**รายงานการวิจัย**

# การศึกษาการทำงานของเอนไซม์ไฮโดรเลสที่ยังไม่มีการศึกษาในข้าว
## Characterization of novel rice hydrolytic enzymes.

**ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี**

ผลงานวิจัยเป็นความรับผิดชอบของผู้อำนวยการชุดโครงการวิจัยแต่เพียงผู้เดียว

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## Characterization of novel rice hydrolytic enzymes.

ผู้วิจัย

ผู้อำนวยการชุดโครงการ

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**Professor Dr. James R. Ketudat-Cairns**

หัวหน้าโครงการ

**1. Identification of rice-beta-glucosidases hydrolyzing gibberellin glucoconjugates**

**Dr. Yanling Hua, Center for Scientific and Technological Equipment**

**2. Cloning and functional characterization of rice Os1BGlu4 beta-glucosidase.**

**Associate Professor Dr. Mariena Ketudat-Cairns, Institute of Agricultural Technology,**

**School of Biotechnology**

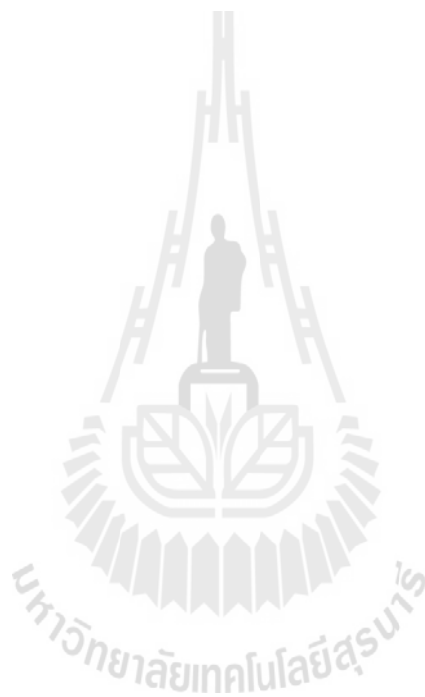**3. Characterization of a glycoside hydrolase family 1 group 6 hydrolase.**

**Professor Dr. James R Ketudat-Cairns, Institute of Science, School of Biochemistry**

มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของผู้อำนวยการชุดโครงการวิจัยแต่เพียงผู้เดียว

กันยายน  2556

# Acknowledgement

# บทคัดย่อ

เบต้ากลูโคซิเดส (β-D-glucopuranoside glucohydrolase, E.C. 3.2.1.21) เป็นเอนไซม์ที่สามารถ
สลายพันธะไกลโคซิดิกจากปลายที่ไม่มีหมู่รีดิวซ์ เพื่อปลดปล่อยหมู่ไกลโคซิลออกจากโมเลกุลของไกล
โคไซด์และโอลิโกแซคคาไรด์ จากความสัมพันธ์ของลำดับกรดอะมิโน พบว่าเอนไซม์เบต้ากลูโคซิเด
สจากพืชส่วนใหญ่ จัดอยู่ในกลุ่มไกลโคไซด์ไฮโดรเลส กลุ่มที่ 1 (GH1) เมื่อพิจารณาถึงยีนในกลุ่ม GH1
จำนวน 34 ยีน ของข้าว พบว่าสามารถแบ่งออกเป็น 8 กลุ่มย่อย โดยแต่ละกลุ่มย่อยจะมีลำดับกรดอะมิโน
คล้ายกับโปรตีนจาก *Arabidopsis thaliana* มากกว่าโปรตีนจากข้าวที่อยู่ในกลุ่มย่อยอื่น ในชุด
โครงการวิจัยนี้ประกอบไปด้วย 3 โครงการที่ศึกษาสมบัติของเอนไซม์ในกลุ่ม GH1 ของข้าวที่ยังไม่มี
การศึกษาหน้าที่ตามธรรมชาติ โดยโครงการแรกได้ทำการสังเคราะห์สาร gibberellin glucosides และสาร
gibberellin $GA_4$ glucosyl eater ($GA_4$-GE) ที่สังเคราะห์ขึ้นถูกใช้เพื่อตรวจหาเอนไซม์ที่สามารถย่อย
สารประกอบเหล่านี้จากรีคอมบิแนนท์เอนไซม์ในกลุ่ม GH1 ที่ผลิตขึ้นโดย *Escherichia coli* และเอนไซม์
ที่สกัดได้จากข้าวโดยตรง เอนไซม์ Os3BGlu6 จากกลุ่มย่อย At/Os1 มีความสามารถในการสลาย GA4-
GE ได้ค่อนข้างดี ซึ่งเอนไซม์ที่สกัดได้จากข้าวก็มีความสามารถในการย่อยสลายสารนี้เช่นกัน อย่างไรก็
ตาม ยังไม่สามารถแยกเอนไซม์บริสุทธิ์ที่มีกิจกรรมสูงได้ ในโครงการที่สอง เอนไซม์ Os1BGlu4 ซึ่งเป็น
เอนไซม์เบต้ากลูโคสิเดสเพียงชนิดเดียวจาก GH1 ของข้าวในกลุ่มย่อย At/Os3 ที่มีการทำนายว่าอาจมีการ
แสดงออกและมีเป้าหมายอยู่ในไซโตพลาสซึมของเซลล์ โดยการศึกษาสมบัติของเอนไซม์ดังกล่าวทำโดย
อาศัยรีคอมบิแนนท์เอนไซม์ Os1BGlu4 ที่ผลิตขึ้นโดย *E. coli* จากการศึกษาพบว่า Os1BGlu4 มี
ความสามารถในการย่อยโอลิโกแซคคาไรด์ของกลูโคส และไกลโคไซด์อื่น ๆบางชนิดได้ดี และใน
โครงการสุดท้ายได้ทำการศึกษากิจกรรมของเอนไซม์ Os9BGlu31 ซึ่งเป็นเอนไซม์ในกลุ่ม GH1 กลุ่มย่อย
At/Os6 พบว่าเอนไซม์นี้มีความสามารถในการเกิดเร่งปฏิกิริยาทรานไกลโคซิเลชั่น โดยมีความสามารถ
ค่อนข้างสูงต่อสารในกลุ่ม phenolic 1-O-acyl β-glucosyl ester เช่น feruloyl glucose ที่ทำหน้าที่เป็นตัวรับ
กลูโคส และมี aglycones ทำหน้าที่เป็นตัวให้กลูโคส รูปแบบการแสดงออกของยีนนี้ชี้ให้เห็นว่าเป็นยีนที่
น่าจะมีบทบาทสำคัญต่อการแก่ของใบ การพัฒนาของเมล็ด และการงอกของเมล็ด รวมไปถึงการ
ตอบสนองต่อความเครียด เช่น ความแห้งแล้ง และความเครียดจากฮอร์โมนต่าง ๆ ที่เกี่ยวข้อง

โครงการชุดนี้ได้พัฒนาองค์ความรู้เกี่ยวกับเอนไซม์เบต้ากลูโคซิเดส และกลูโคซิลคอนจูเกตใน
ข้าว ซึ่ง ณ ปัจจุบันสามารถนำองค์ความรู้ที่ได้ตีพิมพ์ในวารสารวิชาการระดับนานาชาติได้ 2 เรื่อง และ
คาดว่าจะมีการตีพิมพ์เพิ่มเติมอีกในโครงการต่อเนื่อง อีกทั้งชุดโครงการนี้ยังได้มีการพัฒนานักวิจัยรุ่น
ใหม่เพิ่มขึ้นอีกหลายท่าน

# Abstract

Beta-glucosidases (β-D-glucopyranoside glucohydrolases, E.C. 3.2.1.21) are enzymes that release nonreducing terminal glucosyl residues from glycosides and oligosaccharides via hydrolysis. Based on amino acid sequence relationships, most characterized plant β-glucosidases belong to glycoside hydrolase family GH1. The thirty-four active rice (*Oryza sativa*) GH1 genes that have been identified in the rice genome have been divided into 8 phylogenetic clusters, the members of which each have more amino acid sequence similarity with related proteins from the dicot *Arabidopsis thaliana* than with other rice proteins outside that cluster. In this project set, the three projects characterized the activities of GH1 enzymes for which the natural functions have yet to be defined. In the first project, gibberellin glucosyl conjugates were synthesized and the gibberellin $GA_4$ glucosyl ester ($GA_4$-GE) produced was used to screen for enzymes that hydrolyze it among GH1 enzymes produced in recombinant *Escherichia coli* and in rice extracts. The rice GH1 At/Os cluster 1 β-glucosidase Os3BGlu6 was identified as an enzyme with relatively high $GA_4$-GE hydrolysis ability, while such ability was identified in rice tissues, but the responsible enzyme has yet to be purified and identified. In the second project, Os1BGlu4, the only rice GH1 enzyme predicted to be expressed in the cytoplasm and a member of At/Os cluster 3, was expressed in *E. coli* and its enzymatic activity characterized. Os1BGlu4 was found to be a β-glucosidase with relatively high activity toward gluco-oligosaccharides and a few glycosides. In the third project, we characterized the activity of Os9BGlu31, a GH1 At/Os cluster 6 representative was found to be a transglucosidase with broad specifity and with high activity toward phenolic 1-O-acyl β-glucosyl esters, such as feruloyl glucose, as acceptors and the corresponding aglycones as donors. Its expression pattern suggests a role in senescing leaves, developing seeds and germinating seeds, as well as in response to stress, such as drought, and to stress related phytohormones. These studies have advanced the understanding of β-glucosidases and glucosyl conjugates in rice, and have contributed to publication of two international journal papers, with more expected from continuing work, in addition to training several scientists.

# CONTENTS

# CONTENTS (Continued)

# LIST OF TABLES

# LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

## 1.1. Importance and background of research project

Rice is the most important food crop in the world and is critical to the culture and economy of Thailand. However, even with the availability of the rice genome sequence and many molecular tools to study rice, the molecular mechanisms regulating rice germination and growth, development, and defense against pests remain to be determined. Germination and elongation of stems and roots are regulated by phytohormones, including gibberillins and abscissic acid (ABA), which are often found in relatively inactive glucoconjugates [Buchanan et al., 2000]. However, no rice beta-glucosidases that release active gibberellins or abscissic acid from these glucoconjugates has been identified at the molecular level to allow molecular breeding to target these activities, although recently a tuberonic acid beta-glucosidase has been identified. In addition, the defense mechanisms that allow rice to avoid herbivores and microbial infections are yet to be determined in detail. Since beta-glucosidases are known to play roles in phytohormone regulation, defense, lignification to build strong cell walls for defence, secondary metabolism and cell wall metabolism, determining their functions is bound to lead to new strategies to improve rice productivity and stress resistance.

We previously identified 34 glycoside hydrolase family 1 (GH1) genes that are likely to produce beta-glucosidases in rice [Opassiri et al., 2006]. These could be grouped into 8 clusters (Figure 1), of which we have characterized the activities of 1 of 2 from cluster 1 [Seshadri et al., 2009], 3 of 5 from cluster 4 [Opassiri et al., 2003, 2004, Kuntothom et al., 2009], and 1 of 12 from cluster 7 [Opassiri et al., 2006, 2010; Himeno et al., 2013]. Most of the genes that have been characterized for their function in *Arabidopsis thaliana*, the dicot model plants, fall in a cluster of crucifer-specific genes [Opassiri et al., 2006]. So, it is unclear what genes carry out these important functions, which include control of active abscissic acid levels, defense against nonspecialist fungi, adaptation to endophytes, and response to phosphate

starvation [Malboobi MA, Lefebvre, 1997; Lipka et al., 2005; Sherameti et al., 2008; Bednarek et al., 2009]. Since Cluster 6, with 11 genes, was still uncharacterized at the beginning of this project, we proceeded to characterize a member of this group, Os9BGlu31. Unexpectedly, Os9BGlu31 turned out to be a transglucosidase with high activity toward phenolic acids and their 1-O-acyl glucose esters, which has a wide range of implications for possible roles in glucose transfer in plants [Luang et al., 2013].

In addition, only one rice β-glucosidase predicted from the genes in the database is predicted to reside in the cytoplasm, rather than enter the secretory pathway, which was Os1BGlu4, the sole rice member of At/Os Cluster 3. Neither this enzyme nor any other member of this phylogenetic cluster, which includes one *A. thaliana* protein and proteins from other plants that are also predicted to be cytoplasmic, had been characterized. The unique location and conservation of this isoenzyme across plant species points to an important, as yet uncharacterized role, so we tried to characterize its biochemical function.

