รหัสโครงการ SUT1-102-54-12-22

รายงานการวิจัย

การศึกษาการทำงานของไกลโคไซด์ไฮโดรเลส ตระกูลที่ 1 กลุ่ม 6

Characterization of a glycoside hydrolase family 1 group 6

ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

รหัสโครงการ SUT1-102-54-12-22

รายงานการวิจัย

การศึกษาการทำงานของไกลโคไซด์ไฮโดรเลส ตระกูลที่ 1 กลุ่ม 6

Characterization of a glycoside hydrolase family 1 group 6

ผู้วิจัย

หัวหน้าโครงการ

ศาสตราจารย์ ดร. เจมส์ เกตุทัต-คาร์นส์

Professor Dr. James R. Ketudat-Cairns

สาขาวิชาเคมี/ชีวเคมี มหาวิทยาลัยเทคโนโลยีสุรนารี

รองศาสตราจารย์ ดร. มารินา เกตุทัต-คาร์นส์

Associate Professor Dr. Mariena Ketudat-Cairns

สาขาวิชาเทคโนโลยีชีวภาพ มหาวิทยาลัยเทคโนโลยีสุรนารี

ได้รับทุนอุดหนุนการวิจัยจากมหาวิทยาลัยเทคโนโลยีสุรนารี ปีงบประมาณ พ.ศ. 2554
ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

กันยายน 2556

1. Preliminary

1.1 Acknowledgement

This work could not have been completed without the contributions of several talented scientists. The enzymatic characterization in this grant was primarily conducted by Dr. Sukanya Luang with assistance from Ms. Kannika Phasai and Ms. Juthamath Komvongsa. Prof. Yoshihiro Ozerki, Dr. Nobuhiro Sasaki and Dr. Yuki Matsuba are gratefully acknowledged for providing the 1-O-acyl glucose esters used to establish the donor specificity of the Os9BGlu31 transglucosidase. The collaboration of Dr. Rodjana Opassiri and Dr. Takashi Akiyama in northern blot analysis of gene expression and Prof. Jong-Seong Jeon, Dr. Jung-Il Cho, and Mr. Bancha Mahong in quantitative real-time reverse-transcription polymerase chain reaction (Q-RT-PCR) analysis of gene expression is also gratefully acknowledged. We are also grateful for advice, encouragment and support from Prof. Dr. M.R. Jisnuson Svasti and other colleagues from the Center for Protein Structure and Function Mahidol University and the Laboratory of Biochemistry, Chulabhorn Research Institute. Dr. Mariena Ketudat-Cairns is thanked for her thoughtful advice throughout the project.

Suranaree University of Technology is acknowledged for the financial support of this project.

Prof. James R. Ketudat-Cairns
Head of the project
September, 2013

1.2. Thai Abstract (บทคัดย่อ)

โครงการวิจัยนี้ คณะผู้วิจัยได้ศึกษาการทำงานของเอนไซม์เบต้า-กลูโคสิเดส Os9Bglu31 จากข้าว เอนไซม์นี้แสดงกิจกรรมในการเคลื่อนย้ายกลูโคส (transglucosidase) มากกว่าการสลายพันธะเบต้า-กลูโคไซด์ (β-glucosidase) พบว่าเอนไซม์ Os9BGlu31 สามารถใช้ 4-nitrophenyl (4NP) β-D-glucopyranoside, 4NP β-D-fucopyranoside และ 4NP β-D-xylopyranoside เป็นสารตั้งต้นในการให้หมู่น้ำตาล และเคลื่อนย้ายหมู่น้ำตาลให้แก่ nucleophiles ได้แก่ acetate หรือ azide ได้ดีกว่าน้ำที่ใช้ในการเกิดปฏิกิริยาไฮโดรไลซีส โดยในสภาวะที่มีสารตั้งต้นในการให้หมู่น้ำตาลดังกล่าวสามารถพบการเกิดปฏิกิริยาไฮโดรไลซิสได้เล็กน้อย นอกจากนี้ เอนไซม์ Os9BGlu31 ยังสามารถใช้สารจำพวก 1-*O*-acyl เอสเทอร์ของกลูโคส ได้แก่ 1-*O*-β-D-feruloyl glucose, 1-*O*-β-D-(4-coumaroyl) glucose, 1-*O*-β-D-vanillyl glucose, 1-*O*-β-D-(4-hydroxybenzoyl) glucose, 1-*O*-β-D-sinapoyl glucose และ gibberellins GA4 β-D-glucosyl ester ตลอดจน phloridzin และ apigenin 7-*O*-β-D-glucoside เป็นตัวให้กลูโคสได้ดีกว่าการใช้ 4NP-glycosides เอนไซม์ Os9BGlu31 สามารถเคลื่อนย้ายกลูโคสให้แก่แอลกอฮอล์และสารตัวรับจำพวก carboxylate ได้หลากหลายเช่นเดียวกับการใช้ azide อย่างไรก็ตาม เอนไซม์นี้จะให้ค่ากิจกรรมในการเคลื่อนย้ายกลูโคสได้สูงสุดเมื่อใช้สารจำพวก phenolic carboxylates ได้แก่ ferulic acid และ 4-coumaric acid นอกจากที่กล่าวมาแล้วเอนไซม์นี้ยังสามารถใช้สารในกลุ่มฮอร์โมนจากพืช ได้แก่ auxin (indole acetic acid และ naphthalene acetic acid) และ gibberellins (GA4) เป็นสารตั้งต้นตัวรับกลูโคส ในกรณีของการศึกษาสารยับยั้งการเกิดปฏิกิริยาของเอนไซม์ นอกเหนือจากไอออนของทองแดงและปรอทแล้ว พบว่าไอออนของโลหะหนักอื่น ๆ ไม่มีผลยับยั้งการทำงานของเอนไซม์ ซึ่งรวมถึงสารที่มีผลยับยั้งกิจกรรมของเอนไซม์เบต้า-กลูโคสิโดสโดยทั่วไป เช่น δ-gluconolactone, phenylethyl glucoimidazole, deoxy-nojirimycin, isofagamine, conduritol B epoxide, cyclophellitol และ 2,4-dinitrophenyl-β-D-deoxy-2-fluoro-glucopyranoside ก็ไม่แสดงการผลในการยับยั้งกิจกรรมของเอนไซม์ Os9Bglu31 พบว่าเอนไซม์ชนิดนี้มีการแสดงออกสูงที่สุดในใบธง (senescing flag leaf) และเมล็ดที่กำลังพัฒนา โดยการแสดงออกในต้นกล้าถูกเหนี่ยวนำได้โดยสภาวะเครียดต่าง ๆ และฮอร์โมนพืช ดังนั้น การศึกษาเอนไซม์เบต้า-กลูโคสิเดส Os9BGlu31 นี้จึงมีบทบาทสำคัญในทางชีววิทยาและชีวเคมีเป็นอย่างมาก ผลจากโครงการวิจัยนี้ได้ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ Journal of Biological Chemistry และมีการนำเสนอผลงานในการประชุมวิชาการระดับนานาชาติ รวมถึงมีการพัฒนาบุคลากรวิจัยหลังปริญญาเอก ผู้ช่วยวิจัย และนักศึกษา นอกจากนี้ยังมีการวางแผนเพื่อพัฒนาผลงานวิจัยนี้ไปสู่การใช้งานทางเกษตรกรรมและอุตสาหกรรมยาในอนาคต

1.3. English Abstract

In this project, we characterized the putative rice β-glucosidase Os9BGlu31 and showed that it is a transglucosidase, rather than a β-glucosidase. The Os9BGlu31 enzyme was found to utilize 4-nitrophenyl (4NP) β-D-glucopyranoside, 4NP β-D-fucopyranoside and 4NP β-D-xylopyranoside as donor substrates and to transfer the sugars onto nucleophiles, such as acetate or azide better than to water in hydrolysis. In the presence of such donors, negligible amounts of hydrolysis were detected. Os9BGlu31 can use the 1-*O*-acyl glucose esters 1- *O*-β-D-feruloyl glucose, 1-*O*-β-D-(4-coumaroyl) glucose, 1-*O*-β-D-vanillyl glucose, 1-O-β-D-(4-hydrolxybenzoyl) glucose, 1-O-β-D-sinapoyl glucose, and gibberellin GA_4 β-D-glucosyl ester, as well as the glycosides phloridzin and apigenin 7-O-β-D-glucoside as donors with higher activity than the 4NP-glycosides. Os9BGlu31 can transfer glucose to a wide range of alcohol and carboxylate acceptors, as well as azide, but had highest activity with phenolic carboxylates, such as ferulic acid and 4-coumaric acid. Other notable substrates, from the plant function perspective included the phytohormones auxin (indole acetic acid and naphthalene acetic acid) and gibberellin (GA_4). Except for mercury and copper, Os9BGlu31 activity was not affected by metal ions and the typical β-glucosidase inhibitors δ-gluconolactone, phenylethyl glucoimidazole, deoxy-nojirimycin, isofagamine, conduritol B epoxide, cyclophellitol, and 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside did not inhibit it. The enzyme is highly expressed in senescing flag leaf and developing seed, and its expression in seedlings was induced by a variety of stress conditions and phytohormones. Thus, the enzyme displays several interesting biological and biochemical properties that give it potentially useful biological functions and applications. This work contributed to the publication of a paper in the Journal of Biological Chemistry, presentations at international meetings, and training of a postdoc, an assistant and a student. It sets the foundation for a future studies with implications for agriculture and pharmaceutical modification.

1.4. CONTENTS

1.4. CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

Section 2.1: Introduction

2.1.1. Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

2.1.1.1. Rice as an experimental model

Rice (*Oryza sativa* L.) is one of the most highly produced crop in the world, along with its fellow monocots maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), and rice is the most important food crop in the world. Due to its relatively small genome, rice was designated as the first monocot to have its genome sequenced [International Rice Genome Project, 2005]. This, along with the production of expressed sequence tags (ESTs) and full-length cDNA from rice RNA extracted from various tissues under various conditions has provided a wealth of information on the genes that are expressed and the sequences of their protein products [Kawasaki et al., 2001; Kikuchi et al., 2003]. Many microarray experiments have also been done with much of the data obtained available in public data bases [Rensink and Buell, 2005]. Thus, rice provides a wealth of resources for investigation of important processes in plants, particularly monocots, which include many of the world's most important crops.

Besides for the sequence-related knowledge about rice, many experimental techniques have been generated for rice, including transformation with exogenous DNA via Agrobacteria, RNA interference-mediated gene knock-down and generation of gene-disrupted rice lines by activation of endogenous retrotransposons [Hirochika et al., 2001] or insertion of T-DNA [Jeon et al., 2000]. Rice gene insertion lines can often be identified by a search of publicly available databases, and the functions of the disrupted genes can then be investigated by ordering and growing the lines or by collaboration with those in the consortium or laboratory that generated the lines. As such, there are many tools available for the investigation of rice gene functions.

2.1.1.2. Glycoside hydrolases

Plants produce a vast array of polysaccharides and glycoconjugates, the production and breakdown of which are catalyzed by glycosyltransferases (GT) and glycoside hydrolases (GH), respectively. GH can be classified according to their enzymatic activities by the International Union for Biochemistry and Molecular Biology (IUBMB) Enzyme Commission (E.C.) numbers starting with 3.2.1, or by their sequence similarity and presumed evolutionary relationship, as was systemized by Henrissat [1991, Henrissat & Baroch 1993, 1996]. The latter approach can be used to analyze large amounts of sequence data that has become

available with genomic sequencing projects and is used to give a clue to the functions of various gene products [International Rice Genome Project, 2005]. However, predicting the function based on sequence similarity leads to misannotation of the public databases due to the assumptions of functions being made, and further studies of protein functions are necessary to determine their exact functions (and sometimes even their general functions). Nonetheless, the grouping of related GH into related groups that tend to have related functions can provide useful clues as to how to investigate their functions. So far, more than one hundred GH families have been identified (CAZY, carbohydrate active enzyme website: www.cazy.org), and many of these have been grouped into clans of families that have similar 3-dimensional structures and, in general, utilize similar catalytic mechanisms [Coutinho & Henrissat, 1999; Cantarel et al., 2009]. Despite their similar sequences and mechanisms, members of GH families generally include enzymes with a few to several different substrate specificities. For instance, GH family 2 (GH2) contains β-D-galactosidases, β-D-glucuronidases and β-D-mannosidases, while GH3 contains exoglucanases, β-D-glucosidases, β-D-xylosidases and α-L-arabinosidases (CAZY).

2.1.1.3. Clan A beta-glycosidases

The GH families have been grouped into larger catagoies called Clans, based on similarity in overall structural fold, mechanism and catalytically important amino acids, with Clan A being the largest. Clan A β-glycosidases have catalytic domains that form $(\beta/\alpha)_8$-barrels with two catalytic carboxyl residues, the catalytic acid/base on the end of β-strand 4 and the nucleophile on the end of β-strand 7 of the barrel [Jenkins et al., 1995; Henrissat et al., 1995; Chuenchor et al., 2008]. They generally catalyze hydrolysis by a shared retaining mechanism in which the stereochemistry of the anomeric carbon in the released sugar is initially conserved with that in the substrate [Ketudat Cairns and Esen, 2010; Ketudat Cairns et al., 2012]. This is achieved by a two step mechanism in which the sugar is initially attacked by the catalytic nucleophile to displace the aglycone, which leaves with acid assistance from the catalytic acid/base, resulting in glycosylation of the enzyme. The covalent bond with the sugar residue is broken in the subsequent deglycosylation step in which a water molecule, or another nucleophile, attacks the anomeric carbon with basic assistance from the catalytic acid/base to displace the nucleophilic carboxyl residue. Many enzymes with diverse specificities fall in clan A, including those of GH families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 50, 51, 53, 59, 72, 79, 86, and 113, making it the largest clan both in terms of numbers of families and different specificities. These enzymes are almost all specific to β-D-glycosidic

linkages, although some also hydrolyze α-L-arabinoside and α-L-iduronides, for which the glycone sugar residue is shaped similar to β-D-glycosidic residues. It is notable that these types of sugar linkages account for most of the structural polysaccharides of plants [Carpita, 1996; Cosgrove, 1997], as well as many glycosides in plant secondary metabolism [Chuankhayan et al., 2005].

2.1.1.4. Functions of plant β-glucosidases.

We have previously annotated the enzymes of GH1 in rice and given them systematic names based on their chromosomal location, from Os1BGlu1 to Os12BGlu38 (where the number after the Os is the rice chromosome and the number after the BGlu is a running number, starting from the top of chromosome 1), to begin to unravel the functions of these β-D-glycosidases in plants [Opassiri et al., 2006]. In general, β-glucosidase functions that have been described include cell wall metabolism and recycling, phytohormone activation, release of defense compounds from inactive glycosides, activation of intermediates in metabolic pathways, and release of volatile compounds, such as fragrances [Ketudat Cairns and Esen, 2010]. They also play unknown roles in response to abiotic and biotic stresses and establishment of mutualistic relationships with endophytes.

2.1.1.4.1. Cell wall metabolism.

Plant cell walls are made up of a combination of cellulose, hemicelluloses, pectins and related polysaccharides, glycoproteins, and lignins, as well as silica and other components, depending on the type of cell wall [Carpita, 1996; Cosgrove, 1997]. In order to grow and develop, plants constantly remodel and turnover their cell wall polysaccharides, and in processes like breakdown of endosperm cell walls in germination, completely disassemble them. This breakdown depends on endoglycosidases, especially endoglucanases such as cellulases, but also on the action of glycosidases to breakdown the released oligosaccharides to prevent them from inhibiting the endoglycosidases and to release glucose and other sugar residues for use by the plant. In addition to release of sugar residues from oligosaccharides, some exoglycosidases can also release external residues from cell wall polysaccharides, making the polysaccharides more accessible to the endoglucanases, xylanases, galactanases and mannanases that hydrolyze the polysaccharide backbones. So, plants generally have a wide range of enzymes with different specificities to act at different points in cell wall degradation and recycling.

Several GH1 enzymes from rice have been found to hydrolyze β-linked oligosaccharides, suggesting this may be a major component of their role in the plant

[Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006; Kuntothom et al., 2009]. The rice BGlu1 (Os3BGlu7), Os3BGlu8 and Os7BGlu26 isoenzymes could also hydrolyze β-linked mannooligossacharides, as does a closely related enzyme from barley [Hrmova et al., 1996, 1998, 2006], suggesting they could have roles in recycling of multiple cell wall components. A similar β-mannosidase/β-glucosidase has been described from Arabidopsis [Xu et al., 2004], suggesting this may be a general feature among plant exoglycosidases.

Microorganisms also have evolved enzymes to metabolize plant cell walls as an energy source [Gilbert et al., 2008]. Some bacteria and fungi have developed a wide range of genes related to cell wall breakdown, thereby dedicating a significant portion of their genomes to this process. As such, these organisms and enzymes derived from them are commonly used for biomass conversion. The opportunistic breakdown of plant cell walls by these organisms has evolved separately and is fundamentally different from that which occurs in plants.

In addition to their roles in cell wall breakdown, plant β-glucosidases also act in the development of secondary cell walls by their release of lignin precursors sites of lignin formation [Dharmawardhana et al., 1995]. The presence of monolignol glucoside β-glucosidases has been shown in Arabidopsis [Escamilla-Treviño et al., 2006], as well as pine trees, and a cluster of 3 closely related genes and two gene fragments is also found in the rice genome [Opassiri et al., 2006]. Thus, β-glucosidases may affect the mechanical properties and digestibility of cell wall through the depositing of lignin, in addition to their roles in polysaccharide recycling. Since decreasing lignin content is one of the main goals of genetic engineering of plants for bioconversion to fuels [Sticklen, 2006], the effect of these β-glucosidases on cell wall lignin content is especially of interest.

2.1.1.4.2. Phytohormone activation

There has been some debate over the years as to the roles of phytohormone glycosides and the β-glycosidases (mainly β-glucosidases) that hydrolyze them [Buchanan et al., 2000]. These glycosides have been suggested to be deadend by-products of plant down-regulation of the phytohormones, though their high levels and availability suggested that they could be a source for rapid release of phytohormones in response to biotic or abiotic stress. Enzymes have been identified that can hydrolyze glycosides of gibberrilins [Schliemann, 1984], cytokinin [Brzobohatý et al., 1993], auxin [Jakubowska & Kowalczyk, 2005], jasmonic acid derivatives [Seto et al., 2009], and abscissic acid [Lee et al., 2006]. The demonstration that maize β-glucosidase could hydrolyze and activate cytokinin β-glucosides was taken as evidence for the role of β-glucosidases in phytohormone activation [Brzobohatý et al., 1993],

but others noted that the more abundant DIMBOA-glucoside, which can serve as a defense compound, was likely the major substrate for this enzyme [Babcock & Esen, 1994]. However, the demonstration that an Arabidopsis β-glucosidase could aggregate and be activated to release ABA in response to drought stress [Lee et al., 2006; Xu et al., 2012], suggests that at least some of these glycosylated forms are stored for the release by β-glucosidases under stress conditions. In rice, Schliemann [1984] demonstrated the presence of β-glucosidases that hydrolyzed gibberellin glucosides and glucosyl esters, but these enzymes have yet to be identified at the molecular level. Recently, it has also been shown that a rice enzyme can hydrolyze tuberillic acid (TA) glucoside (TAG), thereby modulating its level in the rice plant [Wakuta et al., 2010].

2.1.1.4.3. Defense

A large number of β-glucosidases have been shown to release toxic compounds from inactive glycosides in response to herbivory, fungal invasion and damage [Morant et al., 2008]. Cyanogenic glycosides, which rapidly react to release HCN upon release from their glucosyl blocking group by β-glucosidases [Poulton, 1990]. As noted above, hydroxaminic acids, like DIMBOA and DIBOA can be released from their glucosides in maize [Babcock and Esen, 1994], wheat and rye [Sue et al., 2006] by β-glucosidases. In addition, the nonhost-type resistance of *Arabidopsis thaliana* to powdery mildew and other fungi has been shown to be mediated by PEN2, a peroxisomal thioglucosidase/β-glucosidase that hydrolyzes glucosinolates as part of the cellular response to fungal invasion [Lipka et al., 2005; Bednarek et al., 2009]. In addition, the endoplasmic reticulum body GH1 glycosyl hydrolase PYK10 is necessary for Arabidopsis to set-up a mutualistic interaction with the endophytic fungus *Piriformospora indica* [Sherameti et al., 2008]. In Arabidopsis lines deficient in PYK10, the *P. indica* overpopulates the roots and elicits different defensive proteins, suggesting that PYK10 may defend against overpopulation of the roots to achieve the balance needed for the mutualistic interaction.

