance at 405 nm.

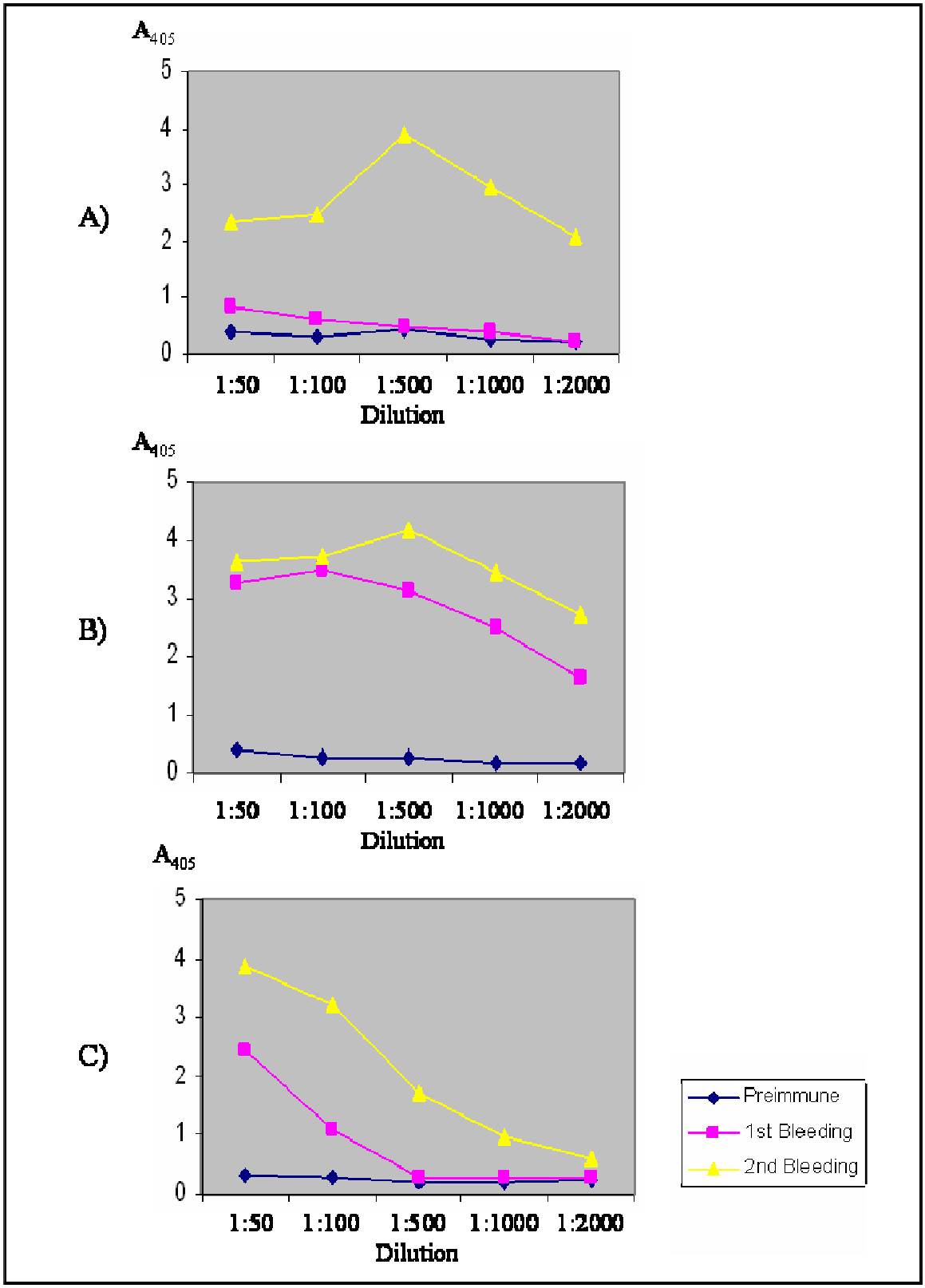


Figure 3.28 Titer determination of polyclonal anti-OsBGal2 antibodies from rabbits M4 (A), M5 (B) and M6 (C). 5 mg/ml OsBGal2 synthetic peptide was used to coat the wells

of a 96-well plate, and 100 µl of each dilution (1:50, 1:100, 1:500, 1:1000 and 1:2000) of pre-immune serum (—◆—), anti-OsBGal2 serum from the first bleeding (—■—) and anti-OsBGal2 serum from the second bleeding (—▲—) were added to each well. The plates were incubated at room temperature for 2 h, followed by washing 3 times with PBS and reacted with a 1:5000 dilution of HRP-conjugated goat anti-rabbit antibody in PBS (100 µl/well) at room temperature for 1 h. After washing 3 times with PBS buffer, the antigen-antibody reaction was visualized by adding 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (0.1 mg TMB in 100 µl DMSO with 9.9 µl of sodium citrate, pH 6.5, and 5 µl of 30% H_2O_2) to each well, then measuring absorbance at 405 nm.

Then, OsBgal 1 and OsBgal 2 antibodies were affinity purified with immobilized peptides and shown to bind to their respective proteins on immuno-dot-blots, while preimmune rabbit sera did not react to any protein. This blot showed that OsBgal1 could bind to the OsBGal1 synthetic peptide, but no reactive spot was seen for the negative control (lysozyme). When 10 μg of total protein extracted from the soluble and cell wall bound fractions from rice seedlings (5 days) were spotted and detected with the OsBGal1 and preimmune antibodies, they showed only weak reactive spots on the blot, though they seemed a little more intense than the control. When, purified OsBGal2 antibody was used to detect the OsBGal2 synthetic peptide and crude protein expressed in *E. coli*. Only in the synthetic OSBGal2 peptide was detected in the dot blot.

3.6.2 Western blotting analysis in rice plant

Immunoblot analysis of total protein extracted from root and shoot of 7-day-old seedling and root, leaf sheath and leaf blade of one-month-old rice was done to investigate the size and abundance of OsBGal1 and OsBGal2 expressed in the rice plant, as shown in Figure 3.8. OsBGal1 was found as a single band in all 5 tissues at approx. 90 kDa, though very little was seen in 1-month rice leaf blade. This is approximately the same as the size predicted from the amino acid sequence, suggesting that posttranslational processing of the enzyme in the plant may be limited. OsBGal2 was found to have a major band at around 55 kDa and a minor band at approx. 80 kDa in all tissues, except for 1-month rice root, where the 55 kDa band was seen, but a more intense band was seen at 45 kDa.

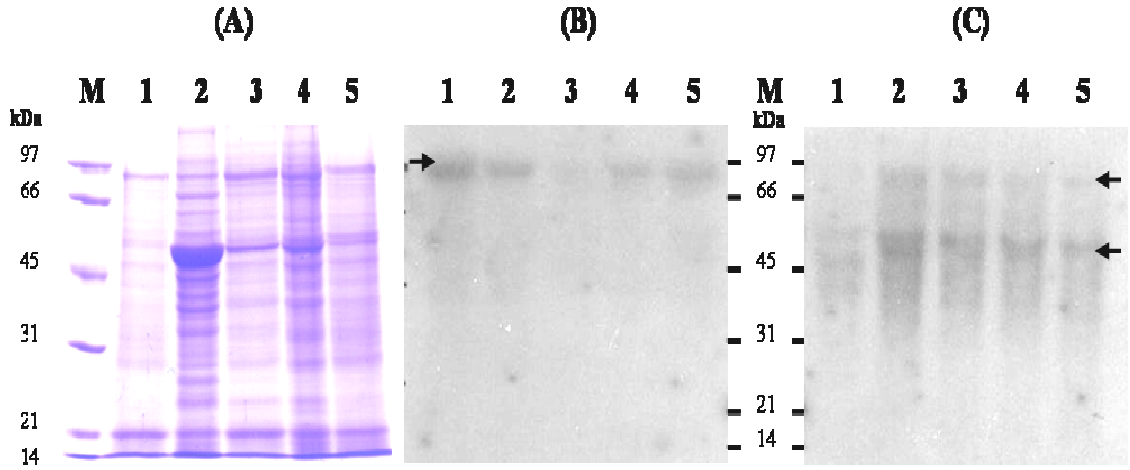


Figure 3.29. 10% SDS-PAGE gel (A) and immunoblot (B) and (C) of total protein extracted from rice plants at 7 days and one month after soaking. Twenty-five micrograms of proteins was loaded in each lane. (A). Coomassie Brilliant Blue stained gel. Lane M, Molecular weight marker; lane 1, total protein extracted from rice root at 1 month; lane 2, total protein extracted from rice leaf sheath at 1 month; lane 3, total protein extracted from leaf blade at 1 month; lane 4, total protein extracted from rice root at 7 days; and lane 5, total protein extracted from rice shoot at 7 days. Immunoblots with affinity-purified antibodies against catalytic domain peptides are shown in (B) for OsBGal1 and (C) for OsBGal2 with the lanes the same as in (A). The western shows a 90 kDa band for OsBGal1 (B), as expected for the predicted full-length protein, while OsBGal 2 gave a major band at 50-55 kDa, with other bands at approx. 80 kDa in leaf, shoot and seedling root (C, lanes 2, 3, 4 and 5) and at 45 kDa in the mature root (C, lane 1).

3.6.3 Immunolocalization of OsBGal1 and OsBGal2 in rice root

In order to determine the localization of the rice β-galactosidases in the plant, which may help to elucidate their functions, we performed immuno-histochemical localization by light microscopy. Immunolocalization of OsBGal1 and OsBGal2 was carried out with the purified polyclonal antibodies raised against the synthetic peptides. In longitudinal sections of rice seeds, the antibodies showed localization of OsBGal1 and OsBGal2 around the embryo from 0 to 7 days after soaking, as demonstrated by the red stain (Figure 3.29), which was absent in the control sections stained without the primary antibody. The OsBGal1 antibody stained diffusely and in a few rather diffuse spots in the embryo, while the OsBGal2 antibody intensely stained several bands in the embryo and stained the aleurone close to the embryo as well at day 0. At day 3, the staining for both isozymes was more widespread in the embryo and intense at the emerging radicle. OsBGal1 and OsBGal2 were also detected in root at days 5-7. At day 5, only OsBGal1 was detected around shoot, but only OsBGal2 was localized at the shoot tip at day 7. Strong staining along the roots and root hairs was seen with antibodies against both β-galactosidases at days 5 and 7.