On the other hand, one role that has not been attributed to a specific isoenzyme is hydrolysis of gibberellin glucoconjugates [Schliemann, 1984], as noted above. Therefore, we synthesized the β-D-glucosyl esters and β-D-glucosides of gibberellins $GA_3$ and $GA_4$ and tested Os1BGlu4, Os3BGlu6, Os3BGlu7, Os4BGlu12, Os4BGlu18 and Os9BGlu31, which were expressed in recombinant *Escherichia coli* in our lab, to see whether they could hydrolyze $GA_4$ glucosyl ester ($GA_4$-GE). Since Os3BGlu6 hydrolyzed the $GA_4$-GE much better than the other isoenzymes, its hydrolysis of the substrate was characterized and this was combined with the characterization of acid/base mutants and structures of the enzyme achieved in another project for publication [Hua et al., 2013]. We also used the $GA_4$-GE to identify rice extracts containing enzymes that hydrolyze it, and partially purify the enzymes. This set the basis for further purification and identification of the $GA_4$-GE β-glucosidase in the next project. Thus, this set of projects provided a great deal of information on what glycoside hydrolase family GH1 enzymes are doing in rice.
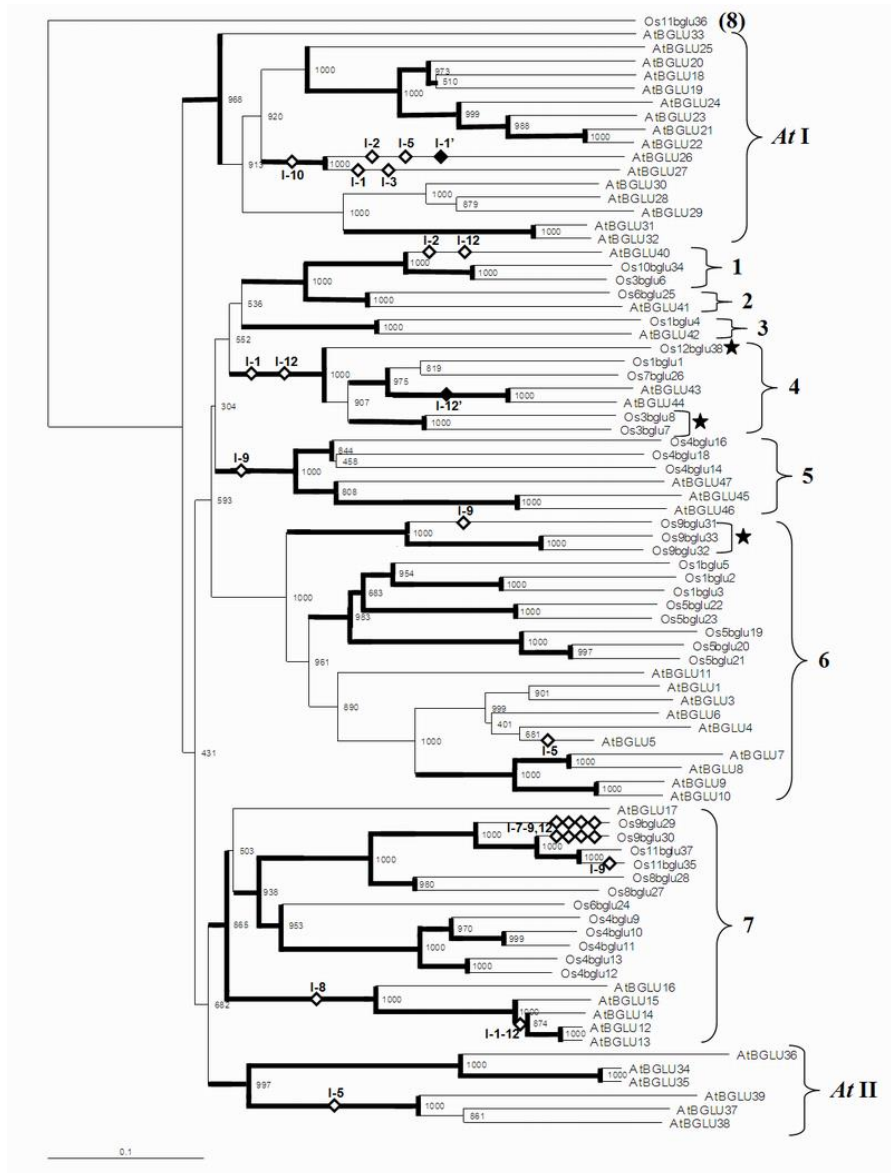
**Figure 1**. Phylogenetic tree of the derived protein sequences from glycoside hydrolase family 1 (GH1) genes found in the rice (*Oryza sativa* L.) and Arabidopsis (*Arabidopsis thaliana*) genomes. The bootstrap values are shown at the head of each cluster. The tree was rooted by the Os11BGlu36 (rice 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. The figure is from Opassiri et al. [2006].

This set consisted of 3 projects to address the issues noted above, which were:

1. Identification of rice-beta-glucosidases hydrolyzing gibberellin glucoconjugates

(การบ่งชี้เบตากลูโคไซเดสจากข้าวที่ย่อยกลูโคไซด์ของจิบเบอริลิน);

2. Cloning and functional characterization of rice Os1BGlu4 beta-glucosidase.

(การโคลนและการศึกษาการทำงานของ Os1BGlu 4 เบตากลูโคไซเดสจากข้าว); and

3. Characterization of a glycoside hydrolase family 1 group 6 hydrolase.

(การศึกษาการทำงานของไกลโคไซด์ไฮโดรเลส ตระกูลที่ 1 กลุ่ม 6 ).

## 1.2. Research objectives

The main objectives of this research set, summarizing those of each project, were:

1.2.1. To synthesize the gibberellin $GA_3$ and $GA_4$ beta-glucosyl esters and beta-glucosides and use them to identify which rice enzymes may hydrolyze them to regulate gibberellin activity in the plant.

1.2.2. To express Os1BGlu4, the only cytoplasmic beta-glucosidase in rice, in a recombinant system and determine its substrate specificity in order to learn about its probable role in rice.

1.2.3. To express a representative of GH1 Cluster 6, Os9BGlu31, in a recombinant system and determine its substrate specificity, in order to try to determine its function in the plant.

## 1.3. Scope

In this project, we could only accomplish a limited amount in one year, so the scope was limited somewhat, although a large amount was accomplished. Although all $GA_3$ and $GA_4$ glucoconjugates were synthesized, only $GA_4$-GE was produced in large amounts and used to screen recombinant enzymes in the laboratory, as well as extracts from rice tissues. Os1BGlu4 was expressed in *E. coli* and the protein purified and characterized for hydrolysis of available substrates. The Os9BGlu31 enzyme was found to be a transglucosidase, so it's activity for transfer of glucose from *p*NPGlc to various alcohols and carboxylic acids to make glycosides and 1-O-acyl glucose esters was characterized, as well as the sugar specificity for the donor substrate and the donor specificity with 1-acyl-O-glucosyl esters and other possible natural substrates. In addition, our collaborators in Japan and Korea helped to determine the expression profile of Os9BGlu31 by northern blot and quantitative real time reverse-transcription polymerase chain reaction (Q-RT-PCR), respectively.

**1.4. Conceptual framework**

Rice is the world's most important food crop, and also an important model system for the study of crop processes due to the availability of the genome sequence and many other resources for molecular studies. Many physiological processes that regulate plant growth and resistance to environmental stresses that are important to crop productivity are still not understood at a molecular level, which has limited the progress that has been made in improving crop traits. Beta-glucosidases of the GH1 family are known to play a variety of roles in plants, including release of defense compounds, regulation of phytohormones, release of monolignols for cell wall lignification, breakdown of cell-wall-derived oligosaccharides, release of active intermediates in secondary metabolism, and release of volatile aromatics to the environment [Morant et al., 2008; Ketudat Cairns and Esen, 2010; Ketudat Cairns et al., 2012]. However, the enzymes for few of these activities have been thus far identified in rice. Most of the work on rice GH1 beta-glucosidases has focused on their activity in break-down of cell wall oligosaccharides [Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006, 2010], with the only phytohormone beta-glucosidase so far identified at the molecular level being tuberonic acid beta-glucosidase (TAGG1 or Os4BGlu13, [Wakuta et al., 2010]), while no defensive β-glucosidases have been positively identified in rice.

Previously, analysis of the rice genomic sequences available in the public databases identified 40 genes, including 2 endophyte genes, 2 gene fragments, and 2 pseudogenes, with the remaining 34 being likely to encode endogenous rice β-glucosidases or related hydrolases [Opassiri et al., 2006]. Sequence-based phylogenetic analysis showed that these 34 genes can be grouped into 8 clusters (Figure 1). At the onset of this project, representatives of At/Os clusters 1, 4, and 7 had been characterized, with the largest remaining cluster without a representative being At/Os cluster 6, which is most closely related to soy bean hydroxyisoureate hydrolase (HIUH), among the enzymes that have thus been characterized. On the other hand, cluster 3 contains only one representative in rice, Os1BGlu4, which is clearly conserved with a single Arabidopsis homologue and proteins expressed in other plants for which large amounts of sequence data is available, however, none of these proteins has been characterized to date. In fact, Os1BGlu4 is the only cytoplasmic GH1 enzyme in rice, which suggests it may have an important role in basic or secondary metabolism. Therefore, understanding its function is important to understanding plant cell function. Although sequence based methods are very useful for producing proteins with known sequences, they cannot

always identify the enzyme with a specific function. Since gibberellins are important for regulating rice development, it is important to know what enzymes release gibberellins from their abundant glucoconjugates (glucosyl esters and glucosides) and, thereby regulate the levels of active phytohormone in the plant. Thus, we have set-out to synthesize the gibberellin $GA_3$ and $GA_4$ glucosyl esters and glucosides to use them to screen and determine which enzymes release the free gibberellin. However, only $GA_4$ (not $GA_3$, which is synthesized and used to treat plants) and its glucoconjugates have been identified in the plant, so with limited time and resources, we concentrates on the $GA_4$ glucosyl ester ($GA_4$-GE) Since gibberellins control seed germination, stem elongation and flower development, genetic manipulation of such enzymes might be critical for increasing rice productivity and successful response to stress.

Based on this, the three projects proposed here were meant to characterize representatives of two GH1 phylogenetic clusters in order to determine new functions, and to determine which enzyme(s) are responsible for the putative function of regulation of free gibberellin levels via release of $GA_4$ from $GA_4$-GE. Standard methods of protein purification, enzyme assays, and recombinant expression were be combined with synthesis of the novel substrate and recombinant protein expression to characterize these enzymes.

## 1.5. Benefits of the research and expected beneficiaries

### 1.5.1. Benefits in addressing the problems of the institution

This project benefited the university by helping to train 3 graduate students, 2 assistants and a postdoctoral fellow. It increased the research community, in addition to providing the research results for papers. Two papers resulting from this work have been published so far and this number should increase, as the projects are continued on longer term funding and other results are further analyzed and written up.

### 1.5.2. Generation of new knowledge

The roles of the enzymes characterized in this work were unknown, so the identification of new biochemical functions led to a better understanding of what they are doing in the plant. The identification further mutagenesis of Os9BGlu31 helped to show that the same catalytic groups seem to be required for transglucosidases as β-glucosidases, despite their different prefrences for reaction

products. The characterization of the Os1BGlu4 enzyme showed that it could hydrolyze some substrates not expected for a cytoplasmic enzyme, suggesting some previously unconsidered functions, such as internal break-down of oligosaccharides. Identifying Os3BGlu6 as a $GA_4$-GE β-glucosidasse, not only gave a new possible biological function, but also allowed us to use it as a model enzyme to look at the action of β-glucosidases in 1-O-acyl β-D-glucose ester hydrolysis. This knowledge can be applied for application of these enzymes to synthesize useful compounds, as well as improving our understanding of what GH1 enzymes are doing in the plant. This understanding of basic rice biology allows basic knowledge approach to rice breeding in the future.

### 1.5.3. Providing knowledge to the people

Aside from serving as a training project for graduate students, research assistants and research fellows, this project had provided important knowledge of the molecular function of rice, which is an important part of Thai culture. Discovery of this new knowledge attracts the attention the general citizens, allowing them to acquire new knowledge about how rice grows in various local forums, including the Thailand Research Expo organized by NRCT in the year 2012.

### 1.5.4. Production of knowledge for business, product production and product quality

Although this project did not produce a commercial product or an immediate means to one, the work sets the basis for improving rice production in the future, which will help agro industry. The knowledge gained in this project set can be used for production of higher quality rice seed in the future. During the project, it provided for high quality graduates of SUT. In addition, the transglucosidase enzyme is being explored for application to synthesis of bioactive glycosides that may be of use in the future.

### 1.5.5. Benefits to target groups

Plant scientists have benefit from this work by gaining new knowledge in the function of rice β-glucosidases and transglucosidases and their functions in rice growth and development. In the end, this knowledge will be supplemented with further discoveries and transferred to molecular breeders. This knowledge should allow improvement of rice varieties, which will benefit the farmers and the rice consuming public.

# CHAPTER II

## EXPERIMENTAL METHODS

The methods are described in detail in the appropriate project reports, so here we will simply describe briefly what was done.

### 2.1. Characterization of gibberellin glucosyl conjugate β-glucosidase

#### 2.1.1. Synthesis of gibberellin glucosyl conjugates

The $GA_3$-glucosyl ester ($GA_3$-GE) and $GA_4$-glucosyl ester ($GA_4$-GE) were synthesized essentially according to the method of Hiraga et al. (1974, Figure 2). Acetylated $GA_4$-GE was producedwith 43.7% yield (0.870 g), while acetylated $GA_3$-GE was obtained in 18.3% yield. The deacetylation proceded to give to yield 0.504 g of $GA_4$-GE (92.3% yield, 40.3% for the full synthesis), while $GA_3$-GE was obtained in 60% yield (11% yield for the full synthesis).
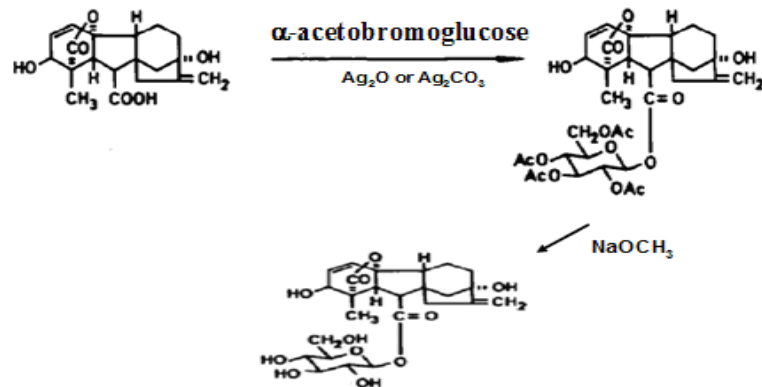


**Figure 2.** Reaction scheme for synthesis of gibberellin glucosyl esters (Hiraga et al., 1974).

Synthesis of the glucosides was begun with methyl esterification of the carboxyl groups of $GA_3$ and $GA_4$ by the method of Lombardi [1990], followed by glucosylation of the alcohols with $\alpha$-bromoglucose tetraacetate, and deblocking of the acetyl and methyl groups, as describe by Scheiber et al. [1969] (Figure 3). Starting from one millimole of $GA_3$-OMe or $GA_4$-OMe, the yield for tetra

acetylated β-D-glucopyranosyl gibberellin $A_3$ methyl ester (GA$_3$-OMe-Glc-Ac$_4$) was 54.3%, and for tetra acetylated β-D-glucopyranosyl gibberellin $A_4$ methyl ester (GA$_4$-OMe-Glc-Ac$_4$) was 50.0%. However, the deacetylation yields for β-D-glucopyranosyl gibberellin $A_3$ methyl ester (GA$_3$-OMe-Glc) and for β-D-gluco-pyranosyl gibberellin $A_4$ methyl ester (GA$_4$-OMe-Glc) were approximately 10%, so the overall yields were too low to continue to demethylation. The structures of the synthesized compounds were verified by LC-MS and NMR.
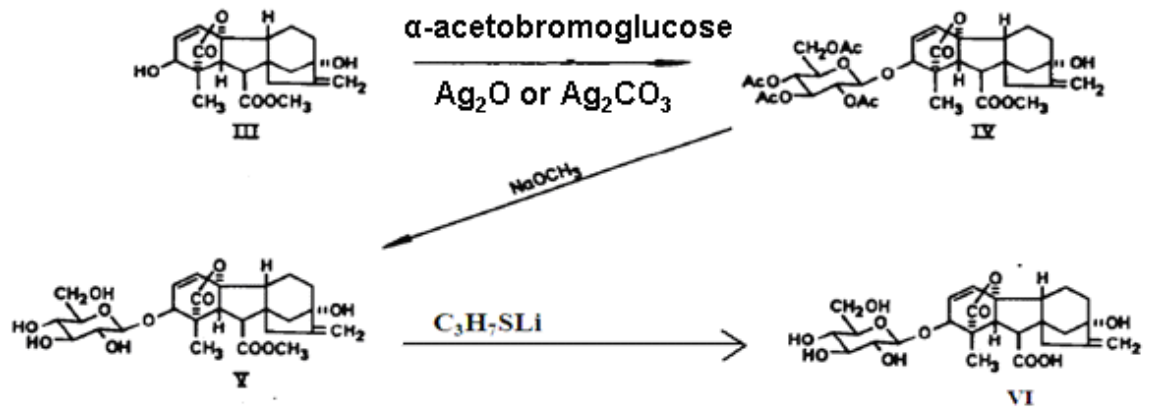


**Figure 3** Reaction scheme for synthesis of β-D-glucopyranosyl gibberellin methyl ester [Scheiber et al., 1969].

**2.1.2**. **Extraction, purification and characterization of β-glucosidase from rice**

Rice seeds, glumes, 7-day rice seedlings and roots were homogenized with McIlvaine buffer (0.1 M citric acid-0.2 M disodium hydrogen phosphate (Na$_2$HPO$_4$), pH 5.0, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C overnight. After removal of solids the protein was precipitated with 80% saturated ammonium sulphate [(NH$_4$)$_2$SO$_4$] and suspended in 4-fold diluted McIlvaine buffer, pH 5 (buffer A), and dialyzed overnight. The proteins were then fractionated with ConA sepharose chromatography, and Hi-Trap Q-sepharose anion exchange chromatography. The fractions were tested for hydrolysis activities toward *p*NPGlc and GA$_4$-Glc, and the fractions with the GA$_4$-Glc hydrolyzing activity were analyzed by SDS-PAGE to evaluate their complexity, and carried onto further purification steps (repeated anion exchange chromatography, since gel filtration, cation exchange chromatography and phenyl sepharose hydrophobic interaction chromatography did not

prove useful in initial trial runs).

