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รายงานการวิจัย

กลูโคซิลทรานส์เฟอร์เรสในข้าวที่มีศักยภาพในการควบคุมฮอร์โมนพืช และการสังเคราะห์ไกลโคไซด์

Characterization of rice glucosyl transferases with potential for phytohormone regulation and glycoside synthesis.

ได้รับทุนอุดหนุนการวิจัยจาก

มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

ตุลาคม 2560

บทคัดย่อ

ไกลโคไซด์ไฮโดรเลสแฟมิลี่ที่ 1 (GH1) ประกอบด้วยบีตา-กลูโคซิเดสซึ่งสามารถย่อยอนุพันธ์ของกลูโคสได้หลากหลาย และทรานส์กลูโคซิเดสซึ่งย้ายกลูโคสจากโมเลกุลหนึ่งไปยังโมเลกุลอื่น จากการศึกษาก่อนหน้าและในการศึกษานี้ Os9BGlu31 เป็นเอนไซม์จากข้าวที่แสดงกิจกรรมเหมือนทรานส์กลูโคซิเดส ทั้งนี้ วิธีการ 2 วิธี ได้นำมาใช้เพื่อหาสารตั้งต้นทางชีวภาพสำหรับ Os9BGlu31 วิธีที่ 1 สารสกัดจากพืชข้าวด้วยแอลกอฮอล์ถูกใช้เป็นตัวรับในปฏิกิริยาทรานส์กลูโคซิเลชั่นในปฏิกิริยาที่ใช้ Os9BGlu31 เป็นตัวเร่งปฏิกิริยา และสารประกอบที่ได้จากปฏิกิริยาดังกล่าวมีการจัดจำแนกโดยทำให้บริสุทธิ์ และวิเคราะห์โดย mass spectrometry (MS) และ nuclear magnetic resonance (NMR) วิธีที่ 2 สกัดสารจากพืชข้าวที่มีการแทรก T-DNA ในยีน Os9BGlu31 และเปรียบเทียบสารเมแทบอไลท์ที่สกัดได้กับสารสกัดจากพืชข้าวที่ไม่มีการแทรก T-DNA โดยวิธี LCMSMS เมื่อนำข้อมูลจากวิธีการทั้งสองมาแปลผล เราสามารถแสดงให้เห็นว่าเอสเทอร์ของกรดไขมัน, 1-O-oleoyl β-D-glucose และ 1-O-linoleoyl β-D-glucose สามารถทำหน้าที่เป็นผลิตภัณฑ์หรือสารตั้งต้นของ Os9BGlu31 และสร้างในพืชข้าวที่ขาด Os9BGlu31 นอกจากนี้เรายังได้ทำต้นข้าวกลายพันธุ์ที่บริเวณรอบๆ บริเวณเร่งปฏิกิริยาของ Os9BGlu31 เพื่อติดตามว่าเราจะสามารถเปลี่ยนกิจกรรมของ Os9BGlu31 เป็นไฮโดรเลสหรือไม่ และเพื่อทำความเข้าใจพื้นฐานของกิจกรรมทรานส์กลูโคซิเดส แม้ว่าไม่มีต้นข้าวกลายพันธุ์ที่ให้กิจกรรมไฮโดรไลซิสสูงกว่ากิจกรรมทรานส์กลูโคซิเดส แต่การกลายที่ W243N (Tryptophan residue 243 to Asparagine) ทำให้ได้เอนไซม์ที่มีกิจกรรมทรานส์กลูโคซิเดสสูงขึ้นและมีความสามารถในการใช้สารตั้งต้นได้หลากหลายกว่า Os9BGlu31 จากต้นข้าวสายพันธุ์ดั้งเดิม นอกจากนี้ เรายังสามารถระบุจำนวนของไกลโคไซด์และบิส-ไกลโคไซด์ของ flavonol kaempferol ที่สร้างขึ้นโดย Os9BGlu31 W243N โดยอาศัยข้อมูลตามรูปแบบการกระจายตัวของมวลสารสเปกโตรมิเตอร์ งานวิจัยนี้ชี้ให้เห็นว่า Os9BGlu31 สามารถนำมาใช้ในการผลิตสารประกอบต่างๆ รวมทั้ง phytohormone glucoconjugates เพื่อใช้ในห้องปฏิบัติการและนักวิจัยอื่นที่ศึกษาในด้านนี้ การทำงานเพิ่มเติมเกี่ยวกับการโคลนและการแสดงออกของเอนไซม์ GH1 Cluster At / Os6 Os1BGlu5 และ Os6BGlu22 ใน *Escherichia coli* ไม่สามารถตรวจพบกิจกรรมของเอนไซม์จากโปรตีนที่แสดงออก อย่างไรก็ตาม กิจกรรมทรานส์กลูโคซิเดสของ Os9BGlu31 ก็ยังคงมีประโยชน์สำหรับการสังเคราะห์สารที่มีความหลากหลาย รวมถึงยังสามารถปรับเปลี่ยนรูปแบบของกิจกรรมโดยอาศัยการกลายพันธุ์เพื่อใช้ในการสร้างผลิตภัณฑ์ที่มีเอกลักษณ์

Abstract

Glycoside hydrolase family 1 (GH1) contains β-glucosidases that hydrolyze various glucoconjugates and transglucosidases, which instead transfer glucose from one molecule to another. Os9BGlu31 is the rice enzyme that has been shown to function as a transglucosidase during this and previous work. In order to determine the biological substrates for Os9BGlu31, two approaches were used. First, alcoholic extracts from rice plants were used as transglycosylation reaction acceptors in reactions with Os9BGlu31 and the compounds that were transglycosylated identified by purification, mass spectrometry (MS) and nuclear magnetic resonance (NMR). Second, rice plants containing a T-DNA insertion in the gene for Os9BGlu31 were extracted and the metabolites compared to those of wild type by LCMSMS. By combining these methods, we were able to show that the fatty acid esters, 1-O-oleoyl β-D-glucose and 1-O-linoleoyl β-D-glucose, can serve as products or substrates of Os9BGlu31 and build-up in plants deficient in Os9BGlu31. We also mutated Os9BGlu31 at sites around the active site to see if we could change it to a hydrolase, in order to understand the basis for transglucosidase activity. Although none of the mutations made hydrolysis activity higher than transglucosidase activity, the W243N (Tryptophan residue 243 to Asparagine) mutation resulted in an enzyme with higher activity and greater substrate range than wild type Os9BGlu31. Moreover, we could identify a number of glycosides and bis-glycosides of the flavonol kaempferol that were created by Os9BGlu31 W243N, based on their mass spectrometric fragmentation patterns. This work suggested that Os9BGlu31 could be used to produce a number of compounds, including phytohormone glucoconjugates, of use to our lab and other investigators in the field. Additional work on expression of the other relatively highly expressed GH1 Cluster At/Os6 enzymes Os1BGlu5 and Os6BGlu22 were cloned and expressed in *Escherichia coli*, but no activities could be detected from the expressed proteins. Nonetheless, the transglucosidase activity of Os9BGlu31 is useful for synthesis of various compounds and can be modulated by mutations to produce unique products.