The defense related β-glucosidases and thioglucosidases are often stored in a different compartment from their glycoside substrates, and the two only come together upon compromising of the cell membranes during herbivory and microbial invasion [Morant et al., 2008]. For instance, the plant hydroxaminic acid glycoside β-glucosidases are stored in the chloroplasts, while their substrates are found in an alternative position, possibly the vacuole [Esen and Stetler, 1993; Nikus et al., 2003], as is also the case of saponin β-glucosidases of oat [Nisius, 1988]. The enzyme and substrate sometimes appear to be in separate layers of cells or types of cells, as is seen in thioglucosidases or myrosinases, which are found in

myrosin granuoles of myroblasts, while their substrates are found in other cells [Höglund et al., 1992]. In many eudicots, cyanogenic β-glucosidases are found in the apoplast, while the substrates are found in the vacuole [Morant et al., 2008 and references therein]. Aside from localization, another common feature of defensive β-glucosidases is the presence of a cytoplasmic aggregating factor that binds to them upon cell wall disruption [Blanchard et al., 2001]. These proteins are thought to affect the localization after the cells are broken open to make the β-glucosidases most effective against the herbivore or pathogen.

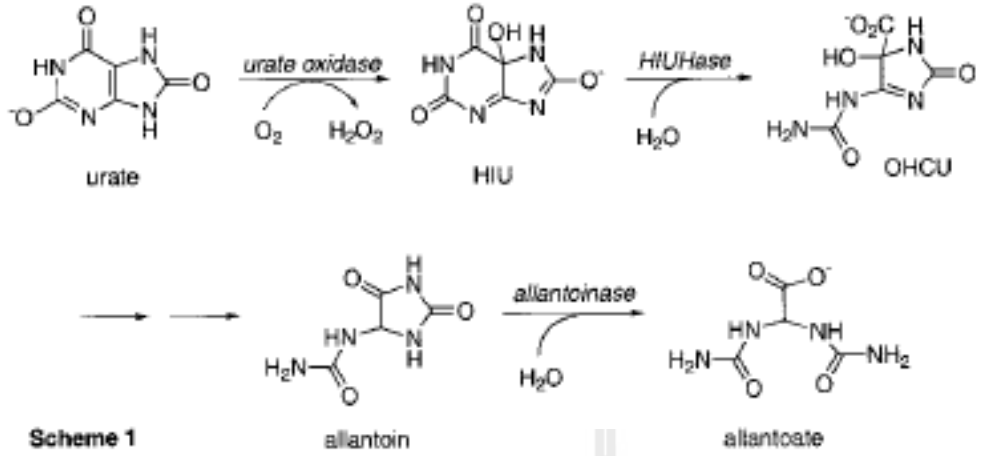
2.1.1.4.4. Response to abiotic and biotic stress

In many cases β-glucosidases have been noted to be increased in response to abiotic or biotic stress, although their roles are often unknown. As mentioned above, ABA-GE β-glucosidase (designated AtBG1) is encoded by a cDNA isolated from a salt-stressed Arabidopsis subtraction library and was shown to hydrolyze ABA-GE [Lee et al., 2006]. Moreover, Arabidopsis plants mutant in this gene were impaired in the ability to close stomata in response to drought. It was found that the AtBG1 was aggregated upon water deficiency, which resulted in a 4-fold increase in activity, suggesting a means by which active ABA levels could be adjusted according environmental conditions. Although this is a fairly well defined mechanism of β-glucosidase action in response to stress, the mechanisms of other β-glucosidases are not so clear. A β-glucosidase has been identified from an Arabidopsis mutant *sensitive to freezing 2 (sfr-2)*, in which the plants could not recover from cold stress [Thorlby et al., 2004]. The enzyme is found in the chloroplast and seems to be distantly related to other plant GH1 β-glucosidases, but its natural substrates have yet to be identified. Other β-glucosidases have been found to be upregulated in responses to stresses like salt [Kawasaki et al., 2001], phosphate starvation [Malboobi & Lefebvre, 1997], and whitefly infestation [van de Ven et al., 2000].

2.1.1.5. Hydroxyisourate hydrolase

Hydroxyisourate hydrolase is an enzyme thought to be involved in ureide metabolism in legumes, which was first identified in soy bean (*Glycine max*) root nodules [Sarma et al., 1999]. The enzyme hydrolyzes the reaction of 5-hydroxyurate (HIU), generated from urate by urate oxidase, to form 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline, which then undergoes nonenzymatic decarboxylation and tautomerization to form alantoin, as shown in scheme 1 [Raychaudhuri and Tipton, 2002]. Although HIU is unstable and is hydrolyzed the pathway can occur nonenzymatically, the half life of HIU is 30 min and nonenzymatic hydrolysis would result in a racemic mixture of alantoin, while the alantoinase

requires S-alantoin, so enzymatic hydrolysis is required for the ureide pathway to proceed at a reasonable rate [Sarma et al., 1999].



When the soy HIUH was cloned, it was found that it is clearly related to GH1 plant β-glucosidases, with the conserved position of the catalytic acid/base in a sequence of NEP and the catalytic nucleophile in the sequence HENG, which is similar to the typical T/SENG motif seen in GH1 β-glucosidases [Raychaudhuri and Tipton, 2002]. However, the sugar-binding residues that are conserved in GH1 β-glucosidases are not conserved in HIUH. Mutation of the residues corresponding to the catalytic acid/base and catalytic nucleophile, resulted in a large loss in activity. Further analysis showed that addition of small nucleophiles could rescue the acid/base mutant and supported a hydrolysis mechanism in which these residues act as the catalytic acid/base and nucleophile, similar to hydrolysis of glycosides [Raychaudhuri and Tipton, 2003].

Although ureide metabolism is most critical for transporting nitrogen that is fixed in root nodules to other parts of the plant in legumes, other plants contain a group of related enzymes that has been designated GH1 Cluster 6 in rice (Figure 1) [Opassiri et al., 2006]. The rice enzymes in this cluster generally contain the conserved glucose-binding residues of the catalytic glycon-binding site, so they could well act as β-glucosidases, rather than HIUH enzymes. Of these enzymes, only the subcluster containing Os9BGlu31, Os9BGlu32 and Os9BGlu33 shares the HENG sequence around the nucleophile with HIUH.

2.1.1.6. Transglycosidases.

Glycosidic bonds are generally created by nucleotide-phosphate or lipid-phosphate glycoside-dependent glycosyltransferases, but they may also be produced by transfer of a sugar from an existing non-phospho glucoside or glucosyl ester by a transglycosidase

[Lairson and Withers, 2004]. Transglycosidases are generally related to GH, and many GH can also catalyze transglycosylation reactions in the presence of high concentrations of glycoside substrates and acceptors.

Recently, two different types of transglycosidases have been identified as GH1 enzymes. The galactolipid:galactolipid galactosyl transferase (GGGT) in chloroplasts was identified as the Sensitive to Freezing 2 (SFR2) protein, which is critical to Arabidopsis survival in freezing conditions [Moellering et al., 2010]. SFR2 acts to transfer galactosyl residues from monogalactosyl diacyl glycerol (MGDG) to another MGDG or longer galactolipids, such as digalactosyl diacylglyceride (DGDG) to make DGDG or longer oligo-galactolipids and release diacylglycerol that can be converted to triacylglycerides and removed from the membrane. In another case, the anthocyanin monoglycosides in carnation and delphinium flowers were found to be converted to diglycosides by acyl glucose-dependent transglycosidases instead of the expected glycoyltransferases [Matsuba et al., 2010]. Purification and cloning of the cDNA for these enzymes showed them to be GH1 Cluster 6 enzymes, which like HIUH contain the IHENG sequence around the catalytic nucleophile. Since certain plant GH1 β-glucosidases, such as rice BGlu1 (Os3BGlu7) have been shown to have high transglycosidase activity [Opassiri et al., 2004], it is perhaps not surprising that some GH1 enzymes act primarily as transglycosidases.

2.1.1.7. Rice β-glucosidases

Although β-glucosidase activity has been reported in rice for over 30 years [Palmiano and Juliano, 1973], progress into assessing what the enzymes might be doing and identifying them has come more recently. Schliemann [1984] reported characterization of β-glucosidases in dwarf rice seedlings that could hydrolyze synthetic glucoconjugates, but he did not completely purify it at characterize it at the protein level. Akiyama and colleagues [1998] purified a cell-wall-bound β-glucosidase from rice seedlings and showed that it was active against cell-wall-derived oligosaccharides, as well as determining the amino-terminal amino acid sequence. Later, two β-glucosidase cDNA were cloned from rice seedlings and used to express recombinant proteins in *E. coli* [Opassiri et al., 2003]. The protein designated BGlu1 was found to be highly expressed in flower and shoot and also hydrolyzed β-(1,3)- and β-(1,4)-linked gluco-oligosaccharides. Further analysis showed that BGlu1 had 6 subsites for binding β-(1,4)-linked gluco-oligosaccharides and had relatively high transglycosylation activity [Opassiri et al., 2004]. Subsequently, the rice genome sequences were analyzed and 40 glycoside hydrolase (GH) family 1 (GH1) genes were identified, although they included

two genes likely to be derived from endophytes, two pseudogenes and two gene fragments [Opassiri et al., 2006]. Expressed sequence tag (EST) analysis showed that most of the remaining 34 genes are expressed in rice and that the gene for BGlu1 (*Os3bglu7*) was most abundantly expressed. Subsequently it was found that other isoenzymes in the same phylogenetic cluster as Os3BGlu7, Os3BGlu8 and Os7BGlu26, also hydrolyzed oligosaccharides, but Os7BGlu26 preferred mannosides to glucosides, while Os3BGlu8 was more similar Os3BGlu7 [Kuntothom et al., 2009]. A cDNA for the gene that was most similar to the sequence of the cell-wall-bound β-glucosidase described by Akiyama et al. [1998], *Os4bglu12*, was cloned and used to express a recombinant Os4BGlu12 protein, which was found to hydrolyze oligosaccharides, similar to Os3BGlu7. However, further it was found to hydrolyze 4-nitrophenyl glycosides more efficiently and also hydrolyze steroid and flavonoid beta-glucosides with similar efficiency [Opassiri et al., 2010]. Another isoenzyme, Os3BGlu6, has also been expressed and found to hydrolyze octyl β-D-glucoside and (1,3)- and (1,2)-β-linked disaccharides, but not cellooligosaccharides [Seshadri et al., 2009]. Currently, the structures of Os3BGlu6, Os3BGlu7 and Os4BGlu12 have been determined and it can be seen that small differences in their active sites can account for their differences substrate specificities.

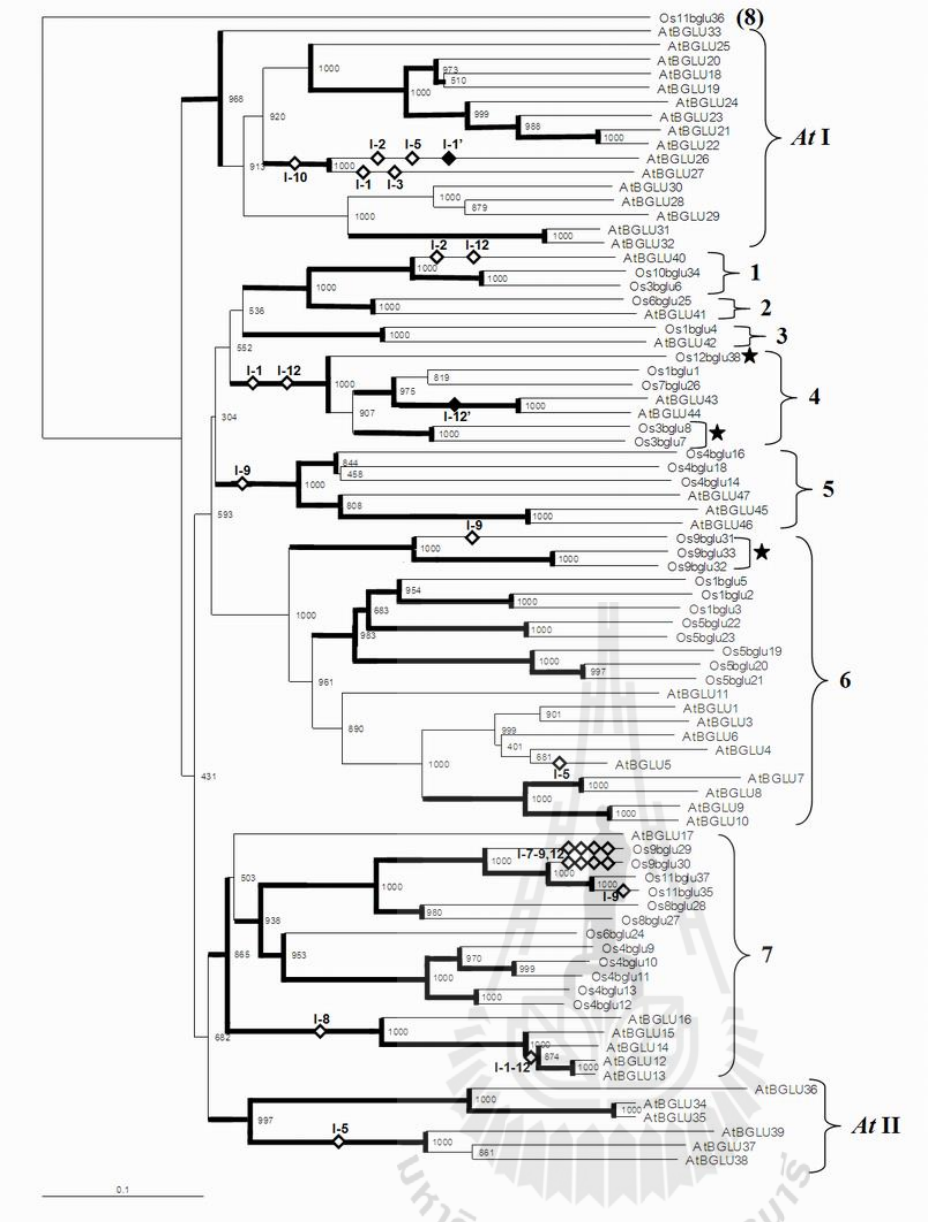


Figure 1: Phylogenetic tree of the derived protein sequences from Glycosyl Hydrolase family 1 (GH1) genes found in the rice (*Oryza sativa* L.) and Arabidopsis (*Arabidopsis thaliana*) genomes. The bootstrap values are shown at the heads of each cluster. The tree was rooted by the Os11BGlu36 (rice SFR2) sequence, which is as similar to bacterial GH1 proteins as to plants. The Eight clusters in which both rice and Arabidopsis protein sequences are clearly clustered with a closer relationship to each other than to the sequences from the same plant outside the cluster are numbered 1-8. The two clusters found only in Arabidopsis are At I, which includes the ABA-GE β-glucosidase BGL1 or AtBGlu23, along with the symbiosis-related ER body β-glucosidase PYK10 and fungal defense myrosinase PEN2, along with a phosphate starvation-induced β-glucosidase, and At II, which includes classical myrosinases. Enzymes previously characterized in our group include Os3BGlu6 (Cluster 1), Os3BGlu7 (Cluster 4), Os3BGlu8 (Cluster 4), Os4BGlu12 (Cluster 7), Os6BGlu26 (Cluster 4) and Os9BGlu31 (Cluster 6), which has only been partially characterized. The figure is from Figure 1 of Opassiri et al. [2006].

Although *Arabidopsis thaliana* has been promoted as the plant molecular model, the Arabidopsis β-glucosidases that have been shown to hydrolyze ABA-glucosyl ester (ABA-GE BG or BG1) and to be involved in plant microbe interactions (PYK10 and PEN2) belong to a

Brassicaceae-specific phylogenetic cluster (Figure 1) [Opassiri et al., 2006], so the enzymes that might play similar functions in rice are yet to be discovered. Seto et al. [2009] also showed that rice makes β-glucoside of tuberonic acid (a jasmonic acid derivative), which can be hydrolyzed by a β-glucosidase. Recently, the isoenzyme most closelty related to Os4BGlu12 (Os4BGlu13) was found to hydrolyze tuberonic acid beta-glucoside (TAG), and was designated TAG beta-glucosidase I (TAGG1) [Wakuta et al., 2010]. Later, Os4BGlu12 was purified as a second TAGG isoenzyme and designated TAGG2 [Wakuta et al., 2011]. This, along with the gibberellin β-glucosidase work of Schlieman [1984] and the report of ABA-GE β-glucosidase in barley [Dietz et al, 2000] suggests that several rice β-glucosidases that hydrolyze phytohormones may exist. Similarly, it is not know if any enzymes in rice catalyze conversion of HIU to alantoin, as does HIUH in soy beans. Nor have any enzymes in rice been characterized that act in secondary metabolism or that are directly involved in defense. So investigation of the substrate specificity of rice GH1 enzymes that have not yet been characterized is critical to understanding these many functions in rice.

2.1.2. Research Objectives (วัตถุประสงค์ของการวิจัย)

The objectives of this research project were:

2.1.2.1. To express Os9BGlu31 in *E. coli* and purify the protein.

2.1.2.2. To test the activity of Os9BGlu31 protein against 4-nitrophenyl (4NP) glycosides, including 4NP-β-D-glucopyranoside (4NPGlc), 4NP-β-D-galactopyranoside, 4NP-β-D-mannopyranoside, 4NP-β-D-xylopyranoside, etc.

2.1.2.3. To test Os9BGlu31 against a range of natural glycosides.

2.1.2.4. To determine the kinetics of hydrolysis of glycosides which are hydrolyzed at a reasonable rate by Os9BGlu31.

However, these objectives had to be adjusted somewhat when it was discovered that Os9BGlu31 is indeed a transglucosidase, to accomodate the characterization of transglycosylation rather than hydrolysis.

2.1.3. Scope of Research (ขอบเขตของการวิจัย)

At the beginning of this project, Os9BGlu31 had been cloned into a pET32a-derived expression vector and preliminary expression and identification of apparent β-glucosidase activity achieved, so this project went to further characterize the enzyme, as well as trying to optimize the expression. A number of bacterial strains were tested for expression and an

alternative expression vector with a tobacco etch virus protease cleavage site was tested for expression. Moreover, the expressed enzyme was purified and characterized for transfer of glucose and other monosaccharides from 4NP glycosides and various natural substrates. The pH optimum was determined for various acceptors with 4NPGlc donor. Glucose acceptor and glucose donor substrates were identified by thin layer chromatography (TLC). The kinetics for transfer from various natural glucose esters and transfer to various acceptors was tested, along with the kinetics of use of 4NPGlc. The relative activities of various donor substrates for transfer of glucose to active phenolic acid acceptors were also determined. This information provides the basis for understanding catalytic activity of Os9BGlu31 and its potential role in the plant.

2.1.4. Short Description of Methods (ข้อตกลงเบื้องต้น)

In this work, the Os9BGlu31 enzyme was expressed as an N-terminal thioredoxin fusion protein with a His-tag in between the thioredoxin and Os9BGlu31 in Origami B(DE3) *Escherichia coli*. The Os9BGlu31 fusion protein was purified from the expression cell pellet by immobilized metal affinity chromatography, anion exchange chromatography and hydrophobic interaction chromatography and characterized for its enzymatic properties.