The protein from the HiTrap Q-Sepharose column purification was separated on an 8% polyacrylamide SDS-PAGE gel, the main bands were exercised, chopped, washed, reduced with dithiothreitol, modified with iodoacetamide and digested with trypsin.  The tryptic peptides were assessed by nanoscale liquid chromatography electrospray ionization tandom mass spectrometry at the National Center for Biotechnology and Genetic Engineering (BIOTEC).  The results were evaluated with MASCOT.

### 2.1.3. Screening of rice GH1 enzymes for GA$_4$-glucosyl ester hydrolysis

Five glycoside hydrolase family 1(GH1) enzymes that have been expressed in our lab, Os3BGlu6 [Seshadri et al., 2009], Os3BGlu7 (BGlu1, [Opassiri et al., 2003]), Os4BGlu12 [Opassiri et al., 2006], Os3BGlu18 (Baiya et al., unpublished) and Os9BGlu31 [Luang et al., 2013] were tested for the hydrolysis activity to $p$NPGlc and GA$_4$-GE.

## 2.2. Characterization of Os1BGlu4 beta-glucosidase

### 2.2.1. Os1BGlu4 cloning and expression

Rice cDNA was prepared for *Os1blgu4* gene amplification. The PCR reaction amplified the full coding region with a 1:1 mixture of *Pfu* and *Taq* polymerases utilizing a 5' primer including the start codon and an *Nco*I site and a reverse primer including the stop codon and an *EcoRI* site.  Utilizing these restriction sites, the insert was cloned into pET32a(+). The recombinant plasmid (pET32a(+)*Os1blgu4*) was sequenced to confirm the correct reading frame and sequences.

To produce recombinant thioredoxin-Os1BGlu4 fusion protein, the plasmid pET32a(+) *Os1blgu4* was transformed into Origami B(DE3) *E. coli*, grown overnight at 37°C with shaking was and inoculated into LB broth containing the same antibiotics and then incubated at 37°C with shaking until the optical density at 600 nm reached 0.5-0.6. IPTG final concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5 mM were added to the culture flasks to induce the expression of recombinant protein. The cultures were cultured further at 20°C, 25°C and 30°C for different induction times of 4, 8, 12 and 16 h. The cells were collected by centrifugation at 4,000 x *g* for 10min at 4°C, then kept at -70°C until extraction.

The cell pellets were extracted as previously described [Opassiri et al., 2003]. The soluble protein was purified by immobilized metal affinity chromatography (IMAC) on BD TALON cobalt resin, equilibrated with equilibration buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0). The column was washed with 4 bed volumes of equilibration buffer, and then with 8 bed volumes of washing buffer (10 mM immidazole in an equilibration buffer). The bound protein fractions were eluted from a column with 4 bed volumes of elution buffer (250 mM immidazole in an equilibration buffer). The protein fractions were kept at 4°C. The protein contents of the fractions were evaluated on 15% SDS polyacrylamide gels, which were stained in Coomassie brilliant blue, as well as by assaying β-glucosidase activity with $p$NPGlc [Opassiri et al., 2003]. Protein concentrations were estimated with the Bio-RAD Bradford Coomassie staining assay, with bovine serum albumin as a standard.

### 2.2.2. Optimum pH and temperature and pH and temperature stability

To determine the pH optimum of the Trx-rOs1BGlu4 fusion protein enzyme, $p$NPGlc hydrolysis assays were set-up in buffers ranging from pH 3.5 to 10.5 (formate pH 3.5-4; sodium acetate pH 4.5-5.5; sodium phosphate pH 6-8.5; and CAPS pH 9-10.5), at 0.5 pH unit increments. The pH stability for the Trx-rOs1BGlu4 were determined by incubating the enzymes in buffers ranging from pH 4 to 10 as above at increments of 1.0 pH unit for 10 min, 1, 3, 6, 12 and 24 h at room temperature. After incubation, the enzyme was diluted 20 fold in 50 mM buffer at the optimum pH, pH 5.0, and the aliquots of enzyme were assayed for activity with 1 mM $p$NPGlc, and the products released were measured [Opassiri *et al*., 2003].

`The optimum temperature for enzyme activity was determined by assaying the Trx-rOs1BGlu4 with 1 mM $p$NPGlc in 50mM buffer, pH 5.0, at temperatures ranging from 5-90°C at $5^{\circ}$C increments for 20 min, and then stopping the reaction with one-half volume of 1 M $Na_2CO_3$ and measuring the absorbance at 405 nm (as described by Opassiri [2003]). Thermostability was evaluated by incubating the enzyme in 50 mM buffer at the optimum pH, at different temperatures in the range of 20-70°C at 10°C intervals for 15, 30, 45 and 60 min, then evaluating the activity in the standard assay 1 mM $p$NPGlc at $37^{\circ}$C for 20 min.

### 2.2.3. Evaluation of Os1BGlu4 enzyme specificity and kinetics study

The enzyme glycone specificity was evaluated by release of *p*-nitrophenol (*p*NP) liberated from the *p*NP-glycosides *p*NP-β-D-glucoside, *p*NP-β-L-fucoside, *p*NP-α-D-glucoside, *p*NP-β-D-cellobioside, *p*NP-β-D-mannoside, *p*NP-α-L-arabionoside, *p*NP-β-D-xyloside, *p*NP-β-D-fucoside, and *p*NP-β-D-galactoside, by the standard assay described for pNPGlc (pH 5, for 5 min at 37$^{o}$C). The Os1BGlu4 was also tested with polysaccharides, oligosaccharides, and glycosides. In the assay, 1-5 μg enzyme was incubated separately with 0.5% (w/v) laminarin and cellulose, including laminaripentaose, laminaritetraose, laminaritriose, laminaribiose, laminari, cellohexaose, cellopentaose, cellotetraose, cellotriose and cellobiose, or 1 mM concentrations of various glycosides at pH 5, 37$^{o}$C for 30-60 min. The reactions were stopped by boiling and the increase of glucose was measured colorimetrically by the peroxidase/glucose oxidase assay [Opassiri *et al*., 2003]. The products of Trx-Os1BGlu4 hydrolysis of cello- and laminari-oligosaccharides and glycosides were detected by TLC. The oligosaccharide reactions mixtures were spotted on silica-gel 60 F254 plates and chromatographed vertically with a solvent of ethylacetate, acetic acid and water (2:1:1, by volume). The carbohydrate products were detected by spraying with 10% $H_2SO_4$ in ethanol and baked at 120°C.

Kinetic parameters, $K_m$ and $V_{max}$ of purified Trx-Os1BGlu4 with *p*NP-glycosides and oligosaccharides were determined in triplicate reactions. The initial velocity of hydrolysis for each substrate was initially determined using various protein concentrations and incubation times (5-20 min) to find conditions that yield 0.1 to 1.0 absorbance units. The reactions containing buffer at 5-7 different substrate concentrations ranging from 0.1-4 fold the apparent $K_m$ value were pre-incubated at 37°C for 10 min, and then the reactions were started by adding enzyme. The reactions were incubated as above at 37°C for 5-15 min, depending on each substrate, to establish the initial velocity ($V_0$). The kinetic parameters were calculated by nonlinear regression of the Michaelis-Menten curves with the Grafit program.

The inhibition of Os1BGlu4 activity by many chemicals was studied with 1 mM *p*NPGlc as the substrate. Various inhibitors were mixed with substrate in 50 mM buffer, pH 5, followed by adding Os1BGlu4 β-glucosidase and incubated for 10 min at 37°C, then the reactions were stopped and processed as usual.

## 2. 3. Characterization of Os9BGlu31 transglucosidase

### 2.3.1. Protein expression plasmid production and protein expression

A 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 specific 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 plasmids were extracted, checked by restriction digest and recombinant plasmids that gave correct patterns were sent for automated sequencing (Macrogen Corp., Seoul, Korea).

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 lysed in 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, and insoluble components removed by centrifutation at 4°C for 15 min. Three steps were used to purify the Os9BGlu31 fusion protein. First, crude protein was purified with $CoCl_2$-equilibrated IMAC resin (GE Healthcare) with sequential washes of 5 mM, 10 mM, and 20 mM imidazole in equilibration buffer, followed by elution with 250 mM imidazole in equilibration buffer. Then, the recombinant protein was purified by anion exchange chromatography on a Q-Sepharose column eluted with a linear gradient of 0-0.5 M NaCl in 50 mM Tris-HCl, pH 8.0. The NaCl concentration of the Os9BGlu31 fraction pool was adjusted to 2 M, and it was onto a Phenyl-Sepharose column that 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, 20 mM Tris-HCl, pH 8.0.

### 2.3.2. pH optimum determination

To determine the pH dependence, the activity of Os9BGlu31 was measured with 5 mM *p*NPGlc 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 *p*NP released was quantified from the absorbance at 405 nm of its 4-nitrophenolate ion.

### 2.3.3. Enzymatic characterization of Os9BGlu31

The preference of Os9BGlu31 for glucose donors was evaluated by incubating 0.5 mM of glucose donor with 0.2 mM 4-hydroxybenzoic acid (*p*HB) as glucose acceptor with 1 μg of Os9BGlu31 in 50 mM citrate, pH 4.5, at 30°C. For 1-O-(4-hydroxybenzoyl)-β-D-glucose ester (*p*HBG) donor, 0.2 mM ferulic acid was used as the acceptor instead of *p*HB. After 10 min, the reactions were stopped by adding phosphoric acid to 1% final concentration. Transglycosylation products were separated by C18 reverse phase HPLC 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 were assayed with 0.2 mM glucose acceptor, 5 mM *p*NPGlc 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 *p*NP, so the released *p*NP was separated from other reaction components by C18 reverse phase HPLC and quantified with by its absorbance (peak area) at 360 nm. Relative activity toward *p*NP-glycosides was determined with 0.5 mM of *p*NP-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 2 M $Na_2CO_3$ and the *p*NP released quantified by the absorbance at 405 nm.

The $K_m$ and $V_{max}$ values of *p*NPGlc in the presence of various acceptors were determined by varying the concentration of *p*NPGlc in the range 2-30 mM with 0.2 mM glucose acceptor 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 *p*NPGlc in 50 mM citrate, pH 4.5. The release of *p*NP product was quantified as described above. The kinetic parameters were determined by

15

nonlinear regression of the Michaelis-Menten plots with the Grafit 5.0 computer program (Erithacus Software, Horley, UK).

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 $30^{\circ}$C for 10 min for EDTA and metal ions or 2 h for organic inhibitors. Activity was then assayed by incubating pretreated enzyme with 5 mM $p$NPGlc substrate at $30^{\circ}$C for 15 min. The reactions were stopped and released 4NP measured as described above.

### 2.3.4. Evaluation of gene expression

Rice (cv. Yukihikari) seeds were sterilized, soaked in water overnight, and germinated in the dark for 4 days at $27^{\circ}$C, then in a 12 h light-12 h dark cycle from day 4 to day 10 at $28^{\circ}$C on adsorbant paper moistened with sterile distilled water. Some 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.

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. 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. Total RNA was isolated from rice tissues by the method of Bachem et al. (1996).

Total RNA was denatured and electrophoresed on 1.5% formaldehyde–agarose gels and transferred onto nylon membrane by standard procedures [Sambrook et al. 1989]. A gene-specific probe for *Os9BGlu31* was amplified from a rice genomic DNA as the template with primers derived from the 3'-untranslated region of the gene and *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN). The *Os9BGlu31* probe was labeled with α-[$^{32}$P]dCTP (GE Healthcare) and was hybridized with RNA blots. The blots were then washed and exposed to a Fuji film imaging plate, which was developed with a Fuji Film BAS 1000 BioImaging Analyzer to show the radioactive gene-specific bands.