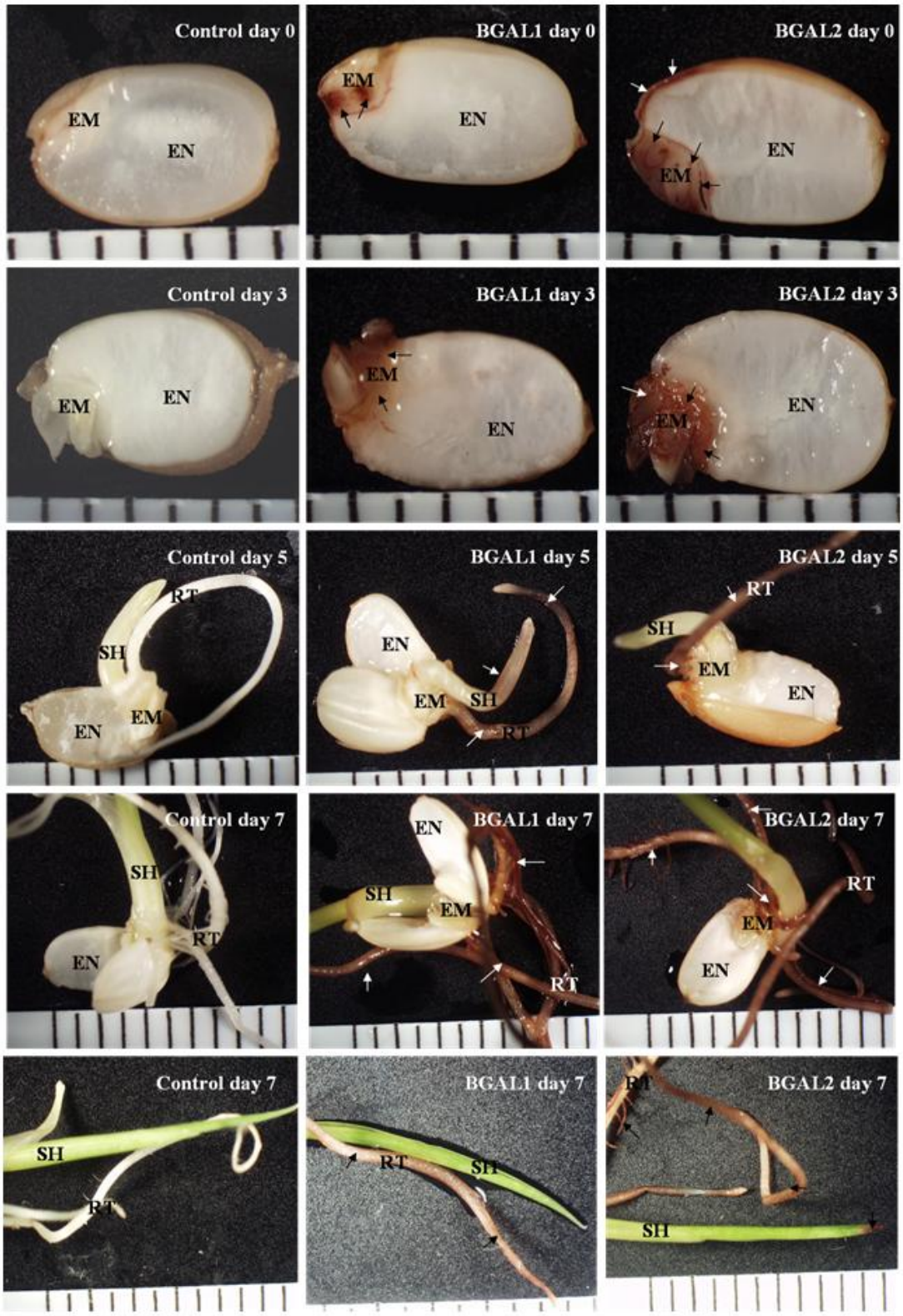


Figure 3.29 Immunohistochemical localization of OsBGal1 and OsBGal2 in rice seedlings 0-7 days after seed soaking. The seeds were cut in half and intrinsic

peroxidases inactivated with hydrogen peroxide before immunostaining with OsBGal1 and OsBGal2 specific antibodies, horse radish peroxidase-linked secondary antibodies and an aminoethyl carbazole substrate kit, as described in the Materials and Methods. The embryo is stained for both OsBGal1 and OsBGal2, while some aleurone also stains for OsBGal2. Surface staining of the roots and root hairs is seen for both proteins, while the 5-day shoot shows staining for OsBGal1 and the 7-day shoot tip is also stained for OsBGal2.

Since staining was seen in the root and root hairs for OsBGal1 and OsBGal2, embedded sections of five-day root were stained and examined at the microscopic level. The sections incubated with purified anti-OsBGal1 IgG and anti-OsBGal2 IgG and antigen-antibody detected with HRP-linked secondary antibody and *NOVA Red*^{TM} SUBSTRATE, which gave a red product that signified the β-galactosidase localization. For the negative control, sections treated with preimmune rabbit serum IgG or PBS were also done. Finally, the sections were viewed under a light microscope. We found that the sections incubated with preimmune serum IgG (Figure 3.30 A) and PBS (Figure 3.30 B) did not reveal any reactive cells, but staining could be seen with the specific antibodies (Figure 3.31).

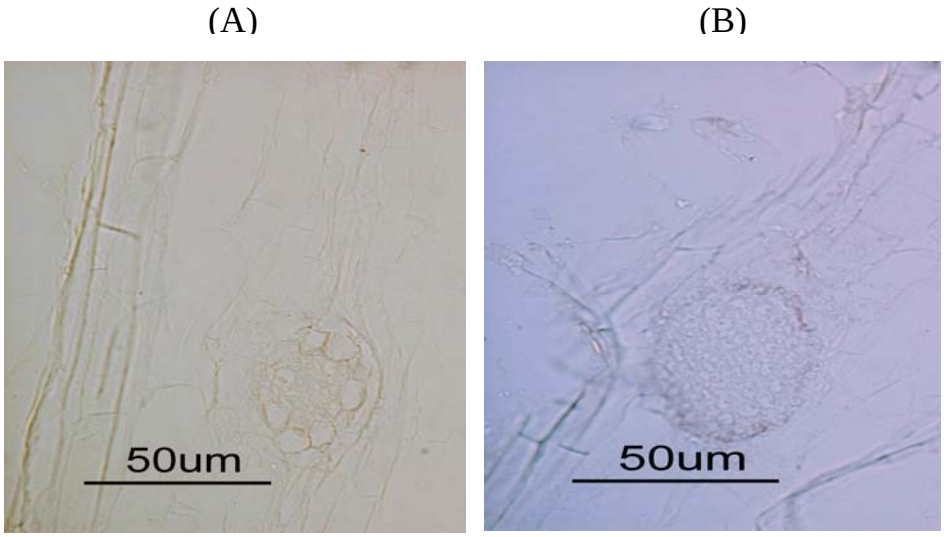


Figure 3.30 Control for immunohistochemical localization of OsBGal1 and OsBGal2 in rice root at 5 days after seed soaking. Frame A was stained with preimmune rabbit serum and frame B with PBS in place of primary antibody. Both are sections of root at the elongation zone. Fixed root sections were then stained with HRP-conjugated secondaryantibody and the peroxidase reaction developed with NovaRed to give a redish brown color.

In longitudinal sections of root, immunohistochemical analysis with OsBGal1 specific antibodies showed accumulation of OsBGal1, as depicted by the red stain, along the sieve tube around zone of differentiation of root (Figure 3.31), which did not occur in the control section stain with pre-immune serum. OsBGal2 was also immunolocalized to endodermis, which develop to the lateral root in the zone of differentiation. In contrast, in longitudinal sections of shoot, no staining for OsBGal1 and OsBGal2 was seen and the antibody stained section were similar to the negative control with pre-immune serum (Figure 3.30 A and B).

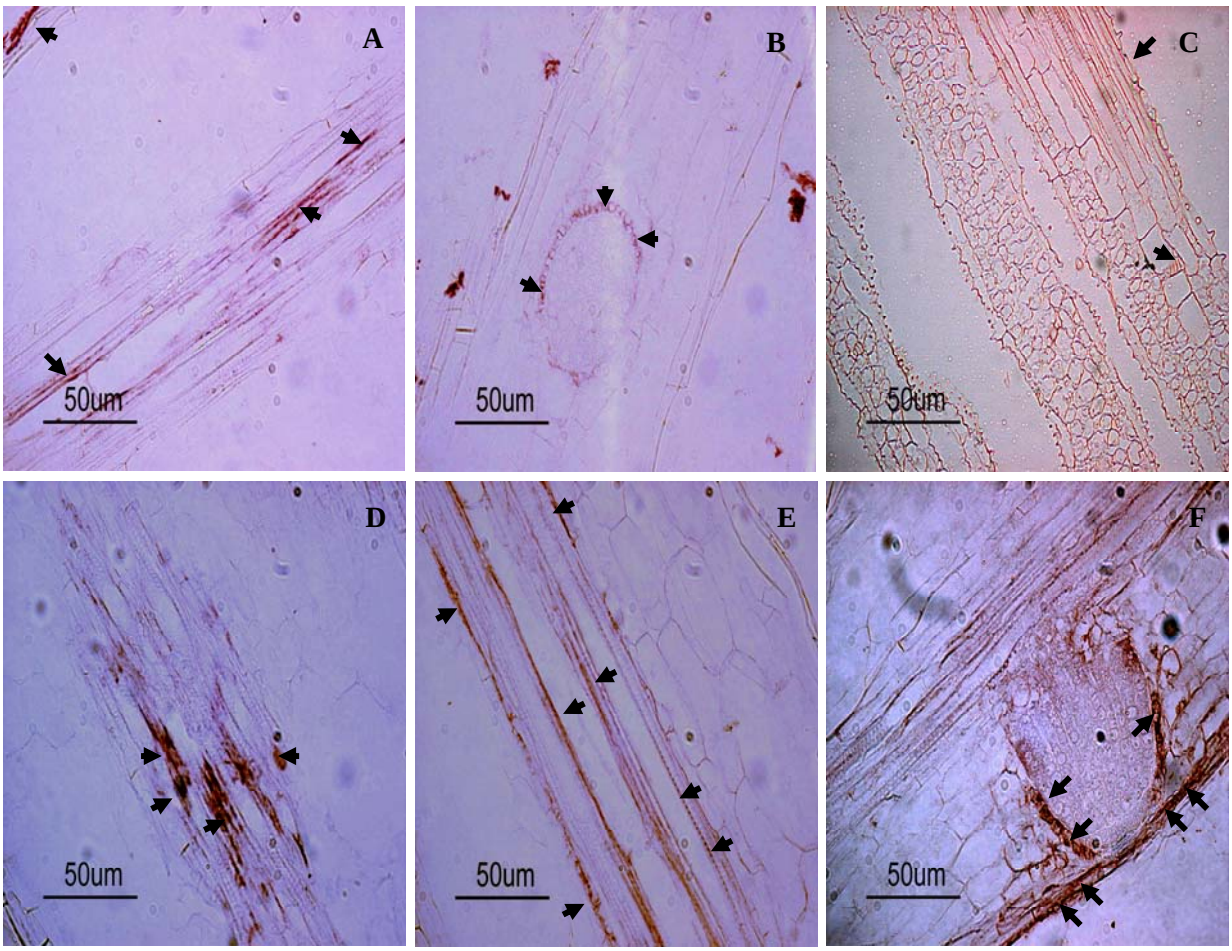


Figure 3.31 Immunolocalization of OsBgal1 and OsBgal2 β-galactoidases in rice seedling roots. Fixed root sections are stained by specific antibodies, followed by the

corresponding HRP-conjugated secondary antibody and NovaRed substrate to give a redish brown color. Frames A, B, and C are stained with affinity purified antibody against OsBGal1, frames D, E, and F with antibody against OsBGal2. Frames A), D) and E) are maturation zone of the root, frames B) and F) are the elongation zone, and frame C) is the meristem region. The arrows indicated the location of OsBGal1 and OsBGal2 expressed in the rice root, as detected with the antibodies.