Initial characterization of the catalytic properties focused on testing the enzyme for hydrolysis of available glucosides, but it was found that little glucose release could be detected from various substrates. The pH-activity profile was determined with 4-nitrophenyl β-D-glucoside (4NPGlc) as substrate in over-lapping buffers, in which acetate yielded much higher activity. Therefore, thin layer chromatography (TLC) of the reactions was used to identify products. Transglycosylation of glucose from 4NPGlc onto various alcohols and carboxylic acids was determined by TLC and release of 4-nitrophenol (4NP) from 4NPGlc. Relative activity toward different 4NP glycosides was determined by transglycosylation onto 4-naphthalacetic acid. A set of phenolic acid esters was obtainted from Prof. Ozeki and colleagues at the Tokyo Univerity of Agriculture and Technology and tested as donor substrates along with glucosides and glucosyl esters found in the laboratory or commercially available. After initial determination by TLC, the reaction rates were determined by either measuring the absorbance of 4NP released with 4NPGlc as a donor, or the production of glucosyl ester products by C18 reverse phase high performance liquid chromatography (HPLC). Time courses with different acceptor concentrations were used to find initial rates and kinetic parameters determined for various acceptors with 4NPGlc as a donor.

2.1.5. Benefits and output from this research project (ประโยชน์ที่ได้รับจากการวิจัย)

2.1.5.1. Knowledge. This work resulted in the identification of Os9BGlu31 as a relatively broad specificity transglycosidase and the characterization of the enzyme. This suggests a new mechanism by which the amounts of bioactive compounds and their glycoconjugates can be regulated in plants via equilibration by Os9BGlu31 and similar transglycosidases. This new knowledge will provide a basis for better understanding of the way that phytohormones and other bioactive compounds can be regulated and have cross-talk in their regulation. Further understanding of this process may allow better crop development in the future.

2.1.5.2. Human Resources Development. One postdoctoral fellow, one student and one research assistant have trained on this project and learned more skills.

2.1.5.3. Research Publication. This work was presented at one invited talk at the symposium of the Japanese Society for Applied Glycosciences and as an oral presentation at the 50th Meeting of the Phytochemistry Society of North America. A manuscript has since been published in the Journal of Biological Chemistry [Luang et al., 2013], which is a prestigious journal in the field of biochemistry with an impact factor of approx. 4.7. Much of the work for this paper was done on this grant, although a small amount had to be completed on a subsequent grant.

2.2. Materials and Methods

2.2.1. Materials

Glucose esters of phenylpropanoids were generously provided by Prof. Yoshihiro Ozeki and Dr. Nobuhiro Sasaki of the Tokyo University of Agriculture and Technology. Prof. Jisnuson Svasti and Dr. Kriengkrai Lirdprapamonkol of the Chulabhorn Research Institute provided vanillin and its derivatives. Other chemicals used for potential substrates were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA) and other chemicals and supplies were purchased from a variety of suppliers.

2.2.2. Experimental methods

2.2.2.1. Protein expression plasmid production

The plasmid construct containing a full-length cDNA encoding the Os9BGlu31 protein was acquired from the Rice Genome Resource Center, Tsukuba, Japan (http://www.rgrc.dna.affrc.go.jp/, Genbank accession AK121679). The cDNA fragment encoding mature *Os9BGlu31* was amplified with the BGlu31F (CAC CAT GGC GGC GGG GAT CAC CAG) and BGlu31R (CTC GAG AAC CTT GAT CAC TGG GAG TAG GCT C) primers and *Pfu* DNA polymerase. The PCR product was cloned into the pENTR™/D-TOPO Gateway® system entry vector (Invitrogen) and subcloned into the pET32a/DEST expression vector (Opassiri et al., 2006) by LR Clonase (Invitrogen) reaction to make the plasmid pET32a/DEST/Os9BGlu31. The recombinant plasmids were transformed into *E. coli* strain DH5α and selected on Lenox LB broth containing 50 μg/ml ampicillin. The plasmids were extracted, checked by restriction digest and recombinant plasmids that gave correct patterns were sent for automated sequencing (Macrogen Corp., Seoul, Korea).

2.2.2.2. Protein expression and purification

The thioredoxin-Os9BGlu31 fusion protein was expressed from the recombinant pET32a/DEST/Os9BGlu31 plasmid in *E. coli* strain Origami B(DE3) by induction with 0.4 mM IPTG at 20°C overnight, followed by collection of the cells as previously described [Opassiri et al., 2003]. The cells were incubated with lysis buffer (20 mM Tris-HCl, pH 8.0, 0.2 mg/mL lysozyme, 1% Triton-X 100, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.40 mg/mL DNase I and 0.1 mg/mL soybean trypsin inhibitor) at room temperature for 30 min. Soluble protein was separated from the broken cell pellet by centrifugation at 12,000 x g at

4°C for 15 min. Three steps were used to purify the Os9BGlu31 fusion protein. First, crude protein was mixed with $CoCl_2$-equilibrated immobilized metal affinity chromatography (IMAC) resin (GE Healthcare) with equilibration buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0) at 4°C for 30 min. The resin suspended in the crude protein solution was loaded into a column and washed sequentially with 10 column volumes (CV) of 5 mM imidazole in equilibration buffer, 5 CV each of 10 mM imidazole and 20 mM imidazole in equilibration buffer. The Os9BGlu31 fusion protein was eluted with elution buffer (250 mM imidazole in equilibration buffer). The fractions with activity were pooled and imidazole was removed by dialysis with 50 mM Tris-HCl, pH 8.0, at 4°C. Then, the recombinant protein was loaded onto a Q-Sepharose column pre-equilibrated with 50 mM Tris-HCl, pH 8.0, at a flow rate of 1 mL/min and unbound protein was washed from the column with 10 CV of 50 mM Tris-HCl, pH 8.0. Os9BGlu31 was eluted with a linear gradient of 0-0.5 M NaCl in 50 mM Tris-HCl, pH 8.0. The fractions containing activity were pooled and the NaCl concentration adjusted to 2 M. The protein was loaded onto a Phenyl-Sepharose column equilibrated with 2 M NaCl in 50 mM Tris-HCl, pH 8.0. Unbound protein was washed from the column with 10 CV of 2 M NaCl in 50 mM Tris-HCl, pH 8.0. Os9BGlu31 fusion protein was eluted with a linear gradient of 2 to 0 M NaCl in 50 mM Tris-HCl, pH 8.0, followed by 0-50% ethylene glycol in 50 mM Tris-HCl, pH 8.0. Finally, the buffer of the Os9BGlu31-containing fraction pool was changed to 150 mM NaCl in 20 mM Tris-HCl, pH 8.0, by dialysis.

2.2.2.3. pH optimum determination

To determine the pH dependence, the activity of Os9BGlu31 was measured with 5 mM 4NPGlc as substrate in 0.1 M citric acid-0.2 M disodium hydrogen phosphate (McIlvaine buffer) over the pH range of 3.0-10.0 alone or with addition of 5 mM of azide, acetate, formate, fluoride or ascorbate. The reaction was incubated at 30°C for 1 h and stopped by addition of 2 M sodium carbonate. The 4NP released was quantified from the absorbance at 405 nm of its 4-nitrophenolate ion.

2.2.2.4. Enzymatic characterization of Os9BGlu31

The preference of Os9BGlu31 for glucose donors (Table 1) was evaluated by incubating 0.5 mM of glucose donor with 0.2 mM 4HB as glucose acceptor with 1 μg of Os9BGlu31 in 50 mM citrate, pH 4.5, at 30°C. For 4HBG donor, 0.2 mM ferulic acid was used as the acceptor instead of 4HB. After 10 min, the reactions were stopped by adding phosphoric acid to 1% final concentration. Transglycosylation products were detected by

HPLC on an Agilent 1100 system with an XDB-C18 HPLC column (Agilent). The substrate and products were separated at 1 mL/min with a linear gradient from 5% to 12% acetonitrile in 1.5% phosphoric acid for 20 min, followed by 12% to 80% acetonitrile in 1.5% phosphoric acid in 5 min and detected by absorbance at 254 nm on a diode array detector (DAD).

To establish the acceptor specificity of Os9BGlu31, activities with various glucose acceptors (Table 2) were assayed with 0.2 mM glucose acceptor, 5 mM 4NPGlc as glucose donor, and 2 μg of Os9BGlu31 in 50 mM citrate, pH 4.5. The reactions were incubated at 30°C for 1 h and then stopped by adding phosphoric acid to 1%, as described above. Some of the acceptors have absorbance spectra overlapping that of 4NP, so the released 4NP was separated from other reaction components by HPLC with an XDB-C18 column and elution with a linear gradient from 20% to 55% acetonitrile in 1.5% phosphoric acid over 20 min (1 mL/min), then from 55% to 80% acetonitrile in 5 min. Released 4NP was monitored with a DAD and quantified by its absorbance (peak area) at 360 nm. For the assay of 0.2 mM 4NP as an acceptor, 0.5 mM FG was used as the donor instead of 5 mM 4NPGlc and the relative activity of 4NP was compared to 0.2 mM 4HB as acceptor with 0.5 mM FG as donor. The 4NPGlc formed in this reaction was detected by HPLC with the same solvent system as the glucose donor preference determination.

Relative activity toward 4NP-glycoside substrates was determined by incubating a 50 μL reaction containing 10 μg of Os9BGlu31 with 0.5 mM of 4NP-glycoside and 0.2 mM of 4HB in 50 mM citrate, pH 4.5, at 30°C for 1 h. The reaction was stopped by adding 100 μL of 2 M Na_2CO_3 and the 4NP released quantified by the absorbance at 405 nm.

2.2.2.5. Kinetic parameter determination

The K_m and V_{max} values of 4NPGlc in the presence of various acceptors were determined by varying the concentration of 4NPGlc in the range 2-30 mM with 0.2 mM glucose acceptor and 1.2-2.4 μg of Os9BGlu31 in 50 mM citrate, pH 4.5. The K_m and V_{max} values of the glucose acceptors were determined by varying their concentrations between 0.02-0.5 mM in the presence of 30 mM 4NPGlc with 1.2-2.4 μg of Os9BGlu31 in 50 mM citrate, pH 4.5. The release of 4NP product was quantified as described above. The kinetic parameters were determined by nonlinear regression of the Michaelis-Menten plots with the Grafit 5.0 computer program (Erithacus Software, Horley, UK).

2.2.2.6. Effect of metals and inhibitors

The effects of various metal ions and inhibitors on enzyme activity were determined by preincubating the enzyme with an individual chemical in 50 mM sodium acetate, pH 4.5, at 30°C for 10 min for EDTA and metal ions or 2 h for organic inhibitors. Activity was then assayed by incubating 2.5 µg of pretreated enzyme with 5 mM 4NPGlc substrate at 30°C for 15 min. The reactions were stopped with 100 µL of 2 M Na_2CO_3 and released 4NP measured as described above.

2.2.2.7. Northern blot analysis

Rice (*Oryza sativa* L. cv. Yukihikari) seeds were sterilized, soaked in water overnight, and germinated in the dark for 4 days at 27°C. The seedlings were then grown in a 12 h light-12 h dark cycle from day 4 to day 10 at 28°C on adsorbant paper moistened with sterile distilled water. Some 10-day-old seedlings were harvested and dissected into separate parts (shoot, root, and endosperm). Other seedlings were transferred to soil and grown for an additional 4-5 weeks to reach the flowering stage. Rice plants were harvested and separated to different parts (flower, stem, root, node, leaf blade, and leaf sheath).

To test for the effects of stress, ten-day-old rice seedlings were exposed to various abiotic stresses and plant hormones for an additional 2 days. The abiotic conditions were: 1) cold temperatures (5°C and 12°C); 2) drought (no water added); 3) salt stress, (0.3 M NaCl solution); 4) flooding, by submerging the seedlings in distilled water; 5) 0.1 mM ABA; 6) ethylene, (10 mM ethephon), 7) 0.1 mM each of methyl jasmonic acid, GA, and kinetin and 8) 10 mM 2,4-D. To determine the effect of ethephon in rice tissues, 10-day-old seedlings were treated with ethephon for 2 days and rice seedlings were dissected into separate parts (shoot, root, and endosperm). All plant samples were kept at -70°C for RNA isolation.
A gene-specific probe for *Os9BGlu31* was amplified from a rice genomic DNA as the template with the BGlu31-3'UTRf (5'-AGGTTCTTCTTCCTCGCAC-3') and BGlu31-3'UTRr (5'-TTCAAACTACGAGAGACTTC-3') primers derived from the 3'-untranslated region of the gene. The amplification was performed with 30 cycles of 94°C 45 s, 52°C 45 s, and 72°C 1 min, using *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA).

Total RNA was isolated from rice tissues by the method of Bachem et al. (1996). Thirty micrograms of total RNA was denatured and electrophoresed on 1.5% formaldehyde–agarose gels and transferred onto Hybond N+ nylon membrane (GE Healthcare, Buckinghamshire, UK) by standard procedures [Sambrook et al. 1989]. The *Os9BGlu31* probe was labeled by Rediprime II random priming with α-[^{32}P]dCTP (GE Healthcare) and

was hybridized with RNA blots for 16 h at 42°C. The blots were then washed once in 0.1% SDS, 2 x SSC for 30 min at 65°C and washed twice in 0.1% SDS, 0.1 x SSC for 15 min at 65°C, then exposed to a Fuji film imaging plate for 16 h at room temperature. The positions of radioactive bands were visualized with a Fuji Film BAS 1000 BioImaging Analyzer (Fuji Photo Film Co., Ltd, Tokyo, Japan).

2.2.2.8 Quantitative real-time reverse transcription-PCR analysis of gene expression

For quantitative real-time PCR, all samples were collected from greenhouse-grown *japonica* rice cultivar Dongjin, as described previously (25-27). Flag leaves were harvested at four different developmental stages, 15 days before flowering (DBF), 15 days after flowering (DAF), 40 DAF, and 50 DAF, respectively. Total RNA was isolated from harvested samples with Trizol reagent (Invitrogen) and reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). The expression level of the rice ubiquitin 5 (*OsUBQ5*) gene was used to normalize the cDNA quantity (28). Gene-specific primers used for quantitative real-time PCR were the BGlu31RT-f and BGlu31RT-r primers for *Os9BGlu31* and OsUBQ5RT-f and OsUBQ5RT-r primers for *OsUBQ5* (Supplemental Table S2). All experiments were conducted in triplicate with the SYBR Premix Ex *Taq* (Takara) and an ABI PRISM 7500 sequence detector (Applied Biosystems) according to the manufacturer's instructions and changes in gene expression were calculated by the comparative cycle threshold (ΔΔCt) method with Sequence Detector Systems version 1.2 software (Applied Biosystems).

2.3. Results

2.3.1. Os9BGlu31 is primarily a transglucosidase

Initially, Os9BGlu31 fusion protein expressed in *E. coli* was found to have weak activity toward 4NPGlc in 50 mM sodium acetate buffer. The protein was purified by IMAC, followed by anion exchange and hydrophobic interaction chromatography to give a protein that was approximately 90% pure (Figure 2).

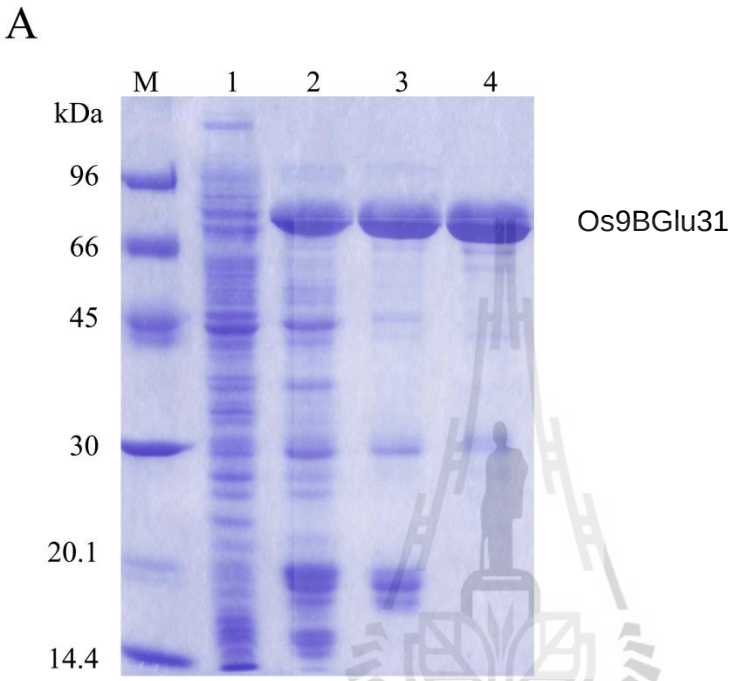


Figure 2. SDS-PAGE analysis of purification of Os9BGlu31 fusion protein expressed in *E. coli*. Three-step purification for kinetics: soluble protein extract of induced Origami B (DE3) cells (lane 1), and Os9BGlu31 purified via immobilized metal (Co^{2+}) affinity chromatography (IMAC; lane 2), Q-Sepharose chromatography (lane 3) and Phenyl-Sepharose chromatography (lane 4). Bio-Rad low molecular weight markers are shown for comparison (lane M).

Attempts to find natural substrates resulted in no detection of glucose by glucose-oxidase assays, except in the case of some unstable substrates, such as dhurrin, that broke down on their own. To determine at least a few properties of the enzyme, we set-up a pH profile with overlapping buffer solutions and found that the acetate buffer had much higher activity than other buffers (Figure 3 A). When the products of reactions in various buffers were run on thin layer chromatography (Figure 3 B), a transglycosylation product with a mobility slightly lower than 4NPGlc was seen in the acetate buffer. Given the retaining mechanism of GH1 enzymes, this was presumed to be acetyl β-D-glucopyranose. Due to the instability of this product, it could not readily be purified and its structure verified. However, a series of alkyl acids (formic, acetic, propionic and buteric acids) gave similar results, with

apparent transglycosylation products detected for each of them except for formic acid, the glucosyl ester of which may have been too unstable to detect.

When the pH optimum curve was repeated in the presence of azide, the different buffers gave much more compatible results, as seen in Figure 4 A. Inspection of the products of these reactions on TLC showed that the reactions in which the 4NP release was detected contained a product that apparently corresponded to β-D-glucosyl azide, in addition to the β-D-glucosyl acetate seen in the reaction with acetate buffer (Figure 4 B).

When the pH optimum was determined in citrate/phosphate buffer, in which no transglycosylation product was detected, the activity of Os9BGlu31 in the absence of an additional nucleophile was highest at pH 4.5, the activity decreased to approx. 70% at pH 3.5 and pH 5.0, and there was no activity at pH 6.5 or higher (Figure 4). The release of 4NP increased approx. 2.9-, 3.3- and 4.2-fold in the presence of 5 mM azide, formate and acetate, respectively, which gave pH optima in the range of 4 to 4.5 (Figure 5), while fluoride and ascorbate had no effect on enzyme activity (data not shown).