For quantitative real-time PCR, all samples were collected from greenhouse-grown *japonica* rice cultivar Dongjin. 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. Gene-specific primers were used for quantitative real-time PCR. 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. Changes in gene expression were calculated by the comparative cycle threshold ($\Delta\Delta$Ct) method.
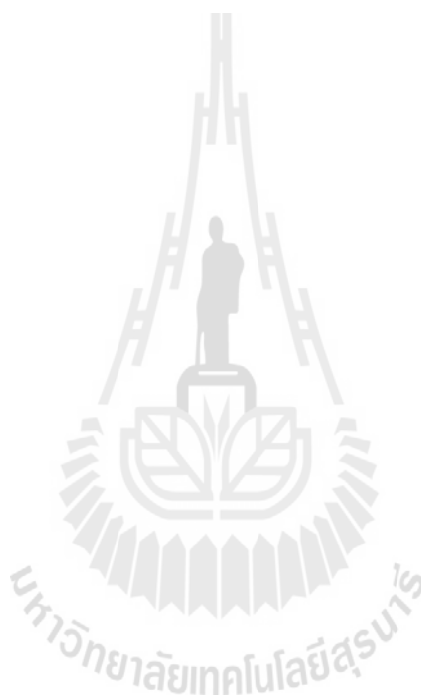
# CHAPTER III

# RESULTS AND DISCUSSION

## 3.1. Identification of rice gibberellin β-glucosidase

### 3.1.1. Syntheses of GA-glucosyl conjugates

Acetylated and deacetylated $GA_4$ Glc esters were synthesized as described in section 2.2.1. The acetylated and deacetylated $GA_4$-GE were obtained with 43.7% and 40.5% yields, respectively. The synthesized acetylated and deacetylated $GA_4$-GE structures were confirmed by NMR spectra The ^{1}H NMR was consistent with the published data for $GA_4$-GE [Hiraga et al., 1974]. The identity of the deacetylated $GA_4$-GE was also confirmed from its mass spectrum. In the positive mode, we detected $[M+Na]^+$ at m/z 517.1, $[M+H-H_2O]^+$ at m/z 477.2, and $[M+H-Glc]^+$ at m/z 315.5.

The structures of acetylated and deacetylated $GA_3$ Glc esters were confirmed by NMR and mass spectrometry. In the ^{1}H NMR spectra of the acetylated $GA_3$-Glc ester, the peak for the H1 proton on the glucosyl ring was located at 5.81 ppm, with a coupling constant of 7.8 Hz, which confirmed that the acetylated $GA_3$-Glc ester had a β-configuration. The deacetylated $GA_3$-Glc ester ($GA_3$-GE) was confirmed from its negative mode mass spectrum peaks, $[M+^{35}Cl]^-$ at *m/z* 543.4, $[M+^{37}Cl]^-$ at *m/z* 545.2 and $[M+HCO_2]^-$ at *m/z* 553.3. The β-configuration of the $GA_3$-Glc ester was confirmed from the peak for the anomeric H1 proton on the glucosyl ring, which was located at 5.55 ppm, with $J_{1,2}$=8.4 Hz. The ^{1}H NMR of $GA_3$-Glc ester was also consistent with the published data (Hiraga et al., 1974).

$GA_3$-OMe and its two glucosides (Figure 4) were synthesized and identified with mass and NMR spectrometry as described for the esters. $GA_3$-OMe was confirmed from its positive mode mass spectrum peaks: $[M+Na]^+$ at *m/z* 383.3 and $[2M+Na]^+$ at *m/z* 743.5. The ^{1}H NMR spectrum was consistent with $GA_3$-OMe. The two $GA_3$-OMe glucosides were separated by LC-MS (Figure 5), and their molecular masses were confirmed in their mass spectra. $[M+HCO_2]^-$ were found as the base peaks, *m/z* at 567.2, for both glucosides, although they had different fragment patterns.

When the ^{1}H NMR spectra for these two glucosides were compared with the spectrum of $GA_3$-OMe, differences were seen mainly for H2 and H17 on the skeleton of $GA_3$. HPLC peak 2 in Figure 5 was assigned as $GA_3$-OMe-3-O-Glc, since its chemical shift value for the peak of H2 was

higher than the one on $GA_3$-OMe and $GA_3$-OMe-13-O-Glc, because of the glucosyl linkage at carbon position 3, as shown in Figure 6.

Similarly, the structures of $GA_4$-OMe and $GA_4$-OMe-Glc were mainly confirmed by their negative mode mass spectra, in which the peaks for the $GA_4$-OMe and $GA_4$-OMe-Glc formate adducts $[M+HCO_2]^-$ were identified as the base peaks at $m/z$ 391.1 and 553.3, respectively.
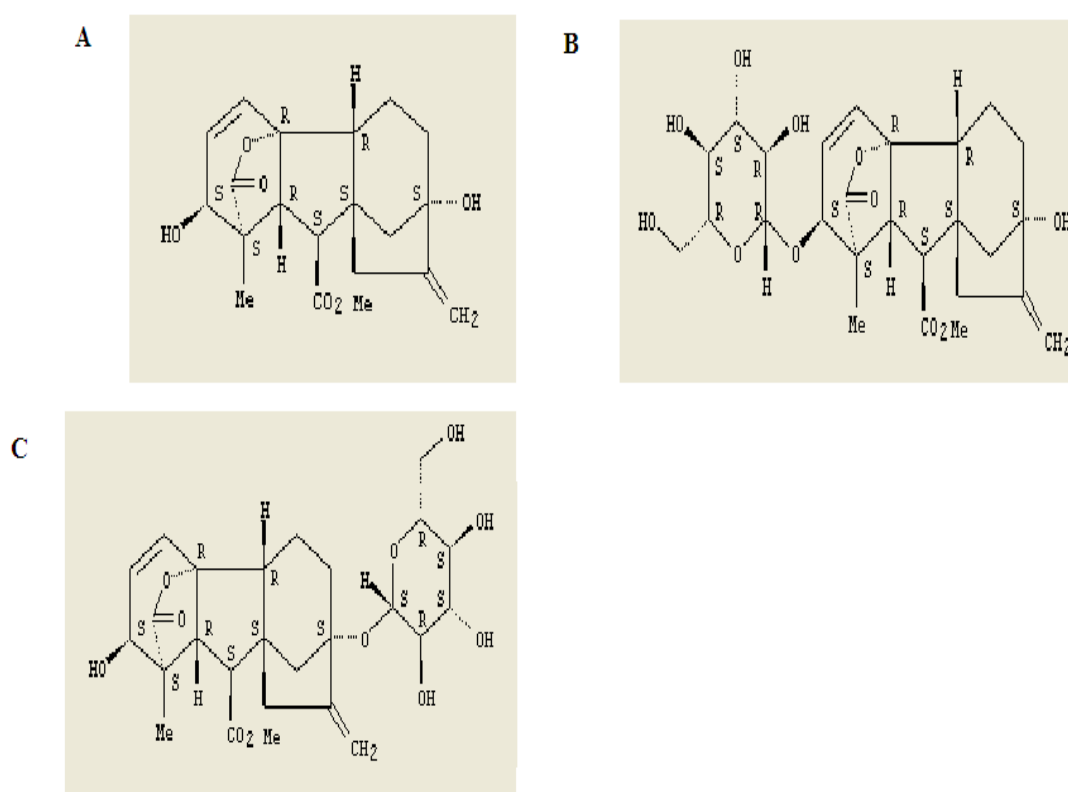


**Figure 4** The structures of the $GA_3$-OMe (A), $GA_3$-OMe-3-O-Glc (B) and $GA_3$-OMe-13-O-Glc (C).
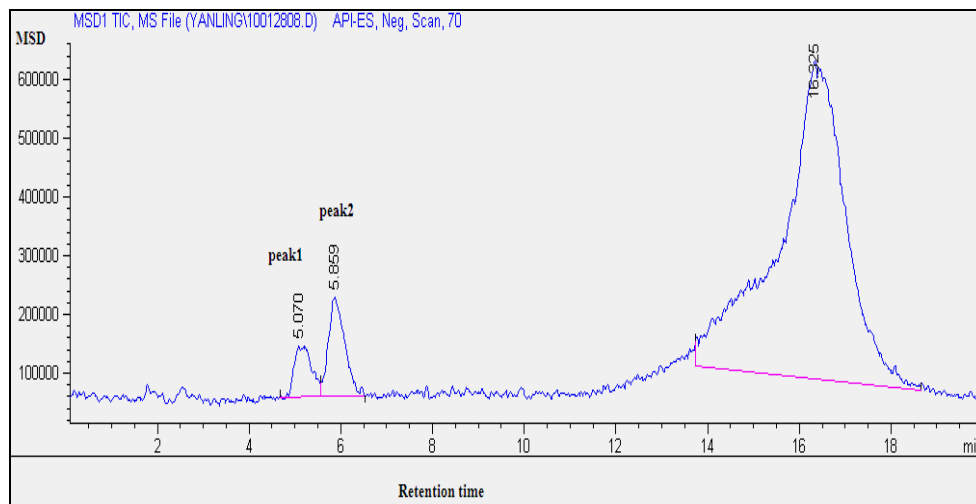
**Figure 5** The LC-MS chromatogram of the GA$_3$-OMe-glucosides. The compounds were separated on a ZORBAX Eclipse XDB-C18, 4.6*150 mm, 5 Micron column (Aglient). Peak 1 :GA-$_3$OMe-13-O-β-D-Glc; Peak 2 :GA-$_3$OMe-3-O-β-D-Glc.
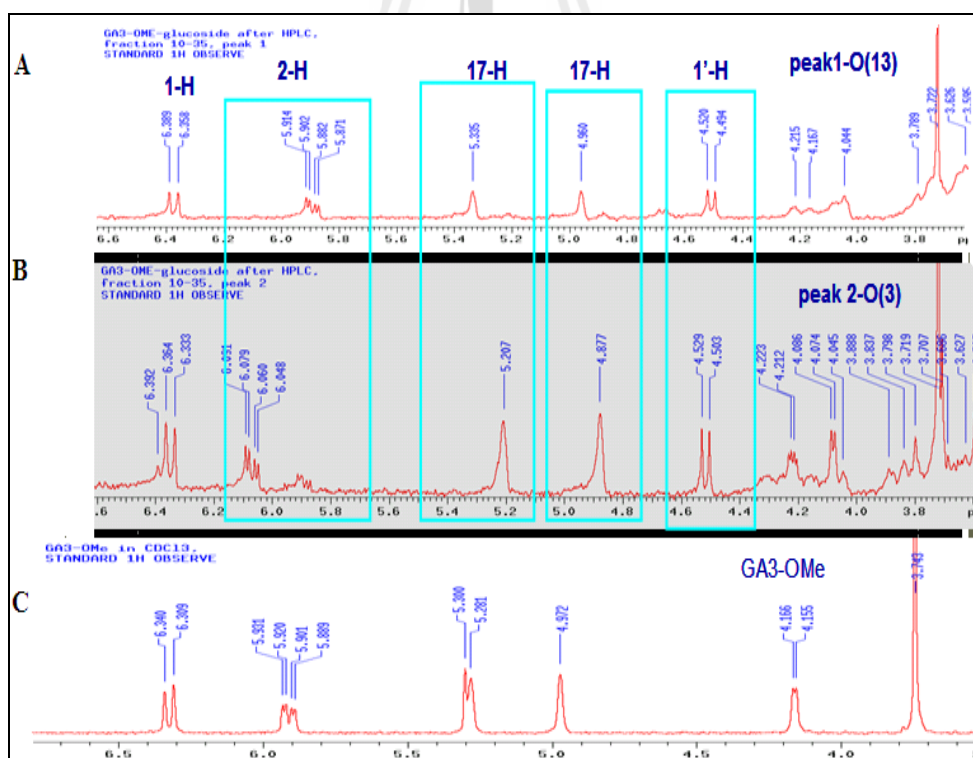


**Figure 6** Overlay of the NMR spectra of GA$_3$-OMe and its glucosides. A, peak 1, GA$_3$-OMe-13-O-Glc; B, peak 2, GA$_3$-OMe-3-O-Glc; C, GA$_3$-OMe.

### 3.1.2 Extraction of $GA_4$-glucosyl ester β-glucosidase from rice

The proteins were extracted from whole rice seeds, glumes, 7-day rice seedlings and roots, and then partially purified by ammonium sulfate precipitation and ion exchange chromatography. The β-glucosidase activities were found in the crude extracts and fractions from the ion exchange columns. The root extract was found to have the highest specific activity to $p$NPGlc, but the glume extract had the highest specific activity to the $GA_4$-GE (Figure 7).
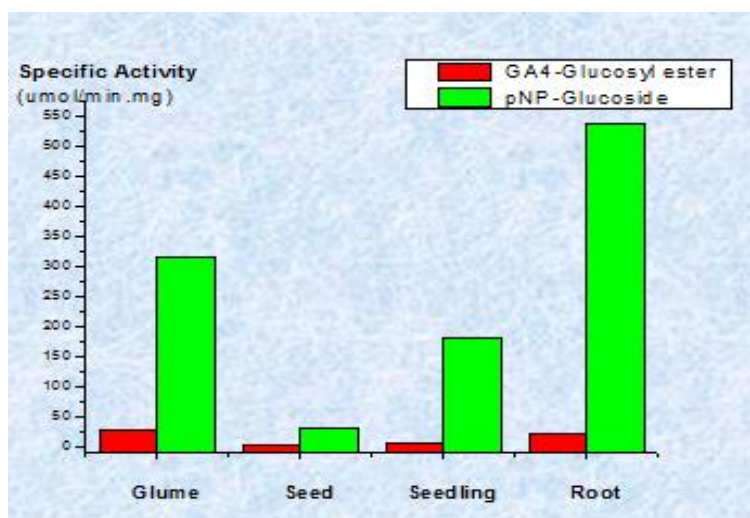


**Figure 7** The specific activities of different rice tissue extracts.

The crude protein extracted from glumes after $(NH_4)_2SO_4$ precipitation and dialysis with McIlvaine buffer was fractionated with a Con A-Sepharose column, and the eluate showed higher activities than flow-through and crude for hydrolyzing $p$NPGlc (Table 1).

**Table 1.** Specific activities of fractions before and after Con A column.

| Fractions | Total protein mg | Specific Activity *p*NP-Glucoside μmol/min.mg | Fold Purification |
|---|---|---|---|
| Extraction solution | 21,800 | $2.02*10^{-3}$ | 1 |
| Pellets, after dialysis | 3,137 | $1.69*10^{-2}$ | 8 |
| Protein after Con A column | 145 | $9.41*10^{-2}$ | 47 |

When the active fractions from the Con A-Sepharose column were concentrated, dialyzed and purified with a Q-Sepharose column, four peaks were detected in elution of the bound proteins. Although each peak contained activity to hydrolyze *p*NPGlc and $GA_4$-GE, peak 3 showed the highest activities to hydrolyze $GA_4$-GE (0.0244 μmol/min(mg protein) and 5.6% relative activity to $GA_4$-GE compared to *p*NPGlc) and peak 4 had the highest relative activity for hydrolysis of $GA_4$-GE compared to pNPGlc (9.6%, 0.0151 μmol/min(mg protein)), so these peaks were used for further purification. Since other purification methods did not result in increased specific activities, they were fractionated over further Q-sepharose columns, which gave similar profiles and somewhat higher specific activities in the third and fourth peaks. After a third Q-sepharose chromatography step on a smaller, higher resolution column, the peak fractions Q4Q4Q3 and Q4Q4Q4 had relatively high specific activities of 0.0795 and 0.087 mmol/min(mg protein), as shown in Table 2, although they were still not pure, as shown in the SDS-PAGE analysis in Figure 8.