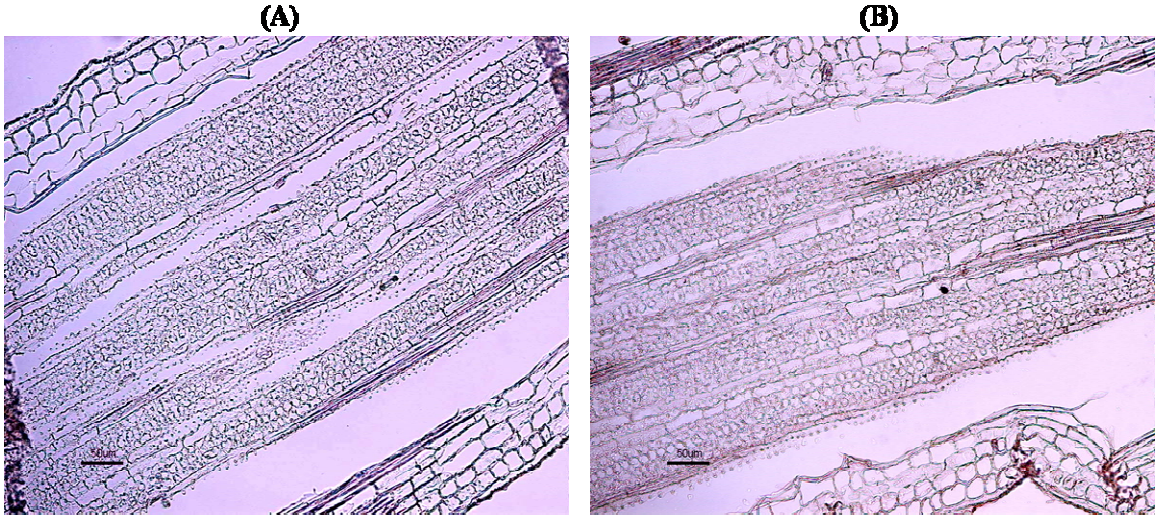


Figure 3.32 Immunolocalization of OsBgal1 and OsBgal2 β-galactoidases in rice seedling shoot. Fixed root sections are stained by specific antibodies, followed by the corresponding HRP-conjugated secondary antibody and NovaRed substrate to give a redish brown color. Frames A and B are stained with affinity purified antibody against OsBGal1 and OsBGal2, respectively. The figure indicated no localization of OsBGal1 and OsBGal2 expressed in rice shoot under microscope.

CHAPTER IV

DISCUSSION

In this study, genes for fifteen isozymes were identified in the rice genome database and their gene structures and representation in the dbEST database were confirmed. In addition, 2 cDNA for rice β-galactosidase, *OsBGal1* and *OsBGal2*, were further characterized and used to produce recombinant proteins. The two genes were found to be relatively highly expressed in seedling root and shoot and in leaf sheath in 15-30 day-old plants. Both were also expressed at low levels in flowers and immature seeds, but only the *OsBGal2* transcript was found in mature seed. The function of these genes was confirmed by characterization of β-galactosidase activity in recombinant proteins produced in *E. coli*. The β-galactosidase specific activity in crude extracts of OrigamiB (DE3) cells expressing OsBGal1 or OsBGal2 was much higher than in extracts from *E. coli* which had no Os*BGal1* or *OsBGal2* gene. To examine possible natural substrates for rice β-galactosidases, the crude protein extracts of the recombinant *E. coli* were also tested for hydrolysis of various galactose-containing substrates. In addition, the biochemical properties of purified OsBGal1 and immunolocalization of both OsBGal1 and OsBGal2 were investigated.

4.1 Rice glycosyl hydrolase family 35 genes and their phylogenetic relationship

Several β-galactosidases are found in plants, as indicated by the 7 cDNAs cloned from ripening tomato (Smith and Gross, 2000) and the 18 GH35 genes found in *Arabidopsis* (Iglesias, 2006). Glycosyl Hydrolase family 35 was found to have 15 genes in the rice databases, with all of these being present in the final Rice Genome Project japonica rice genome draft (International Rice Genome Project, 2005), and all of them being expressed in rice. The genes that were identified are shown in Table 3.1. The sequences for all these genes were identified and the gene structures determined and compared to the annotated mRNA in the databases. In several cases, the annotations were wrong, either due to misprediction of splicing or to aberrantly spliced cDNA sequences in the database, evidently due to alternative splicing in some cases. Our lab was able to obtain a full-length cDNA for *OsBGal 15* (Tanthanuch and Ketudat-Cairns, unpublished data), for which no previous cDNA sequence was available, thereby confirming its predicted gene structure as shown in Figure 3.1. The gene structures that were predicted for rice GH35 showed 8 different patterns with pattern 1, which contains 19 exons and 18 introns, appearing to be the ancestral pattern from which the patterns 2-7 were derived by loss of introns. In each case, the splice sites were almost completely conserved with just a loss of one or more sites, which led to consolidation of exons. The gene structure of *OsBGal 9* was different with only one exon (exon 2) appearing to have been in common with the others, though a few other splice sites were in similar positions. The *OsBGal 9* sequence is also at least as similar to animal and microbial β-galactosidases as to plant enzymes. Thus, it appears that *OsBGal 9* diverged from the other plant genes very early,

perhaps before the divergence of plants from other organisms. As shown in the phylogenetic tree based on the protein sequences in Figure 3.2, the genes with similar gene structures generally grouped together. For instance, the OsBGal2 and OsBGal7, both of which lack the C-terminal putative carbohydrate binding domain and have similar though not identical gene structures, were most similar to each other. Similarly, the phylogenetic analysis and gene structure comparison of OsBGal5, OsBGal12, OsBGal14, and OsBGal15 shows they group together and have the same gene structure, which suggests that they might be recently diverged from a single ancestral gene.

4.2 Expression pattern of β-galactosidase genes in rice plant

Inspection of the EST databases indicated that a large number of ESTs were released in 2006, which helped to increase the usefulness of EST analysis in assessing GH35 gene expression. These results are summarized in Table 3.1. Some genes, like *OsBGal 10* and *OsBGal 14* were found at relatively high levels in flowers and panicles at flowering stage. In fact, *OsBGal 14* was in the top 15 genes for gene expression in one rice flower library and represented 0.35% of the transcripts in the flower tissue based on the number of ESTs in the library from the *OsBGal 14* gene. However, these results are inconclusive in telling relative abundances between tissues in many cases due to differences in the number of ESTs generated from each tissue. So, experimental approaches were used to determine the relative expression levels.

Attempts at northern blot analysis of the mRNAs of *OsBGal1* and *OsBGal2* gave high background, despite the use of specific 3' UTR probes, so semiquantitative RT-PCR was used to probe their expression pattern. Analysis of expression of all

fifteen GH35 genes was done by RT-PCR (Tanthanuch et al., unpublished). *Actin* and *Ubq6* control genes were co-amplified for each gene from each sample, though the reactions were split, since the control genes sometimes affected the amplification of the experimental genes and vice-versa. The reactions of control and experimental genes were run on the same gel and the bands quantified at the same time with all reactions in triplicate to allow calculation of standard errors. The results of these estimates of relative expression of *OsBGal1* and *OsBGal2* in the different vegetative and reproductive tissues are shown in Figure 4.1.

For the GH35 enzymes, *OsBGal1*, *OBGal2*, *OsBGal4*, *OsBGal7*, *OsBGal8*, and *OsBGal13* were found to be expressed throughout the life cycle, while *OsBGal3*, *OsBGal6* and *OsBGal9* were predominantly expressed in vegetative tissues and *OsBGal5*, *OsBGal10*, *OsBGal11*, *OsBGal12*, *OsBGal14*, and *OsBGal15* were predominantly expressed in reproductive tissues (Tanthanuch and Ketudat-Cairns, unpublished data). Of these, *OsBGal5*, *OsBGal12*, and *OsBGal14* were nearly panicle specific (except some *OsBGal14* was found in immature seed), while *OsBGal10*, *OsBGal11* and *OsBGal15* were highly expressed in both flower and panicle. The absence of *OsBGal12* in flowers is strange, given its high abundance in flower ESTs, but it may be due to different preparations of the organs, which contained different parts (ie. it was quite high in panicle at flowering stage in the RT-PCR study). The anther was the tissue tested with the fewest genes expressed, as might be expected since it is the most specialized tissue tested, and neither *OsBGal1* nor *OsBGal2* was expressed in anther.

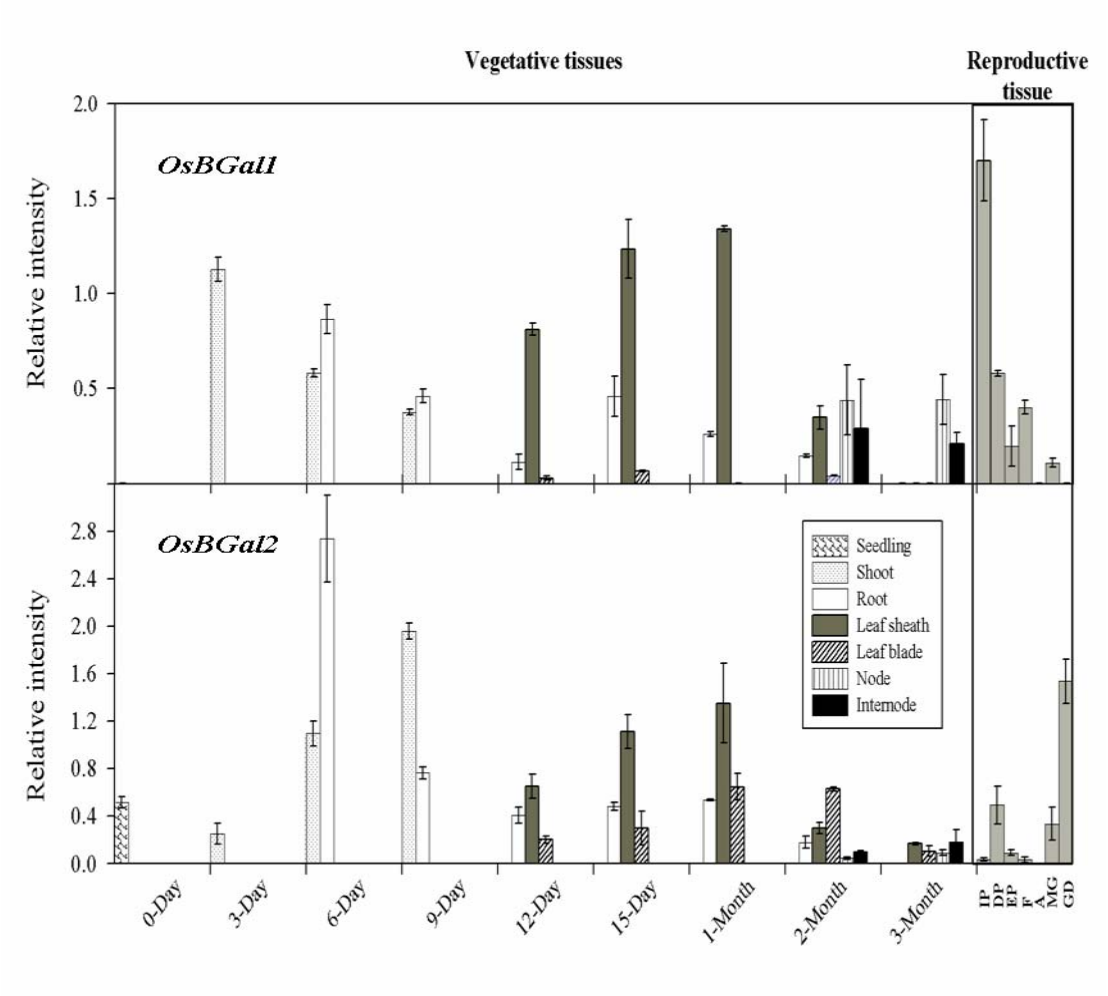


Figure 4.1 Relative expression levels of *OsBGal1 and OsBGal2* genes in different tissues determined by semiquantitative RT-PCR. Signals were quantified and normalized to *UBQ6*. Each error bar shows the standard error in triplicate reactions for each sample. The left side are vegetative tissues given by the figure legend, while the right side are reproductive tissues, abbreviated as IP, initiating panicle; DP, differentiating panicle; EP, exerting panicle; F, flower; A, anther; MG, milk grain; GD, grain during dry down.