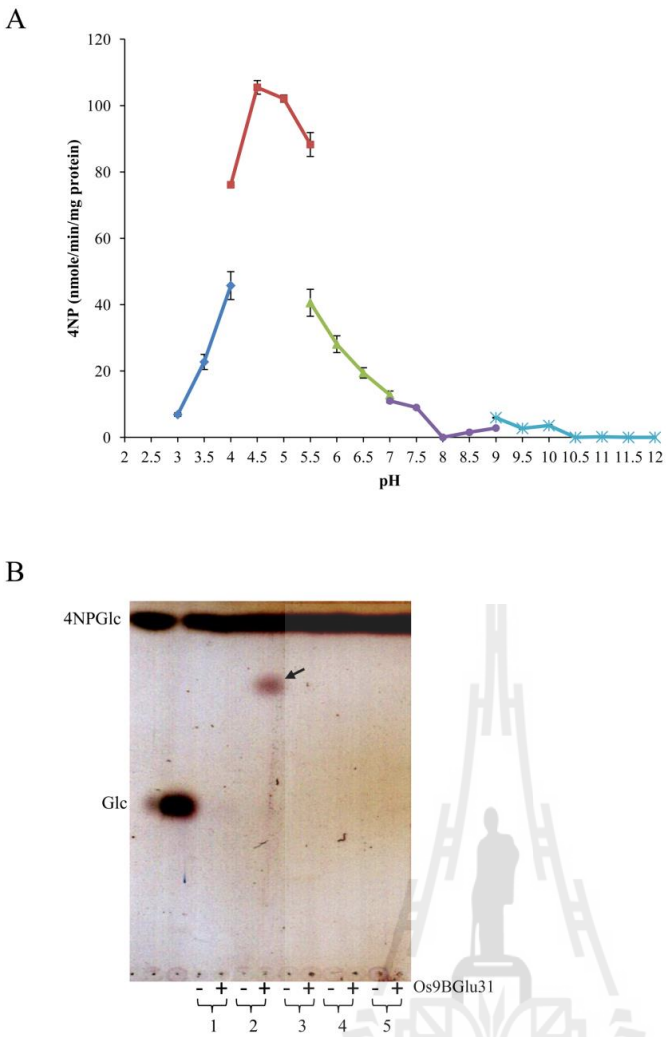


Figure 3. Identification of Os9BGlu31 as a transglucosidase. The pH profile of Os9BGlu31 release of 4NP from 4NPGlc in a series of overlapping 50 mM pH buffers: citrate pH 3.0-4.0 (♦), acetate pH 4.0-5.5 (■), MES pH 5.5-7.0 (▲), Tris-HCl pH 7.0-9.0 (●), and sodium phosphate pH 9.0-12.0 (*) is shown in (A). (B) shows thin layer chromatography analysis of the products of reactions of 5 mM 4NPGlc in 50 mM citrate, pH 4.0 (lane 1), acetate, pH 4.5 (lane 2), MES, pH 5.5 (lane 3), Tris-HCl, pH 7.0 (lane 4), and sodium phosphate, pH 9.0 (lane 5) with Os9BGlu31 (+) or without Os9BGlu31 (-). The substrates and products of the reactions were separated on silica-gel TLC with a solvent system of ethyl acetate:methanol:water (7:2.5:1 v/v/v). The developed plates were stained with 10% H_2SO_4 in ethanol and charred at 110°C.

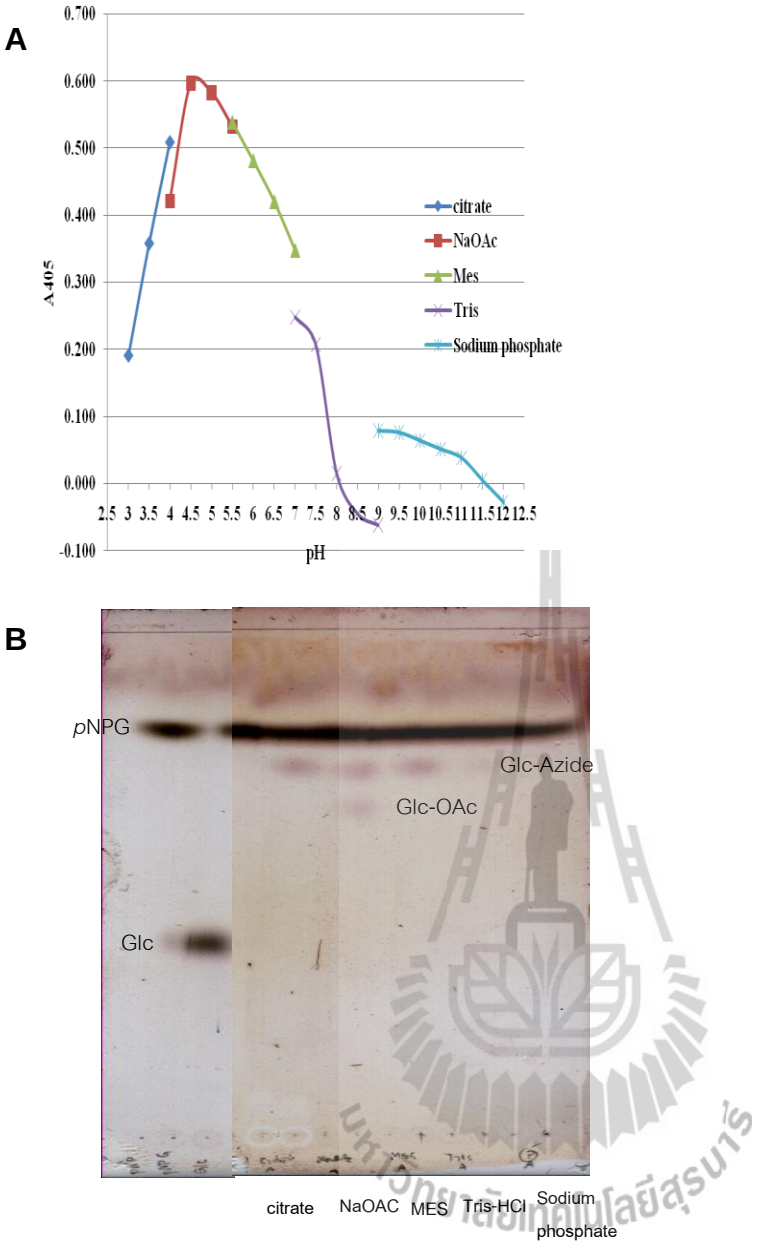


Figure 4. pH Optimum with different buffers in the presence of sodium azide. A. pH optimum curve in the presence of 50 mM sodium azide and an overlapping set of buffers, as described in Figure 3. B. TLC of the products of the reactions in different buffers, separated as described in Figure 3.

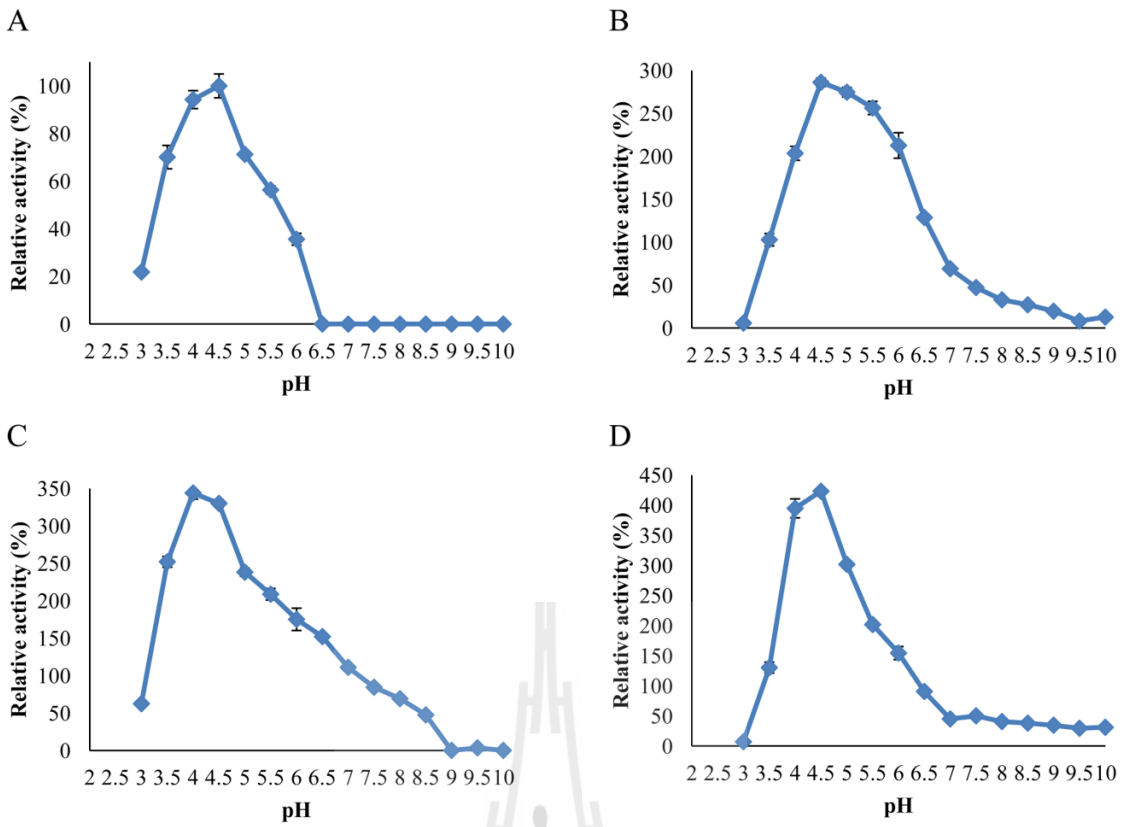


Figure 5. Profile of Os9BGlu31 activity over the pH range of 3.0-10.0. The hydrolysis of 5 mM 4NPGlc was monitored in citrate/phosphate buffer alone (A), or in the presence of 5 mM azide (B), 5 mM formate (C) or 5 mM acetate (D) by wildtype Os9BGlu31.

2.3.2. Characterization of Os9BGlu31 transglucosidase activity

2.3.2.1. Glycosyl donor substrate specficity

Based on the TLC analysis of the reaction with acetate and other potential nucleophilic acceptors described in Section 2.3.1, Os9BGlu31 acts to transfer the glucosyl moiety from a donor substrate to an acceptor. The glycon (sugar) specificity of Os9BGlu31 was determined by transfer from 4NP-glycosides to 4-hydroxybenzoic acid (4HB). Os9BGlu31 transferred glucose from 4NPGlc better than β-D-fucose and β-D-xylose from their respective 4NP-glycosides (Table 1), and could not use 4NP-β-D-galactoside, 4NP-β-D-mannoside, 4NP-β-D-N-acetylglucosaminide, 4NP-β-D-glucuronide, 4NP-α-D-glucoside, 4NP-α-D-galactoside or 4NP-α-L-arabinoside as substrates.

In addition to 4NP-glycosides, Os9BGlu31 used phenolic glucose esters as donor substrates with highest activity to 1-*O*-β-D-feruloyl-glucose (FG), 1-*O*-β-D-4-coumaroyl-glucose (4CG), 1-*O*-β-D-4-hydroxybenzoyl-glucose (4HBG), 1-*O*-β-D-sinapoyl-glucose (SG),

and 1-*O*-β-D-vanillyl-glucose (VG) (Table 1 and Figure 6). Os9BGlu31 also used the flavonoid glucosides, phloridizin and apigenin 7-*O*-glucoside, and the gibberellin GA_4-β-D-glucose ester (GA_4-GE) as glucose donors, although with lower efficiency.

.

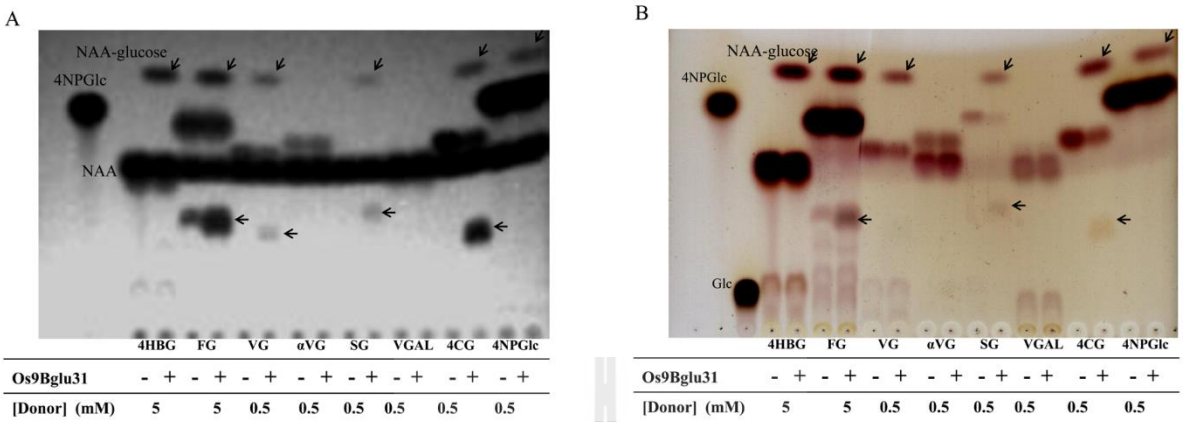


Figure 6. Thin layer chromatogram of Os9BGlu31 transfer of glucose from phenolic glucose esters to 1-naphthalene acetic acid (NAA) acceptor. 1-*O*-β-D-4-hydroxybenzoyl-glucose (4HBG), 1-*O*-β-D-feruloyl-glucose (FG), 1-*O*-β-D-vanillyl-glucose (VG), 1-*O*-α-D-vanillyl-glucose (αVG), 1-*O*-β-D-sinapoyl-glucose (SG), 1-*O*-β-D-vanillyl-galactose (VGAL), 1-*O*-β-D-4-coumaroyl-glucose (4CG), and 4NP-β-D-glucoside (4NPGlc) were used as donors with 5 mM NAA as acceptor in the presence of 1 µg enzyme in 50 mM citrate, pH 4.5, at 30°C for 1 h. The transglycosylation product 1-O-(naphthalene acetic acid) β-D-glucose ester (NAA-glucose), glucose (Glc), and substrates were separated on silica gel F_{254} TLC with chloroform:methanol:ammonia (7:2.8:0.2). The products were observed under UV light (A) and stained with 10% H_2SO_4 in ethanol, followed by charring (B).

Table 1. Relative activities of Os9BGlu31 on various donors.

Donor	Relative activity (%)
Phenol glucose esters and glucosides:	
1-*O*-β-D-Feruloyl-glucose	100.0
1-*O*-β-D-4-Coumaroyl-glucose	96.3 ± 1.0
1-*O*-β-D-Vanillyl-glucose	93.4 ± 1.9
1-*O*-α-D-Vanillyl-glucose	nd
1-*O*-β-D-4-Hydroxybenzoyl-glucose	82.0 ± 2.1
1-*O*-β-D-Sinapoyl-glucose	51.4 ± 0.5
Phloridizin	49.2 ± 1.7
Apigenin 7-glucoside	32.8 ± 0.9
Hormone glucose esters:	
Gibberellin A_4-glucose	12.5 ± 0.5
Flavonoid/isoflavone/flavon glucosides:	
Quercetin 3-β-D-glucoside	nd
Naringin	nd
Arbutin	nd
Gossypin	nd
Cytokinin glucoside:	
Trans-Zeatin glucoside	nd
Nucleoside diphosphate sugar:	
Uridine 5'-diphosphoglucose	nd
Coumarin glucoside:	
Esculin	nd
4NP-*glucosides and synthetic glucoside:*	
4NP-β-D-glucoside	3.8 ± 0.05
4NP-β-D-fucoside	2.3 ± 0.03
4NP-β-D-xyloside	1.8 ± 0.03
4NP-β-D-galactoside	nd
4NP-β-D-mannoside	nd
4NP-α-D-glucoside	nd
4NP-α-D-galactoside	nd
4NP-β-D-glucosiduronide	nd
4NP-α-L-arabinoside	nd
4NP-N-acetylglucosaminide	nd
2NP-β-D-glucoside	nd
4-methylumbelliferyl β-D-glucoside	nd
n-octyl glucoside	nd
n-heptyl glucoside	nd

Donor preferences of Os9BGlu31 using 4HB as acceptor or ferulic acid as acceptor for 4HBG as donor. Daidzin and genistin showed showed small mounts of donor activity by thin layer chromatography when reactions with 5 mM glucose donor were incubated overnight. There was no activity to monolignol glucosides (4-coumary alcohol glucoside and coniferin), and cyanogenic glucosides (linamarin, (D)-amygdalin, and dhurrin) as glucose donors. ‘nd’ indicates ‘not detected’.

2.3.2.2. Acceptor specificity of Os9BGlu31 transglucosidase

The TG activity of Os9BGlu31 determined with a range of potential acceptors is summarized in Table 2. When transglycosylation reactions of various acceptors with Os9BGlu31 and 4NPGlc donor were inspected by TLC, spots of 4NP and a single transglycosylation product were seen in each case (Figure 7), except esculetin had two transglycosylation products. One esculetin product migrated to a position similar to esculin (esculetin-6-O-β-D-glucoside), while the other product was located between esculetin and esculin and only the mass of 339.0 m/z of esculin and no diglucoside mass could be detected on LCMS, suggesting the other product was esculetin-7-O-β-D-glucoside. Glucose was also transferred to the carboxylic group of alkyl organic acids, with increasing relative activity from formic to butyric acid, and to hydroxyl groups of alcohols, especially aromatic alcohols like catechin and esculetin, and certain flavonoid alcohols. Glycoside products of methanol, ethanol, 1-butanol, 1,3-butanediol, 4-methyl-2-pentanol, 2-methyl-1-pentanol, 1-hexanol, 2-hexanol, and 1-octanol glucoside could also be detected on TLC, although the addition of these alcohols did not increase the release of 4NP from the donor more than the buffer alone (data not shown).

Quantitation of 4NP released from 4NPGlc in the presence of different acceptors by HPLC allowed the apparent kinetic parameters of transglucosylation to be determined, as shown in Table 3. It was noted that at high concentrations (>0.5 mM) most of the phenolic acids that acted as exceptional acceptors exhibited substrate inhibition of the enzyme. Os9BGlu31 had the highest relative activity and apparent k_{cat} (1.21 s^{-1}) for ferulic acid, but had the highest k_{cat}/K_m value for 4-coumaric acid (33.3 $mM^{-1}s^{-1}$), due to its low K_m, followed by ferulic acid (25.4 $mM^{-1}s^{-1}$) and sinapic acid (14.2 $mM^{-1}s^{-1}$), respectively (Table 3). Os9BGlu31 could glycosylate the auxins indole acetic acid and naphthalene acetic acid with 43% the relative activity of ferulic acid, and had significant activity toward abscisic acid (ABA) and GA_4, as well.

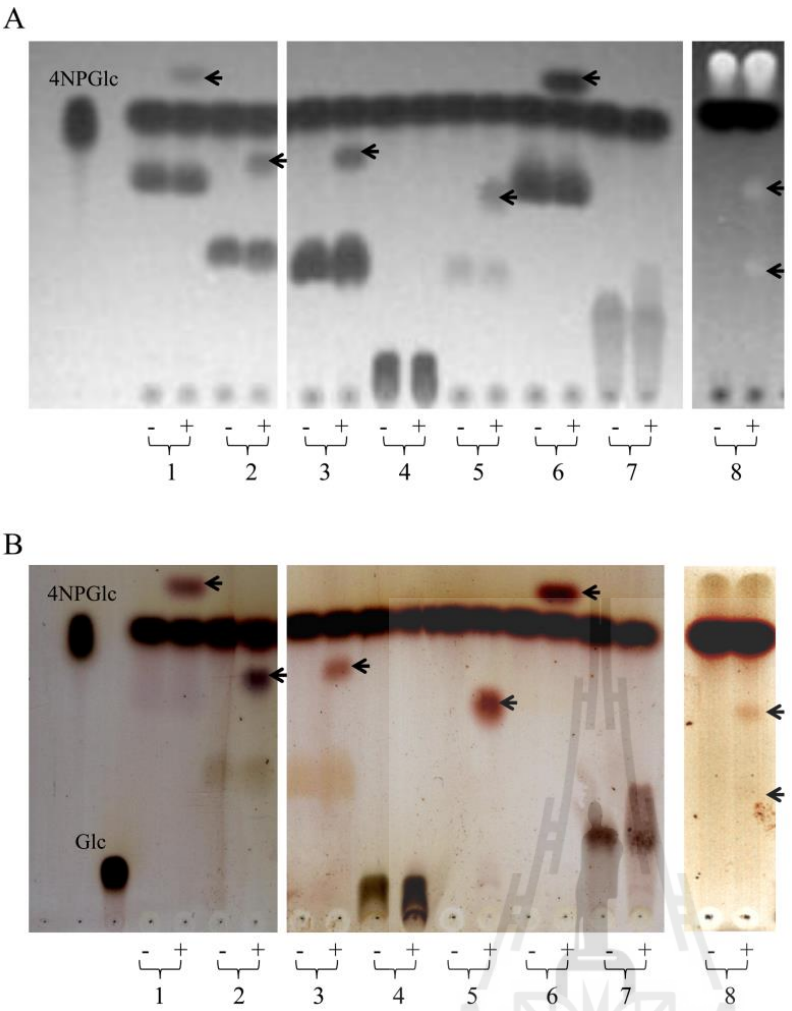


Figure 7. Thin layer chromatography analysis of Os9BGlu31 transfer of glucose from 4NPGlc to acceptors. Reactions containing 5 mM 4NPGlc, 5 mM acceptors, and 3 µg of enzyme in 50 mM citrate, pH 4.5, were incubated at 30°C for 1 h. The acceptors 1-naphthalene acetic acid (lane 1), syringic acid (lane 2), 4-coumaric acid (lane 3), gallic acid (lane 4), 4-hydroxybenzoic acid (lane 5), trans-cinnamic acid (lane 6), caffeic acid (lane 7), and esculetin (lane 8) were incubated in reactions with (+) and without (-) Os9BGlu31 enzyme. The substrates and products of the reactions were separated on silica-gel F_{254} TLC with a solvent system of chloroform:methanol:ammonia (7:2.8:0.2). The developed plates were observed under UV (A) and then stained with 10% H_2SO_4 in ethanol and charred at 110°C (B).