กิตติกรรมประกาศ

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ผู้วิจัย

ตุลาคม 2560

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CHAPTER 1. Introduction

1.1 Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

Transfer of sugars onto other molecules is an important way of regulating molecular recognition and bioactivity. The products of these reactions include important molecules, such as glycoproteins and glycolipids, as well as glycosides of phytohormones and other biologically or chemically active moieties. In plants, these compounds are of considerable economic importance, since they can regulate growth and development of crops, and some glycosides, such as the antioxidant arbutin, are of economic importance as products as well.

Most glycosyltransferases operate by transferring sugars from alpha-linked nucleotide (i.e. uridine) diphosphate (i.e. UDP) glycosyl conjugates onto various acceptors, including alcohols, carboxylic acids, lipids and proteins. The nucleotide diphosphate is a high energy intermediate, which provides the energy for the formation of the new bond. On the other hand, glycoside hydrolases cut these bonds by taking a sugar from one glycoconjugate and releasing it to water, but many can also catalyze transglycosidase reactions to put the sugar onto a new acceptor other than water, thus conserving the energy of the glycosidic bond and producing potentially useful products. For instance, plants use xyloglucan transferases, which belong to the same gene family as certain xyloglucan hydrolases to link xyloglucan chains and thereby cross-link the cell wall (Eklöf & Brumer, 2010). In addition, reaction conditions with high acceptor and/or low water concentrations are often used to generate glycosyl transfer products, such as alkyl glucosides (Shaik & Withers, 2008), and certain mutations of the catalytic amino acid residues can alter glycoside hydrolases to give glycosynthases and thioglycoligases that give transfer reactions with no or little hydrolysis.

Plant glycoside hydrolase family 1 (GH1) enzymes include a broad range of enzymes with different reaction and substrate specificities, including β-glucosidases, β-mannosidases, disaccharidases, and hydoxyisourate hydrolase, which is not a glycoside hydrolase at all (Ketudat Cairns & Esen, 2010). Rice and Arabidopsis GH1 enzymes have been split into 8 phylogenetic clusters found in both plants, along with 2 found only in Arabidopsis and other crucifers (Opassiri et al., 2006). In our efforts to characterize the functions of rice GH1 enzymes, we recently discovered that one enzyme, Os9BGlu31, from GH1 At/Os cluster 6 acted not as a hydrolase, but was primarily a glucosyl transferase, which preferred to transfer glucose to carboxylic acids, though it could also transfer to certain alcohols (Luang et al., 2013). About the time we first discovered this, Matusuba et al. (2010) published the characterization of two glycosyl transferases (transglucosidases) from carnation and delphinium that belonged to the same phylogenetic cluster and used aromatic glucosyl esters, such as feruloyl glucose and cinnamoyl glucose to anthocyanin 3-glucoside. We have shown that the rice enzyme we characterized used similar donors and could also transfer to them as acceptors, as well as to phytohormones, such as indole acetic acid and gibberellin GA_4 (Luang et al., 2013). Since the rice Os9BGlu31 enzyme appears more promiscuous in its donor and acceptor substrates, it allows us to synthesize various glucose esters and glycosides, with little hydrolysis. Thus, it is of interest to further investigate the role of the Os9BGlu31 and other GH1 At/Os cluster 6 enzymes in the plant and their application to glycoconjugate synthesis.

In this work, we tried to answer the critical questions that remained to be answered about Os9BGlu31 and the other members of GH1 At/Os cluster 6. These included: What is the basis for glycosyltransferase vs. hydrolase activity and substrate specificity in general? What are predominant and important substrates in the plant? What is the biological

significance of their transfer of glucose to and from phytohormones, phenolic acids and other compounds in the plant? Do other cluster 6 enzymes also act as transferases and do they show different substrate specificity? Can Os9BGlu31 be effectively used for production of economically valuable glycoside products? We were able to make significant progress on several of these issues (Komvongsa et al, 2015a, 2015b).

1.2 Research Objectives (วัตถุประสงค์ของการวิจัย)

The objectives of this research project were:

1.2.1 To investigate the biological substrates of Os9BGlu31 glucosyl transferase and determine the enzyme's likely function in the plant.

1.2.2 To determine what amino acids in Os9BGlu31 are responsible for its preference for transfer reactions over hydrolysis reactions.

1.2.3 To determine whether other rice GH1 At/Os cluster 6 enzymes that have different sequences around their catalytic nucleophile act as transferases or hydrolases.

1.2.4 To determine whether the Os9BGlu31 glucosyl transferase can be used to efficiently and economically synthesize arbutin or other economically valuable products.

1.3 Scope of Research (ขอบเขตของการวิจัย)

In this project, we completed the characterization of Os9BGlu31 and made mutations around the active site and substrate-binding cleft to see whether it could be converted to a hydrolase. We also determined the structures and identities of two substrates found in rice extracts and found that the knockout of this enzyme in rice plants, resulted in the increase in the amounts of their glucose esters. In addition, we cloned the cDNA for Os1BGlu5 and Os6BGlu22 into the pET32a expression vector, along with a synthetic gene for Os5BGlu19, and found that no activities could be detected. We will also tried to apply the Os9BGlu31 enzymes to production of arbutin from 4-hydroxy phenol, but found it was not economical, and found that it could also produce octyl glucoside, a biologically degradable nonionic detergent, though at low levels. The Os9BGlu31 W243N mutant was found to be effective at transglycosylation of many compounds, so development of an effective process for production and purification of interesting products was the subject of the next project.

1.4 Short Description of Methods (ข้อตกลงเบื้องต้น)

1.4.1 Identification of natural substrates of Os9BGlu31 in rice.

To identify rice substrates for Os9BGlu31, rice seedlings were extracted with alcohol and fractionated by reverse phase and silica gel chromatography, as described in Komvongsa et al. (2015b). The fractions were tested by incubation with recombinantly produced Os9BGlu31 and new product spots and substrate spots identified by thin layer chromatography, followed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) of the purified compounds to identify the structures. A liquid chromatography-tandem mass spectrometry (LCMSMS) multiple reactant monitoring (MRM) methods were then developed to monitor the levels of unsaturated fatty acids and their glucose esters and feruloyl glucose (FAG) and ferulic acid (FA) in rice extracts.

Meanwhile, our collaborators in Kyung Hee University, Korea, Bancha Mahong and Jong-Seong Jeon bred Os9BGlu31 knockout insertion lines to identify the effects of the mutation. The flag leaves of the plants were extracted and measured for the amounts of oleic acid, linoleic acid, their 1-O-glucose esters, ferulic acid and feruloyl glucose to identify the effect of Os9BGlu31 or its defect on these compounds.