4.3 Recombinant protein expression of OsBGal1 and OsBGal2 in *E. coli*

Purification of β-galactosidases from rice is difficult, since multiple isozymes of β-galactosidases are found, with up to 15 genes identified (see section 3.1). Therefore, it may be hard work to purify to homogeneity each isozyme from rice. So, the recombinant expression system allows the production of large amounts of enzyme for further characterization and confirms the importance of the fusion tag in the production of the active recombinant protein. The β-galactosidase specific activities in crude extracts of OrigamiB (DE3) cells expressing OsBGal1 and OsBGal2 were 8 fold and 55 fold, respectively, higher than in extracts from *E. coli*, that had no Os*BGal1* or *OsBGal2* gene. In fact, Origami (DE3) and other expression strains tested had high β-galactosidase background, which made the activity of proteins expressed in those cells difficult to judge. The fact that the activity is due to the recombinant protein was further confirmed by purification of the OsBGal1 protein, which unlike endogenous *E. coli* β-galactosidase does not hydrolyze lactose and has a low pH optimum of 3.0-3.5.

Most of the recombinant OsBGal1 and OsBGal2 were found to aggregate as inclusion bodies when produced as mature protein. Very small amounts of soluble and active rice OsBGal1 and OsBGal2 were obtained when the proteins were produced by fusion with thioredoxin (TRX) in the OrigamiB (DE3) *E. coli*. TRX might help to reduce aggregation of insoluble protein. Unfortunately, the recombinant clone OsBGal2 which produced active protein lost expression of

recombinant protein over time. Therefore, an in-depth characterization could not be performed.

4.4 Purification of OsBGal1

OsBGal1 produced in *E. coli* proved difficult to purify because of its apparent susceptibility to degradation, which resulted in multiple bands in the final preparation. In fact, if the crude extract was kept at 4ºC overnight, no active protein could be purified by IMAC. Freshly extracted protein was purified as 2 major bands that contained the catalytic domain and bound to the IMAC resin, one at 95 kDa and one at 66 kDa. In addition, there were several smaller bands that did not bind anti-catalytic domain antibodies. Several β-galactosidases have been purified with molecular masses in the range of 35 to 50 kDa or as multiple subunits (Konno and Katoh, 1992; Li et al., 2001; Giannakouros et al., 1991; Triantafillidou and Georgatsos, 2001). When Kaneko and Kobayashi (2003) purified a different β-galactosidase isozyme from rice suspension cell media, it was made up of two subunits, the N-termini of which they sequenced. A comparison of the sequence with the GenBank accession no. **AK067479** full-length cDNA (Kaneko and Kobayashi, 2003) indicates that both these subunits are derived from the precursor protein translated from this gene. So, it may be that OsBGal1 is cleaved by bacterial proteases, but the protein fragments remain associated, as appears to happen to other isozymes that have been isolated from the plant. Mass spectrum analysis of the major bands in the SDS-PAGE of the purified product were derived from the expressed OsBGal1 fusion protein, though some minor bands likely correspond to bacterial contaminants.

4.5 Substrate specificity for rice β-galactosidase

Kotake *et al.* (2005) suggested that plant β-galactosidases could be divided into at least 2 classes: those that hydrolyze 1,4-linked β-galactans, and those that prefer *p*NPGal. When investigating a radish β-galactosidase of this second class, they found it to hydrolyze β-(1→3)- and β-(1→6)-linked oligogalactans, such as those found in the carbohydrates of arabinogalactan proteins, but not β-(1→4)-linked oligogalactans, such as are found in side-chains of the pectic polysaccharide rhamnogalacturonan-I. OsBGal1 is similar, in that it seems to hydrolyze *p*NPGal best and prefers β-(1→3) to β-(1→4)-linked disaccharides, but unlike the radish enzyme, it hydrolyzes β-(1→6)-galactobiose only slowly. Curiously, OsBGal1 showed no ability to decrease the M_r of larger β-(1→4)-galactan oligosaccharides (degree of polymerisation 5, 8 and 11). It also failed to release galactose from the xyloglucan-oligosaccharide, XLLG, in which the galactose residues are β-(1→2)-linked to α-D-xylose.

When the crude rice β-galactosidase extracts were tested for activity against various substrates including maize AIR, rice coleoptile AIR and rice root AIR, ribose was found as the product, which suggested that the recombinant rice β-galactosidase might have an unexpected activity on a ribose-containing polymer. However, we found that all crude extracts of *E. coli* contain ribosidase activity, which can hydrolyze uridine and adenosine to release ribose. In several experiments, this release appeared higher in OsBGal extracts than in control extracts. The purified OsBGal1 was used to test for hydrolysis of zeatin-riboside, since it was the phytohormone that might be a target of the enzyme, as well as adenine. No ribose was released from

these substrates, which suggests that the ribose released when using the crude *E. coli* extract was due to the ribosidase activity of the *E. coli*, and not the action of rice β-galactosidase.

Initially, we were unable to detect any polymeric substrate for OsBGal1 in rice or maize. However, with extended digest with a large amount of purified OsBGal1, some galactose appeared to be released from rice coleoptile and root AIR and arabinogalactan. So, it is possible OsBGal1 could play a role in degradation of cell wall arabinogalactans in the plant, though its activity on these substrates is slow. Like the radish enzyme, OsBGal1 still showed a much higher preference for *p*NP-Gal compared to any of the putative natural substrates tested, so it is unclear if such oligosaccharides or related carbohydrates serve as natural substrates for the enzyme in the plant. The fact that the enzyme has an extremely low pH optimum of 3.0 to 3.5, which is similar to the optima of 3.2 and 3.6 reported for mango β-galactosidases (Ali, 1995) and 3.6 seen for a β-galactosidase from mung bean seedlings (Li, 2001), suggests that it may function in an acidic compartment. To achieve this low pH optimum, the catalytic nucleophile and catalytic base (glutamate residues) are likely to be stabilized in their functional, deprotonated forms by contacts with positively charged residues. OsBGal1 is targeted to the secretory pathway, so this might be an acidic vacuole involved in recycling, since the pH optimum is lower than the expected pH of around 5 for the apoplast. However, OsBGal1 maintains nearly 50% activity at pH 5.0 and could be activated by acidification of the apoplast during cell wall remodeling. Its specificity also suggests that it does not function to degrade apoplastic xyloglucan oligosaccharides, as suggested for *Arabidopsis thaliana* β-galactosidases (Iglesias, 2006). The OsBGal1 enzyme may act to degrade β-(1→3)-,

β-(1→4)- and β-(1→6)-galacto-oligosaccharides, or glycoprotein carbohydrates, or even glycolipids in such an acidic compartment or in the acidified apoplast. Golden (1993) noted that β-galactosidase from ripe coffee berries alone failed to release galactose from pectin, but did so from arabinogalactan and galactan, which is similar to our results in which OsBGal1 acted on short oligosaccharide, but showed no ability to hydrolyze larger oligosaccharides. OsBGal1 b-galactosidase appeared to hydrolyze arabinogalactan (Figure 3.25), which is in contrast to apple β-galactosidase, which did not hydrolyze this type of substrate (Gross and Wallner, 1979).

4.6 Inhibition studies and possible mechanism of metal inhibition

The effects of several metal ions and sugar derivatives on β-galactosidase activity were investigated (Tables 3.6 and 3.7). OsBGal1 had decreased activity when it was incubated with increasing concentrations of IPTG, imidazole, δ-gluconolactone and δ-galactonolactone. OsBGal1 activity was strongly inhibited by Hg^{2+}, Fe^{3+} and Cu^{2+} metal ion, as are others plant β-galactosidase (Hashimoto, 1994), which suggests that a free sulphydryl group may be important for the enzyme function. Other cations tested, including $CoCl_2$, $MgCl_2$, $CaCl_2$, and $ZnSO_4$, appeared to have slight effects on β-galactosidase activity, whereas $MnCl_2$, $NiSO_4$ and histidine did not affect on β-galactosidase activity. The purified OsBGal1 enzyme also showed inhibition by galactose and lactose like other β-galactosidases (Suginta, 1993; Hashimoto, 1994), but this inhibition was very weak. The nature of this inhibition was competitive for all the sugar for other β-galactosidases (Wodzinski, 1990).

4.7 Localization of β-galactosidase in rice

Rice seed and seedling staining with X-Gal showed localization of β-galactosidase activity to the aleurone layer and the embryo in the seed and to the shoot and root tip in the seedling (Chantarangsee, 2001). The aleurone layer is the site of production of many hydrolases during and after germination (Palmiano and Juliano, 1973). Germination of rice is likely to require rupture of the seed coat, allowing the shoot and root to emerge, while growth of the cotyledon, radicle, shoot and roots requires cell wall remodelling. Cell wall recycling often involves the loss of galactose residues from polysaccharides in dicots (Carey et al., 1995), though this is not so clear for rice. In our study, β-galactosidase activity was seen in the seed, roots and shoots at 0-7 days after seed-soaking, suggesting that it might be involved in this cell wall remodelling processes. Enzyme activity hydrolyzing *p*NP-β-D-galactopyranoside has been shown to peak at 6 days in whole IR8 rice seedlings (Palmiano and Juliano, 1973), and we have seen a similar peak in the activity in the rice used in this study (Chantarangsee, 2001). In the IR8 seeds, many enzyme activities, including phosphatase, protease, esterase, peroxidase, catalase, β-glucosidase, and α- and β-galactosidase, along with soluble protein, increased by 4 days and had peak activity by 5–7 days.