Table 2. Relative activities of Os9BGlu31 on various acceptors.

Acceptor[a]	Relative activity (%)
Citrate buffer alone	9.3 ± 0.4
Phenolic compounds:	
Ferulic acid	100.0
Vanillic acid	85.5 ± 0.4
4-Hydroxybenzoic acid	78.3 ± 2.6
Syringic acid	78.3 ± 2.1
Trans-cinnamic acid	78.3 ± 0.4
Caffeic acid	78.3 ± 1.9
Sinapic acid	64.1 ± 0.04
Benzoic acid	64.1 ± 0.7
4-Coumaric acid	57.0 ± 0.1
Isovanillic acid	49.9 ± 1.1
Dihydroxybenzoic acid	42.7 ± 0.3
Scopoletin	28.5 ± 0.2
Esculetin	28.5 ± 3.0
Vanillin	14.2 ± 0.1
1-Naphthol	14.2 ± 0.3
2-Naphthol	nd[b]
Apocynin	7.1 ± 0.5
Vanillyl alcohol	7.1 ± 0.02
Dihydroxybenzaldehyde	7.1 ± 0.1
4-nitrophenol[c]	2.0 ± 0.01
Phytohormones:	
Napthalene acetic acid	42.7 ± 1.0
Indole acetic acid	42.7 ± 0.6
2,4-dichlorophenoxyacetic acid	29.9 ± 0.4
Abscisic acid	14.2 ± 0.1
Gibberellin A_3	7.1 ± 0.02
Gibberellin A_4	14.2 ± 0.1
Flavonoids/flavonoid glucoside/Anthocyanidin glucoside/ Vitamin glucoside/Monolignol glucoside:	
(-)-Catechin	35.6 ± 0.1
Quercitin 3-glucoside	28.5 ± 1.0
Apigenin	28.5 ± 1.7
Kaemferol	21.4 ± 1.2
Nariginin	14.2 ± 0.3
Pyridoxine 5-glucoside	14.2 ± 0.1
Cyanidin 3-glucoside	nd
4-Coumaryl alcohol 4-glucoside	16.9 ± 0.3
Alkyl acids and chemical nuclophiles:	
Butyric acid	28.5 ± 0.3
Propinoic acid	21.4 ± 0.2
Azide	9.7 ± 0.08
Acetate	6.2 ± 0.04
Formate	4.2 ± 0.04
Thiosulfate	nd
Thiocyanate	nd
Cyanide	nd

[a]Acceptor preferences of Os9BGlu31 with 4NPGlc as donor. Relative activity is shown in comparison to ferulic acid, which is arbitrarily set as 100%. [b]nd indicates 'not detected'.[c]4-nitrophenol (4NP) was assayed with 0.5 mM feruloyl glucose, rather than 5 mM 4NPGlc as a donor, so the comparison is not equitable. However, it is included to show that the reaction can go in reverse.

Table 3. Kinetic parameters of Os9BGlu31 on phenolic compounds and phytohormones as acceptor substrates.

Acceptor	Kinetic parameters for Acceptor at 30 mM 4NPGlc			Kinetic parameters for 4NPGlc at 0.25 mM Acceptor		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Citrate buffer alone	nm[a]	nm	nm	nm	nm	nm
Phenolic compounds:						
Ferulic acid	0.05 ± 0.004	1.21 ± 0.06	25.42 ± 1.52	9.33 ± 0.62	1.21 ± 0.2	0.13 ± 0.01
Vanillic acid	0.11 ± 0.012	0.43 ± 0.04	3.82 ± 0.4	5.92 ± 0.28	0.36 ± 0.023	0.06 ± 0.005
4-Hydroxybenzoic acid	0.21 ± 0.02	0.49 ± 0.002	2.31 ± 0.22	2.88 ± 0.27	0.33 ± 0.01	0.21 ± 0.01
Syringic acid	0.13 ± 0.012	0.35 ± 0.03	2.70 ± 0.25	1.18 ± 0.13	0.24 ± 0.02	0.20 ± 0.02
Trans-cinnamic acid	0.10 ± 0.01	0.32 ± 0.02	3.26 ± 0.08	1.62 ± 0.17	0.12 ± 0.004	0.07 ± 0.002
Caffeic acid	0.03 ± 0.003	0.25 ± 0.005	7.62 ± 0.6	7.70 ± 0.6	0.25 ± 0.02	0.03 ± 0.0004
Sinapic acid	0.02 ± 0.002	0.29 ± 0.01	14.15 ± 0.4	7.06 ± 0.74	0.28 ± 0.02	0.05 ± 0.001
Benzoic acid	0.12 ± 0.014	0.35 ± 0.038	2.87 ± 0.5	2.00 ± 0.17	0.28 ± 0.02	0.14 ± 0.04
4-Coumaric acid	0.01 ± 0.001	0.34 ± 0.03	33.3 ± 3.9	3.31 ± 0.3	0.30 ± 0.03	0.10 ± 0.008
Isovanillic acid	0.042 ± 0.003	0.22 ± 0.001	5.13 ± 0.5	1.91 ± 0.2	0.17 ± 0.02	0.10 ± 0.007
Dihydroxybenzoic acid	0.05 ± 0.004	0.12 ± 0.01	2.59 ± 0.2	0.64 ± 0.006	0.12 ± 0.01	0.19 ± 0.02
Phytohormones:						
Napthalene acetic acid	0.09 ± 0.007	0.12 ± 0.003	1.46 ± 0.02	0.27 ± 0.03	0.11 ± 0.001	0.39 ± 0.034
Indole acetic acid	0.05 ± 0.005	0.09 ± 0.001	2.05 ± 0.21	0.33 ± 0.02	0.1 ± 0.002	0.31 ± 0.01

[a]nm indicates 'not measured'.

2.3.2.3. Effects of metal ions and potential inhibitors on Os9BGlu31 transglucosidase

The effects of EDTA, several metal ions and β-glucosidase inhibitors on Os9BGlu31 activity were determined (Table 4). Little or no inhibition of Os9BGlu31 activity was seen with 1 mM EDTA, Ni^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ca^{2+}, while 1 mM Fe^{3+} and Cu^{2+} decreased the activity by 25% and 34%, respectively. Mercury (Hg^{2+}) had the greatest effect on Os9BGlu31, decreasing activity by approx. 90% at 1 mM. Surprisingly, Os9BGlu31 was not inhibited when it was preincubated with the covalent inhibitors conduritol B epoxide, cyclophellitol, and 2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside, which completely inhibit most GH1 β-glucosidases, and the β-glucosidase transition state mimic inhibitors glucono δ-lactone, 1-deoxy-nojirimycin, isofagomine and phenylethyl glucoimidazole.

Table 4. Effects of EDTA, metal salts and inhibitors on Os9BGlu31 activity.

Metal ions/inhibitors	concentration	Relative activity (%)
control	-	100
EDTA	1 mM	93.8 ± 8.6
Metal ions:		
$HgCl_2$	1 mM	10.7 ± 1.0
$MgCl_2$	1 mM	90.2 ± 7.4
$MnCl_2$	1 mM	90.8 ± 1.7
$FeCl_3$	1 mM	65.9 ± 0.6
$NiSO_4$	1 mM	103.9 ± 5.3
$ZnSO_4$	1 mM	97.0 ± 4.7
$CuSO_4$	1 mM	74.8 ± 3.6
$CoCl_2$	1 mM	88.8 ± 7.4
$CaCl_2$	1 mM	87.7 ± 3.6
Inhibitors:		
Conduritol B epoxide	1mM	102 ± 6.8
Cyclophellitol	1 mM	95 ± 5.6
Isofagomine	1 mM	98 ± 4.3
2,4-dinitrophenyl-β-D-2-deoxy-2-fluoro-glucopyranoside	1 mM	95.6 ± 1.7
Glucono δ-lactone	5 mM	98.2 ± 1.7
1-Deoxynojirimycin	7 µM	99.2 ± 3.8
Phenylethyl glucoimidazole	30 µM	98.7 ± 2.7

.

2.3.3. Analysis of gene expression

2.3.3.1. Northern blot analysis

We analyzed *Os9BGlu31* expression by northern blotting in collaboration with Dr. Rodjana Opassiri and Dr. Takashi Akiyama. The *Os9BGlu31* mRNA was detected at a relatively high level in the leaf blade and at a low level in the stem of mature plants (6-week-old), while low levels were detected in endosperm, shoot and root of 10-day-old seedlings (Figure 8A). When rice seed was germinated, *Os9BGlu31* expression was highest during the first day (0, 12, and 24 h), and then decreased at day 2 (Figure 8B). When 10-day-old seedlings were subjected to 2 days of low temperature (5 or 12°C), drought, salinity or flooding, *Os9BGlu31* transcript abundance increased in drought and increased slightly in NaCl (0.3 M) compared with the control condition (Figure 8C). Additionally, treatments with the phytohormones ABA, ethephon (which releases ethylene), and 2,4-dichlorophenoxyacetic acid (2,4-D; a synthetic auxin) significantly increased *Os9BGlu31* mRNA in rice seedlings, while methyljasmonate and kinetin induced small increases. Figure 8D shows that ethephon appears to induce the gene in roots of seedlings, but not in shoot and endosperm.

2.3.3.2. Quantitive real-time RT-PCR analysis.

The expression pattern of *Os9BGlu31* at various stages in various tissues was further examined by real-time PCR throughout rice development (Figure 9). The relative expression levels of *Os9BGlu31* normalized to the *OsUBQ5* gene were high in young leaf (YL), root (R), developing seed at 7-8 day after fertilization (DAF; Stage S4) and 9-10 DAF (S5) and flag leaves (FL1 to FL4), and displayed the highest expression in samples harvested from senescing flag leaves, FL3 (30 DAF flag leaf) and FL4 (40 DAF flag leaf), suggesting that *Os9BGlu31* may have a function in mature and senescent leaf tissues.

.

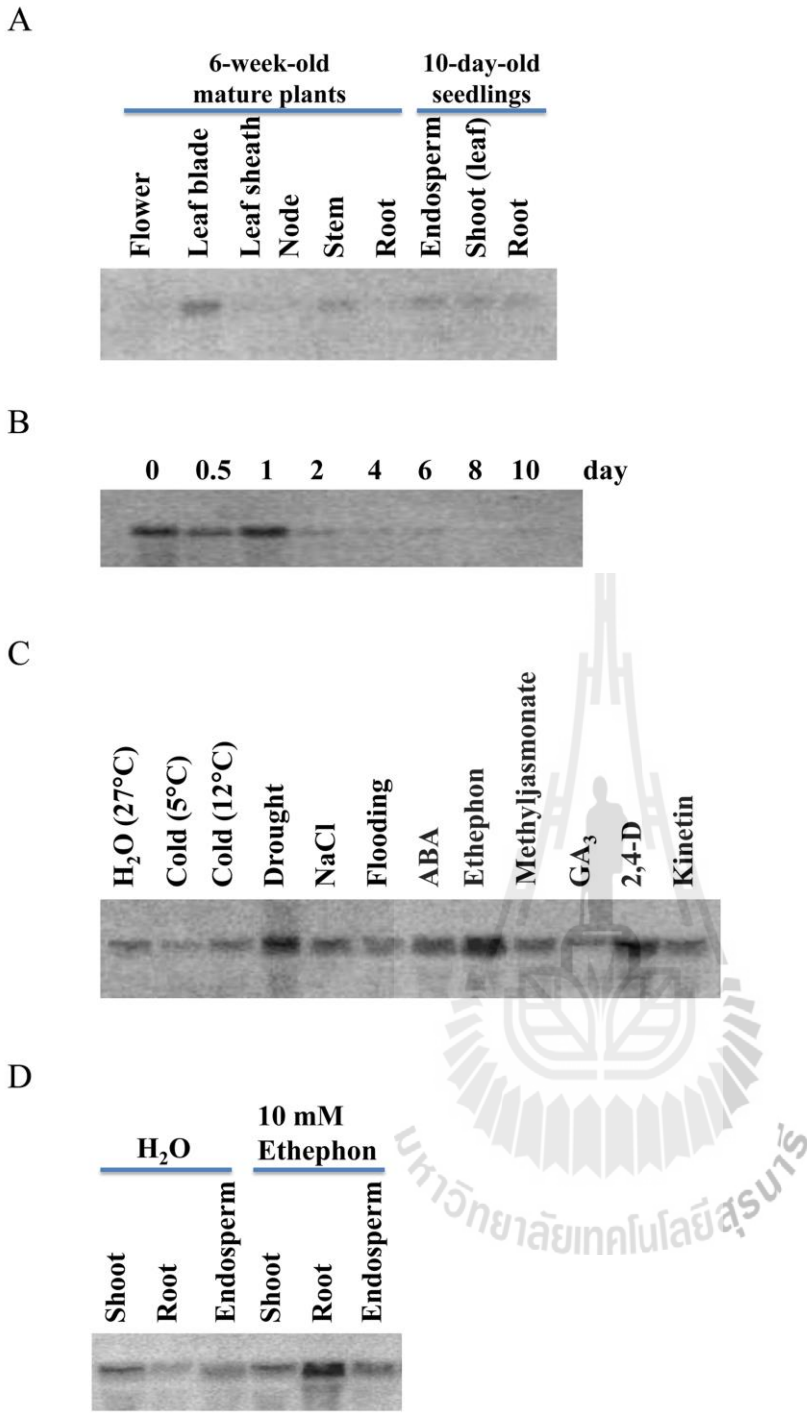


Figure 8. Northern blot analysis of *Os9BGlu31* gene expression in rice cv. Yukihikari plants and seedlings. (*A*) Blot of RNA extracted from various tissues of 6-week-old mature plants or 10-day-old rice seedlings. (*B*) Total RNA was extracted from whole seeds or seedlings germinated and grown at 27°C for the indicated periods of time. (*C*) Effects of stresses and phytohormones on *Os9BGlu31* gene expression in 10-day old rice seedlings. RNA gel blots of 10-day-old rice seedlings treated for an additional 2 days with the indicated stresses or phytohormones: H_2O (control); cold (5°C and 12°C); drought; 0.3 M NaCl; flooding; 0.1 mM abscisic acid (ABA); 10 mM ethephon; 0.1 mM methyljasmonate; 0.1 mM gibberellin A_3 (GA_3); 10 mM 2,4-dichlorophenoxyacetic acid (2,4-D); 0.1 mM kinetin. (*D*) Effects of ethephon on different tissues of 10-day old rice seedlings. As in part (*C*), 10-day-old rice seedlings were treated an additional 2 days with water (control) or 10 mM ethephon.

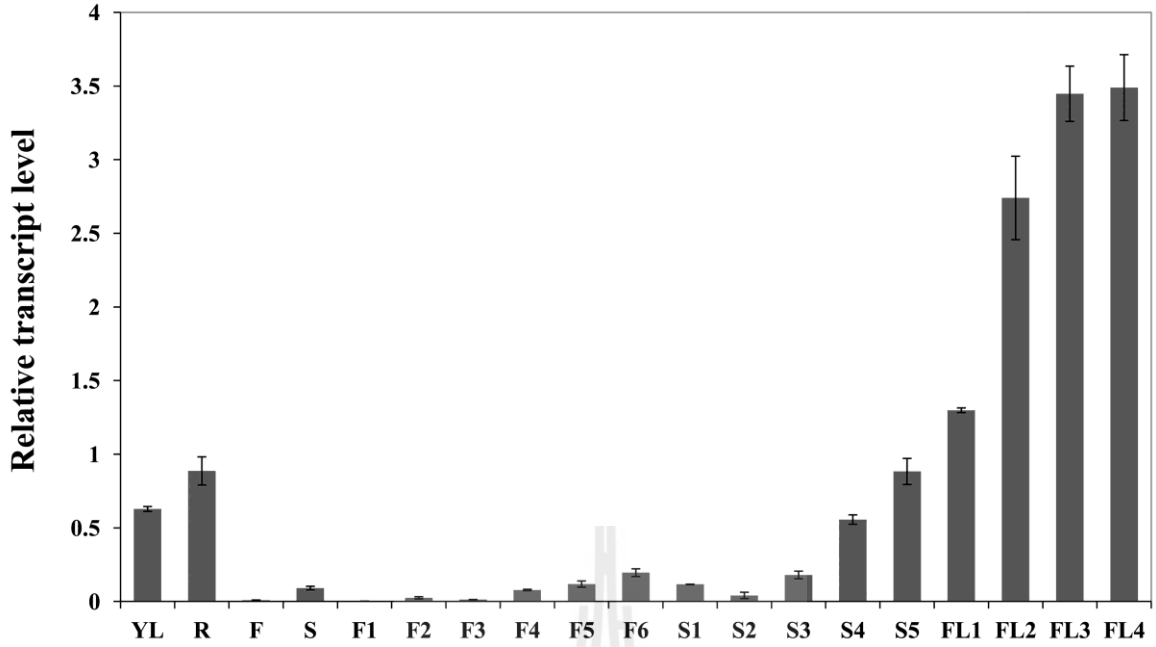


Figure 9. Expression pattern of the *Os9BGlu31* gene in rice cv. Dongjin, determined by real-time PCR. The relative expression level of *Os9BGlu31* was compared with that of *OsUBQ5* and error bars indicate standard deviations from three separate experiments. YL, young leaf; FL, flag leaf; R, root; F, flower; S, seed; I1, 0-4 cm inflorescence; I2, 4-8 cm inflorescence; I3, 8-12 cm inflorescence; I4, 12-16 cm inflorescence; I5, 16–20 cm inflorescence; I6, >20 cm inflorescence; S1, 1-2 DAF seeds; S2, 3-4 DAF seeds; S3, 5-6 DAF seeds; S4, 7-8 DAF seeds; S5, 9-10 DAF seeds; FL1, 15 DBF flag leaf; FL2, 15 DAF flag leaf; FL3, 30 DAF flag leaf; FL4, 40 DAF flag leaf.

.

Section 2.4 Analysis

2.4.1 Discussion:

In this project, we started with the idea to characterize a putative β-glucosidase from GH1 Os/At cluster 6, but found that Os9BGlu31 was instead a transglucosidase that transfers glucosyl groups to a range of acceptors, with highest activity toward phenolic acids and their glucose esters. This meant that instead of simply characterizing the activity toward hydrolytic or "donor" substrates, we had to characterize the specificity toward both donor and acceptor substrates, and that the activity was more unique and interesting than expected.

Os9BGlu31 transglucosidase showed greater than 10-fold activity for transfer of glucose to ferulic acid compared to transfer to water (hydrolysis, Table 3) and no glucose from hydrolysis was detected when acceptor substrates other than water were present in the reaction. The release of pNP in citrate buffer was assumed to be hydrolysis, since the citrate concentration no effect on release of pNP and glucose was detected on TLC analyses of these reactions. It is notable that no hydrolysis was reported for previously reported GH1 transglucosidases [Matsuba et al., 2010; Moellering et al., 2010]. However, those enzymes could not use pNP glycosides for substrates. The acyl glucose-dependent cyanidin-3-glucoside glucosyl transferases (which are really transglucosidases) could only transfer glucose from certain phenolic acid glucose esters, such as vanillyl β-D-glucose and feruloyl β-D-glucose to anthocyanin 3-O-glucoside, while the galactolipid galactolipid galactosyl transferase (again, a transgalactosidase) was only reported to transfer galactose between galactolipids. In contrast, Os9BGlu31 has a broader specificity, although its favorite donor substrates seem to be the same types of phenolic acid 1-O-acyl glucose esters utilized by the anthocyanidin 3-O-glucoside transglucosidases.