**Table 2.** Specific activities of peak Q4Q4's fractions from Q sepharose FF 1 ml column.

| Fractions | Specific Activity *p*NP-Glucoside μmol/min.mg | Specific Activity $GA_4$-GE ester μmol/min.mg | Fold Purification | Relative Activity $GA_4$-GE/*p*NP-Glc (%) |
|---|---|---|---|---|
| Q4Q4 | 0.480 | $3.05*10^{-2}$ | 238 | 6.4 |
| Q4Q4Q3 | 1.28 | $7.95*10^{-2}$ | 634 | 6.2 |
| Q4Q4Q4 | 1.63 | $8.7*10^{-2}$ | 807 | 5.3 |
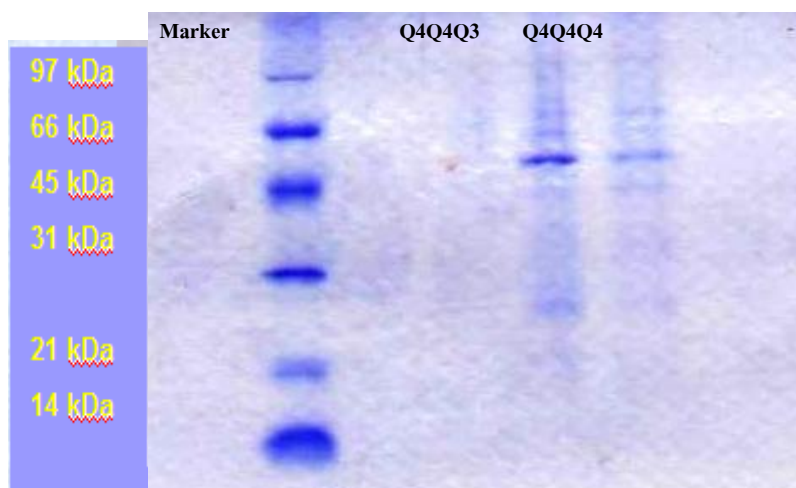
**Figure 8** SDS-PAGE of fractions from Q sepharose FF 1 ml column

The protein bands from **Q sepharose** column **fraction** were identified by LCMS of tryptic peptides generated from their SDS-PAGE gel bands. The MASCOT search of the Genbank non-redundant (nr) protein database **(Tables 3)** did not match any obvious β-glucosidase**s**.

### 3.1.3 Screening of rice GH1 enzymes for GA$_4$-glucosyl ester hydrolysis

Five rice GH1 enzymes that have been expressed in our lab were tested for the hydrolysis of $p$NPGlc and GA$_4$-GE. As shown in Table 4, Os3BGlu6 had the highest hydrolysis activity to GA$_4$-GE among these enzymes. Although Os9BGlu31 had a higher ratio of activity toward GA$_4$-GE compared to $p$NPGlc (0.267 vs. 0.07 for Os3BGlu6), it is primarily a transglycosidase [Luang et al., 2013] and has low activity toward both substrates.

**Table 3.** MASCOT search results for protein band Q4Q1.

| Name (NCBI gi) | % Probability | SDS-PAGE band | Description |
|---|---|---|---|
| 1. 125577329 | 17.7 | Q4Q1 | hypothetical protein OsJ 032760 *Oryza sativa* japonica cultivar group |
| 2. 125534579 | 17.6 | Q4Q1 | hypothetical protein OsI 035086 *Oryza sativa* indica cultivar group |
| 3. 108864437 | 16.2 | Q4Q1 | Glycosyl hydrolases family 38 protein expressed *Oryza sativa* japonica cultivar group |
| 4. 115485699 | 16.1 | Q4Q1 | Os11g0525600 *Oryza sativa* japonica cultivar group |
| 5. 113645215 | 16.1 | Q4Q1 | Os11g0525600 *Oryza sativa* japonica cultivar group |
| 6. 77551210 | 16.1 | Q4Q1 | Glycosyl hydrolases family 38 protein expressed *Oryza sativa* japonica cultivar |
| 7. 115435074 | 15.6 | Q4Q4Q3 | Os01g0196600 *Oryza sativa* japonica cultivar group |
| 8. 14209591 | 15.6 | Q4Q4Q3 | putative nucleotide diphosphatase *Oryza sativa* Japonica Group |
| 9. 125569379 | 15.6 | Q4Q4Q3 | hypothetical protein OsJ 000719 *Oryza sativa* japonica cultivar group |
| 10. 113531826 | 15.6 | Q4Q4Q3 | Os01g0196600 *Oryza sativa* japonica cultivar group |
| 11. AF245483.1 | 15.6 | Q4Q4Q3 | OSE4 *Oryza sativa* |
| 12. | 15.6 | Q4Q4Q3 | nucleotide pyrophosphatase precursor |
| 13. 125524775 | 6.51 | Q4Q4Q3 | hypothetical protein OsI 000736 *Oryza sativa* indica cultivar group |
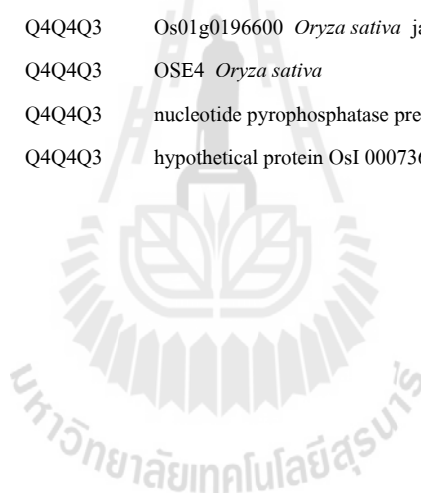
**Table 4.** GA$_4$-GE hydrolysis by recombinantly expressed rice GH1 enzymes.

| Enzyme | Activity toward GA$_4$-GE (µmol Glc released/min/mg) | Activity toward $p$NPGlc (µmol $p$NP released/min/mg) | Ratio of activity toward GA$_4$-GE/$p$NPGlc |
|---|---|---|---|
| Os3BGlu6 | 0.185 | 2.6 | 0.07 |
| Os3BGlu7 (BGlu1) | 0.02 | 4.0 | 0.005 |
| Os4BGlu12 | 0.035 | 130 | 0.003 |
| Os4BGlu18 | N.D. | 0.94 | - |
| Os9BGlu31 | 0.02* | 0.075* | 0.267 |

N.D. means not detectable.

* Activity is primarily transglycosylation, rather than hydrolysis [Luang et al., 2013].

## 3.2. Characterization of Os1BGlu4

### 3.2.1. Recombinant protein expression conditions

The coding sequence of *Os1bglu*4 gene was amplified from 14 day rice seedlings cDNA library, and cloned into the pET32a(+) plasmid to generate pET32a(+)*Os1bglu*4 for expression of Os1BGlu4 in *E. coli*. The *E.coli* strain Origami B(DE3) appeared to give best expression and the expression in these cells was optimized by varying the IPTG concentration, induction time and induction temperature. Although the amount of cells from 10$^{\circ}$C and 20$^{\circ}$C treatments were lower than that of 30$^{\circ}$C, the crude extract had higher 'activity' at the lower temperatures (Figure 9), so 20$^{\circ}$C was selected as the best temperature for expression. This phenomenon was also observed in the expression of Os3BGlu6 and Os3BGlu7 [Seshadri *et al.* 2009; Opassiri et al., 2003]. This is likely caused by the higher percentage of insoluble protein which were not properly folded due to fast expression at higher temperature. At 20$^{\circ}$C, β-glucosidase activity in the crude extracts was similar from 12-24 h at 0 to 0.5 mM IPTG concentration, so the protein was expressed without IPTG at this temperature for further studies.

3.2.2. **Recombinant protein extraction and purification**

The recombinant Trx-His6-rOs1BGlu4 was purified by IMAC on BD Talon$^{TM}$ (immobilized cobalt) metal affinity resin to obtain approximately 85% pure protein. And an intense band at 66 kDa was observed on SDS-PAGE (Figure 9). After concentrated and changing the buffer, the concentration of Trx-His6-rOs1BGlu4 was 3.4 mg/mL. Approximately 2.8 mg of purified Trx-His6-rOs1BGlu4 could be obtained per liter of bacterial expression culture. The purified Trx-His6-rOs1BGlu4 was cut by the enterokinase and the recombinant Os1BGlu4 (rOs1BGlu4) which is about 55 kDa, and the thioredoxin tag were released (Figure 9). The size of rOs1BGlu4 by experimental estimate is almost identical to the predicted molecular weight (55.3 kDa). After a second IMAC purification step, the pure rOs1BGlu4 was obtained as a single band on SDS-PAGE. After concentrating and changing the buffer of the rOs1BGlu4, the concentration was 2.2mg/mL. This rOs1BGlu4 was aliquotted and kept in -20$^{o}$C and used to characterize the biochemical properties.
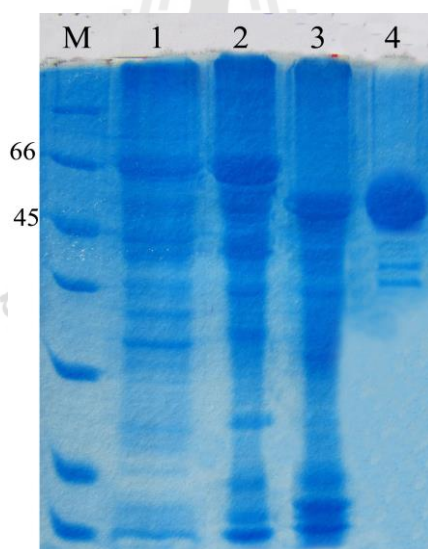


**Figure 9**          SDS-PAGE profiles of Trx-His6-rOs1BGlu4 recombinant protein at different steps of purification. M, standard protein marker (Bio-RAD); Lane 1, crude Trx-His6-rOs1BGlu4; lane 2, Trx-His6-rOs1BGlu4 purified over one IMAC step; lane 3, Trx-His6-rOs1BGlu4 digested with enterokinase; lane 4, purified rOs1BGlu4 after second IMAC step.

**3.2.3.** Characterization of Os1BGlu4 enzymatic activity

rOs1BGlu4 was most active at pH 6.5, with similarly high activity from pH 6 to 7 (Figure 10). rOs1BGlu4 had almost no activity when the enzyme was assayed in buffers with pH below 5.0, and its

activity also decreased dramatically at pH above 8.0. The pH optima of most β-glucosidases ranged between pH 4 and 7.5, depending on their source and cellular location [Ketudat-Cairns and Esen 2010]. The pH optimum of the purified rOs1BGlu4 was pH 6.5, which is reasonable even it is different to many others β-glucosidases (Os3BGlu8, pH 5.0, Os7BGlu26, pH 4.5, Os3BGlu7, pH 5.0, rHvBII, pH 4.0) [Hrmova *et al.* 1998, Opassiri *et al.* 2003, Kuntothom et al., 2009]. This pH is related to the pH environment of the catalytic reaction, since Os1BGlu4 was predicted to localize to the cytoplasm, the pH of which should be near neutral pH 7 [Berrin *et al.* 2002].
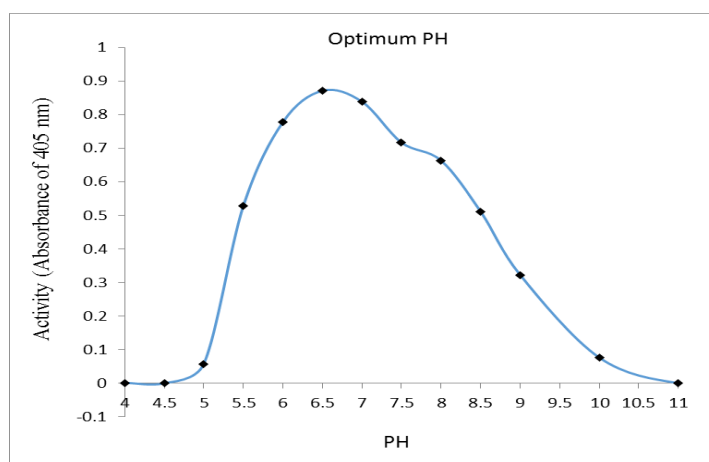


**Figure 10** The activity versus pH profile for rOs1BGlu4 over the pH range of 4.0-11. The buffers used were formate pH 3.5-4.0; sodium acetate pH 4.5-5.5; sodium phosphate pH 6.0-7.5; Tris pH 8.0-9.5; and CAPS pH 10.0-11.0. rOs1BGlu4 (0.25 μg) was assayed with 1 mM *p*NPGlc in different 50 mM pH buffers at $30^{\circ}$C for 10 min.

The temperature optimum for rOs1BGlu4 was determined by incubating the enzyme for hydrolysis of 1 mM *p*NPGlc for 10 min was $45^{\circ}$C. The enzyme was stable at $30^{\circ}$C and maintained 80% of its activity at $40^{\circ}$C for up to 60 min, but rapidly lost activity at $50^{\circ}$C. Since it is a reasonable physiological temperature for rice, 30°C was used as the standard incubation temperature.

The activity of the purified rice rOs1BGlu4 β-glucosidase towards natural and artificial glycosides was characterized. Hydrolysis of *p*NP-glycosides with different glycone moieties was used to assess glycone specificity of rOs1BGlu4, as summarized in Table 5. Among the artificial *p*NP-glycosides, rOs1BGlu4 hydrolyzed *p*NPGlc most rapidly, and *p*NP-β-D-fucopyranoside was hydrolyzed at 82% of the rate of *p*NPGlc. rOs1BGlu4 hydrolyzed *p*NPGlc ($k_{cat}/K_m$, 17.92, $s^{-1}$ $mM^{-1}$) and *p*NP-β-D-fucopyranoside ($k_{cat}/K_m$, 9.34, $s^{-1}$ $mM^{-1}$) with high efficiency. rOs1BGlu4 hydrolyzed *p*NP-β-

D-galactopyranoside, *p*NP-β-D-cellobioside, *p*NP-α-L-arabionopyranoside, *p*NP-β-D-mannopyranoside and *p*NP-β-D-xylopyranoside and at 4.3%, 3.4%, 2.4%, 1.8% and 1.0% the rate of *p*NPGlc, respectively, based on *p*NP relase. Hydrolysis of *p*NP-α-D-glucopyranoside, *p*NP-α-L-fucopyranoside, *p*NP-N-acetyl-β-D-glucosami- nide, *p*NP-β-D-maltoside, *p*NP-β-L-fucopyranoside and 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside was not detectable. A similar ability to hydrolyze pNP-glycosides has been reported in other rice β-glucosidases [Opassiri et al., 2003, 2006, 2007; Seshadri et al., 2009; Kuntothom et al., 2009].