1.4.2 Determination of amino acid residues responsible for transglycosylation and substrate specificity in Os9BGlu31.

To identify the amino acids responsible for transglycosylation activity in Os9BGlu31, a homology model was developed based on the Os3BGlu6 x-ray crystallographic structure and was superimposed on that structure to identify residues different between the residues in Os9BGu31 and Os3BGlu6 active sites. These residues were then mutated and the hydrolysis and transglycosylation activites evaluated, as described by Komvongsa et al. (2015a). Further work was done to characterize the multiple products of transglycosylation by some mutants by mass spectrometric analysis (Komvongsa et al., 2015a).

1.4.3 Cloning and characterization of novel GH1 At/Os cluster 6 enzymes.

The relative expression patterns of the rice enzymes in the same phylogentic cluster with Os9BGlu31 (At/Os 6) were evaluated on the rice XPRO website and those with relatively high expression evidence were chosen for recombinant expression and characterization, including Os1BGlu5, Os5BGlu19, and Os6BGlu22. Two of these, Os1BGlu5 and Os6BGlu22 had previously been cloned from cDNA clones from Japan and were expressed from the pET32a plasmid, while the Os5BGlu19 was synthesized and cloned into the pET32a and pPICZαBNH8 (Toonkool et al., 2006) plasmids for expression in *E. coli* and pichia. The enzymes were expressed in the appropriate systems. The expressed enzymes were tested with 4-nitropheny β-D-glucopyranoside (4NPGlc) donor and acetic acid or ferulic acid acceptor and release of 4NP evaluated. Activity was also assessed by production of FAG in assays with rice extract and FA acceptor by the mass spectrometric analysis described above.

1.5 Benefits and output from this research project (ประโยชน์ที่ได้รับจากการวิจัย)

1.5.1 Knowledge.

In this project, we learned about the natural substrates of Os9BGlu31 and found that they were more diverse than previously expected. We found that the fatty acid glucose esters occur in the plant in low amounts, which suggests they may be investigated further as possible signaling compounds of importance to the plant. Furthermore, the role of FA and FAG is still being further monitored and may have important implications for biomass conversion of rice straw for rice breeders to consider in the future. Moreover, we found that the Os9BGlu31 enzyme and its mutants are very useful for the production of many glycosides, which can be applied to product development in the future.

1.5.2 Human Resources Development.

This project was critical to the development of knowledge in young scientiests, providing for the thesis research of one Ph.D.student and one M.Sc. student, as well as training 2 research assistants in laboratory science. This allowed the assitants to go on to productive careers in government research as well.

1.5.3 Research Publication.

1.5.3.1 International Journal Publications

1.5.3.1.1 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. (finished up work for paper revision on this project).

1.5.3.1.2 Komvongsa J, Luang S, Marques JV, Phasai K, Davin LB, Lewis NG, Ketudat Cairns JR. 2015a. Active site cleft mutants of Os9BGlu31 transglucosidase modify acceptor substrate specificity and allow production of multiple kaempferol glycosides. *Biochimica et Biophysica Acta, General Subjects* **1850** (7), July 2015, 1405–1414.

1.5.3.1.3 Komvongsa J, Mahong B, Phasai K, Hua Y, Jeon JS, Ketudat Cairns JR. 2015b. Identification of fatty acid glucose esters as Os9BGlu31 transglucosidase substrates in rice flag leaves. *Journal of Agricultural and Food Chemistry* **63**(44):9764-9769.

1.5.3.1 International and National Conference Presentations

1.5.3.1.1 Ketudat Cairns JR, Komvongsa J, Luang S, Phasii K, Lewis NG. (2014) Acceptor and product specificity in rice Os9BGlu31, a glycoside hydrolase family 1 transglucosidase. 27th International Carbohydrate Symposium. Indian Institute of Science, Bangalore, India 12-17 January, 2014. Invited Lecture II-B-IL-9. Proceedings Pg. 144.

1.5.3.1.2 Ketudat-Cairns JR, Pengthaisong S, Tankrathok A, Komvongsa J, Svasti J. 2014. Protein-carbohydrate interactions leading to specificity in glycoside hydrolase 1 enzymes. The 4th Asia Pacific Protein Association (APPA) Conference, 17-20 May, 2014, Jeju International Convention Center, Jeju, South Korea. Invited lecture, Symposium 4A. Proceedings Pg. 104.

1.5.3.1.3 Ketudat Cairns JR. 2014. Biochemical functions of rice glycoside hydrolase family 1 enzymes. 2014 Annual Meeting of the Korean Society for Plant Biotechnology. Kyung Hee University, Suwon, South Korea, 15-16 May, 2014. Invited Lecture S5-2; Proceedings Pg. 49

1.5.3.1.4 Ketudat-Cairns JR, Pengthaisong S, Tankrathok A, Hua Y, Komvonsa J, Rimlumnduan T, Baiya S, Prawisut A, Ekkhara W. Phasai K, Charoenwattanasatien R, Svasti J. 2014. Protein-carbohydrate interactions in β-D-glycosidases and transglycosidases. The 7the Asia Oceania Human Proteome Organization Congress and 9th International Symposium of the Protein Society of Thailand "Frontiers in Protein and Proteomic Research". 6-8 August, 2014. Miracle Grand Convention Hotel, Bangkok, Thailand Invited Lecture 9; Proceedings Pg. 17.

1.5.3.1.5 Phasai K, Ketudat Cairns JR. 2014. Identification of transglucosylation products for Os9BGlu31 transglucosidase and its W243N mutant. The 7th Asia Oceania Human Proteome Organization Congress and 9th International Symposium of the Protein Society of Thailand "Frontiers in Protein and Proteomic Research". 6-8 August, 2014. Miracle Grand Convention Hotel, Bangkok, Thailand; Poster Presentation PP154-59, Proceedings Pg. 127

1.5.3.1.6 Komvongsa J, Luang S, Phasai K, Marque J, Lewis NG, Ketudat-Cairns JR. 2014. Single point mutations at tryptophan residue 243 increase Os9BGlu31

transglucosidase activity on hydroxyl groups of kaempferol. The 7th Asia Oceania Human Proteome Organization Congress and 9th International Symposium of the Protein Society of Thailand "Frontiers in Protein and Proteomic Research". 6-8 August, 2014. Miracle Grand Convention Hotel, Bangkok, Thailand; Poster Presentation PP297-132; Proceedings Pg. 200.

1.5.3.1.7 Komvongsa J, Phasai K, Ketudat-Cairns JR. 2013. Rice Os9BGlu31 transglucosidase activity changed in a single point position around the active site mutation. 52nd Annual Meeting of the Phytochemical Society of North America. Oregon State University, Corvallis OR, USA. 3-7 August, 2013. Poster P07.

1.5.3.1.8 Komvongsa J, Luang S, Phasai K, Ketudat-Cairns JR. 2013. Site-directed mutagenesis and inhibition studies of rice Os9BGlu31 transglucosidase mechanism and substrate specificity. The 8th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute, Bangkok, Thailand. 5-7 August, 2013. Poster Presentation 1

1.5.3.1.9 Phasai K, Luang S, Kittakoop P, Ketudat Cairns JR. 2013. Transglucosylation of fatty acids by rice Os9BGlu31 transglucosidase. The 8th International Symposium of the Protein Society of Thailand, Chulabhorn Research Institute, Bangkok, Thailand. 5-7 August, 2013. Poster Presentation 21.