The Os9BGlu31 transglucosidase was similar to the rice GH1 β-glucosidases that have been characterized in its pH optimum and sensitivity to metal ions, but quite different in its sensitivity to organic inhibitors. Most β-glucosidases are completely inhibited by preincubation with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside, conduritol epoxide or cyclophelitol, which serve as suicide substrates [Withers et al., 1990; Penthaisong et al., 2012], but Os9BGlu31 is insensitive to these inhibitors. Lack of inhibition by 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside was not due to its rapid turnover as a substrate, so Os9BGlu31 did not show signs of binding this inhibitor in the active site. What is more, Os9BGlu31 was insensitive to the putative transition state mimics δ-gluconolactone,

isofagamine, deoxynojirimycin and phenylethyl glucoimidazole at concentrations that inhibit other β-glucosidases. Although these differences do not preclude the same double displacement retaining mechanism seen in GH1 β-glucosidases, they do suggest significant differences in the way Os9BGlu31 transglucosidase interacts with potential substrates and inhibitors.

The broad specificity of Os9BGlu31 transglucosidase makes its potential significance in plant metabolism more general, and yet more difficult to interpret. We subsequently found that Os9BGlu31 is found in the vacuole, similar to the acyl glucose dependent anthocyanin glucosyl transferases (AA5GT and AA7GT). It would evidently be able to equilibrate the levels of feruloyl glucose and other phenolic acids, if these are moved to the vacuole or Os9BGlu31 is moved to the vacuole. There are many glucose esters and glucosides that are stored in the vacuole, so a wide range of compounds could be equilibrated. In fact, the glucose esters of the phytohormones ABA (ABA-GE) and auxin (IAA-GE) are moved to the vacuole and Os9BGlu31 could equilibrate these levels, since it acts on these and other phytohormone conjugates, such as GA_4-GE. In this scheme, a high level of ABA in the vacuole could result in the release of IAA from its glucosyl ester, via transfer of the glucosyl moiety from IAA-GE. Similarly, high levels of glucose esters could blunt the release of phytohormones by resulting in their glucosylation from the available donor substrates. In addition, although Os9BGlu31 could not be detected to act on cyanidin 3-O-glucoside, it did have activity toward apigenin 7-O-glucoside, so other flavonoids may also be involved in this equilibrium. Clearly, much work remains to determine the biological function of the Os9BGlu31 transglucosidase, and those of similar enzymes in rice and other plants, but the potential impact is high. We are currently collaborating with Prof. Jong-Seong Jeon's group in Korea to characterize Os9BGlu31 knockout rice to get a better handle on what the enzyme is doing in the plant.

In addition to the intriguing possibilities for biological function, Os9BGlu31 has potential for application to the production of glycosides and glucose esters of interest. Of course, production of phytohormone glucoconjugates, which are generally difficult to purify from plants due to their low levels and can be difficult to synthesize is one potential application. These compounds could then be used to characterize their metabolism and action in the plant more thoroughly, as well as for identifying β-glucosidases and other enzymes that use them as substrates. In addition, much work has recently been done on the glycosylation of certain drug molecules for improving their pharmacological properties [Gant et al., 2011;

Singh et al., 2012]. In these experiments, glycosyl transferases, which require nucleotide sugar donors, are generally used, but the use of a transglucosidase, like Os9BGlu31, could potentially eliminate the need for these expensive substrates and therefore be advantageous. Mutagenesis of the enzyme active site cleft might allow for changes in the specificity to achieve better production of glucoconjugates of interest. So, much work can be done in the future in the application of Os9BGlu31 for glucoconjugate production.

2.4.2. Conclusions and Comments

2.4.2.1. Conclusions

In conclusion, we have found that Os9BGlu31 is a transglucosidase that can move the glucosyl moiety between different glucoconjugates, thereby fulfilling many potential roles in the plant and having a potential for the synthesis of glucoconjugates of interest.

Although it was first mistaken for a β-glucosidase based on sequence homology, the Os9BGlu31 enzyme turned out to be a transglucosidase that could transfer a glucosyl moiety from 4NPGlc to a nucleophilic acceptor, such as acetate in the acetate buffer originally used to measure the activity. Later it was found to utilize phenolic acids, such as 4-hydroxybenzoic acid and ferulic acid as the best acceptors, although it could also transfer glucose to various acids and alcohols, including the phytohormones IAA, NAA, ABA, and GA_4, as well several flavonoids, short chain fatty acids, and even simple alcohols, although the latter did not cause an increase in breakdown of the donor substrate. Although it could use 4NPGlc, as well as 4NP-β-D-fucoside and 4NP-β-D-xyloside, Os9BGlu31 showed much higher activities with 1-O-acyl glucose esters, such as feruloyl β-D-glucosie, 4-hydroxycoumaroyl β-D-glucose, and vanillyl β-D-glucose, as well as the flavonoid glucosides phloridzin and apigenin 7-O-β-D-glucoside and GA_4-GE. Although it is in the GH1 family, it was not inhibited by any of the substrates that inhibit GH1 β-D-glucosidases, such as 2,4-dinitrophenyl 2-deoxy-2-fluorglucoside and cyclophelitol. This suggested there may be some small mechanistic differences that may also relate to the preference of transglycosylation over hydrolysis.

The Os9BGlu31 transglucosidase is a vauolar enzyme that is expressed in seedling, particularly at the beginning of germination, but is most highly expressed in senescing flag leaves and maturing seeds. Its expression in induced by drought, ABA, methyl jasmonate, the

cytokinin kinetin and the artificial auxin 2,4-D in seedling shoots. Thus, Os9BGlu31 has potential to act in modulating the response to various stress conditions via interactions with hormones that can induce its production and act as substrates. Much work remains to be done in teasing out the biological function of this interesting enzyme.

2.4.2.1. Closing Comments:

Currently, we are continuing to work on Os9BGlu31 in a new project to tease out important aspects of its function. This includes investigating the basis for acceptor substrates specificity by mutagenesis, investigation of more inhibitors, and looking at the effects of knockout of the gene on the metabolite levels in the rice plant. Hopefully, these efforts will bring us a better understanding of the biochemical and biological function of the enzyme. A few of the early efforts on this new project were combined with those of this project to produce a publication (Luang et al., 2013). We also plan to make an effort to apply this enzyme to synthesis of glucoconjugates of potential medical significance. Thus, this project served to catalyze a whole line of investigations and will have lasting impact.

2.5. References

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

Babcock GW, Esen A. (1994) Substrate specificity of maize β-glucosidase. Plant Sci**.** 101: 31-39

Bednarek P, Piślewska-Bednarek M, Svatoš A, Bernd Schneider B, Doubský J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323: 101-106

Blanchard DJ, Cicek M, Chen J, Esen A. (2001) Identification of beta-glucosidase aggregating factor (BGAF) and mapping of BGAF binding regions on Maize beta-glucosidase. J. Biol. Chem. 276: 11895-11901

Brzobohatý B, Moore I, Kristoffersen P, Bako L, Campos N, Schell J, Palme K. (1993) Release of active cytokinin by a β-glucosidase localized to the maize root meristem. Science 262: 1051-1054

Buchanan BB, Gruissem W, Jones RL. (2000) "Biochemistry and Molecular Biology of Plants." John Wiley & Sons, Somerset, NJ, USA. 1367 pages.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. (2009) Nucleic Acids Res. 37: D233-D238.

Carpita NC. (1996) Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol. Mol. Biol. 47: 445-476.

Chuankhayan P, Hua Y, Svasti J, Sakdarat S, Sullivan PA, and Ketudat Cairns JR. (2005) Purification of an isoflavonoid 7-O-β-apiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurz. Phytochemistry 66: 1880-1889

Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR, Esen A, Chen C-J, Opassiri R, Svasti J, Ketudat Cairns JR. 2008. Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation. J. Mol. Biol. 377: 1200-1215

Cosgrove DL. (1997) Assembly and enlargement of the primary cell wall in plants. Annu. Rev. Cell Dev. Biol. 13: 171-201.

Coutinho PM, Henrissat B. (1999) Carbohydrate-active enzymes: an integrated database approach. In "Recent Advances in Carbohydrate Bioengineering", H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., pp. 3-12, The Royal Society of Chemistry, Cambridge, UK.

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

Dietz K-J, Sauter A, Wichert K, Messdaghi D, and Hartung W. (2000) Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. J. Exp. Bot. 51: 937–944

Escamilla-Treviño LL, Chen W, Card ML, Shih M-C, Cheng CL, Poulton JE. (2006) *Arabidopsis thailiana* β-glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. Phytochemistry 67: 1651-1660

Esen A, Stetler DA (1993) Subcellular localization of maize β-glucosidase. Maize Genet Coop News Lett 67: 19–20

Gantt RW, Peltier-Pain P, Cournoyer WJ, Thorson JS. (2011) Use of simple donors to drive the equilibria of glycosyltransferase-catalyzed reactions. Nat. Chem. Biol. 7: 685-691.

Gilbert HJ, Strålbrand H, Brumer H. (2008) How the walls come crumbling down: recent structural biochemistry of plant polysaccharide degradation. Curr. Opin. Plant Biol. 11: 338-348

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280: 309–316

Henrissat, B., Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based in amino acid sequence similarities. Biochem. J. 293: 781-788

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

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc Natl Acad Sci USA 92: 7090–7094

Himeno N, Saburi W, Takeda R, Wakuta S, Matsuura H, Nabeta K, Sansenya S, Ketudat Cairns JR, Imai R, Mori H, Matsuda H. 2013. Identification of rice β-glucosidase with high hydrolytic activity towards salicylic β-glucoside. Biosci.Biotech..Biochem. 77: 934-939.

Hirochika, H, Miyao A, Yamazaki S, Takeda A, Abe K, Hirochika R, Agrawal GK, Watanabe T, Sugimoto K, Sasaki T, Murata K, Tanaka K, Onosato K, Miyazaki A, Yamashita Y, Kojima N. (2001) Retrotransposons of rice as a tool for the functional analysis of genes. In: *Rice Genetics IV*, Khush, Brar and Hardy Editors, Scientific Publishers Inc. Enfield, USA. pg 279-292.

Höglund AS, Lenman M, Falk A, Rask L (1991) Distribution of myrosinase in rapeseed tissues. Plant Physiol. 95: 213–221

Hrmova M, Harvey AJ, Wang J, Shirley NJ, Jones GP, Stone BA, Hoj PB, Fincher GB. (1996) Barley β-D-glucan exohydrolase with β-D-glucosidase activity: purification, characterization, and determination of preliminary structure from a cDNA clone. J. Biol. Chem. 271, 5277-5286

Hrmova M, MacGregor EA, Biely P, Stewart RJ, Fincher, G. B. (1998) Substrate binding and catalytic mechanism of a barley β-D-glucosidase/(1,4)-β-D-glucan exohydrolase. J. Biol. Chem. 273, 11134-11143

Hrmova M, Burton RA, Biely P, Lahnstein J, Fincher GB. (2006) Hydrolysis of (1,4)-β-D-mannans in barley (*Hordeum vulgare* L.) is mediated by the concerted action of (1,4)-β-D-mannan endohydrolase and β-D-mannosidase. Biochem. J. 399, 77-90

International Rice Genome Project (2005) The map-based sequence of the rice genome. Nature 436: 793-800

Jakubowska A, Kawalczyk S. (2005) A specific enzyme hydrolyzing 6-O(4-O)-indole-3-ylacetyl-β-D-glucose in immature kernels of *Zea mays*. J. Plant Physiol. 162: 207-213.

Jenkins J, Lo Leggio L, Harris G, Pickersgill R (1995) Beta-glucosidase, beta-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold beta/alpha architecture and with two conserved glutamates near the carboxy-terminal ends of beta-strands four and seven. FEBS Lett 362: 281–285

Jeon J-S, Lee S, Jung K-H, Jun S-H, Jeong D-H, Lee J-W, Kim C, Jang S, Lee S-Y, Yang K, Nam J, An K, Han M-J, Sung R-J, Choi H-S, Yu J-H, Choi J-H, Cho S-Y, Cha S-S, Kim S-I, An G. (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J. 22, 561–570

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ. (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13: 889–905

Ketudat Cairns JR, Esen A. (2010) β-Glucosidases. Cell. Mol. Life Sci. 67: 3389-3405.

Ketudat Cairns JR, Pengthaisong S, Luang S, Sansenya A, Tankrathok A, Svasti J. (2012) Protein-carbohydrate interactions leading to hydrolysis and transglycosylation in plant glycoside hydrolase family 1 enzymes. J.Appl. Glycosci. 59: 51-62

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, Hotta I, Kojima K, T. Namiki , E. Ohneda, W. Yahagi, K Suzuki, C.J. Li, K. Ohtsuki, T. Shishiki, Y. Otomo, K. Murakami, Y. Iida, S. Sugano, T. Fujimura, Y. Suzuki, Y. Tsunoda, T Kurosaki, T. Kodama, H. Masuda, M. Kobayashi, Q. Xie, M. Lu, R. Narikawa, A. Sugiyama, K. Mizuno, S. Yokomizo, J. Niikura, R. Ikeda, J. Ishibiki, M. Kawamata, A. Yoshimura, J. Miura, T. Kusumegi, M. Oka, R. Ryu, M. Ueda, K. Matsubara, J. Kawai, P. Carninci, J. Adachi, K. Aizawa, T. Arakawa, S. Fukuda, A. Hara, W. Hashizume, Hayatsu N, Imotani K, Ishii Y, M. Itoh, I. Kagawa, S. Kondo, H. Konno, A. Miyazaki, N. Osato, Y. Ota, R. Saito, D. Sasaki, K. Sato, K. Shibata, A. Shinagawa, T. Shiraki, M. Yoshino, Y Hayashizaki, A. Yasunishi, Rice Full-Length cDNA Consortium; National Institute of Agrobiological Sciences Rice Full-Length cDNA Project Team; Foundation of Advancement of International Science Genome Sequencing & Analysis Group; RIKEN. (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice, Science. 301: 376-379

Kuntothom T, Luang S, Harvey AJ, Fincher GB, Opassiri R, Hrmova M, Ketudat Cairns JR. (2009) Rice family GH1 glycosyl hydrolases with β-D-glucosidase and β-D-mannosidase activities. Arch. Biochem. Biophys. 491: 85-95.

Lairson LL, Henrissat B, Davies GJ, Withers SG. (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu. Rev. Biochem. 77: 521-555.

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

Lipka V, Dittgen J, Pawel Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S. Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P. (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183.

Luang S, Cho J-I, Mahong B, Opassiri R, Akiyama T, Phasai K, Komvongsa J, Sasaki N, Hua Y, Matsuba Y, Ozeki Y, Jeon J-S, Ketudat Cairns JR. (2013) Os9BGlu31 is a transglucosidase with the capacity to equilibrate phenolpropenoid, flavonoid and phytohormone glycoconjugates. *J.Biol. Chem.* 288: 10111- 10123

Malboobi MA, Lefebvre DD. (1997) A phosphate-starvation inducible beta-glucosidase gene (psr3.2) isolated from Arabidopsis thaliana is a member of a distinct subfamily of the BGA family. Plant Mol. Biol. 34: 57-68

Matsuba Y, Nobuhiro Sasaki N, Tera M, Okamura M, Abe Y, Okamoto E, Nakamura H, Funabashi H, Takatsu M, Saito M, Matsuoka H, Nagasawa K, Ozeki Y. (2010) A novel glucosylation reaction on anthocyanins catalyzed by acyl-glucose–dependent glucosyltransferase in the petals of carnation and delphinium. Plant Cell 22: 3374-3389

Moellering ER, Muthan B, Benning C. (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science 330: 226-228

Morant AV, Jørgensen K, Jørgensen C, Paquette SM, Sánchéz-Perez R, Møller BL, Bak S. (2008) β-Glucosidases as detonators of plant chemical defense. Phytochemistry 69: 1795-1813

Nikus J, Esen A, Jonsson LMV. (2003) Cloning of a plastidic rye (*Secale cereale*) β-glucosidase cDNA and its expression in *Escherichia coli*. Physiol. Plant. 118: 337-345

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

Opassiri R, Ketudat Cairns JR, Akiyama T, Wara-Aswapati O, Svasti J, and Esen A. 2003. Characterization of a rice β-glucosidase genes highly expressed in flower and germinating shoot Plant Sci. 165: 627-638

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

Opassiri R, Pomthong B, Okoksoong T, Akiyama T, Esen A, Ketudat Cairns JR. 2006. Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β-glucosidase. BMC Plant Biol. 6: 33

Opassiri R, Maneesan J, Akiyama T, Pomthong B, Jin S, Kimura A, Ketudat Cairns JR. 2010. Rice Os4BGlu12 is a wound-induced β-glucosidase that hydrolyzes cell wall-β-glucan-derived oligosaccharideds and glycosides. Plant Sci. 179: 273-280.

Palmiano EP, Juliano BO. 1973. Changes in activity of some hydrolases, peroxidase, and catalase in rice seed during germination. Plant Physiol. 52: 274-277.

Pengthaisong S, Chen C-F, Withers SG, Kuaprasert B, Ketudat Cairns JR. (2012), Rice BGlu1 glycosynthase and wild type transglycosylation activities distinguished by cyclophellitol inhibition Carbohyd. Res. 352: 51-59

Poulton JE. (1990) Cyanogenesis in plants. Plant Physiol. 94: 401-405.

Raychaudhuri A, Tipton PA. (2002) Cloning and expression of the gene for soybean hydroxyisourate hydrolase. Localization and implications for function and mechanism. Plant Physiol. 130: 2061-2068.

Raychaudhuri A, Tipton PA (2003) A familiar motif in a new context: the catalytic mechanism of hydroxyisourate hydrolase. Biochemistry 42: 6848-6852.

Rensink WA, Buell CR. (2005) Microarray expression profiling resources for plant genomics. Trends Plant Sci. 10: 603-609.

Sarma AD, Serfozo P, Kahn K, Tipton PA. (1999) Identification and purification of hydroxyisourate hydrolase: a novel ureide-metabolizing enzyme. J. Biol. Chem. 278: 33863-33865.