**Table 5** Relative activities of purified rOs1BGlu4 in the hydrolysis of *p*NP-derivatives.

| No. | Substrate | Activity[a] (μmole/min/mg) | Relative activity[b] (%) |
|-----|-----------|-----------------------------|---------------------------|
| 1 | *p*NP-β-D-glucopyranoside | 5.69 | 100 |
| 2 | *p*NP-β-D-fucopyranoside | 4.6 | 80.9 |
| 3 | *p*NP-β-D-galactopyranoside | 0.25 | 4.32 |
| 4 | *p*NP-β-D-cellobioside | 0.19 | 3.39 |
| 5 | *p*NP-α-L-arabinopyranoside | 0.14 | 2.40 |
| 6 | *p*NP-β-D-mannopyranoside | 0.1 | 1.80 |
| 7 | *p*NP-β-D-xylopyranoside | 0.06 | 1 |
| 8 | *p*NP-α-D-galactopyranoside | n.d[c]. | n.d. |
| 9 | *p*NP-α-D-mannopyranoside | n.d. | n.d. |
| 10 | *p*NP-β-L-arabinopyranoside | n.d. | n.d. |
| 11 | *p*NP-β-D-maltoside | n.d. | n.d. |
| 12 | *p*NP-α-L-fucopyranoside | n.d. | n.d. |
| 13 | *p*NP-N-acetyl-β-D-glucosaminide | n.d. | n.d. |
| 14 | 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyranoside | n.d. | n.d. |
| 15 | *p*NP-α-D-galactopyranoside | n.d. | n.d. |

[a] The assay contained 1 mM substrate in 50 mM sodium phosphate, pH 6.5, at $30^{\circ}$C

[b] Percentage activity relative to *p*NP released from *p*NP-β-D-glucopyranoside.

[c] n.d. means not detected

The relative activity of rOs1BGlu4 in hydrolysis of various kinds of oligosaccharides is summarized in the Table 6. The rOs1BGlu4 hydrolyzed the β-1,3-linked oligosaccharide laminaribiose and laminaritriose, but not laminaritetraose and laminaripentaose, and β-1,4-linked oligosaccharides cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose were hydrolyzed at different rates. The rOs1BGlu4 did not hydrolyze chitopentaose and the β-1,6-linked disaccharide gentiobiose. rOs1BGlu4 showed high hydrolytic efficiency with β-(1, 3)-linked oligosaccharides with DP of 2-3. The hydrolysis rates toward while those of cellotriose and cellotetraose are similar, about 70% of the rate of laminaribiose, while those for cellopentaose and cellohexaose were about 28% of the rate of laminaribiose. In comparison, cellobiose was the most poorly hydrolyzed substrate.

Cello-oligosaccharides and laminari-oligosaccharides are released from cell wall polysaccharides by endoglucanases and are likely to be hydrolyzed by β-glucosidases which may be involved in cell-wall related processes. It was unexpected that Os1BGlu4, which was predicted to be localized in the cytoplasm, can hydrolyze the cello-oligosaccharides and laminari-oligosaccharides.

**Table 6** Relative activities of purified rOs1BGlu4 in the hydrolysis of oligosaccharides.

| No. | Substrate | Activity[a] (µmole/min/mg) | Relative activity[b] (%) |
|---|---|---|---|
| 1 | Laminaribiose | 1.81[c] | 100.00 |
| 2 | Laminaritriose | 1.50 | 82.77 |
| 3 | Laminaritetraose | n.d.[d] | n.d. |
| 4 | Laminaripentaose | n.d. | n.d. |
| 5 | Cellobiose | 0.10 | 5.52 |
| 6 | Cellotriose | 1.27 | 69.96 |
| 7 | Cellotetraose | 1.39 | 76.91 |
| 8 | Cellopentaose | 0.53 | 29.22 |
| 9 | Cellohexaose | 0.48 | 26.61 |
| 10 | Gentiobiose | n.d. | n.d. |
| 11 | Chitopentaose | n.d. | n.d. |

[a] The assay contained 1 mM substrate in 50 mM sodium phosphate, pH 6.5, at 30 °C

[b] Percentage activity relative to hydrolysis of laminaribiose.

[c] The released glucose of laminaribiose and cellobiose was divided by 2, since one cut produced two glucose molecules.

[d] n.d. means not detected.

The activity of rOs1BGlu4 was tested with available natural glycosides and evaluated for hydrolysis on TLC, as shown in Table 7. The result indicated that salicin, esculin and *para*-coumaryl alcohol glucoside (*p*CAG) can be hydrolyzed by rOs1BGlu4 (Figure 15). As judged from the TLC plate, the glucose released from the esculin at the identical time is more than that from salicin and *p*CAG. That suggested that esculin can be hydrolyzed more efficiently than salicin and *p*CAG.

**Table 7** Activity of rOs1BGlu4 on available natural substrates.

| No. | Substrate | Glucose | No. | Substrate | Glucose |
|---|---|---|---|---|---|
| 1 | Salicin | + | 14 | n-octyl-β-D-glucoside | - |
| 2 | Esculin | + | 15 | $GA_4$ glucose ester | - |
| 3 | Linamarin | - | 16 | α-lactose | - |
| 4 | D-amygdalin | - | 17 | Sinigrin monohydrate | - |
| 5 | Trans-zeatin glucoside | - | 18 | Maltose | - |
| 6 | Daidzin | - | 19 | Metyl-β-D-glucopyranoside | - |
| 7 | Genistin | - | 20 | Arbutin | - |
| 8 | Naringin | - | 21 | Palatinose | - |
| 9 | Queretin-3-glucoside | - | 22 | mangiferin | - |
| 10 | *p*-coumaryl alcohol glucoside | + | 23 | lactulose | - |
| 11 | Coniferin | - | 24 | epigenin-7-glucoside | - |
| 12 | Indoxyl-β-D-glucoside | - | 25 | uridine | - |
| 13 | n-hepty-β-D-glucoside | - | | | |

'+' stands for glucose was detected, '-' stands for glucose was not detected

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) for rOs1BGlu4 hydrolysis of various *p*NP-β-D-glycosides are summarized in Table 8. *p*NPGlc can be hydrolyzed by rOs1BGlu4 with the $k_{cat}/K_m$ value 17.9 $s^{-1}mM^{-1}$. *p*NP-β-D-fucoside can be hydrolyzed about 2-fold less efficiently than *p*NPGlc by rOs1BGlu4. However, the $K_m$ value of *p*NP-β-D-fucopyranoside and *p*NPGlc were similar at about 0.71±0.02 mM, and the $k_{cat}$ value of *p*NPGlc is 2-fold that of *p*NP-β-D-fucopyranoside. The *p*NP-β-D-cellobioside was hydrolyzed more slowly, with the catalytic efficiency ($k_{cat}/K_m$) value 3.92 $s^{-1}$ $mM^{-1}$in terms of *p*NP release, although this likely reflects two hydrolytic steps. *p*NP-β-D-mannoside, *p*NP-α-L-

arabinopyranoside and $p$NP-β-D-galactopyranoside were hydrolyzed very slowly, with similar $k_{cat}/K_m$ value about 0.5 s$^{-1}$ mM$^{-1}$. The $p$NP-α-L-arabinopyranoside has the lowest $K_m$ value, that means this substrate easily saturates rOs1BGlu4, but is not rapidly turned over.

**Table 8** Apparent kinetic parameters of rice rOs1BGlu4 in the hydrolysis of $p$NP-derivatives.

| Substrate | $k_{cat}$ (S$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (S$^{-1}$ mM$^{-1}$) |
|---|---|---|---|
| $p$NP-β-D-glucoside | 12.76±0.18 | 0.71±0.02 | 17.92 |
| $p$NP-β-D-fucoside | 6.61±0.080 | 0.71±0.02 | 9.34 |
| $p$NP-β-D-cellobioside | 2.06±0.062 | 0.53±0.03 | 3.92 |
| $p$NP-α-L-arabinoside | 0.52±0.0043 | 0.38±0.02 | 0.43 |
| $p$NP-β-D-galactoside | 3.16±0.065 | 7.33±0.32 | 0.43 |
| $p$NP-β-D-mannoside | 1.25±0.025 | 2.24±0.03 | 0.56 |

The kinetic parameters of rOs1BGlu4 hydrolysis of oligosaccharides are summarized in Table 9. rOs1BGlu4 hydrolyzed laminaribiose most efficiently, with the $k_{cat}/K_m$ value of 12.45 s$^{-1}$mM$^{-1}$, followed by the cellotetraose, the $k_{cat}/K_m$ value is 8.73s$^{-1}$mM$^{-1}$. Laminaritriose, cellotriose, cellopentaose and cellohexaose are hydrolyzed in order of decreasing efficiencies. Cellobiose was hydrolyzed most slowly, with the $k_{cat}/K_m$ value only 0.03 s$^{-1}$mM$^{-1}$. In contrast, laminaribiose can be hydrolyzed 415-fold more efficiently than cellobiose. This behaviour is fairly similar to Os4BGlu12, which is thought to be a cell wall β-glucosidase, but also hydrolyzes phytohormone glycosides [Opassiri et al., 2010, Himeno et al., 2013].

The kinetic parameters of rOs1BGlu4 enzyme in the hydrolysis of esculin were determined. The hydrolysis product of esculin was esculetin, a yellow compound, with the absorbance at 405 nm, do it could be assayed similarly to pNPGlc. The kinetic parameters for Os1BGlu4 hydrolysis of esculin were $k_{cat}$ equal to 2.13±0.04 s$^{-1}$, $K_m$ equal to 0.55±0.02 mM and the $k_{cat}/K_m$ equal to 3.86 s$^{-1}$mM$^{-1}$. This indicated that esculin was hydrolyzed about 3.8 fold less efficiently than $p$NPGlc.

**Table 9** Apparent kinetic parameters of rOs1BGlu4 in the hydrolysis of oligosaccharide.

| Substrate | $k_{cat}$ (S$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (S$^{-1}$ mM$^{-1}$) |
|---|---|---|---|
| Laminaribiose | 4.67±0.09 | 0.38±0.02 | 12.45 |
| Laminaritriose | 3.37±0.55 | 0.6±0.03 | 5.63 |
| Cellobiose | 0.58±0.01 | 19.0±0.5 | 0.03 |
| Cellotriose | 2.74±0.06 | 0.59±0.03 | 4.64 |
| Cellotetraose | 2.27±0.03 | 0.26±0.01 | 8.73 |
| Cellopentaose | 2.15±0.03 | 1.07±0.04 | 2.01 |
| Cellohexaose | 1.08±0.02 | 1.1±0.05 | 0.99 |

As shown in Table 10, compounds that could strongly inhibit rOs1BGlu4 included $HgCl_2$, delta-glucono-lactone, $FeCl_3$, and $CuSO_4$. $HgCl_2$, delta-glucono- lactone, $FeCl_3$ were able to inhibit almost 100% of the hydrolysis activity of rOs1BGlu4 at 10 mM. Besides these, 1% SDS also had strong inhibitory effect on rOs1BGlu4. The salts $PbCl_2$ and $CuSO_4$ also have relatively strong inhibitory effects. EDTA, $CoSO_4$, KCl, $CaCl_2$, and $MnSO_4$ had no inhibitory effects on the hydrolysis activity of rOs1BGlu4. The rest of the chemicals tested had inhibitory effects to the hydrolysis activity of rOs1BGlu4 ranging from 38% to 10%. $HgCl_2$ is a very strong inhibitor to the hydrolysis activity of rOs1BGlu4, when the concentration deceased to 0.05 mM, the inhibitory effect was still 100%. The activity recovered to 18% when the concentration was diluted to 0.01 mM. For the delta-glucono-lactone, 62% of the activity was recovered when it was diluted to 0.1 mM.

**Table 10** The activity of rOs1BGlu4 in the presences of 10 mM inhibitors.

| No. | Inhibitor | Activity | Relative activity remaining (%) |
| --- | --- | --- | --- |
| 1 | Control | 8.1 | 100 |
| 2 | $HgCl_2$ | 0 | 0 |
| 3 | Delta-glucono-lactone | 0.02 | 0 |
| 4 | $FeCl_3$ | 0.12 | 2 |
| 5 | 1% SDS | 0.43 | 5 |
| 6 | $CuSO_4$ | 2.49 | 31 |
| 7 | $PbCl_2$ | 4.16 | 51 |
| 8 | L-Arabinose | 4.96 | 61 |
| 9 | Imidazole | 5.92 | 73 |
| 10 | Urea | 5.91 | 73 |
| 11 | D-Mannose | 5.91 | 73 |
| 12 | LiCl | 5.88 | 73 |
| 13 | $NiSO_4$ | 6.4 | 79 |
| 14 | $ZnCl_2$ | 6.36 | 79 |
| 15 | D-Glucosamine | 6.82 | 84 |
| 16 | D-Xylose | 6.9 | 85 |
| 17 | $CdCl_2$ | 6.98 | 86 |
| 18 | $MgCl_2$ | 7.16 | 88 |
| 19 | D-Glucose | 7.29 | 90 |
| 21 | $CaCl_2$ | 7.8 | 96 |
| 22 | KCl | 7.88 | 97 |
| 23 | L-Histidine | 8.01 | 99 |
| 24 | $MnSO_4$ | 8.11 | 100 |
| 25 | $CoSO_4$ | 8.16 | 101 |
| 26 | EDTA | 8.35 | 103 |

The reactions contained 1 mM $p$NPGlc in 50 mM sodium phosphate, pH 6.5, at 30°C. The same reaction without the inhibitor was used as the control.

### 3.3. Characterization of Os9BGlu31

### 3.3.1. Os9BGlu31 is primarily a transglucosidase

Initially, Os9BGlu31 fusion protein expressed in *E. coli* was found to have weak activity toward *p*NPGlc 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 11).
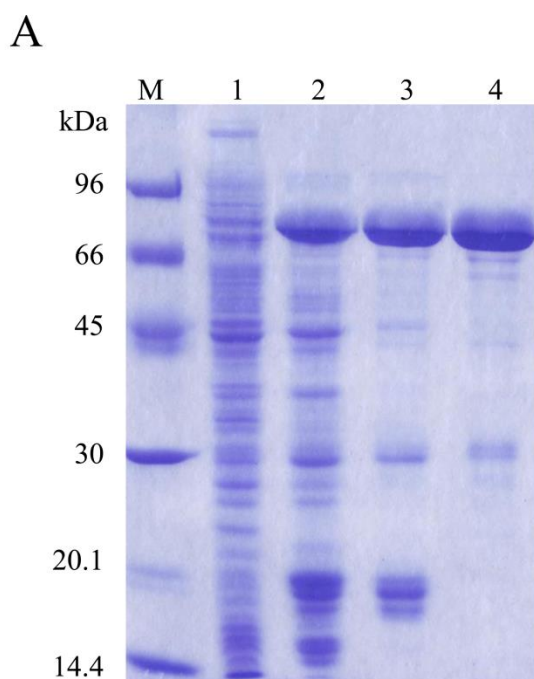


**Figure 11.** SDS-PAGE analysis of purification of Os9BGlu31 fusion protein expressed in *E. coli*. Three-step purification for kinetics: soluble protein extract of induced Origami B (DE3) cells (lane 1), and Os9BGlu31 purified via immobilized metal ($Co^{2+}$) affinity chromatography (IMAC; lane 2), Q-Sepharose chromatography (lane 3) and Phenyl-Sepharose chromatography (lane 4). Bio-Rad low molecular weight markers are shown for comparison (lane M).