CHAPTER 2. Literature Review

Carbohydrate active enzymes include glycoside hydrolases (GT), glycosyltransferases (GT), polysaccharide lyases (PL), and carbohydrate esterases (CE), according to the CAZY website (www.cazy.org, Cantarel et al., 2009; Coutinho et al., 1999). Of these enzymes, the GHs and GTs are most responsible for the addition and removal of glucosyl residues from various complex carbohydrates, polysaccharides and glycoconjugates. Most GTs utilize monosaccharides put onto nucleotide carriers, such as UDP-glucose, as donor substrates for glycosylation, but some are known to transfer from lipid donors (Lairson et al., 2008). Recently, aromatic acid glucose esters, such as feruloyl glucose and vanillyl glucose have been found to serve as the source of glucose for transfer to the anthocyanin cyaniding 3-glucoside (Matsuda et al., 2010). Although the process of addition and removal of monosaccharides can follow analogous mechanisms, it is thought that this is not always the case. Inverting GH and GT, in which the stereochemistry at the anomeric carbon of the donor substrate and product are the same, are thought to act through the same S_{N2} single-displacement mechanism, in which acceptor attacks from the opposite side as the nucleotide or other donor substrate (Lee et al., 2011). However, while retaining GH are thought to act through a double displacement mechanism with formation of a covalent glycosyl-enzyme intermediate, the GT are thought to act through a oxocarbenium ion intermediate, with the phosphate of the nucleotide acting as a catalytic base. Nonetheless, the presence of hydrolases and transferases in the same families, in the case of xyloglucan endotransferases, suggests that for these transglycosidases (TG), which do not use nucleotide sugar donors, the mechanisms may be more similar to GH. The presence of both in GH1 suggests the retaining GH and TG enzymes in this family may share a common mechanism, but this remains to be proven.

GH1 is one family of GH clan A, which is composed of enzyme families with similar $(\beta/\alpha)_8$ structures and retaining mechanisms (Henrissat, 1991, Henrissat et al., 1995, Jenkins et al., 1995). The proteins in this family range from broad specificity glycosidases, β-glucosidases, phospho-β-glycosidases, and β-mannosidases in bacteria and archaea, to glycolipid β-glucosidases, β-galactosidases, and hormonal regulators in animals, to β-glucosidases, β-mannosidases, thioglucosidases, disaccharidases, glycolipid galactosyl transferases and hydroxyisourate hydrolase in plants (Henrissat, 1991; Cantarel et al., 2009; Ketudat Cairns and Esen, 2010). In Arabidopsis, there are 48 genes in this family (Xu et al., 2004), while in rice 40 genes have been identified in genome sequences, although these include 2 likely endophyte genes, 2 pseudo genes and 2 gene fragments (Opassiri et al., 2006). The rice and Arabidopsis protein sequences where found to fall in 8 phylogenetic clusters containing both rice and Arabidopsis genes more closely related to each other than to genes from the same plant outside that cluster, along with two clusters found only in Arabidopsis and other crucifers. These crucifer-specific clusters include one for classical myrosinases (thioglucosidases) and another with nonclassical myrosinases and β-glucosidases found in endoplasmic reticulum (ER) derived bodies. Curiously, most Arabidopsis genes that have been functionally characterized fall into these two crucifer-specific clades (e.g. Lee et al., 2006), except for two monolignol glucoside hydrolyzing β-glucosidases (from At/Os cluster 5, Escamilla-Treviño et al., 2006), a β-mannosidase (from At/Os cluster 4, Xu et al., 2004), and a galactolipid galactosyl transferase (SFR2, which makes up At/Os cluster 8, Moellering et al., 2010). Rice enzymes for which the characterization has been published include Os3BGlu6 β-glucosidase from Os/At cluster 1 (Seshadri et al., 2009), Os3BGlu7 and Os3BGlu8 β-glucosidases and Os7BGlu26 β-mannosidase from At/Os cluster 4 (Opassiri et al., 2003, 2004; Chuenchor et al., 2008; Kuntothom et al., 2009), and Os4BGlu12 (cell wall

associated β-glucosidase; tuberonic acid β-glucosidase 2, TAGG2, Akiyama et al., 1998; Opassiri et al., 2006, 2010, Sansenya et al., 2011; Wakuta et al., 2011) and Os4BGlu13 (TAGG1, Wakuta et al., 2010).

At/Os cluster 6 is the phylogenetic cluster that contains hydroxyisourate hydrolase (HIUH), which has the unusual sequence of HENG around the nucleophile, instead of the usual TENG, and lacks the sugar binding residues (Raychaudburi and Tipton, 2002; 2003). Recently, we characterized Os9BGlu31 from At/Os cluster 6 in our laboratory and found it to be glucosyl transferase with little hydrolase activity and no HIUH activity. About the time we discovered the glycosyltransferase activity, Matsuda et al. (2010) reported two glycosyltransferases from carnation and delphinium that transfer glucose from phenolic glucosyl esters, such as feruloyl glucose and vanillyl glucose to the anthocyanin cyanidin 3-O-glucoside. These enzymes, like Os9BGlu31, have the HENG sequence at the catalytic nucleophile, but contain the conserved sugar-binding residues missing in the purine hydrolase HIUH. Upon obtaining ferulic acid and feruloyl glucose, we found that these compounds served as excellent substrates and other compounds serving as substrates included various cinaminic acids and their glucosyl esters, indole acetic acid (IAA), naphthalene acetic acid (NAA) and gibberellic acid GA4 (Luang et al., 2013). Since these substrates suggest cross-talk between feruloyl glucose and phytohormone glycosyl conjugates is possible, we are continuing to try to determine likely substrates and their effects in the plant.

Both GT and GH can be used to synthesize oligosaccharides and glycosides (Shaik and Withers, 2008). GT can be used to produce these through their natural reaction, but the requirement of nucleotide-sugars by most makes this process rather expensive. What is more, many classical GT are membrane-bound and difficult to express and manipulate. On the other hand, GH can achieve synthesis of these compounds by reverse hydrolysis or transglycosylation under high substrate and/or low water conditions. For instance, synthesis of glycosides of primary alcohols has been achieved in good yield using Thai rosewood dalcochinin β-glucosidase (Lirdprapomongkol and Svasti, 2000), while the use of cassava β-glucosidase allows synthesis of secondary and tertiary alcohols in good yields (Svasti et al., 2003).