Schliemann W. (1984) Hydrolysis of conjugated gibberellins by β-glucosidases from dwarf rice (*Oryza sativa* L. cv. Tan-ginbozu). J Plant Physiol. 116: 123-132

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

Seto Y, Shigeki Hamada S, Hideyuki Matsuura H, Matsushige M, Satou C, Takahashi K, Masuta C, Ito H, Matsui H, Nabeta K. (2009) Purification and cDNA cloning of a wound inducible glucosyltransferase active toward 12-hydroxy jasmonic acid. Phytochemistry 70: 370-379

Sherameti I, Venus Y, Drzewiecki C, Tripathi W, Dan VM, Nitz I, Varma A, Grundler F, Oelmüller R. (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thanliana* and the endophytic fungus *Piriformospora indica*. Plant J. 54: 428-439

Singh S, Phillips GN, Thorson JS. (2012) The structural biology of enzymes involved in natural product glycosylation. Nat. Prod. Rep. 29: 1201-1237

Sticklin, M. (2006) Plant genetic engineering to improve biomass characteristics for biofuels. *Curr. Opinion Biotechnol.* **17**, 315-319

Sue M, Yamazaki K, Yajima S, Nomura T, Matsukawa T, Iwamura H, Miyamoto T. (2006) Molecular and structural characterization of hexameric β-D-glucosidases in wheat and rye. Plant Physiol 141: 1237-1247

Thorlby G, Fourrier N, Warren G. (2004) The SENSITIVE TO FREEZING2 gene, required for freezing tolerance in *Arabidopsis thaliana*, encodes a beta-glucosidase. Plant Cell 16: 2192-203

van de Ven WT, LeVesque CS, Perring TM, Walling LL (2000) Local and systemic changes in squash gene expression in response to silver winged whitefly feeding. Plant Cell 12: 1409-1423

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

Wakuta S, Hamada S, Ito H, Imai R, Mori H, Matsuura H, Nabeta K, Matsui H. (2011) Comparison of enzymatic properties and gene expression profiles of two tuberonic acid β-glucosidases from *Oryza sativa* L. J. Appl. Glycsci. 58, 67-70

Withers SG, Warren RAJ, Street IP, Rupitz K, Kempton JB, Aebersold R. (1990) Unequivocal demonstration of the involvement of a glutamate residue as a nucleophile in the mechanism of a retaining glycosidase. J. Am. Chem. Soc. 112: 5887-5889

Xu Z, Escamilla-Treviño LL, Zeng L, Lalgondar M, Bevan DR, Winkel BSJ, Mohamed A, Cheng C, Shih M, Poulton JE, Esen A. (2004) Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1. Plant Mol. Biol. 55: 343-367

Xu Z-Y, Lee KH, Dong T, Jeong JC, Jin JB, Kanno Y, Kim DH, Kim SY, Seo M, Bressan RA, Yun D-J, Hwang I. (2012) A vacuolar β-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in Arabidopsis. Plant Cell 24: 2184–2199

3. Publications

3.1. International Journal Publication

Luang S, Cho J-I, Mahong B, Opassiri R, Akiyama T, Phasai K, Komvongsa J, Sasaki N, Hua Y, Matsuba Y, Ozeki Y, Jeon J-S, Ketudat Cairns JR. (2013) Os9BGlu31 is a transglucosidase with the capacity to equilibrate phenolpropenoid, flavonoid and phytohormone glycoconjugates. *J.Biol. Chem.* 288: 10111- 10123

Ketudat Cairns JR, Pengthaisong S, Luang S, Sansenya A, Tankrathok A, Svasti J. 2012. Protein-carbohydrate interactions leading to hydrolysis and transglycosylation in plant glycoside hydrolase family 1 enzymes. *J. Appl. Glycosci.* **59**, 51-62.

3.2. Meeting Presentations

Ketudat Cairns JR, Luang S, Opassiri R, Akiyama T, Sasaki N, Ozeki Y, Matsuba Y. (2011) A stress induced rice enzyme that equilibrates glucosyl conjugates. Phytochemical Society of North America 50th Annual Meeting. Fairmont Orchid, Kona, Hawaii, USA, 10-15 December, 2011. Oral presentation O5.7.

Luang, S., Ketudat Cairns, J.R. 2011. Os9BGlu31, a glycoside hydrolase family 1 enzyme that is primarily a glucosyl transferase. The Third Asia Pacific Protein Association Conference in conjunction with the Third Symposium of the Chinese Protein Society. Shanghai University, Shanghai, China. 6-9 May, 2011. Poster.

4. Curriculum Vitae

4.1. Head of Project

1. Name: Professor Dr. James Ketudat-Cairns

ชื่อ นาย ศ ดร เจมส์ นามสกุล เกตุทัต-คาร์นส์

2. รหัสประจำตัวนักวิจัยแห่งชาติ 38-30-0103 เลขที่ passport: USA 442032163
3. Current Position: Professor ตำแหน่งปัจจุบัน ศาสตราจารย์
4. Institutional Address: หน่วยงานที่อยู่ที่ติดต่อได้

Institute of Science, School of Chemistry
Suranaree University of Technology
111 University Avenue,
Suranaree Subdistrict, Muang District
Nakhon Ratchasima 30000

โทรศัพท์ 044-224304

โทรสาร 044-224185

E-mail cairns@sut.ac.th

5. ประวัติการศึกษา

Ph.D. University of California, San Diego, USA, 1993, Biology

B.Sc. University of Puget Sound, USA, 1986, Biology

6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

Biochemistry, bioinformatics, structural biology

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

7.1 Journal Publications

7.1.1. Cairns JR, Williamson MK, Price PA. 1991. Direct identification of γ-carboxyglutamic acid in the sequencing of vitamin K-dependent proteins. *Analytical Biochemistry* **199**, 93-97.

7.1.2. Cairns JR, Price PA. 1994. Direct demonstration that the vitamin K-dependent bone Gla protein is incompletely γ-carboxylated in humans. *Journal of Bone and Mineral Research* **9**, 1989-1997.

7.1.3. Svasti J, Srisomsap C, Surarit R, Techasakul S, and Ketudat-Cairns JR. 1998. Characterization of a novel rotenoid-β-glucosidase and its natural substrate from Thai rosewood. *Journal of Pure and Applied Chemistry* **70** (11).

7.1.4. Ketudat Cairns JR, Chantarangsee M, Chaiwangrad S, Phawong J. 1999. Primary structure-based screening for Glycosyl hydrolases in Thai plants. *Thai Journal of Biotechnology* **1**, 20-30.

7.1.5. Ketudat Cairns JR, Champattanchai V, Srisosap C, Thiede B, Wittman-Liebold B, Svasti J. 2000. Sequence and Expression of Thai Rosewood β-Glucosidase/β-Fucosidase, a Family 1 Glycosyl Hydrolase Glycoprotein. *Journal of Biochemistry* **128**, 999-1008.

7.1.6. Svasti S, Yodsowon B, Sriphanich R, Winichagoon P, Boonkhan P, Suwanban T, Sawangareetrakul P, Srisomsap C, Ketudat-Cairns J, Svasti J, Fucharoen S. 2001. Association of Hb Hope [β136(H14)Gly → Asp] and Hb H Disease. *Hemoglobin* **25**, 429-435.

7.1.7. Kaomek M, Mizuno K, Fujimura T, Sriyotha P, Ketudat Cairns JR. 2003. Cloning, Expression and Characterization of an Anti-Fungal Chitinase from *Leucaena leucocephala* de Wit. *Bioscience, Biotechnology and Biochemistry* **67**, 667-676.

7.1.8. Opassiri R, Ketudat Cairns JR, Akiyama T, Wara-Aswapati O, Svasti J, and Esen A. 2003. Characterization of a rice β-glucosidase genes highly expressed in flower and germinating shoot *Plant Science* **165**, 627-638.

7.1.9. Champattanachai V, Ketudat Cairns, JR, Shotelersuk V, Kerratichamreon S, Sawangareetrakul P, Srisomsap C, Kaewpaluek V, Svasti J. 2003. Novel mutations in a Thai patient with methylmalonic acidemia. *Molecular Genetics and Metabolism* **79**, 300-302.

7.1.10. Opassiri R, Hua Y, Wara-Aswapati O, Akiyama T, Svasti J, Esen A, and Ketudat Cairns JR. 2004. β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. *Biochemical Journal* **379**, 125-131.
DOI: 10.1042/BJ20031485.

7.1.11. Chuankhayan P, Hua Y, Svasti J, Sakdarat S, Sullivan PA, and Ketudat Cairns JR. 2005. Purification of an isoflavonoid 7-O-β-apiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurz. *Phytochemistry* **66**, 1880-1889.
DOI:10.1016/j.phytochem.2005.06.024 (ISI IF 2.78, 2005)

7.1.12. Ketudat Cairns JR, Keeratichamroen S, Sukcharoen S, Champattanachai V, Ngiwsara L, Lirdprapamongkol K, Liammongkolkul S, Srisomsap C, Surarit R, Wasant P and Svasti J. 2005. The molecular basis of mucopolysaccharidosis Type 1 in two Thai patients. *Southeast Asian Journal of Tropical Medicine and Public Health* **36**, 1308-1312.

7.1.13. Toonkool P, Metheenukul P, Sujiwattanarat P, Paiboon P, Tongtubtim N, Ketudat-Cairns M, Ketudat-Cairns J, Svasti, J. 2006. Expression and purification of dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. *Protein Expression and Purification* **48**, 195-204, doi: 10.1016/j.pep. 2006.05.011 (ISI IF 1.867, 2006)

7.1.14. Chuenchor W, Pengthaisong S, Yuvaniyama J, Opassiri R, Svasti J and Ketudat Cairns JR. 2006. Purification, Crystallization and Preliminary X-ray Analysis of Rice BGlu1 β-Glucosidase with and without 2-deoxy-2-fluoro-β-D-glucoside Inhibitor *Acta Crystallographica Section F* **62**, 798-801. doi:10.1107/S1744309106027084. (ISI IF 0.606, 2008).

7.1.15. Wattanasirichaigoon D., Svasti J., Cairns JRK, Tangnararatchakit K, Visudtibhan A, Keeratichamroen S, Ngiwsara L, Khowsathit P, Onkoksoong T, Lekskul A, Mongkolsiri D, Jariengprasert C, Thawil C, Ruencharoen S. 2006. Clinical and Molecular Analysis of an Extended Family with Fabry Disease. *Journal of the Medical Association of Thailand*. **89** (9), 1528-1535

7.1.16. Opassiri R, Pomthong B, Okoksoong T, Akiyama T, Esen A, Ketudat Cairns JR. 2006. Analysis of Rice Glycosyl Hydrolase Family 1 and Expression of Os4bglu12 β-Glucosidase. *BMC Plant Biology* **6**, 33.

7.1.17. Chuankhayan P, Rimlumduan T, Svasti J, Ketudat Cairns JR. 2007. Hydrolysis of Soybean Isoflavonoid Glycosides by *Dalbergia* β-Glucosidases. *Journal of Agricultural and Food Chemistry* **55**, 2407-2412. (ISI IF 2.322, 2006)

7.1.18. Keeratichamroen S, Ketudat Cairns JR, Sawangareetrakul P, Liammongkolkul S, Champattanachai V, Srisomsap C, Kamolsilp M, Wasant P, Svasti J. 2007. Novel

mutations found in two genes of Thai patients with isolated methylmalonic acidemia. *Biochemical Genetics* **45**, 421-430. doi: 10.1007/s10528-007-9085-y (ISI IF 0.876, 2006)

7.1.19. Hommalai G, Withers SG, Chuenchor W, Ketudat Cairns JR, Svasti J. 2007. Enzymatic synthesis of cello-oligosaccharides by mutated rice β-glucosidases *Glycobiology* **17**, 744-753. doi: 10.1093/glycob/cwm039 (ISI IF 3.668, 2006)

7.1.20. Chantarangsee M, Payakapong W, Fujimura T, Fry SC, Ketudat Cairns JR. 2007. Molecular cloning and characterization of β-galactosidases from germinating rice *(Oryza sativa) Plant Science* **173**, 118-134. doi:10.1016/j.plantsci.2007.04.009 (ISI IF 1.631, 2006)

7.1.21. Suwannarat P, Keeratichamroen S, Wattanasirichaigoon D, Ngiwsara L, Ketudat Cairns J R, Svasti J, Visudtibhan A, Pangkanon S. 2007. Molecular characterization of type 3 (neuronopathic) Gaucher disease in Thai patients**.** *Blood Cells, Molecules and Diseases* **38,** 348-352. doi:10.1016/j.bcmd.2007.06.015 (ISI IF 2.678, 2006)

7.1.22. Opassiri R, Pomthong B, Akiyama T, Nakphaichit M, Onkoksoong T, Ketudat-Cairns M, and Ketudat Cairns JR. 2007. A stress-induced rice β-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. *Biochemical Journal* **408** (2), 241-249. doi:10.1042/BJ20070734 (ISI IF: 4.1, 2006)

7.1.23. Chuankhayan P, Rimlumduan T, Tantanuch W, Mothong N, Kongsaeree PT, Metheenukul P, Svasti J, Jensen ON, Ketudat Cairns JR 2007. Functional and structural differences between isoflavonoid β-glycosidases from *Dalbergia* sp. *Archives of Biochemsitry and Biophysics* **468** (2), 205-216, doi:10.1016/j.abb.2007.09.015 (ISI IF 2.969, 2006)

7.1.24. Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Oonanant W, Bevan DR, Esen A, Chen C-J, Opassiri R, Svasti J, Ketudat Cairns JR. 2008. Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation. *Journal of Molecular Biology* **377** (4), 1200-1215. doi: 10.1016/j.jmb.2008.01.076 (ISI IF 4.146, 2008).

7.1.25. Keeratichamroen S, Ketudat Cairns JR, Wattanasirichaigoon D, Wasant P, Ngiwsara L, Suwannarat P, Pangkanon S, Tanpaiboon P, Rujirawat T, Svasti J. 2008. Molecular analysis of the iduronate-2-sulfatase gene in Thai patients with Hunter syndrome. *Journal of Inherited Metabolic Disease* doi:10.1007/s10545-008-0876-z (ISI IF 1.574, 2006)

7.1.26. Tantanuch W, Chantarangsee M, Maneesan J, Ketudat Cairns JR. 2008. Genomic and expression analysis of glycosyl hydrolase family 35 genes from rice (*Oryza sativa* L.). *BMC Plant Biology* **8**(1), 84. doi:10.1186/1471-2229-8-84 (ISI IF 4.03, 2008).

7.1.27. Seshadri S, Akiyama T, Opassiri R, Kuaprasert B, and Ketudat Cairns J. 2009. Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosides and (1⟶3)- and (1⟶2)-linked disaccharides. *Plant Physiology*; **151,** 47-58. doi:10.1104/pp.109.139436 (ISI IF 6.110, 2008).

7.1.28. Akiyama T, Jin S, Yoshida M, Hoshino T, Opassiri R, Ketudat Cairns JR. 2009. Expression of an endo-(1,3;1,4)-beta-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. *Journal of Plant Physiology* 166, 1814-1825. doi:10.1016/j.jplph.2009.06.002

7.1.29. Kuntothom T, Luang S, Harvey AJ, Fincher GB, Opassiri R, Hrmova M, Ketudat Cairns JR. 2009. Rice family GH1 glycoside hydrolases with β-D-glucosidase and β-D-mannosidase activities. *Archives of Biochemistry and Biophysics*. 491, 85-95.

7.1.30. Liammongkolkul S, Kuptanon C, Wasant P, Vatanavicharn N, Sawangareetrakul P, Keeratichamroen S, Ketudat Cairns JR, Srisomsap C, Svasti J. 2009. Case report. Novel mutation of methylmalonyl-CoA mutase gene in a Thai infant with methylmalonic acidemia (*mut*^{0}). *Siriraj Medical Journal* 61, 251-253.

7.1.31. Sansenya, S., Ketudat Cairns JR, Opassiri R. 2010. Expression, purification, crystallization and preliminary X-ray analysis of rice (*Oryza sativa* L.) Os4BGlu12 β-glucosidase. *Acta Crystallographica* F**66**, 320-323.

7.1.32. Luang, S., Hrmova, M., Ketudat Cairns, J.R. 2010. High-level expression of barley β-D-glucan exohydrolase HvExoI from a codon-optimized cDNA in *Pichia pastoris*. *Protein Expression and Purification* **73**, 90-98.

7.1.33. Ketudat Cairns, J.R. Esen, A. 2010. Beta-Glucosidases. *Cellular and Molecular Life Sciences* **67**(20), 3389-3405.

7.1.34. Opassiri, R., Maneesan, J., Akiyama, T., Pomthong, B., Jin, S., Kimura, A., Ketudat Cairns, J.R. 2010. Rice Os4BGlu12 is a wounding-induced β-glucosidase that hydrolyzes cell wall-β-glucan-derived oligosaccharides and glycosides. *Plant Science* **179**, 273-280.

7.1.35. Luang, S., Ketudat Cairns JR, Streltsov VA, Hrmova M. 2010. Crystallisation of wild-type and variant forms of a recombinant plant enzyme beta-D-glucan glucohydrolase from barley (*Hordeum vulgare* L.) and preliminary X-ray analysis. *International Journal of Molecular Sciences*. **11**, 2759-69.

7.1.36. Kuntothom, T., Raab, M., Tvaroska, I., Fort, S., Pengthaisong, S., Cañada, F.J., Calle, L., Jimenez-Barbero, J., Ketudat Cairns, J.R., Hrmova, M. 2010. Binding of β-D-glucosides and β-D-mannosides by rice and barley β-D-glycosidases with distinct substrate specificities. *Biochemistry (ACS)* **49**, 8779-8793.

7.1.37. Chuenchor W, Pengthaisong S, Robinson RC, Yuvaniyama J, Svasti J, Ketudat Cairns JR. 2011. The structural basis of oligosaccharide binding by rice BGlu1 beta-glucosidase. *Journal of Structural Biology* **173**, 169-179. doi: 10.1016/j.jsb.2010.09.021 (ISI IF 3.497, 2010).

7.1.38. Negi DS, Kumar A, Sharma RK, Shukla N, Negi N, Tamta ML, Bansal Y, Prasert P, Cairns JRK. 2011. Structure confirmation of rare conjugate glycosides from *Glycosmis arborea* (Roxb.) with the action of β-glucosidases. *Research Journal of Phytochemistry* 5, 32-40.

7.1.39. Sansenya S, Opassiri R, Kuaprasert B, Chen C-J, Ketudat Cairns JR. 2011. The crystal structure of rice (*Oryza sativa* L.) Os4BGlu12, an oligosaccharide and tuberonic acid glucoside-hydrolyzing beta-glucosidase with significant thioglucohydrolase activity. *Archives of Biochemistry and Biophysics* **510**, 62-72.

7.1.40. Pengthaisong S, Withers SG, Kuaprasert B, Svasti J, Ketudat Cairns JR. 2012. The role of the oligosaccharide binding cleft of rice BGlu1 in hydrolysis of cellooligosaccharides and in their synthesis by rice BGlu1 glycosynthase. *Protein Science* **21**, 362-372. doi: 10.1002/pro.2021 (ISI IF 2.798, 2011 JCR)

7.1.41. Sansenya S, Maneesan J, Ketudat Cairns JR. 2012. Exchanging a single amino acid residue generates or weakens a +2 cellooligosaccharide binding subsite in rice beta-glucosidases. *Carbohydrate Research*. **351**, 130-133.

7.1.42. Pengthaisong S, Chen C-F, Withers SG, Kuaprasert B, Ketudat Cairns JR. 2012. Rice BGlu1 glycosynthase and wild type transglycosylation activities distinguished by cyclophellitol inhibition *Carbohydrate Research* **352**(1), 51-59.

7.1.43. Ketudat Cairns JR, Pengthaisong S, Luang S, Sansenya A, Tankrathok A, Svasti J. 2012. Protein-carbohydrate interactions leading to hydrolysis and transglycosylation in plant glycoside hydrolase family 1 enzymes. *Journal of Applied Glycoscience* **59**, 51-62.

7.1.44. Vatanavicharn N, Champattanachai V, Liammongkolkul S, Sawangareetrakul P, Keeratichamroen S, Ketudat Cairns JR, Srisomsap C, Sathienkijkanchai A, Shotelersuk V, Kamolsilp M, Wattanasirichaigoon D, Svasti J, Wasant P. 2012. Clinical and molecular findings in Thai patients with isolated methylmalonic acidemia. *Molecular Genetics and Metabolism***106**, 424-429.

7.1.45. Charoenwattanasatien R., Ketudat Cairns JR, Keeratichamroen S, Sawangareetrakul P, Tanpaiboon P, Wattanasirichaigoon D, Pangkanoon S, Svasti J, Champattanachai V. 2012. Decreasing activities and altered protein processing of human *iduronate-2-sulfatase* mutations demonstrated by expression in COS7 cells. *Biochemical Genetics* **50**, 990-997.

7.1.46. Wang J, Pengthaisong S, Ketudat Cairns JR, Liu Y. 2013. X-ray crystallography and QM/MM investigation on the oligosaccharide synthesis mechanism of rice BGlu1 glycosynthases. *Biochimica et Biophysica Acta Proteins and Proteomics*. **1834** (2), 536-545.

7.1.47. Luang S, Cho J-I, Mahong B, Opassiri R, Akiyama T, Phasai K, Komvongsa J, Sasaki N, Hua Y, Matsuba Y, Ozeki Y, Jeon J-S, Ketudat Cairns JR. 2013. Os9BGlu31 is a transglucosidase with the capacity to equilibrate phenolpropenoid, flavonoid and phytohormone glycoconjugates. *Journal of Biological Chemistry* 288(14), 10111- 10123.