When we set-up a pH profile with overlapping buffer solutions, we found that the acetate buffer had much higher activity than other buffers (Figure 12A).  When the products of reactions in various buffers were run on thin layer chromatography, a transglycosylation product with a mobility slightly lower than *p*NPGlc was seen in the acetate buffer (Figure 12B).  Given the retaining mechanism of GH1 enzymes, this was expected 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 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 13). The release of $p$NP 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.
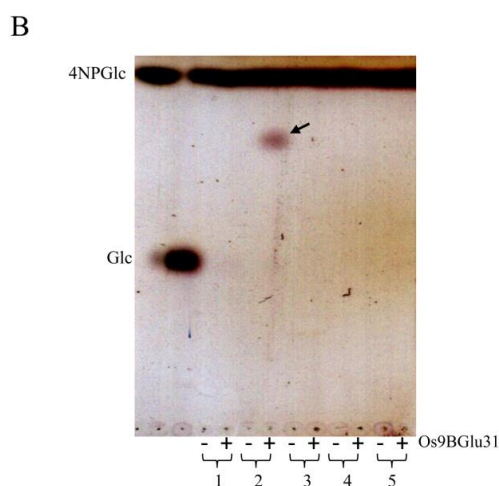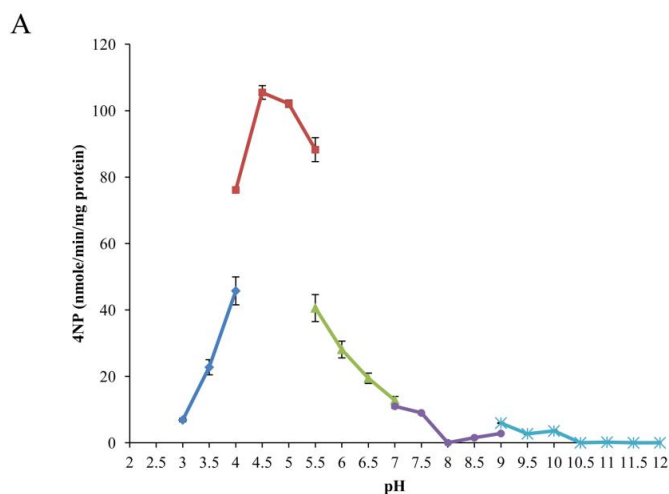
**Figure 12.** Identification of Os9BGlu31 as a transglucosidase. The pH profile of Os9BGlu31 release of 4NP from 4NPGlc in a series of overlapping 50 mM pH buffers: citrate pH 3.0-4.0 (♦), acetate pH 4.0-5.5 (■), MES pH 5.5-7.0 ( ▲ ), Tris-HCl pH 7.0-9.0 (●), and sodium phosphate pH 9.0-12.0 (*) is 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.
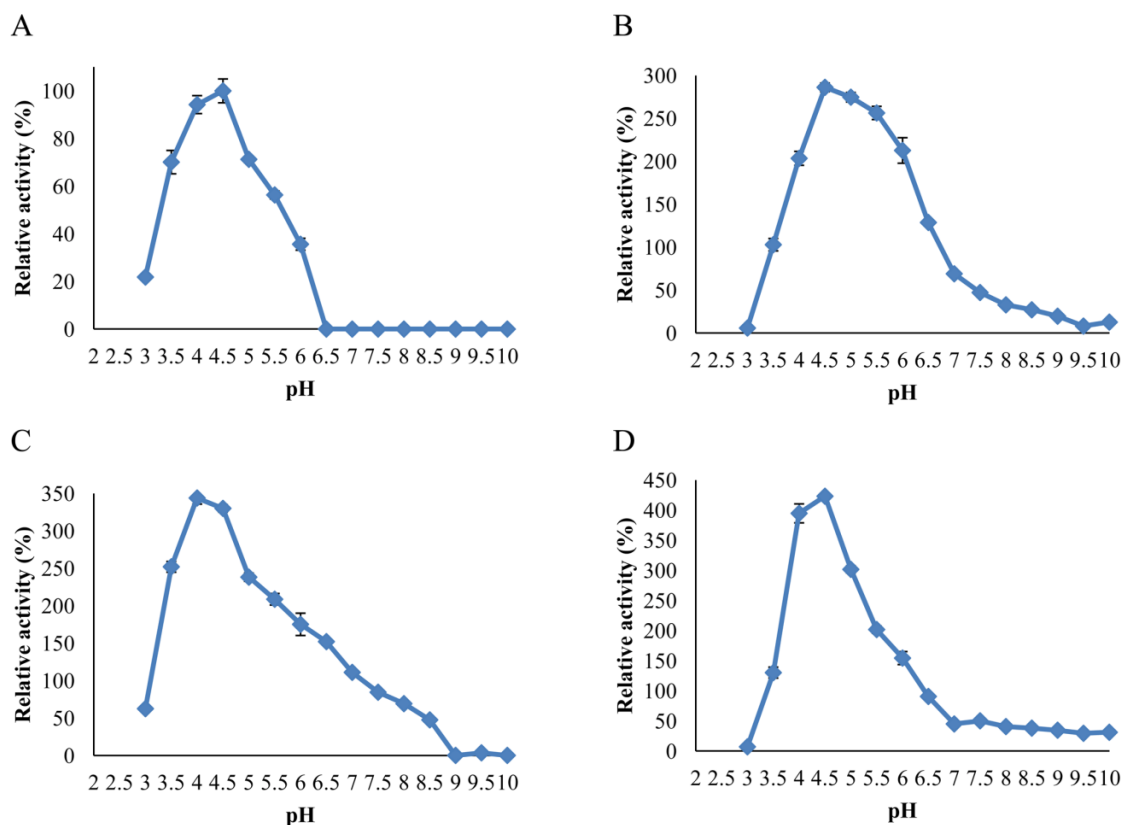
**Figure 13.** Profile of Os9BGlu31 activity over the pH range of 3.0‑10.0. The hydrolysis of 5 mM 4NPGlc was monitored in citrate/phosphate buffer alone (A), or in the presence of 5 mM azide (B), 5 mM formate (C) or 5 mM acetate (D) by recombinantly expressed Os9BGlu31.

### 3.3.2. Characterization of Os9BGlu31 transglucosidase activity

The glycone (sugar) specificity of Os9BGlu31 was determined by transfer from $p$NP-glycosides to $p$-hydroxybenzoic acid ($p$HB). Os9BGlu31 transferred glucose from pNPGlc better than β-D-fucose and β-D-xylose from their respective $p$NP-glycosides (Table 11), and could not use $p$NP-β-D-galactoside, $p$NP-β-D-mannoside, $p$NP-β-D-N-acetylglucosaminide, $p$NP-β-D-glucuronide, $p$NP-α-D-glucoside, $p$NP-α-D-galactoside or $p$NP-α-L-arabinoside as substrates.

In addition to $p$NP-glycosides, Os9BGlu31 used phenolic glucose esters as donor substrates with highest activity to 1-$O$-β-D-feruloyl-glucose (FG), 1-$O$-β-D-$p$-coumaroyl-glucose ($p$CG), 1-$O$-β-D-4-hydroxybenzoyl-glucose (4HBG), 1-$O$-β-D-sinapoyl-glucose (SG), and 1-$O$-β-D-vanillyl-glucose (VG) (Table 11). 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.

**Table 11**. Relative activities of Os9BGlu31 on various donors.

| Donor | Relative activity (%) |
|---|---|
| *Natural 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 |
| Gibberellin $A_4$-glucose | 12.5 ± 0.5 |
| Quercetin 3-β-D-glucoside | nd |
| Naringin | nd |
| Arbutin | nd |
| Gossypin | nd |
| Trans-Zeatin glucoside | nd |
| Uridine 5'-diphosphoglucose | nd |
| Esculin | nd |
| *p*NP-*glucosides and synthetic glucosides:* | |
| *p*NP-β-D-glucoside | 3.8 ± 0.05 |
| *p*NP-β-D-fucoside | 2.3 ± 0.03 |
| *p*NP-β-D-xyloside | 1.8 ± 0.03 |
| *p*NP-β-D-galactoside | nd |
| *p*NP-β-D-mannoside | nd |
| *p*NP-α-D-glucoside | nd |
| *p*NP-α-D-galactoside | nd |
| *p*NP-β-D-glucosiduronide | nd |
| *p*NP-α-L-arabinoside | nd |
| *p*NP-N-acetylglucosaminide | nd |
| *o*NP-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 (*p*-coumary alcohol glucoside and coniferin), and cyanogenic glucosides (linamarin, (D)-amygdalin, and dhurrin) as glucose donors. 'nd' indicates 'not detected'.

The transglucosylation activity of Os9BGlu31 determined with a range of potential acceptors is summarized in Table 12. On TLC, spots of $p$NP and a single transglycosylation product were seen in each case, except esculetin had two transglycosylation products. One esculetin product migrated to a position similar to escu, which appeared to be esculin (esculetin-6-O-β-D-glucoside), and 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 $p$NP from the donor more than the buffer alone.

HPLC quantification of $p$NP released from $p$NPGlc allowed the apparent kinetic parameters of acceptor substrates for transglucosylation to be determined, as shown in Table 13. At high concentrations (>0.5 mM), most of the phenolic acids that acted as acceptors displayed substrate inhibition. 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 $p$-coumaric acid (33.3 mM$^{-1}$s$^{-1}$), due to its low $K_m$, followed by ferulic acid (25.4 mM$^{-1}$s$^{-1}$) (Table 13). In terms of phytohormones, 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.

**Table 12**. Relative activities of Os9BGlu31 on various acceptors.

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

**Table 13**. Kinetic parameters of Os9BGlu31 on as aromatic acid acceptors.

| Acceptor | Kinetic parameters for Acceptor at 30 mM pNPGlc | | | Kinetic parameters for pNPGlc 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 |
| 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 |
| p-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 |
| p-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 |
| 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'.

The effects of several metal ions, EDTA, and β-glucosidase inhibitors on Os9BGlu31 activity are shown in Table 14. No significant 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 highest effect on Os9BGlu31, decreasing activity by approx. 90% at 1 mM. 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, and the β-glucosidase transition state mimic inhibitors glucono δ-lactone, 1-deoxy-nojirimycin, isofagomine and phenylethyl glucoimidazole. These inhibitors can completely inhibit GH1 β-glucosidases at the concentrations used, but had no effect on Os9BGlu31 [Ketudat Cairns and Esen, 2010; Ketudat Cairns et al., 2012].

**Table 14.** Effects of metal salts, EDTA, 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 |
| *Beta-Glucosidase inhibitors:* | | |
| Conduritol B epoxide | 1 mM | 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 |

### 3.3.3. Analysis of gene expression

*Os9BGlu31* expression was analyzed by northern blotting by 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 14A). 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 14B). 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 14C). 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 14D shows that ethephon appears to induce the gene in roots of seedlings, but not in shoot and endosperm.

Real-time RT-PCR was used to further explore expression pattern of *Os9BGlu31* at various stages in various tissues throughout rice development (Figure 15). 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, suggesting that *Os9BGlu31* may have a function in mature and senescent leaf tissues.

Based on the activity, the Os9BGlu31 is a transglucosidase that can transfer glucose between various phenolic acids and alcohols and their glucoconjugates in the vacuole of different rice tissues, since it was localized to the vacuole [Luang et al., 2013]. The expression pattern showed that this action may be important in senescing flag leaves, where ferulic acid and its glucoconjugate are expected to be high, as well as in developing seeds. In the developing seeds, it is possible that phytochemicals, such as gibberellins and auxins that are needed for germination may be the recipients of the glucosyl groups, although the donors are not clear currently. The enzyme expression is found to react to stresses, such as drought, ABA and ethylene, suggesting it may be involved in releasing or reconjugating the ABA released under these conditions. Similarly, it was induced by auxins, in which case it may modulate their effects by transferring glucosyl moieties onto the glucose high concentrations of auxins.