On the other hand, the limitations on GH generated by their hydrolytic activities can be eliminated or minimized by mutations that change or interfere with the normal mechanism of hydrolysis (Shaik and Withers, 2008). Mutations to the catalytic acid/base can allow retaining GH to form covalent intermediates with a sugar from a donor with a leaving group that does not require acid assistance, while a nucleophile can be added to displace the sugar from the enzyme to generate a glycoside. Typically, thiols have been shown to be effective nucleophiles, so these mutants are called thioglycoligases. Converting the catalytic nucleophile to a small amino acid like glycine, alanine or serine prevents the retaining GH from forming a covalent intermediate with the enzyme, but allows a 1-fluoro-glucoside with the opposite anomeric configuration as the normal substrate to substitute for the catalytic intermediate, thereby serving as a donor substrate for transglycosylation by these "glycosynthase" enzymes. Such enzymes have very low hydrolysis activity, so the product is not lost to hydrolysis. For instance, Hommalai et al. (2007) were able to make long pNP-cellooligosaccharides from *p*NP-cellobioside and α-fluoroglucoside with a glycosynthase mutant of rice BGlu1 (Os3BGlu7) β-glucosidase. On the other hand, mutagenesis of other amino acids have also given enzymes with similar glycosynthase activities (Shaik and Withers, 2008) and GH1 enzymes with natural TG activities may be "natural mutants" of GHs to generate TGs, thereby serving as a model for engineering of other enzymes with useful specificities.

As noted above, natural TG enzymes exist in plants, including galactolipid-galactolipid galactosyl transferase (Moellering et al., 2010), acyl glucose-dependent anthocyanin glucosyltransferases (Matsuba et al., 2010) and rice Os9BGlu31, which transfers glucosyl moieties between phenolic acids and their 1-O-acyl β-D-glucosyl esters (Luang et al., 2013). Recently, we characterized Os9BGlu31 variants mutations around the active site and found that the mutations of Trp243, especially Trp243Asn, allowed glycosylation of a broader range of acceptors, including different hydroxyl positions on the flavonoid kaempferol and its glucosides (Komvongsa et al., 2015). Therefore, it is of interest to test these mutants on a broader range of compounds, especially compounds of potential medical significance.

An expanding impact of complex carbohydrates and glycosides in drug discovery supports the development of practical ways to add various sugars onto drug targets, which is sometimes called glycorandomization or glycodiversification (Gantt et al., 2011). The products of these reactions can be screened via low or high throughput discovery-scale bioactivity assays and the reactions used in large-scale production processes. Many carbohydrate-based therapeutics are both orally bioavailable and highly effective. These are often glycosylated natural product-based drugs, which include existing and emerging anticancer and anti-infective agents (Newman and Cragg, 2007).

CHAPTER 3. Materials and Methods

3.1. Materials

3.1.1 Plant material

Rice grown in Thailand were KDML105, including bran from a local rice mill and seedlings grown to the 2-3 week stage in the laboratory, then harvested and extracted in ethanol or frozen at -80 C for later extraction. Rice lines used for extraction in Korea were Dongjin and Nipponbare rice and their T-DNA insertion mutants (see below, Komvongsa et al., 2015b), which were gown in a contained patty field and the flag leaves were harvested during the setting stage after flowering.

3.1.2 Chemicals and laboratory supplies

Ferulic acid, linoleic acid, oleic acid, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc), peroxidase/glucose oxidase assay (PGO), phenylmethylsulfonyl fluoride (PMSF), ampicillin, DNase I, kanamycin, tetracyclin, isopropyl β-D-thiogalactoside (IPTG), lysozyme, methanol-d4, acetone-d6, chloroform-d, deuterium oxide and tetramethylsilane (TMS) were purchased from Sigma-Aldrich (St. Louis, USA). Molecular sieve 4 Å, trifluoroacetic acid (TFA), polyethylene glycol (PEG), 2-morpholinoethanesulfonic acid (MES), Triton X-100, bovine serum albumin (BSA), calcium chloride, metal sodium and formic acid were purchased from Fluka (Steinheim, Switzerland). Silica gel 60 and silica gel 60 F_{254} TLC plates were purchased from Merck (Darmstad, Germany). HPLC-grade water and HPLC-grade methanol were purchased from RCI Labscan (Bangkok, Thailand). Trypsin (sequencing grade) was purchased from Promega (Madison, WI, USA). The Bradford assay kit was purchased from Bio-Rad (Hercules, CA, USA). Imidazole was purchased from USB Corporation (Cleveland, OH, USA). Acetic acid, sulfuric acid, methanol, ethanol, acetone, pyridine, isopropanol, *n*-propanol, acetonitrile, disodium ethylenediamine tetraacetate (EDTA), bromophenol blue, ammonium bicarbonate, calcium chloride (anhydrous), sodium sulfate (anhydrous), magnesium sulfate (anhydrous), ammonium sulfate, sodium bicarbonate, sodium chloride, sodium hydroxide, sodium carbonate, citric acid, disodium hydrogen phosphate, hydrochloric acid, Coomassie brilliant blue R250, Tris(hydroxymethyl)-aminomethane (Tris) and sodium dodecyl sulfate were purchased from CARLO ERBA (Rodano, Milano, Italy). Acrylamide, N,N′,N″,N‴-tetramethyl-ethyllenediamine (TEMED), ammonium persulphate, N,N′-methylenebisacrylamide, Superdex-200 gel filtration resin, immobilized metal affinity chromatography (IMAC) resin, HiPrep CM-Sepharose fast flow column (16/10, 20 ml), HiTrap SP Sepharose XL column (1 ml) and HiTrap Octyl Sepharose 4 fast flow column (1 ml) were purchased from GE Healthcare (Uppsala, Sweden). QuikChange® Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA, USA). Ultra centrifugal filters (Amicon Ultra, regenerated cellulose, 30,000 MWCO) were purchased from Millipore Corporation (Bedford, MA, USA). Other chemicals and laboratory materials used but not listed here were purchased from a variety of suppliers.

3.2 Experimental methods

3.2.1 Identification of Os9BGlu31 substrates in rice

3.2.1.1 Extraction of natural substrates and verification of substrates and products.

The putative acceptor substrates were extracted from rice bran, purified and shown to be oleic acid and linoleic acid in collaboration with Dr. Prasat Kittakoop. The extraction was as described by Komvongsa et al. (2015b). First, 1 g of rice bran was extracted in 50% ethanol overnight, and the supernatant was separated by silica gel thin layer chromatography (TLC) on silica gel plates developed with a 7:2.8:0.2 chloroform/methanol/30% ammonia solvent system, and carbohydrate and lipid spots were detected by staining with 10% sulfuric acid in ethanol and heating at 110 °C. From another TLC without staining, the regions corresponding to the major stained spots were scraped, and each banded fraction was individually eluted in 100% methanol overnight. The crude extract and TLC-purified components were reacted in reactions with 10 μg of recombinant Os9BGlu31 (produced as described by Luang et al., 2013), 10 mM 4NPGlc, and 10% ethanol extract in 50 mM sodium citrate (pH 4.5). The reaction compoents were again separated via TLC, to identify the component serving as the substrate for Os9BGlu31. Once the spots were identified, the substrates were purified from a larger extraction. A 20 g sample of rice bran was extracted with 50% ethanol in water by stirring overnight at room temperature. The mixture was filtered through a 0.45 μm filter. Ten milliliters of supernatant was loaded into a Waters Sep-Pak tC18 resin column (Waters, Milford, MA). The column was washed with 10 mL of water, and 20 mL each of 5, 50, 60, 70, 80, 90, and 100% methanol. Fractions were evaluated via silica gel TLC with a chloroform/methanol/30% ammonia [7:2.8:0.2 (v/v/v)] solvent, stained with 10% sulfuric acid in ethanol, and charred at 110 °C. Fractions eluted in 70, 80, and 90% methanol that appeared to act as substrates were evaluated by ^{1}H NMR (proton nuclear magnetic resonance spectroscopy).