The large size of the OsBGal1 protein compared to the OsBGal2 protein was due primarily to an addition of approximately 100 amino acids at its carboxy terminus. The addition at the carboxy terminus included eight conserved cysteine residues found at the C-terminus of many other plant β-galactosidases. This C-terminal domain is homologous to sea urchin galactose-binding lectin (Trainotti,

2001; Triantafillidou and Georgatsos, 2001). This high concentration of cysteines is similar to many extracellular binding domains, in which they form disulfide bonds to increase stability. This suggests the C-terminal domain may allow efficient binding with galactose-containing substrates, such as cell wall polysaccharides and glycoproteins. Therefore, the difference in lengths of the OsBGal1 and OsBGal2 clones may have an effect on their localization or binding to their substrates. In contrast, Kotake *et al*. (2005) found that this C-terminal domain was not present in purified radish arabinogalactan β-galactosidase, which suggests that it may not be relevant to the final function of that protein.

The expression patterns of *OsBGal1* and *OsBGal2* were somewhat different and suggest different functions for these enzymes, though they were both found in many tissues, like seedling root and shoot. Only the *OsBGal2* transcript was seen in drying seeds, but some immunoreactive protein was seen for both proteins in the seed embryo and after germination. It would seem possible the OsBGal1 protein was produced at some early stage in embryo development and then again after seed soaking. The presence of OsBGal1 and OsBGal2 in the embryo and OsBGal2 in some of the aleurone layer 1 day after soaking and in the root and shoot after germination suggests that it might account for some of the X-Gal staining seen in dry and germinating seeds, though other isozymes may contribute to the activity as well. The presence of OsBGal2 at the shoot tip and tips of the root hairs indicates it may play a role in tissues that are rapidly expanding. What the roles of these enzymes are during and after germination remains to be elucidated.

In mammals, β-galactosidases are known to act in the lysosome to recycle glycolipids, glycoproteins and proteoglycans (Callahan, 1999; Spoel *et al*., 2000). Galactolipids, proteoglycans and galactose-containing glycoproteins certainly exist in plants, but little has been reported about the enzymes that act to recycle them, except for arabinogalactan proteins (Kotake, 2005). Though it is unclear whether OsBGal1 and OsBGal2, which are more closely related to tomato and chickpea β-galactosidases thought to be involved in cell wall recycling than to radish β-galactosidase that appears to be involved in arabinogalactan protein processing or recycling (Figure 3.1), are involved in such functions, the antibodies developed against these proteins may be used to investigate their location inside the cell and probable function. Actually, immunohistochemical analysis by electron microscope was performed to investigate the localization of OsBGal1 and OsBGal2 at the sub-cellular level in rice root, but we could not figure out the location of the proteins since the gold particle localization and the image of the sub-cellular compartment were unclear.

Likely roles of the rice β-galactosidases in the plant, the results obtained from mRNA transcript levels suggested the expression patterns of *OsBGal1* and *OsBGal2* were different, although they were both found in various tissues, like seedling and shoot. Western blot analysis of the 7-day-old seedling shoot and root and 1-month-old root, leaf sheath and leaf blade were consistent with the expression level studied by RT-PCR, with little protein being seen for OsBGal1 in 1 month leaf blades, where no mRNA was detected, and both OsBGal1 and OsBGal2 were found in the other tissues**.** Immunolocalization of OsBGal1 and OsBGal2 in rice root under stereomicroscope indicated the presence of OsBGal2 at the shoot tips of root hairs

indicates it may play a role in rapidly expanding tissue. Therefore, OsBGal1 and OsBGal2 is likely important for helping in rice germination and seedling development.

The substrate specificity of purified OsBGal1 provided a critical basis for assessing its role in biological process. It is capable of acting in degradation of galacto-oligosaccharides, which are components of hemicellulose in the cell wall of higher plants. The low pH optimum of OsBGal1 of 3.0-3.5, suggests that it may function in an acidic compartment. OsBGal1 has a temperature optimum of 50-60°C in a 10 min assay which is unusual condition found in cell compartment, so the protein normally functions at a lower temperature (25-40°C) and this temperature optimum is not physiologically relevant. The previous study of amino acid sequence of OsBGal1 by the PSORT and SignalP programs suggested OsBGal1 may be targeted to outside the cell, which supports a role in cell wall galactosyl modification (Chantarangsee, 2001). However, our substrate specificity test indicated that OsBGal1 failed to hydrolyze the large molecule which is the main components of the cell wall, and only β-(1→3)-, β-(1→4)- and β-(1→6)-linked oligosaccharides were obviously hydrolyzed by OsBGal1. This suggests that OsBGal1 may have to act in concert with other cell wall degrading enzymes in hydrolyzing the cell wall components. However, OsBGal1 had pH optimum of about 3-3.5 and lower activity at pH 5.0, suggests OsBGal1 could be activated by acidification of the apoplast during cell wall remodelling in plant cell, but further work is needed to support this hypothesis. However, the immunolocalization data in this study suggests OsBGal1 and OsBGal2 expressed in alreulone layer, shoot and rice root during 0-7 day of

germination, consistent with β-galactosidase activity hydrolyzing *p*NPGal has been shown to peak at 6 days in whole IR8 rice seedling (Palmiano and Juliano, 1973).

CHAPTER V

CONCLUSION

In this study, a total of 15 family 35 β-galactosidase genes were identified in the rice genome. The gene structure comparisons of β-galactosidase gene family indicated eight different splicing patterns. The relationship of OsBGal1 and OsBGal2 to the other rice isozyme showed that these two isozymes are closely related to each other and some other rice β-galactosidases.

Northern blots with gene specific probes for analysis of the mRNA transcript levels of *OsBGal1* and *OsBGal2* were not successful because of the two genes gave high background, so semi-quantitative RT-PCR were used to study their expression pattern. By RT-PCR, we have found that the mRNA transcription level of *OsBGal1* is high in the initiating panicle, relatively low in other reproductive tissues, and absent in the anther and seed during dry-down. For the *OsBGal2* transcript, RT-PCR revealed low relative expression in the panicle, but significant expression in the seed during dry-down and at the initiation of germination.

cDNA encoding the mature β-galactosidase protein OsBGal1 and OsBGal2 were amplified by polymerase chain reaction (PCR) and cloned into the pET32a (+) vector. OsBGal1 and OsBGal2 were expressed in OrigamiB (DE3) *E. coli* as N-terminal thioredoxin fusion proteins. Both OsBGal1 and OsBGal2 fusion proteins were found in inclusion bodies, though some active soluble proteins were also

produced. The soluble extract from cells containing recombinant plasmids could hydrolyze *p*NP-galactopyranoside at high a level compared to extracts from cells with empty plasmid. In contrast, when the crude extracts from OsBGal1 and OsBGal2 were tested for various galactose-containing substrates with terminal non-reducing β-D-galactopyranosyl residues were not released from them.

The thioredoxin OsBGal1 fusion protein was purified by chelated-metal affinity chromatography and anion exchange chromatography. Purified OsBGal contained two major bands corresponding to 97 and 66 kDa and additional smaller bands were also presented. Purified OsBGal1 was confirmed by mass spectrometry and its substrate specificity and biochemical properties were studied. The enzyme activity had pH and temperature optima at 3.0-3.5 and 50-60°C, respectively. The apparent K_m value of OsBGal1 was 0.32 mM and V_{max} was 2.1 μmol/min/mg protein for *p*NP-β-D-galactopyranoside. The enzyme was completely inactivated by 10 mM $CuCl_2$, and 1 mM $HgCl_2$ and $FeCl_3$, while 10 mM $MgCl_2$, 10 mM EDTA, Delta-galactonolactone, IPTG and δ-gluconolactone also caused inhibition. Galactose and lactose showed little inhibition when these sugars were added at 5 mM or 10 mM to the reaction.

Investigation of the size and abundance of OsBGal1 and OsBGal2 expressed in the rice plant by immunoblot analysis suggested OsBGal1 was found as a single band in total protein extracted from root and shoot of 7-day-old seedling and root, leaf sheath and leaf blade of one-month-old rice at approx. 90 kDa, though very little was seen in 1-month rice leaf blade, while OsBGal2 was found to have a major band at around 55 kDa and a minor band at approx. 80 kDa in all tissues, except for 1-month rice root. Immunolocalization of β-galactrosidase in japonica rice seeds showed localization of OsBGal1 and OsBGal2 around the embryo, shoot and root from 0 to 7

days after soaking, which was absent in the control sections stained without the primary antibody and the microscopic localization in the rice root indicated Os BGal1 localized in the mature zone of root while OsBGal2 located in endodermis of lateral root in elongation zone.

In conclusion, the gene organization of rice β-galactosidase family 35 were identified and the expression pattern of *OsBGal1* and *OsBGal2* genes were also studied. In addition, the biochemical properties and substrate specificity of the purified OsBGal1 were determined and they suggested some possible functions of these enzymes. However, it is still unclear what the natural substrates of this enzyme in the plant are. In the future, the production and purification of the active domain of OsBGal1 might be useful for understanding of the function of this enzyme in rice, and this knowledge might be applied in the biotechnological and food industries.

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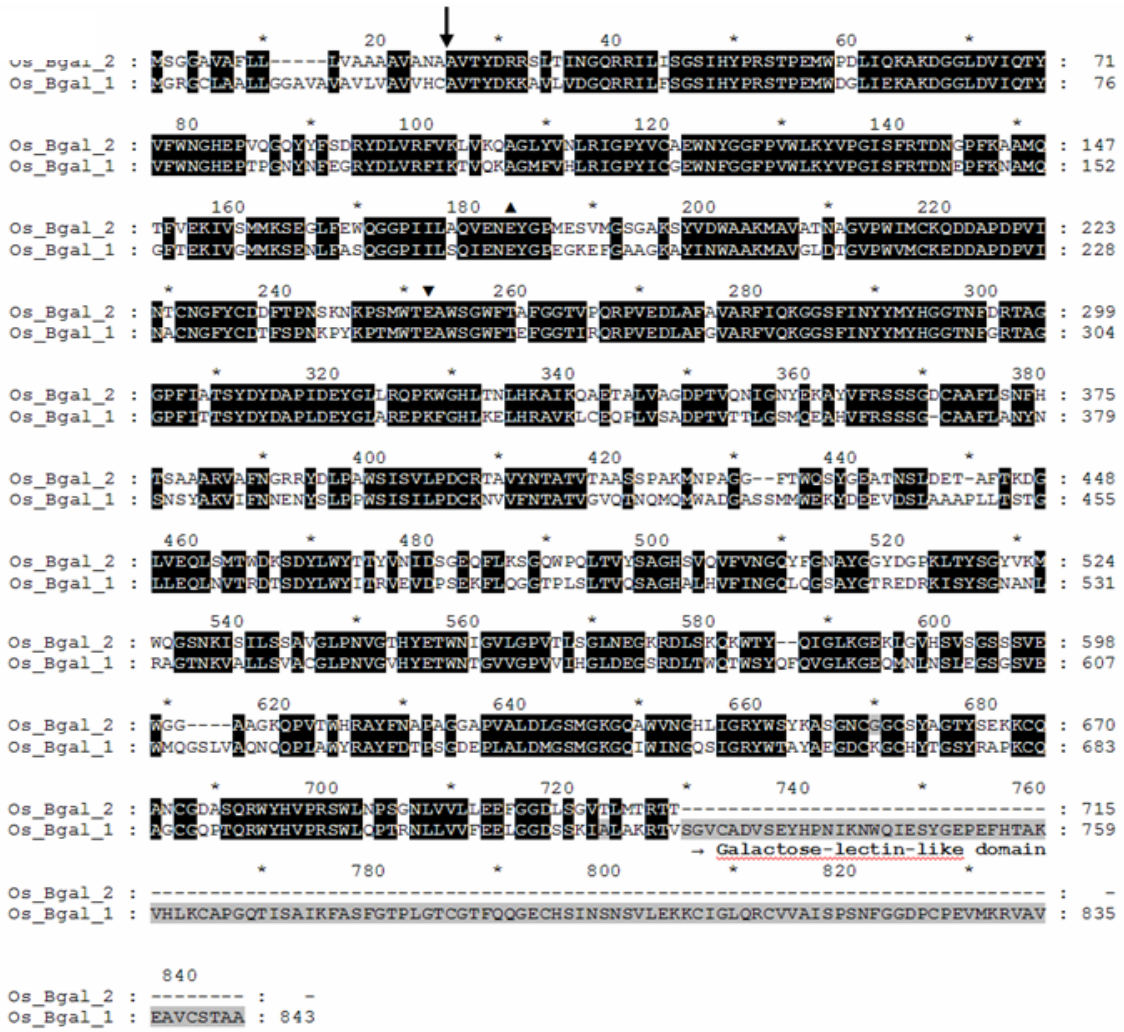
Appendix A