A

**6-week-old mature plants**   **10-day-old seedlings**

Flower | Leaf blade | Leaf sheath | Node | Stem | Root | Endosperm | Shoot (leaf) | Root



B

0  0.5  1  2  4  6  8  10  day



C

H₂O (27°C) | Cold (5°C) | Cold (12°C) | Drought | NaCl | Flooding | ABA | Ethephon | Methyljasmonate | GA₃ | 2,4-D | Kinetin



D

H₂O   **10 mM Ethephon**

Shoot | Root | Endosperm | Shoot | Root | Endosperm
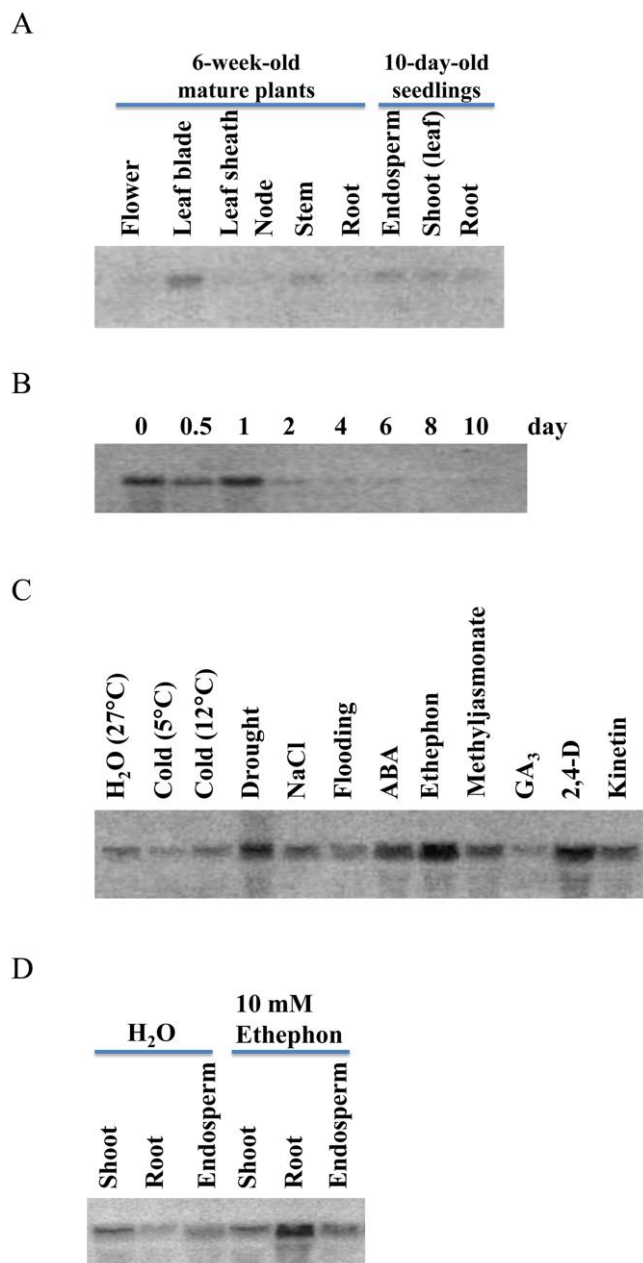


**Figure 14**. Analysis of *Os9BGlu31* gene expression in rice cv. Yukihikari. (*A*) Northern blot of RNA extracted from tissues of 6-week-old mature plants or 10-day-old rice seedlings. (*B*) Total RNA was extracted from whole seeds or seedlings grown for the indicated time periods. (*C*) Effects of stresses and phytohormones on *Os9BGlu31* expression in 10-day old rice seedlings treated for an additional 2 days with: H$_2$O (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 tissues of 10-day old rice seedlings. As in part (*C*), rice seedlings were treated 2 days with water or 10 mM ethephon.
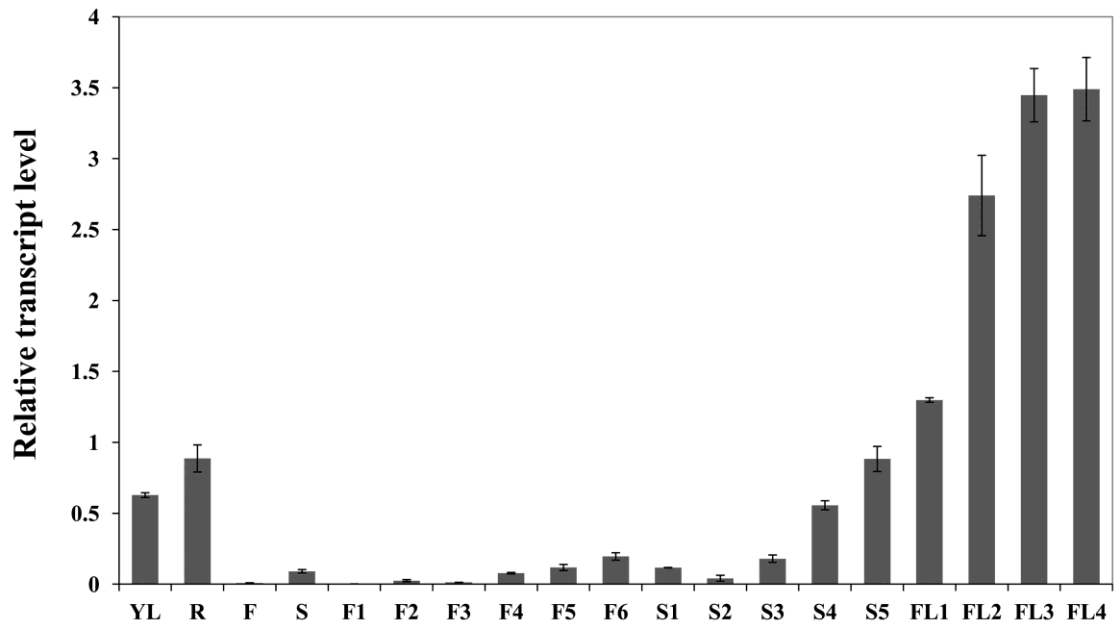
**Figure 15.** Expression pattern of the *Os9BGlu31* gene in rice cv. Dongjin, determined by real-time RT-PCR. The relative expression level of *Os9BGlu31* was compared with that of *OsUBQ5* and error bars indicate standard deviations from three separate experiments. YL, young leaf; FL, flag leaf; R, 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.

**CHAPTER IV**

**CONCLUSION**

This set of research projects investigated 3 aspects of glycoside hydrolase family GH1 enzyme function in rice. Together, these projects gave insight into the functions of GA-GE beta-glucosidase in rice, the activity and function of the cytoplasmic β-glucosidase isoenzyme Os1BGlu4, and the function of a rice GH1 transglucosidase, Os9BGlu31.

In the first project, the glucoconjugates of $GA_3$ and $GA_4$ were synthesized and the $GA_4$-GE was used to screen both rice extract fractions and recombinant rice GH1 enzymes for its hydrolysis. This resulted in the identification of Os3BGlu6 as a rice GH1 enzyme with relatively high hydrolysis activity toward $GA_4$-GE, and this activity was characterized further with mutants and structural analysis in a later project [Hua et al., 2013]. Although $GA_4$-GE hydrolysis activity was seen in extracts of various rice tissues, the purification from rice bran and glumes could not generate a pure protein or one that was readily identified as a likely β-glucosidase by LCMSMS analysis of tryptic fragments. Nonetheless, the techniques and substrate developed could be used more successfully in a subsequent project to extract the proteins from rice seedlings.

In the second project, the recombinant thioredoxin-Os1BGlu4 (Trx-His6-rOs1BGlu4) fusion protein was functionally expressed in Origami B(DE3) and expression conditions optimized. The rOsBGlu4 enzyme had an optimum pH for the hydrolysis of 6.5, and optimum temperature was 45°C (in a 10 min assay), but assays were done at 30°C, at which the enzyme was most stable. rOs1BGlu4 efficiently hydrolyzed β-(1, 3)-linked oligosaccharides with DP of 2 and 3, and β-(1,4)-linked oligosaccharide with DP of 3 and 4, while cellooligosaccharides of DP 5 and 6 were hydrolyzed less well, and cellobiose was hydrolyzed poorly. It is not clear why a cytoplasmic β-glucosidase hydrolyzes cell wall oligosaccharides, but this opens up new aspects of study, possibly related to recovery of cell wall sugars for breakdown or further use. The rOs1BGlu4 was found to hydrolyze pNPGlc most efficiently, although it could also hydrolyze pNP-fucopyranoside and glycosides of other sugars less efficiently. The enzyme could also hydrolyze the natural glycosides salicin, esculin and *p*-CAG, suggesting it might be acting on salicylic acid glucoside and similar compounds involved in acquired resistance. The hydrolysis activity of rOs1BGlu4 was strongly inhibited by $HgCl_2$, delta-glucono-lactone and $FeCl_3$, while other compounds had lesser effects. Although this work did not

result in any clear answers as to what the function of Os1BGlu4 is in the plant, it provided some clues that it might help recycle oligosaccharides and perhaps act on cytosolic salicylate glucosides.

The third project identified Os9BGlu31 as a transglucosidase that transfers glucose between 1-O-acyl glucose esters and certain glycosides and their aglycones [Luang et al., 2013]. One of the best substrates as a donor was feruloyl β-D-glucose, and its aglycone, ferulic acid (ferulate) acted as one of the best acceptors, along with other cinnaminic acids. The enzyme also transfers glucose to and from phytohormones and their esters, including auxins, gibberellin, and abscissic acid, as well as certain flavonoids, such as apigenin 7-O-glucoside. Many of the acceptor substrates appeared to exhibit substrate inhibition at high concentrations, as might be expected for an enzyme exhibiting a ping-pong mechanism. However, several standard mechanism based inhibitors of GH1 β-glucosidases did not inhibit Os9BGlu31. The presence of Os9BGlu31 in seedling tissues, especially in the first day of germination, and its induction in 10-day-old seedlings by auxin, ABA, ethylene and drought, suggested it may be involved in phytophormone metabolism in germination and stress response. It is also highly expressed in developing seed and mature flag leaves, suggesting other roles, perhaps related to feruloyl β-D-glucose, one of its best substrates, which is found in mature leaves. Further work will be necessary to truly understand what roles it is playing in the plant and the implications for glucoconjugate metabolism in grasses in general.

Overall, these three projects have made a significant contribution to our understanding of the roles of GH1 enzymes in rice, as well as their possible applications. They have shown the gibberellins and other phytohormones in rice can be glucosylated and deglucosylated by GH1 enzymes, with the 1-O-acyl glucosyl esters being the particular glycol-conjugates being most relevant to this work. The work also suggested that a cytoplasmic β-glucosidase may contribute to oligosaccharide turnonver, suggesting the plant cells may take oligosaccharides from cell wall break down into the cells to recover the glucose. Further work will be necessary to understand the full significance of these findings for the enzymes' functions, but the current work has already contributed to 2 international paper publications, with another manuscript in preparation, as well as several meeting presentations, and the training of 3 graduate students, several research assistants and a postdoctoral fellow. Two projects are currently following up these results. Therefore, we feel that this one-year seed project was quite successful in production of results and stimulating further scientific investigations.

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

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

## PUBLICATIONS AND PRESENTATIONS

1. **International Journal Publications**

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. *Arch. Biochem. Biophys.* 537(1): 39-48.

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.

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.

Ketudat Cairns, J.R., 2011. Protein-carbohydrate interactions leading to hydrolysis and transglycosylation in plant enzymes belonging to glycoside hydrolase family 1. Japanese Applied Glycoscience Society Meeting on Alpha-Amylases and Related Enzymes. Hokkaido University, Sapporo, Hokkaido, Japan; 28-30 Sept., 2011. Special Invited Foreign Lecture.

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.

Chen, R., Ruamkuson, D., Imsoonthornruksa, S. and Ketudat-Cairns, M. (poster presentation) Expression of Rice Os1BGlu4 β-glucosidase in *Escherichia coli*  Proceeding of the 23rd Annual Meeting and International Conference of the Thai Society for Biotechnology  Imperial Queen's Park Hotel Bangkok Thailand 1-2 Feb 2012 pp 94-95

Chen, R. and Ketudat-Cairns, M. (oral presentation). Amplification of Rice Os1BGlu4 Beta-Glucosidase cDNA and Expression in *Escherichia coli* Proceeding of the 3rd Graduate conference Suranaree University of Technology 21-23 November 2010

Chen, R. and Ketudat-Cairns, M. (oral presentation). Amplification of Rice Os1BGlu4 Beta-Glucosidase cDNA and Expression in *Escherichia coli* Proceeding of the 3rd Graduate conference Suranaree University of Technology 21-23 November 2010

# Curriculum Vitae (ประวัติผู้วิจัย)

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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 : ระบุ สถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัย ในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

    7.1    Journal Publications

7.1.1.    Cairns JR, Williamson MK, Price PA. 1991. Direct identification of $\gamma$-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 $\gamma$-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).

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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.

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.

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

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.

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

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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.

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.

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.

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.

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.

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.

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.

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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.

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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.

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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.

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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.

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.

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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.

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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.  2013. Structural analysis and insights into glycon specificity of the rice GH1 Os7BGlu26 β-D-mannosidase. *Acta Crystallographica Section D* **D69** (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 $\beta$-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 $\beta$-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 และ สถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วงแล้วประมาณร้อยละเท่าใด)

7.4.1. Structure, function and application of plant $\beta$-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.

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

2. <u>Head of Subproject 1</u>

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      **June, 1986: Bachelor of Science**, Department of Chemistry, Nankai University, Tianjin, P.R.China

   6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

                Instrumental analysis on GC, GC-MS/MS, NMR, FT-IR, HPLC, LC-MS, FPLC; multi-step synthesis and structure analysis of organic compounds; extraction of natural product; purification and characterization of proteins, enzyme's activity assay;

   7. Research Experience (ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย  หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น)

      7.1. Journal Publications

        **(1)** Hua Y, Sansenya S, Saetang C, Wakuta S,  Ketudat Cairns JR. *Enzymatic and structural*

*characterization of hydrolysis of gibberellin A4 glucosyl ester by a rice β-D-glucosidase.* Archives of Biochemistry and Biophysics **537**(1) (2013) 39-48.

**(2)** 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. *Os9BGlu31 is a transglucosidase with the capacity to equilibrate phenolpropenoid, flavonoid and phytohormone glycoconjugates.* Journal of Biological Chemistry **288**(14) (2013) 10111- 10123.

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

**(4)** R. Opassiri, Y.L. hua, O. Wara-Aswapati, T.Akiyama, J.Svasti, A.Esen, J.R.Ketudat Cairns, *β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice Bglu1,* Biochemical Journal **379** (2004) 125-131.

**(5)** A.E. Flood, P. Pantaraks, W. Monkaew, Y.L. Hua, *A Study of the Mutarotation Reaction in Solutions of Glucose and Fructose.* Proceedings of the Regional Symposium on Chemical Engineering 1999, November 22-24, Songkhla, Thailand.

**(6)** S.S. Chen, Y.L. Hua, Z.P. Lei, X.K. Yao, The Reaction of 6,6-Dialkylfulvenes with Thienyllithium -- Synthesis and Molecular Structure of Thienyl-Cyclopentadienyl Titanium and Zirconium Derivatives, Progress in Natural Science **1** (1991) 544.

**(7)** S.S. Chen, Y.L. Hua and Z.P. Lei, Steric Effect of the Reaction of 6,6-Dialkylfulvenes with Alkyllithium and Metal Lithium -- Synthesis of Substituted Titanocene Derivatives, Progress in Natural Science **2** (1992) 143.

7.2. Projects as Head of Project or Project Set (หัวหน้าโครงการวิจัย) : None.

7.3. Completed Projects as Head (งานวิจัยที่ทำเสร็จแล้ว : ชื่อแผนงานวิจัย และ/หรือ โครงการวิจัย ปีที่พิมพ์ การเผยแพร่ และสถานภาพในการทำวิจัย) None

7.4. Current Projects as head (งานวิจัยที่กำลังทำ : ชื่อแผนงานวิจัย และ/หรือโครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยลุล่วง แล้วประมาณร้อยละเท่าใด) None

## 3. Head of Subproject 2

### Mariena Ketudat-Cairns

**Position:**     Associate Professor

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**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 Assistant, UCSD

1989-1994    Teaching Assistant, UCSD

1990-1995    Teaching Assistant 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**