The pure substrates oleic acid, linoleic acid and stearic acid were then tested as substrates to verify oleic acid and linoleic acid act as substrates (Komvongsa et al., 2015b). Since the products ran at the same Rf as the product from rice bran, the product of the reaction with oleic acids was purified by silica gel TLC, eluted from the resin in 50% methanol, and submitted to 1H NMR to confirm the structure of 1-O-oleoyl β-D-glucose ester. A similar preparation of 1-O-linoleoyl β-D-glucose gave multiple product spots, because of the instability of this product, so it could not be similarly evaluated. 1H NMR for oleic acid glucosyl ester was performed on a 500 MHz NMR spectrometer (Bruker AVANCE III HD) with a CPP BBO 500 Cryoprobe with CD_3OD as the solvent. The ^{1}H NMR spectra were collected at a frequency of 500.366 MHz. The 1H NMR experiment data were processed with BRUKER TOPSPIN version 3.2.

The production of 1-O-linoleoyl β-D-glucose was confirmed by an NMR time course (Komvongsa et al., 2015b). Enzymatic and control reaction mixtures were prepared in D_2O and monitored by ^{1}H NMR. The 500 μL reaction mixtures contained 20 mM 4NPGlc, 20 mM linoleic acid, and 50 mM citrate (pH 4.5). To start the enzymatic reaction, 280 μg of Os9BGlu31 was added and the reaction mixtures were incubated at 30 °C for 3 and 24 h before being assayed with ^{1}H NMR. The NMR was as described for oleoyl glucose, but the water peak was suppressed.

3.2.1.2 Kinetic evaluation of the fatty acid substrates with Os9BGlu31.

The kinetics of reactions with these glucosyl acceptors were determined as described by Komvongsa et al. (2015b). First, the relative activity toward 4NPGlc donor substrates was determined by incubating 10 μg of Os9BGlu31 with 20 mM 4NPGlc and 0.25 mM fatty acids in 140 μL of 50 mM citrate (pH 4.5) at 30 °C for 1 h. The apparent K_m and V_{max} values of 4NPGlc in the presence of various acceptors were determined by varying the concentration of 4NPGlc in the range of 0.2−10 mM with 0.25 mM fatty acid acceptors and 10 μg of Os9BGlu31 in 50 mM citrate (pH 4.5). The apparent K_m and V_{max} values of the fatty acid acceptors were determined by varying their concentrations between 0.05 and 2 mM in the presence of 8 mM 4NPGlc with 10 μg of Os9BGlu31 in 50 mM sodium citrate (pH 4.5). The reaction was stopped with 100 μL of 2 M Na_2CO_3, and the 4NP released was quantified by the absorbance at 405 nm. The kinetic parameters were calculated by nonlinear regression of the Michaelis−Menten plots with GraFit version 5.0 (Erithacus Software, Horley, U.K.).

3.2.1.3 Identification of the substrates by comparison in mutant rice plants.

The os9bglu31 knockout mutant alleles were identified from the population of T-DNA-tagged mutants and from the Tos17 insertion mutant collection. The japonica cultivar Dongjin is the background genotype of *os9bglu31-1* and *os9bglu31-4*, and and Nipponbare is for *os9bglu31-2* and *os9bglu31-3*. Homozygous lines for insertions in the *Os9BGlu31* gene and wild-type segregant lines from the same heterozygous parents were compared. Homozygous mutants for the insertions were identified by polymerase chain reaction (PCR) analysis of genomic DNA isolated from mature rice leaves with the gene-specific and insertion-specific primers shown in Table 3.1. Note that the Nibonbare Tos17/2 insertion lines, osbglu31-2 and osbglu31-3, were analyzed with the same primers.

Table 3.1 Primers used for identification of homozygous knockout and wild-type lines

Mutant line	Gene specific primers	Insertion-specific primer
os9bglu31-1	5′-CCTCGCTCCCCAATACTATTTTGA-3′ 5′-GAAGACGATGGAAACAAACACATC-3′	5′-ATCCAGACTGAATGCCCACAGG-3′
os9bglu31-2	5′-CTGGAGCACTGTCAATGAGCCTAA-3′	5′-CTGGACATGGGCCAACTATACAGT-3′
os9bglu31-3	5′-TCAACCCGAGTGGGAACTGTTATT-3	
os9bglu31-4	5′-GCTCTTGTGAAATTATACTGTCTG-3′ 5′-ATTCTATATCTATCTTGGACCACC-3′	5′-ATCCAGACTGAATGCCCACAGG-3′

Flag leaves from 3-month-old plants were freeze-dried and ground to powder in a mortar and pestle with liquid nitrogen. Fifty mg samples were extracted with 500 μL of 70% ethanol for UPLC−MS/MS QQQ analysis. The amounts of fatty acids and glucose esters in the extracts were quantified by triple quadrupole (QQQ) LC−ESI MS/MS and NMR, with oleic acid and linoleic acid standards (CalBiochem, La Jolla, CA). To produce the glucose ester standard curves, 10 μg of Os9BGlu31 was used to catalyze the reaction of 20 mM 4NPGlc with 5 mM oleic acid or linoleic acid in 50 mM sodium citrate buffer (pH 4.5) at 30 °C for 1 h. The reactions were stopped with 1% formic acid and the mixtures filtered through a 0.22 μm filter before injection into an Agilent 1290 LC system (Agilent) in line with an electrospray ionization (ESI) Agilent 6400 series QQQ mass spectrometer. Separation was conducted through an Agilent SB-C18 RRHD 1.8 μm, 2.1 mm × 150 mm column (Agilent). The mobile phase consisted of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The gradient varied linearly from 40 to 60% B (v/v) over 13 min and from 60 to 100% B (v/v) over 15 min with a flow rate of 0.3 mL/min. The mass spectrometry analysis was performed in negative ion mode, with a capillary voltage of 3 kV and a gas temperature of 300 °C. The gas flow was set at 16 L/min, nebulizer at 45 psi with the sheath

gas heater at 300 °C, and the sheath gas flow at 11 L/min. The fatty acid product ions were identified for each precursor, and the fragmentation voltage was optimized to yield the highest product ion abundance. Finally, the specific compounds were detected by multiple-reaction monitoring (MRM) with MassHunter software. The abundance of oleic acid and linoleic acid was monitored with a collision energy of 15 V. The product ion at m/z 185.1 generated from the precursor ion at m/z 281 was monitored for oleic acid, while the m/z 211 product ion of the m/z 279 precursor was monitored for linolenic acid. The selected product ions for monitoring the abundance of oleoyl glucose ester and linoleoyl glucose ester were generated with a collision energy of 20 V and were at m/z 281.2 (precursor ion at m/z 489 with formate) and m/z 279 (precursor ion at m/z 441), respectively. The feruloyl glucose and ferulic acid levels were monitored by the same technique, but utilizing their respective masses and fragments.