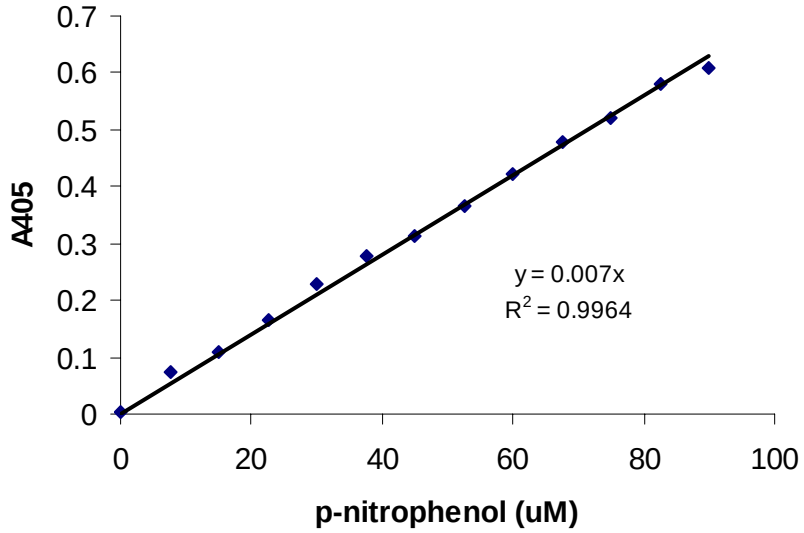
Supplementary Data

1. Sequence alignment of rice OsBGal1 and OsBGal2

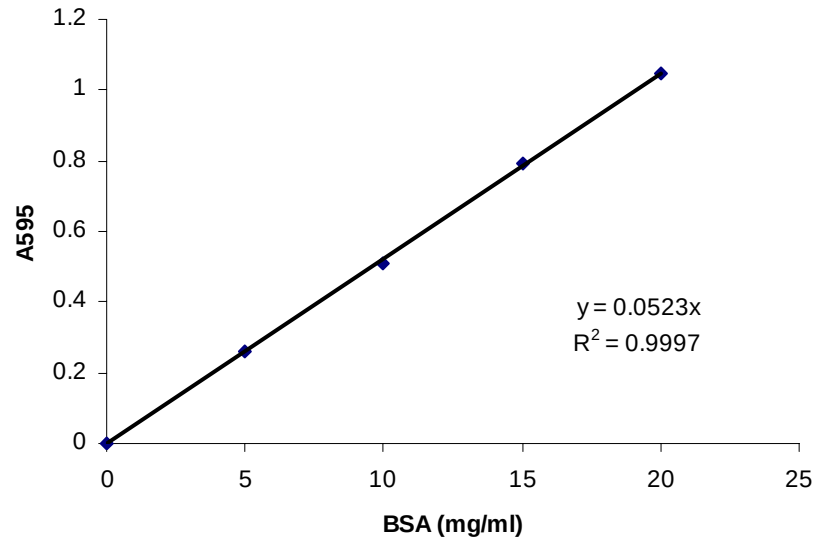
Rice cDNA derived sequences are labeled as OsBGal1 and OsBGal2. Conserved residues are shown in white with black background. An arrow shows the putative signal peptide cleavage site, and the C-terminal galactose-binding-lectin-like domain is shown with a grey background. The putative catalytic acid/base is marked with ▲ and the putative catalytic nucleophile is marked with ▼.



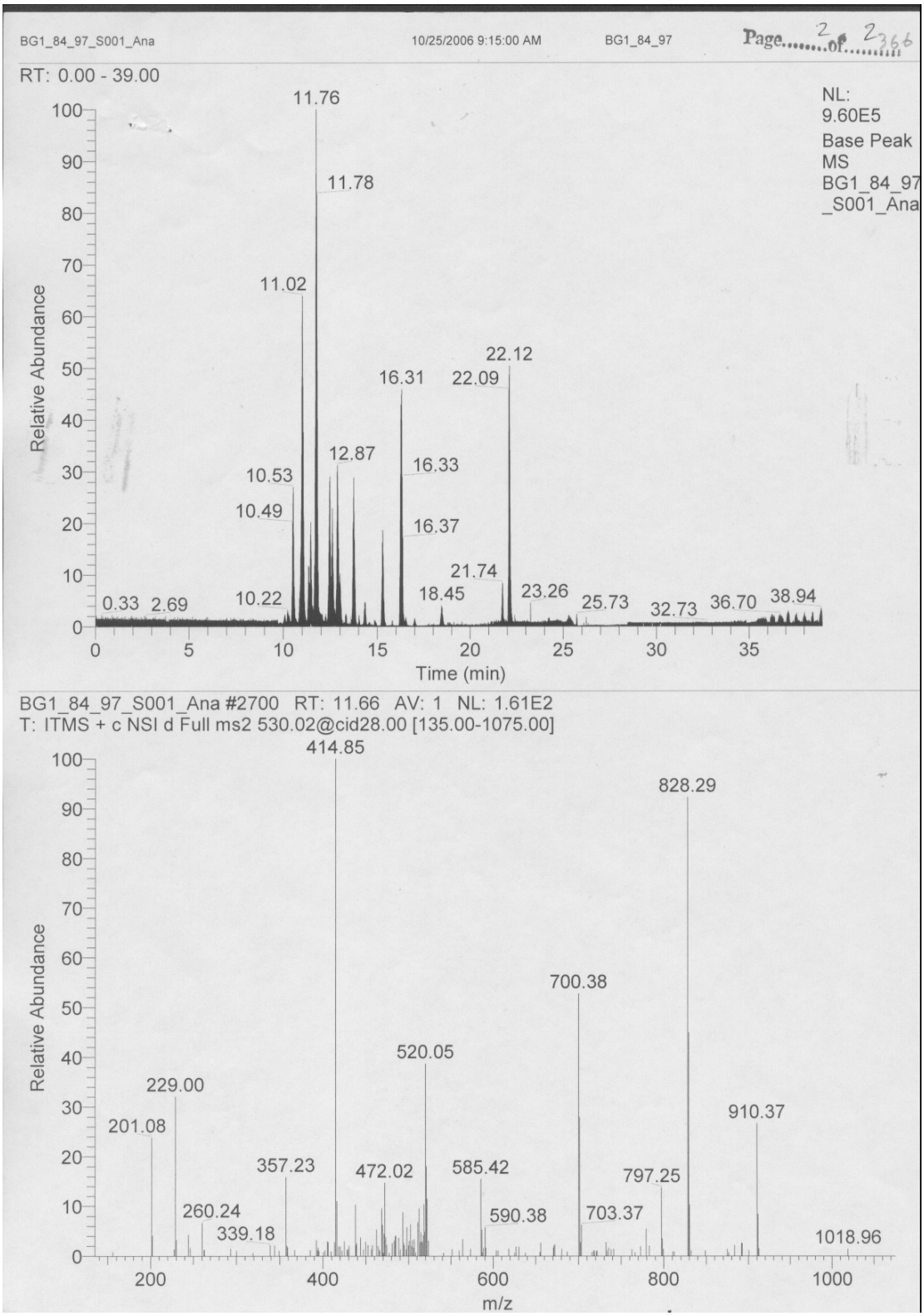
2. Standard curve of *p*-nitrophenol



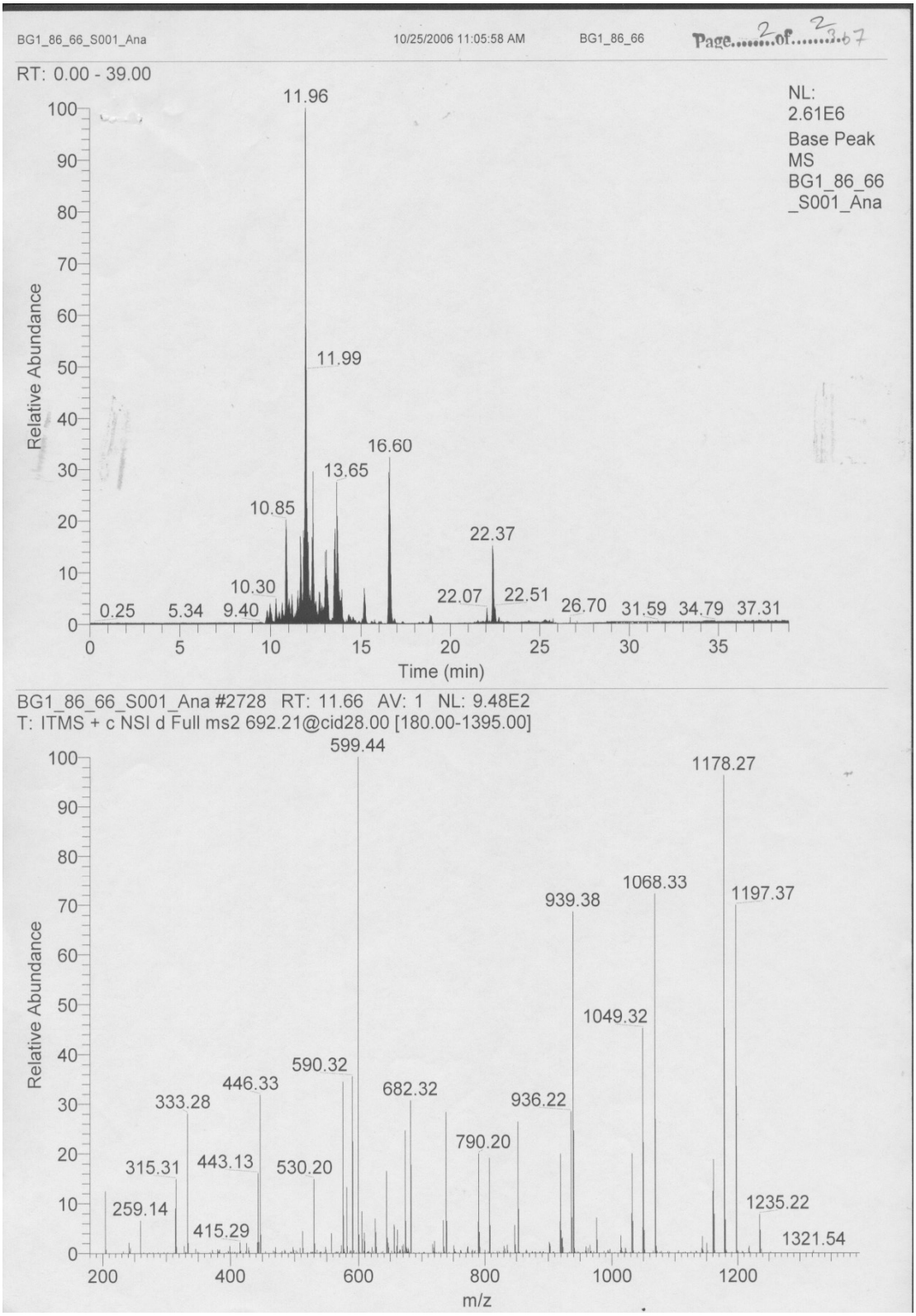
3. Standard curve of protein assay with BSA