7.1.48. Himeno N, Saburi W, Takeda R, Wakuta S, Matsuura H, Nabeta K, Sansenya S, Ketudat Cairns JR, Imai R, Mori H, Matsuda H. 2013. Identification of rice β-glucosidase with high hydrolytic activity towards salicylic β-glucoside. *Bioscience, Biotechnology and Biochemistry* **77** (5), 934-939.

7.1.49. Hua Y, Sansenya S, Saetang C, Wakuta S, Ketudat Cairns JR. 2013. Enzymatic and structural characterization of hydrolysis of gibberellin A4 glucosyl ester by a rice β-D-glucosidase. *Archives of Biochemistry and Biophysics* **537**(1) 39-48.

7.1.50. Tankrathok A, Iglesias-Fernández J, Luang S, Robinson R, Kimura A, Rovira C, Hrmova M, Ketudat Cairns J. (Accepted) Structural analysis and insights into glycon specificity of the rice GH1 Os7BGlu26 β-D-mannosidase. *Acta Crystallographica Section D: Biological Crystallography* **69**(10), 2124-2135.

7.2. Projects as Head of Project (หัวหน้าโครงการวิจัย): ชื่อโครงการวิจัย…

7.2.1. Homology-Based Screening of Glycosidases from Thai Plants, 2539-2542, Thailand Research Fund Young Researcher Development Grant, Completed

7.2.2. Characterization of Glycosidases from Forest Legumes, 2542-2545, SUT/NRCT, Completed

7.2.3. Expression and Characterization of Thai Plant Glycosyl Hydrolases, 2545-2548, Completed

7.2.4. Investigation of Rice Beta-Glycosidase Gene Functions, 2546-2549, National Science and Technology Development Agency, Grant BT-B-06-RG-19-4608, Completed

7.2.5. Enzymatic Screening and Characterization of Thai Plant Glycosides, 2547-2550, SUT/NRCT, Completed

7.2.6. Structure and Function Relationships in Plant Beta-Glucosidases, 2547-2550, Thailand Research Fund Basic Research Grant BRG4780024, Completed

7.2.7. Structural Studies of Carbohydrate Active Enzymes from Rice, 2549-2552, National Synchrotron Research Center, Completed.

7.2.8. Structure and Function Relationships in Plant Beta-Glucosidases II, 2550-2553, Thailand Research Fund Basic Research Grant BRG5080007, Completed 10/2553.

7.2.9. Structural Basis for Substrate-Specificity in Glucooligosaccharide Hydrolyzing β-Glucosidases from Glycosyl Hydrolase Families 1 and 3, 2553-2556, Thailand Research Fund Basic Research Grant BRG53_0017.

7.2.10. Structural Basis for Substrate-Specificity in Glucooligosaccharide Hydrolyzing β-Glucosidases from Glycosyl Hydrolase Families 1 and 3, 2553-2556, Thailand Research Fund Basic Research Grant BRG53_0017. Head of project.

7.2.11. Characterization of a glycoside hydrolase family 1 group 6 hydrolase, 2554. SUT/NRCT, to be completed 2556/09.

7.3. Completed Research Projects (งานวิจัยที่ทำเสร็จแล้ว : ชื่อแผนงานวิจัย และ/หรือ โครงการวิจัย ปีที่พิมพ์ การเผยแพร่ และสถานภาพในการทำวิจัย) See Sections 7.1 and 7.2. (7.2.1-7.2.10), No project sets organized have been completed.

7.4. Projects in progress (งานวิจัยที่กำลังทำ : ชื่อแผนงานวิจัย และ/หรือโครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด)

7.4.1. Structure, function and application of plant β-glucosidases and related enzymes, 2556-2559, Thailand Research Fund Basic Research Grant BRG53800__. Head of project.

7.4.2. Identification and characterization of rice gibberellin beta-glucosidase. 2554-2557, SUT, Budget Bureau, NRCT.

7.4.3. Characterization of rice glucosyl transferases with potential for phytohormone regulation and glycoside synthesis. 2555-2558, SUT, Budget Bureau, NRCT.

4.2. Project participant

Name: Mariena Ketudat-Cairns

.ชื่อ (ภาษาไทย) นาง มาริน่า เกตุทัต-คาร์นส์

Position: Associate Professor

รหัสประจำตัวนักวิจัย 38 40 0999

Address: School of Biotechnology
Institute of Agricultural Technology
Suranaree University of Technology
Nakhon Ratchasima 30000 Thailand

Fax: (66 44) 224 150, 224 154 **Email: ketudat@sut.ac.th**

Phone: (66 44) 224 355, 224 234 ketudatcairns@hotmail.com

Education:

1984 High School Diploma
Math and Science Major
Chulalongkorn University Demonstration School
G.P.A. 3.89

1988 B.Sc. Biology (Plant Science and Technology)
Minor in Chemistry
Chiang Mai University, Thailand
G.P.A. 3.24

1995 Ph.D. Biology (Plant Molecular Biology and Genetic Engineering)
University of California San Diego, USA
G.P.A. 4.00

Awards, Scholarships & International Training courses:

1988 Prof. Dr. Dhab Nelanithi Foundation Award

1988-1990 Scholarship from Institute for Promotion of Teaching Science and Technology (IPST), Thailand

1990-1991 Scholarship from Biology Department UCSD, USA

1991-1992 Graduate Student Fellowship UCSD, USA

1992-1995 Genetics Training Grant from National Institute of Health (NIH), USA

1995 28/8-13/10 International Training Program (ITP) in Biotechnology at Gesellschaft fur Biotechnologische Forschung (GBF), Braunschweig,Germany

2000 21/9 ICRO-UNESCO International Training course on RNA and Biotechnology at Chinese Academic of Science, Shanghai, China

2006 Best paper of the year 2005 Award from Bioprocess and Biosystems Engineering Journal

Experiences:

1988-1995 Research Assistance, UCSD

1989-1994 Teaching Assistance, UCSD

1990-1995 Teaching Assistance Trainer, Department of Biology, UCSD

1995-1998 Instructor, Suranaree University of Technology

1995 4/11-16/12 Head of Business Center for WorldTech'95, Thailand

1997 Secretariat of the JSPS/NRCT Biotechnology Conference, Thailand

1998- 2000 Secretariat of the SUT Biotechnology Graduate Curriculum Development

1998- 2010 Assistant Professor, Suranaree University of Technology

1998- present Thai Society for Biotechnology, committee (7 terms)

2001- 2004 National Bio-safety Subcommittee (Microorganism)

2001- 2003 National Graduate Biotechnology Curriculum Development Project (Thai Society for Biotechnology, TSB & National Science and Technology Development Agency, NSTDA)

2001- present Institute of Agricultural Technology committee (5 terms)

2001- 2009 SUT Academic Senate member (4 terms)

2002- 2006 Department Chair, School of Biotechnology, Institute of Agricultural Technology, SUT

2010- present Associate Professor, Suranaree University of Technology

2011- 2013 SUT Academic Senate member

Membership: Thai Society for Biotechnology (Society committee 1998-2012, 7 terms)

Thai Society for Genetics

American Society of Plant Biology and Plant Physiology

Research Interested: Recombinant Protein Productions

Rice Functional Genomics

Rice Glycosyl Hydrolases

Molecular Biology of Cloned Animal and Stem cells

Reviewer for J. of Biotechnology, Food Control**,** Cellular Reprogramming, J. of the Science of Food and Agriculture, African Journal of Biotechnology, African Journal of Microbiology Research, Science Asia, etc.

International Publications:

Kupradit, C., Ruamkuson, D., Rodtong, S. and **Ketudat-Cairns, M**. (2013) Novel multiplex polymerase chain reaction and an oligonucleotide array for specific detection of the dominant foodborne bacterial pathogens in chicken meat. African Journal of Microbiology Research 7 (24) 3085-3095 DOI: 10.5897/AJMR12.2102

Kupradit, C., Ruamkuson, D., Rodtong, S. and **Ketudat-Cairns, M.** (2013) Oligonucleotide macroarray for specific detection of bacterial foodborne pathogens. Chiang Mai Journal of Science (accepted 4 June 2013)

Kupradit, C., Rodtong, S. and **Ketudat-Cairns, M**. (2013) Development of a DNA macroarray for simultaneous detection of multiple foodborne pathogenic bacteria in fresh chicken meat. World J Microbiol Biotechnol DOI 10.1007/s11274-013-1394-1 (accepted 31 May 2013)

Chittapun, S., Ruamkuson, D. and **Ketudat-Cairns, M**. (2013) Identification and nutritional value of live feeds for ornamental fish from Bangkok metropolitan markets in Thailand. Chiang Mai Journal of Science 40 (3) 364-375

Srirattana, K., Sripunya, N., Sangmalee, A., Imsoonthornruksa, S., Ling, Y-Y., **Ketudat-Cairns, M**., and Parnpai, R. (2012) Developmental potential of vitrified goat oocytes following somatic cell nuclear transfer and parthenogenetic activation. Small Ruminant Research. http://dx.doi.org/10.1016/j.smallrumres.2012.10.011

Imsoonthornruksa, S., Srirattana, K., Phewsoi, W., Tunwattana, W., Parnpai, R. and **Ketudat-Cairns, M.** (2012) Segregation of donor cell mitochondrial DNA in gaur-bovine interspecies somatic cell nuclear transfer embryos, fetuses and an offspring. Mitochrondrion Mitochondrion 12(5): 506–513

Srirattana K., Imsoonthornruksa S., Laowtammathron C., Sangmalee, A., Tunwattana, W., Thongprapai, T., Chaimongkol, C., **Ketudat-Cairns M**. and Parnpai, R. (2012) Full-term development of gaur-bovine interspecies somatic cell nuclear transfer embryos: effect of Trichostatin A treatment. Cellular Reprogramming 14(3): 248-257

Imsoonthornruksa, S., Sangmalee, A., Srirattana, K., Parnpai, R. and **Ketudat-Cairns, M.** (2012) Development of intergeneric and intrageneric somatic cell nuclear transfer (SCNT) cat embryos and the determination of telomere length in cloned offspring. Cellular Reprogramming 14(1): 79-87

Songwattana, P. and **Ketudat-Cairns, M**. (2011) Comparison between serological and molecular detection of citrus canker pathogen (*Xanthomonas axonopodis* pv. *citri*). Molecular Pathogens 2(3) 1-7 doi: 10.5376/mp.2011.02.0003

Ruamkuson, D., Tongpim, S. and **Ketudat-Cairns, M**. (2011) A Model to develop biological probes from microflora to assure traceability of tilapia. Food Control 22: 1742-1747

Rattanasuk, S., Parnpai, R. and **Ketudat-Cairns, M**. (2011) Multiplex polymerase chain reaction used for bovine embryo sex determination. J of Reprod and Dev 57(4) 539-542

Imsoonthornruksa, S., Lorthongpanich, C., Sangmalee, A., Srirattana, K., Laowtammathron, C., Tunwattana, W., Somsa, W., **Ketudat-Cairns, M.,** Nakai, T. and Parnpai R. (2011) The effects of manipulation medium, culture system and recipient cytoplast on *in vitro* development of intraspecies and intergeneric felid embryos. J Reprod Dev 57(3) 385-392

Imsoonthornruksa, S., Noisa, P., Parnpai, R. and **Ketudat-Cairns, M**. (2011) A simple method for production and purification of soluble and biologically active recombinant human leukemia inhibitory factor (hLIF) fusion protein in *Escherichia coli*. Journal of Biotechnology (151): 295-302

Imsoonthornruksa, S., Lorthongpanich, C., Sangmalee, A., Srirattana, K., Laowtammathron, C., Tunwattana, W., Somsa, W., **Ketudat-Cairns, M.** and Parnpai R. (2010) Abnormalities in the transcription of reprogramming genes related to global epigenetic events of cloned endangered felid embryos. Reprod Fertil Dev 22(4): 613-24

Srirattana K., Lorthongpanich C., Laowtammathron C., Imsoonthornruksa S., **Ketudat-Cairns M.**, Phermthai T., Nagai T. and Parnpai R. (2010). Effect of donor cell types on developmental potential of cattle (*Bos taurus*) and swamp buffalo (*Bubalus bubalis*) cloned embryos. J Reprod Dev 56(1): 49-54.

Lorthongpanich, C., Laowtammathron, C., Chan, A. W. S., **Ketudat-Cairns, M**. and Parnpai, R. (2008) Development of interspecies cloned monkey embryos reconstructed with bovine enucleated oocyte. J of Reprod and Dev 54(5) 306-313

Opassiri R., Pomthong B., Akiyama T., Nakphaichit M., Onkoksoong T., **Ketudat-Cairns M.**, and Ketudat-Cairns JR. (2007) A stress-induced rice beta-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. Biochem. J. (408) 241-249

Muenthaisong S., Laowtammathron C., **Ketudat-Cairns, M.**, Parnpai R. and Hochi S. (2007) Quality analysis of buffalo blastocysts derived from oocytes vitrified before or after enucleation and reconstructed with somatic cell nuclei. Theriogenology. 67(4) 893-900

Lorthongpanich, C., Srirattana, K., Imsoonthornruksa, S., Sripunya, N., Laowtammathron, C., Kumpong, O., **Ketudat-Cairns, M.** and Parnpai R. (2007) Expression and distribution of Oct-4 in interspecies-cloned long-tailed monkey (*Macaca fascicularis*) embryo. Reproduction, Fertility and Development 19(1) 149 doi:10.1071/RDv19n1Ab62

Imsoonthornruksa, S., Lorthongpanich, C., Srirattana, K., Sripunya, N., Laowtammathron, C., **Ketudat-Cairns, M.** and Parnpai, R. (2006). Effect of manipulation medium on the development of reconstructed domestic cat embryos. Reproduction, Fertility and Development 19(1) 141

Toonkool, P., Metheenukul, P., Sujiwattanarat, P., Paiboon, P., Tongtubtim, N., **Ketudat-Cairns, M**., Ketudat-Cairns, JR. and Svasti, J. (2006) Expression and purification of dalcochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. Protein Expression and Purification (48) 195-204

Charoenrat, T., **Ketudat-Cairns, M.**, Jahic, M., Veide, A. and Enfors, S.-O. (2006) Increase total air pressure versus oxygen limitation for enhances oxygen transfer and production formation in a *Pichia pastoris* recombinant protein process. Biochemical Engineering Journal. (30) 205-211.

Charoenrat, T., **Ketudat-Cairns, M.**, Enfors, S.-O., Jahic M. and Veide, A. (2006) Recovery of Recombinant β-glucosidase by expanded bed adsorption from *Pichia pastoris* high cell density culture broth. Journal of Biotechnology (122) 86-98

Charoenrat, T., **Ketudat-Cairns, M.**, Stendahl-Andersen, H., Jahic, M. and Enfors, S.-O. (2005) Oxygen limited fed-batch process: An alternative control for *Pichia pastoris* recombinant protein processes. Bioprocess and Biosystems Engineering (27) 399-406 ** *Received Best paper of the year award.* **

Laowtammathron, C., Lorthongpanich, C., **Ketudat-Cairns, M**., Hochi, S. and Parnpai, R. (2005) Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: the effects of hatching stage, linoleic acid-albumin in culture medium, and Ficoll supplementation to vitrification solution. Theriogenology (64) 1185-1196

Lorthongpanich, C., Laowtammathron, C., Muenthaisong, S., Vetchayan, T., **Ketudat-Cairns, M**., Likitdecharote, B. and Parnpai, R. (2004). *In vitro* development of enucleated domestic cat oocytes reconstructed with skin fibroblasts of domestic and leopard cats. Reprod. Fert. Dev. 16: 149.

Carlini, L.E., **Ketudat, M.,** Parsons, R. L., Prabhakar, S., Schmidt, R. J. and Guiltinan, M. J. (1999) The maize bZIP protein orthologue of EmBP-1: Activation of gene expression in yeast from an O2 box and localization of a bipartite nuclear localization signal (NLS). Plant Molec. Biol.41: 339-349. (M. Ketudat and L. Carlini are Co-first authors)

Schmidt, R. J., Pysh, L. D., **Ketudat, M.**, Parsons, R. L. and Hoschek, G. (1994) bZIP proteins regulating gene expression in maize endosperm. In *Molecular Genetic Analysis of Plant Metabolism and Development* (G. Coruzzi and P. Puigdomenech, eds.) NATO ASI Proceedings

Schmidt, R. J., **Ketudat, M.**, Aukerman, M. J. and Hoschek, G. (1992) Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. Plant Cell 4:689-700

Ueda, T., Waverczak, W., Ward, K., Sher, N., **Ketudat, M.**, Schmidt, R. J. and Messing, J. (1992) Mutations of the 22- and 27-kD zein promoters affect transactivation by the Opaque-2 protein. Plant Cell 4:701-709

National Publications:

Kupradit, C., Rodtong, S. and **Ketudat-Cairns, M**. (2013) Novel multiplex PCR to specifically detect bacterial foodborne pathogens. Suranaree J. Sci Technol.

Puangbua, S., Kupradit, C., **Ketudat-Cairns, M**. and Charoenrat, T. (2012) Production of recombinant enterokinase light chain by *Pichia pastoris*. Thammasart Sci and Tech J. 20 (1) 83-97

Parnpai, R., Srirattana, K., Imsoonthornruksa, S. and **Ketudat-Cairns, M.** (2011) Somatic cell cloning for livestock and endangered species. Thai J. Vet Med 41: 77-85

Tam, D.T.T. and **Ketudat-Cairns, M.** (2011) Studying the genomic function of rice β -glucosidases via RNA interference. Thai Journal of Agricultural Science

Wanthanalert, W. and **Ketudat-Cairns, M.** (2011) Knock down *Os*1*bglu*1 β-glucosidase in rice by *Agrobacterium*-mediated transformation. Songklanakarin J. of Sci and Tech 33 (1) 23-32

Rattanasuk, S. and **Ketudat-Cairns, M**. (2009) Genetic diversity of felids' cytochrome b. Suranaree J. Sci Technol 16 (4) 283-290

Ruamkuson, D. and **Ketudat-Cairns, M**. (2009) Optimum conditions for DGGE of 16S rDNA from SUT tilapia intestinal microflora. Suranaree J. Sci Technol 16 (4) 311-317

Kupradit, C. and **Ketudat-Cairns, M**. (2009) The extraction and purification of boar sperm surface protein. Suranaree J. Sci Technol 16 (3) 245-251

Rattanasuk, S. and **Ketudat-Cairns, M**. (2009) Chryseobacterium indologenes, novel mannanase producing bacteria. Songklanakarin J. of Sci and Tech 31(4) 395-399

Kupradit, C., Charoenrat, T. and **Ketudat-Cairns, M**. (2008) Recombinant bovine enterokinase light chain production by *Pichia pastoris*: effect of induction temperature. Thai Journal of Biotechnology 8 (1) 99-105

Phetsom, J., Jung, K., **Ketudat-Cairns, M.** and Ronald, P. (2007) Quality assessment of NSF rice oligonucleotide array. Agricultural Sci. J. 38(6): 11-14.

Charoenrat, T., Vanichsrirattana, V. and **Ketudat-Cairns, M.** (2004) Recombinant β-glucosidase production by *Pichia pastoris*: influence of pH. Thai Journal of Biotechnology 5 (1) 51-55

Kanchanatawee, S., Wanapu, C. and **Ketudat-Cairns, M**. (2000) Biotechnology graduate education in Thailand. Thai Journal of Biotechnology 2 (1): 55-62

Ketudat-Cairns, M. (1998) Biotechnology and daily life. Suranaree J. Sci Technol 5:208-211

Manakasem, Y., Sornsuk, P. and **Ketudat-Cairns, M.** (1998) A survey of the status and problems of the vegetable and fruit production and post-harvest handling system in Nakhon Ratchasima province. Suranaree J. Sci Technol 5:95-100

> 90 Papers Presented at National and International Conferences