3.2.2 Investigation of the effects of amino acid residues near the Os9BGlu31 active site

3.2.2.1 Modeling

The 3-dimensional structure of Os9BGlu31 transglucosidase was modeled based on the most-closely related (in terms of amino acid sequence similarity) structure in the Protein Data Bank (PDB), Os3BGlu6 β-glucosidase (PDB: 3GNO) (Komvongsa et al., (2015a). Modeling was done with the Modeller 9.0 program (Marti-Renon et al., 2000) to produce 5 models, and the model with the lowest DOPE score was used. Since Os3BGlu6 has little transglycosylation activity (Seshadri et al., 2009), the homology model of Os9BGlu31 was further superimposed with its structure in a covalent complex with 2-fluoroglucoside (PDB: 3GNR) to inspect for residues close to a water molecule that is in position to displace the glucosyl-enzyme intermediate.

3.2.2.2 Mutagenesis

Mutations designed based on the 3D model comparison were produced according to the instructions of the QuikChange XL II site-directed mutagenesis kit (Agilent, USA). The pET32a/DEST/Os9BGlu31 vector (Luang et al., 2013) was used as template to generate single point mutation of I172T, L183Q, L241D, and W243 to N, A, D, F, M, and Y. The primers used are given in Table 2.2. The reaction was thermocycled as follows: 95 °C 1 min, then 18 cycles at 95 °C 50 s; 60 °C 50 s and 68 °C 7 min, followed by a final extension at 68 °C 7 min. The templates were digested by *Dpn* I for 3 h and the mutant plasmids were transformed into XL10-Gold Ultracompetent cells. Plasmids were prepared by standard methods and the full inserts were sequenced to verify that they matched the intended sequence.

Table 3.2 Sequences of oligonucleotides used for site-directed mutagenesis.

Primer	Nucleotide sequence (5'-3')
I172T_fwd	GCACTGTCAATGAGCCTAACACCGAGCCGATTGGCGGATACG
I172T_rev	CGTATCCGCCAATCGGCTCGGTGTTAGGCTCATTGACAGTGC
L183Q_fwd	CGGATACGATCAAGGAATCCAACCGCCACGGCGATGCTCATTCC
L183Q_rev	GGAATGAGCATCGCCGTGGCGGTTGGATTCCTTGATCGTATCCG
L241D_fwd	GGACAAATTGGGCTCACATTGGACGGTTGGTGGTACGAGCCCG
L241D_rev	CGGGCTCGTACCACCAACCGTCCAATGTGAGCCCAATTTGTCC
W243A_fwd	GGGCTCACATTGCTCGGTGCGTGGTACGAGCCCGGGACG
W243A_rev	CGTCCCGGGCTCGTACCACGCACCGAGCAATGTGAGCCC
W243D_fwd	GGGCTCACATTGCTCGGTGATTGGTACGAGCCCGGGACG
W243D_rev	CGTCCCGGGCTCGTACCAATCACCGAGCAATGTGAGCCC
W243F_fwd	GGGCTCACATTGCTCGGTTTCTGGTACGAGCCCGGGACG
W243F_rev	CGTCCCGGGCTCGTACCAGAAACCGAGCAATGTGAGCCC
W243M_fwd	GGGCTCACATTGCTCGGTATGTGGTACGAGCCCGGGACG
W243M_rev	CGTCCCGGGCTCGTACCACATACCGAGCAATGTGAGCCC
W243N_fwd	GGGCTCACATTGCTCGGTAATTGGTACGAGCCCGGGACG
W243N_rev	CGTCCCGGGCTCGTACCAATTACCGAGCAATGTGAGCCC
W243Y_fwd	GGGCTCACATTGCTCGGTTACTGGTACGAGCCCGGGACG
W243Y_rev	CGTCCCGGGCTCGTACCAGTAACCGAGCAATGTGAGCCC

3.2.2.3 Protein Expression and purification

The Os9BGlu31 protein and its mutants were expressed and extracted from the Origami B(DE) *E. coli*, as described by Luang et al. (2013). The protein was purified as described by Komvongsa et al. (2015a). Briefly, the Os9BGlu31 protein was purified in 3 steps of IMAC on TALON metal affinity resin (Clontech, S. San Francisco, CA, USA), proteolytic cleavageof the tag from the protein with enterokinase and IMAC to adsorb the

tag, uncut protein, and IMAC-binding contaminants. The protein was concentrated and the buffer changed by centrifugal filtration.

3.2.2.4 Enzymatic comparison of Os9BGlu31 and its mutants

The activities of Os9BGlu31 and it mutants were assayed with various glucose acceptor substrates at 0.25 mM acceptor, 2.5% dimethylsulfoxide (DMSO, as solvent for the acceptors), with 5 mM 4-nitrophenyl β-D-glucopyranoside (4NPGlc) as glucose donor, and 0.25 μg of Os9BGlu31 wild type and W243A, D, M, F, and Y mutants, 0.1 μg of Os9BGlu31 W243N mutant, 1.25 μg of Os9BGlu31 L183Q mutant or 5 μg of I172T mutant, in 50 mM citrate buffer, pH 4.5 (Komvongsa et al., 2015a). For hydrolysis reactions, water replaced the acceptor and DMSO. Reactions were incubated at 30 °C and stopped by adding formic acid to 1% final concentration. Time courses were completed with all acceptors to establish the amount of product that could be produced with a linear response, corresponding to v_0. Then, reactions were run for 15 min, which gave a linear response. The reactions were filtered and separated through a ZORBAX SB-C18 (1.8 μm, 2.1 × 150 mm) column (Agilent) on an Agilent 1290 UPLC. The mobile phase was 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The compounds were eluted with a gradient from 5% to 50% B (v/v) in 13.0 min, 50% to 70% B (v/v) in 1.00 min, and 70% to 100% B (v/v) in 2.00 min at a flow rate of 0.2 mL/min. The diode array detector (DAD) scanned the range from 190–500 nm, but the results were monitored and quantified at 360 nm.

Due to the availability of kaempferol and its glucoside a separate set of reactions was carried out to determine the positional specificity of Os9BGlu31 and its mutants for the hydroxyl groups on the flavonoid rings (Komvongsa et al., 2015a). The products of transglycosylation by Os9BGlu31 and its W243 mutants were generated in reactions with 0.25 mM kaempferol as glucose acceptor, 4NPGlc as donor, and 5 μg of wild type ormutant Os9BGlu31 in 50 mM citrate buffer, pH 4.5. In other reactions, 0.25 mM kaempferol 3-O-glucoside (generously provided by Prof. Dr. Toscahi Amahura, Matsuyama University, Japan) was used as the only substrate of the enzyme. The products were quantitated on an ACQUITY UPLC system (Waters, USA) with a DAD detector and a WATERS Xevo G2 QToF mass spectrometer, which was run in the negative ion mode, with a capillary voltage of 2 kV, a source temperature of 100 °C and desolvation temperature of 200 °C. The desolvation gas flow was set as 600 L/h with the lock spray infusion flow rate set at 20 μL/min and lock spray capillary voltage at 2.50 kV. The data were analyzed with MassLynx Software.