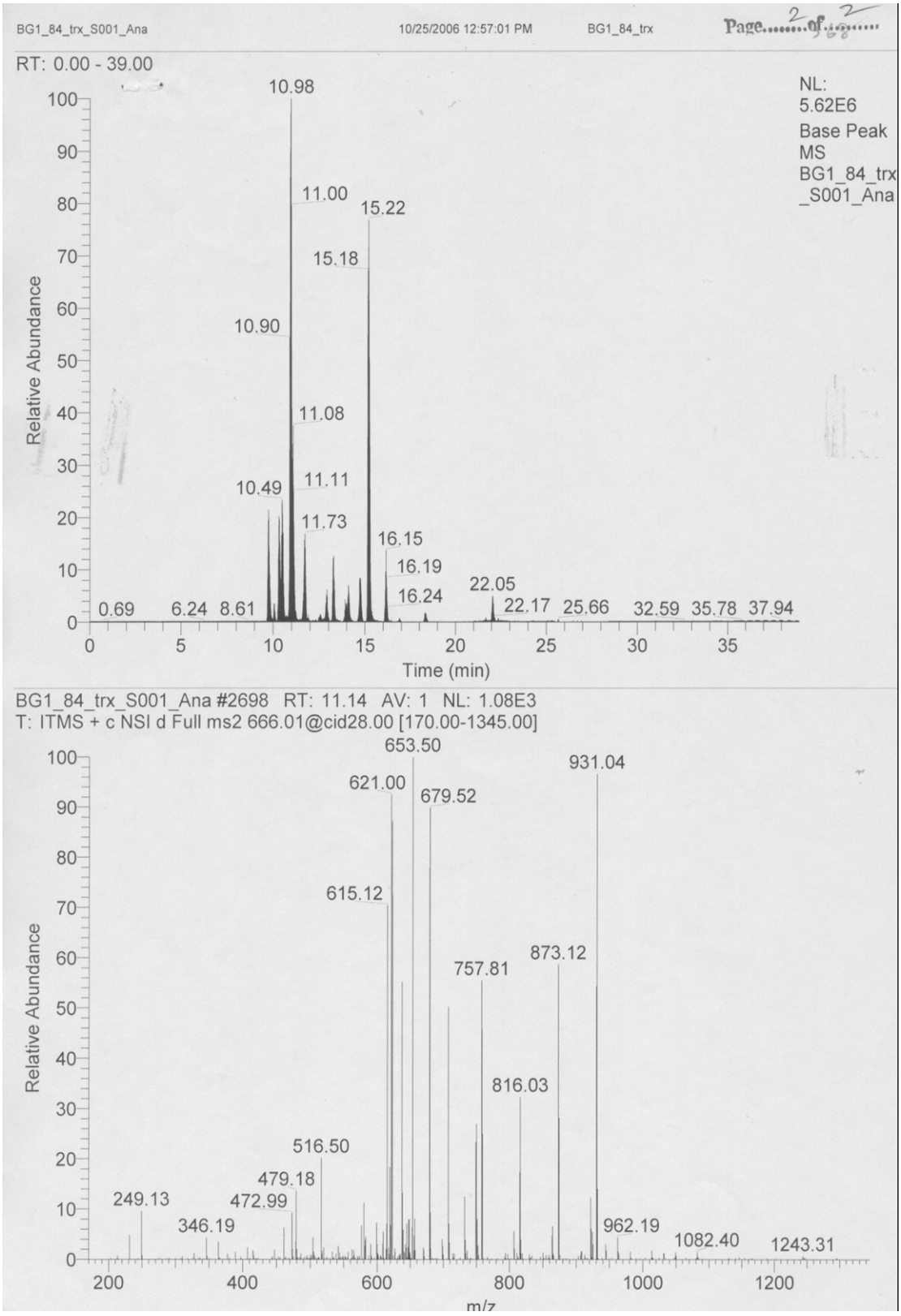
4. Identification of tryptic digests of the 97 kDa band of OsBGal1 by LC/MS/MS.



5. Identification of tryptic digests of the 97 kDa band of OsBGal1 by LC/MS/MS.



6. Identification of tryptic digests of the 29 kDa band of OsBGal1 by LC/MS/MS.

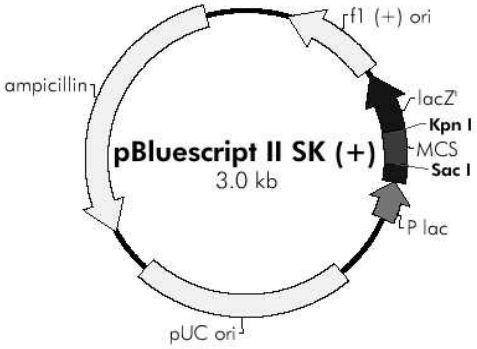


APPENDIX B

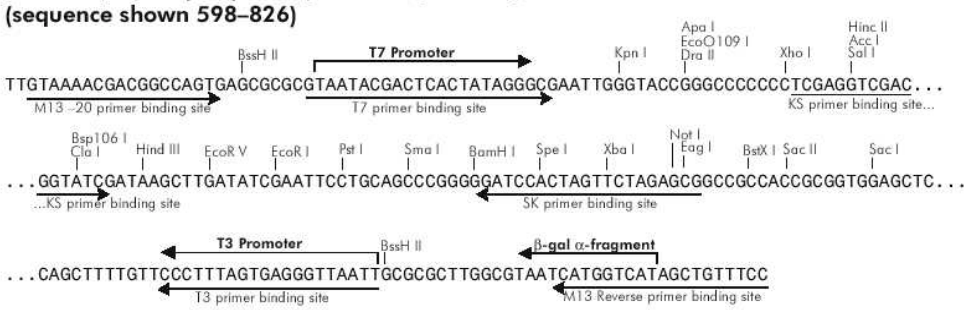
PLASMID MAPS

1. pBluescript SK (+) vector map

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833

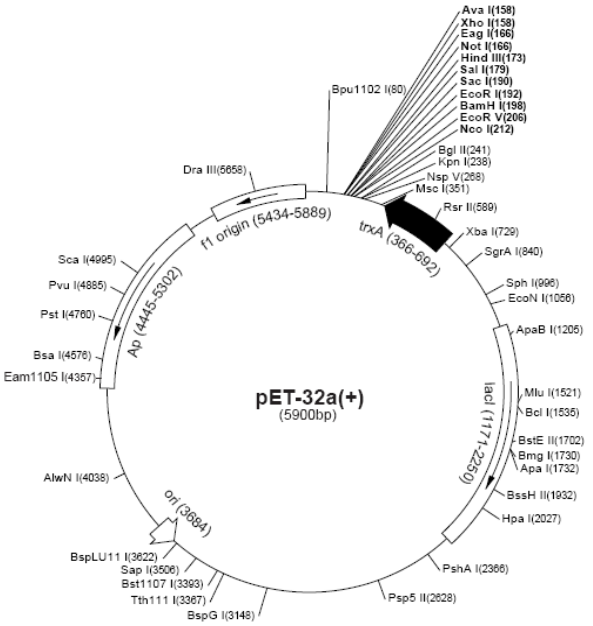


pBluescript II SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)



2. The pET-32a (+) vector map

The pET-32 series is designed for cloning and high level expression of peptide sequences fused with the 109 aa Trx•Tag™ thioredoxin protein. Cloning sites are available for producing fusion proteins also containing cleavable His•Tag® and S•Tag™ sequences for detection and purification. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circle map. The cloning/ expression region of the coding strand transcribed by T7 RNA polymerase is shown below.



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. 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) NaCl

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. Allow the medium to cool to 50°C, then add ampicillin to a final concentration of 100 μg/ml. Pour medium into Petri-dishes. Allow the agar to harden, and keep at 4°C.

1.3 LB agar plate with 50 μg/ml ampicillin, 15 μg/ml kanamycin, 12 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. Allow the medium to cool to 50°C, then add ampicillin to a final concentration 50 μg/ml, kanamycin 15 μg/ml, 12.5 μg/ml tetracycline. Pour medium into Petri-dishes, allow the agar to harden, and keep at 4°C.

1.4 LB plate with 100 mg/ml of ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above. Then spread 50 ml of 100 mM IPTG and 25 ml of 50 mg/mL X-Gal over the surface of the plates and allow to absorb for 10 min before use.

1.5 Antibiotics solution stock

Ampicillin (100 mg/ml):

Dissolve 100 mg ampicillin in 1 ml distilled water.

Kanamycin (15 mg/ml):

Dissolve 15 mg kanamycin in 1 ml distilled water.

Tetracyclin (12.5 mg/ml):

Dissolve 12.5 mg in 1 ml absolute ethanol.

Filter sterile all antibiotic solutions ant kept at -20°C.

1.6 IPTG stock solution (100 mM)

Dissolve 0.12 g IPTG (isopropyl thio-β-D-galatoside) in distilled water and make to 5 ml final volume. Sterile by filter sterile and stored at -20°C.

1.7 X-gal stock solution

Dissolve 50 mg X-gal in 1 mL *N, N*-dimethylformamide (DMF) and store in the dark bottle at -20°C.

2. Reagents for competent cell preparation

2.1 $CaCl_2$ solution (60 mM CaCl2, 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 (autoclave at 100°C, 15 min)

Add sterile distilled water to bring a volume up to 100 ml. Store the solution at 4°C.

2.2 SOC media (1 l)

Dissolve 20 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 mL of 1 M NaCl (5.85 g/100 ml), 2.5 ml of 1 M KCl (7.44 g/100 ml) in distilled water and make to 980 ml final volume. Sterilize by autoclaving at 121°C for 20 min. Allow the medium cool to room temperature, then add 5 ml of 1 M $MgCl_2 \cdot 6H_2O$ (20.33 g/100 ml), 5 ml of 1 M $MgSO_4 \cdot 7H_2O$ (12.30 g/100 ml), 10 ml of 2 M glucose (36 g/100 ml) which were filter sterilized. Store the solution at 4°C.

3. Reagent for isolation plasmid DNA (boiling prep)

3.1 **STET** (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 5% Triton X-100) (100 ml)

Mix 20 ml of 1 M NaCl (sterile), 2 ml of 1 M Tris-HCl pH 8.0 (sterile), 0.4 ml of 0.5 M EDTA pH 8.0 (sterile), 5 ml Triton X-100 and adjust the volume to 100 ml with sterile distilled water. Store at room temperature.

3.2 3 M Sodium acetate pH 4.8 (100 ml)

Dissolved 24.6 g sodium acetate in 80 ml distilled water. Adjust pH to 4.8 with glacial acetic acid and make up the volume to 100 ml with distilled water. Sterilize by autoclaving at 121°C for 20 min. Stored at room temperature.

3.3 RNase A (10 mg/ml)

Dissolve 10 mg *RNase* A in 10 mM Tris-HCl pH 7.4, 15 mM NaCl buffer (sterile). Stored at -20°C.

3.4 1 M Tris-HCl pH 7.4 and pH 8.0 (100 ml)

Dissolve 12.11 g of Tris Base in 80 ml distilled water. Adjust pH with HCl to pH 7.4 or 8.0 and a volume to 100 ml with distilled water and autoclave at 121° C for 20 min. Store at room temperature.

3.5 0.5 M EDTA (pH 8.0) (100 ml)

Dissolve 18.61 g EDTA (disodium ethylene diamine tetraacetate 2•H_2O) in 70 ml distilled water. Sterilize by autoclaving at 121°C for 20 min. Stored at room temperature.

4. Reagent for agarose gel electrophoresis

4.1 50 X TAE for agrose gel electrophoresis (1l)

Mix 424 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.

4.2 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.

5. Reagents for total RNA isolation by using CTAB

5.1 DEPC treated water (1l)

Add 1 ml of diethyl pyrocarbonate (DEPC) in distilled water and make to 1l final volume. Shake vigorously overnight and autoclave the solution at 121°C for 15 min to inactivate the remaining DEPC. Store at room temperature.

5.2 2 X CTAB solution (100 ml) (2% Cetyltrimethylamonium bromide (CTAB), 0.1 M Tris-HCl pH 9.5, 20 mM EDTA, 1.4 M NaCl, 5% 2-mercaptoethanol)

Dissolve 2 ml CTAB, 1.21 g Tris base, 7.44 g EDTA and 8.19 g NaCl in distilled water and adjust the volume to 95 ml. Sterilize by autoclaving at 121°C for 40 min and add 1 ml of 2-mercaptoethanol just prior to use.

5.3 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. Store at room temperature.

5.4 3 M Sodium acetate pH 5.2 (100 ml)

Dissolve 24.6 g sodium acetate in 80 ml distilled water. Adjust pH 5.2 with glacial acetic acid and the volume to 100 ml. Sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

5.5 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. Incubate at 4°C overnight. Remove supernatant. Store at 4°C.

6. Reagents for northern blot analysis

6.1 10 X MOPS buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA) (1l)

Dissolve 41.7 g MOPS free acid, 6.8 g sodium acetate, and 3.7 g EDTA in 700 ml sterile DEPC treated water. Adjust pH to 7.0 with NaOH, and a volume to 1 l with DEPC treated water. Store at 4°C.

6.2 1 X MOPS buffer (Running buffer) (1 l)

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

6.3 1.5% Formaldehyde agarose gel (100 ml)

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

6.4 RNA premix (500 µl)

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

6.5 5 X RNA loading buffer (10 ml)

Mix 16 µl saturated bromophenol blue, 80 ml of 0.5 M EDTA (pH 8.0), 720 µl 37% formaldehyde, 3084 µl formalmide, 4 ml 10 X MOPS buffer, and 2 ml of 100% glycerol. Add DEPC treated water to final volume 10 ml (Stability 3 months at 4°C)

6.6 20 X SSC (3M NaCl, 0.3 M Sodium citrate) (1 l)

Dissolve 175.3 g NaCl, 88.2 g sodium citrate in 800 ml distilled water. Adjust pH to 7.0 with NaOH and the volume to 1 l. Sterilize by autoclaving at 121°C for 20 min. Store at room temperature.

6.7 **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, 35.9 ml of 1 M NaH_2PO_4, and 17.5 g SDS and adjust a volume to 250 ml with distilled water. Sterilize by autoclaving at 121°C for 20 min. Sit to cool down to 60°C and then add 2.5 g BSA. Store at room temperature.

7. Solutions for protein

7.1 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 and 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.

7.2 1.5 M Tris pH 8.8 (100 ml)

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

7.3 0.5 M Tris pH 6.8 (100 ml)

Dissolve 6.06 g Tris Base in 80 ml distilled water. Adjust pH to 6.8 with HCl and the volume to 100 ml with distilled water. Store at 4°C.

7.4 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 h to be homogeneous and filter through Whatman membrane No. 1. Store in the dark bottle at 4°C.

7.5 Tris-Glycine electrode buffer (5 X stock)

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.

7.6 Staining solution with Coomassie brilliant blue for protein

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

7.7 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.

7.8 10% (w/v) Ammonium persulfate (1 ml)

Dissolve 100 mg ammonium persulfate in 1 ml distilled water. Store at -20°C

7.9 12% Separating gel SDS-PAGE (10 ml)

Mix the solution as follow:

1.5 M Tris (pH 8.8)	2.5 ml
Distilled water	3.3 ml
10% (x/v) SDS	0.1 ml

30% acrylamide solution	4.0 ml
10% (w/v) ammonium persulfate	0.1 ml
TEMED	0.004 ml

7.10 5% Stacking gel SDS-PAGE (5 ml)

Mix the solution as follow:

0.5 M Tris (pH 6.8)	1.26 ml
Distilled water	2.77 ml
10% (x/v) SDS	0.05 ml
30% acrylamide solution	0.83 ml
10% (w/v) ammonium persulfate	0.05 ml
TEMED	0.005 ml

7.11 1 X Acetic-KOH resolving gel buffer pH 4.3 (100 ml)

Mix 48 ml 1M KOH and 17.2 ml glacial acetic acid and add distilled water to 100 final volume.

7.12 4 X Acetic-KOH stacking gel buffer pH 6.8 (100 ml)

Mix 48 ml 1 M KOH and 2.9 ml glacial acetic acid and add distilled water to 100 final volume.

7.13 10% Separating gel acidic native (PAGE) (15 ml)

Mix the solution as follow:

4 X acetic-KOH resolving gel buffer pH 4.3	5 ml

Distilled water	7.99 ml
30% Acrylamide solution	6.65 ml
300 mM sodium sulfide	0.2 ml
10% (w/v) ammonium persulfate	0.15 ml
TEMED	0.01 ml

7.14 5% Stacking gel acidic native PAGE (5 ml)

Mix the solution as follow:

4 X acetic KOH stacking gel buffer pH 6.8	1.26 ml
Distilled water	2.86 ml
30% acrylamide solution	0.83 ml
10% (w/v) ammonium persulfate	0.05 ml
TEMED	0.005 ml

8. Solution for western blotting

8.1 1 X PBS (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl)

Dissolve 0.216 g (KH_2PO_4, 1.192 g Na_2HPO_4, 0.199 g KCl, 8 g NaCl in distilled water and make to 1 l final volume.

8.2 1 X PBST (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCL, 137 mM NaCl, 0.05% Tween 20)

Dissolve 0.216 g KH_2PO_4, 1.192 g Na_2HPO_4, 0.199 g KCl, 8 g NaCl, 0.5 ml Tween 20 in distilled water and make to 1 l final volume.

8.3 Fast green staining solution

Dissolve 0.05 g Fast green with 20 ml methanol, 5 ml glacial acetic acid, 75 ml distilled water.

8.4 3 mg/ml 4-Chloronapthol (20 ml)

Dissolve 60 mg 4-chloronapthol in 100% methanol and adjust to 20 ml final volume. Store in the dark bottle at 4°C.

9. Buffer and Reagents for enzyme assay

9.1 10 mM *p*-nitrophenol (10 ml)

Dissolve 0.0139 g *p*-nitrophenol in 50 mM NaOAc pH 5.0 buffer and make to 10 ml final volume.

9.2 50 mM NaOAc pH 4.0 buffer (100 ml)

Dissolve 0.41 g NaOAc in 80 ml distilled water, and adjust pH to 4.0 with glacial acetic acid and the volume to 100 ml with distilled water.

9.3 20 ml Tris-HCl pH 8.0 buffer (100 ml)

Dissolve 0.24 g Tris base in 80 ml distilled water, and adjust pH to 8.0 with HCl and the volume to 100 ml with distilled water.

9.4 0.4 M Na_2CO_3 (100 ml)

Dissolve 0.42 g Na_2CO_3 in distilled water, and adjust the volume to 100 ml with distilled water.

Dissolve 0.24 g Tris base in 80 ml distilled water, and adjust pH to 8.0 with HCl and the volume to 100 ml with distilled water.

9.4 0.4 M Na_2CO_3 (100 ml)

Dissolve 0.42 g Na_2CO_3 in distilled water, and adjust the volume to 100 ml with distilled water.

10. Solution for protein purification

10.1 Buffer 1 (500 mL)

Dissolve 1.51 g Tris 218 g (NH4)2SO4 in 350 mL distilled water, then agjust to pH 8.0 with HCl and added distilled water to 490 mL final volume. Add 5 mL 0.1 M PMSF, 5 mL 1 M ascorbic acid and 0.97 g β-mercaptoethanol just before use.

10.2 Buffer 2 (200 mL)

Dissolve 0.605 g Tris and 2.33 g NaCl in 200 mL distilled water, then adjust to pH 8.0 with HCl and added water to 196 mL final volume. Add 2 mL 0.1 M PMSF, 2 mL 1M ascorbic acid and 0.39 β-mercaptoethanol just before use.

10.3 0.1 M PMSF

Dissolve 0.0871 g PMSF in 5 mL isopropanol. Freshly prepare.

10.4 1 M Ascorbic acid

Dissolve 1.76 g ascorbic acid in 5 mL distilled water. Freshly prepare.

10.5 0.025 M Tris-HCl pH 7.0 (1,500 mL)

Dissolve 3.53 g Tris in 1,400 mL distilled water and adjust to pH 7.0 with HCl and add distilled water up to 1,500 mL

10.6 0.025 M Tris-HCl pH 7.0 + 0.5 M NaCl (500 mL)

Dissolve 1.51 g Tris and 14.61 g NaCl in 400 mL distilled water and adjust to pH 7.0 with HCl and add distilled water up to 500 mL.

10.7 0.050 M Tris-HCl pH 7.0 + 0.3 M NaCl (1,000 mL)

Dissolve 6.057 g Tris and 17.53 g NaCl in 900 mL distilled water and adjust to pH 7.0 with HCl and the volume to 1,000 mL with distilled water.

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