3.2.3 Production and evaluation of uncharacterized GH1 At/Os Cluster 6 enzymes

The Os1BGlu5 and Os6BGlu22 cDNA were cloned into pET32a/DEST in our previous work, but no activity of protein was detected. Therefore, since the possibility of transglucosidase activity was not known at that time, the plasmids were transformed into BL21(DE3), Origami2(DE3), and Origami B(DE3), and expression carried out as described for Os9BGlu31, but with optimization of the time and IPTG concentration at 20 and 25 °C.

For Os9BGlu31, initially primers were designed based on the gene sequence, but no full-length gene could be amplified. So, the gene optimized for expression in *Pichia pastoris* was synthesized and cloned into pUC57 by Genscript Corp. This cDNA was excised from the plasmid with *Pst*I and *Xba*I and cloned into the corresponding sites in pPICZαBNH8and pPICZαBNH8-eGFP. The cDNA was also excised with *Nco*I and *Xho*I and cloned into pET32a, and the sequence confirmed plasmids, pET32a/Os5BGlu19 was transformed into Origami(DE3), Origami B(DE3) and BL21(DE3) *E. coli*. The pPICZαBNH8-Os5BGlu19 and pPICZαBNH8-Os5BGlu19-eGFP were linearized and then electroporated into *Pichia pastoris* strain SMD1168H and selected on 125 μg/mL zeocin.

For protein production, pET32a/Os5BGlu19 containing Origami(DE3), Origami B(DE3) and BL21(DE3) clones were screened by growth in LB media with appropriate antibiotics (ampicillin for pET32a, tetracycline and kanamycin for Origami(DE3) and Origami B(DE3) cells). Starter cultures were grown overnight and diluted 1:200 into protein production cultures. After growth to OD600 of 0.4-0.6, the cells were induced with 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM IPTG and cultured overnight at 20 °C. The cells were lysed and soluble and insoluble fractions analyzed by SDS-PAGE. Lysates were also tested for activity to release 4NP from 4NPGlc in 0.05 M acetate buffer (as described for Os9BGlu31).

For protein expression in *Pichia pastoris*, 50 colonies were screened for activity agains 4NPGlc in acetate buffer. The colonies were grown in 5 mL cultures (as described in the Pichia Manual by Invitrogen) and the cells removed by centrifugation and the media tested for activity. The one that seemed to have the most activity was checked by growing a 25 mL culture and the media used to attempt to purify protein by immobilized metal affinity chromatography, as described by Luang et al. (2010). For those cells transformed with pPICZαBNH8-Os5BGlu19-eGFP, the media was checked for green fluorescent protein fluorescence and the cells examined by fluorescence microscopy.

CHAPTER 4. Results

4.1 Identification of Os9BGlu31 natural substrates from rice

4.1.1 Identification of unsaturated fatty acids and their esters as substrates in rice extracts

To start, we tested seedling and bran extracts for potential acceptor substrates to which Os9BGlu31 could transfer a glucosyl residue (Komvongsa et al., 2015b). Due to pigments and other compounds in shoot leaves, bran was found to give a clearer result, so it was pursued further. A product that was formed from the crude extract was seen on silica gel TLC plates. When that band was TLC-purified, it gave a more intense light brown spot (R_f =0.53) migrating ahead of 4NPGlc (R_f = 0.50) in the chloroform/methanol/ammonia solvent system. The glucosyl conjugate product migrated ahead of the substrate in the ammonia-containing solvent system (Figure 4.1, corresponding to Figure 1 from Komvongsa et al., 2015b), but slower in a system with water in place of ammonia, suggesting the substrate was a carboxylic acid that would be ionized in the ammonia solvent system. Purification of this component by reverse phase chromatography gave a product with NMR peaks consistent with a mixture containing oleic acid and linoleic acid (data not shown, but similar to the peaks for oleic acid listed below).

When oleic acid and linoleic acid were tested for transglycosyaltion in a similar assay, they each produce a spot with an R_f value within error of that of the original product in this system. The reaction with oleic and 4NPGlc showed a new product $(M - H + HCO_2^-)^-$ at m/z 489, and that with linoleic acid at m/z 441 $(M - H^+)^-$ on UPLC−MS/MS QQQ in negative ion mode. The NMR of the purified oleic acid product was consistent with that for the crude mixture (except for linoleic acid peaks in the rice extract mixture). The oleic acid ^{1}H peaks were assigned as follows: δ 0.90 (3H, t, J = 6.9 Hz, H18), 1.28 (20H, m, H4−H7, H12−H17), 1.61 (2H, m, H3), 2.03 (4H, m, H8, H11), 2.36 (2H, t, J = 7.9 Hz, H2), 5.35 (2H, t, J = 5.0 Hz, H9 and H10), 3.35 (1H, m, H2′), 3.57 (1H, m, H4′), 3.67 (1H, m, H3′), 3.84 (1H, m, H5′), 4.06 (1H, m, H6′), 4.14 (1H, m, H6′), 5.48 (1H, J = 8.5 Hz, H1′). The chemical shift of 5.48 ppm and coupling constant of 8.5 Hz for H1′ were consistent with the expected beta-configuration at the anomeric carbon.

The product of the linoleic ester appeared to give an impure product and was lost or degraded in the purification process, so a time course of the reaction was studied by NMR instead. The NMR spectrum was complicated with multiple peaks, but was consistent with the initial product of linoleoyl 1-O-β-D-glucose gradually shifting to linoleoyl 3-O-glucose by acyl migration (Komvongsa et al., 2015b).

When the activity of Os9BGlu31 was tested for relative activity with three fatty acids, stearic acid (18:0), oleic acid ($18:1^{\Delta 9}$) and linoleic acid ($18:2^{\Delta 9,12}$), the activity was seen to be greater found to be approximately 2-fold higher for oleic acid and linoleic acid, compared to stearic acid (Figure 4.2, Komvongsa et al., 2015b). Of these, oleic acid was the most efficient substrate with a k_{cat}/K_m of 0.291 $mM^{-1}s^{-1}$ compared to 0.128 $mM^{-1}s^{-1}$ for linoleic acid, although these were both much lower than the value of 25.4 $mM^{-1}s^{-1}$ for ferulic acid (Komvongsa et al., 2015b).

When the rice extracts of Os9BGlu31 knockout lines and segregated wild type controls were tested for oleic acid, linoleic acid and their 1-O-glucosyl esters, consistent differences were seen for the glucose esters (Figure 4.3). Although the *os9bglu1*-1 line also showed higher oleic acid and linoleic acid concentrations compared to control, the other 3 lines had oleic acid and linoleic acid levels within error of controls (Komvongsa et al., 2015b).

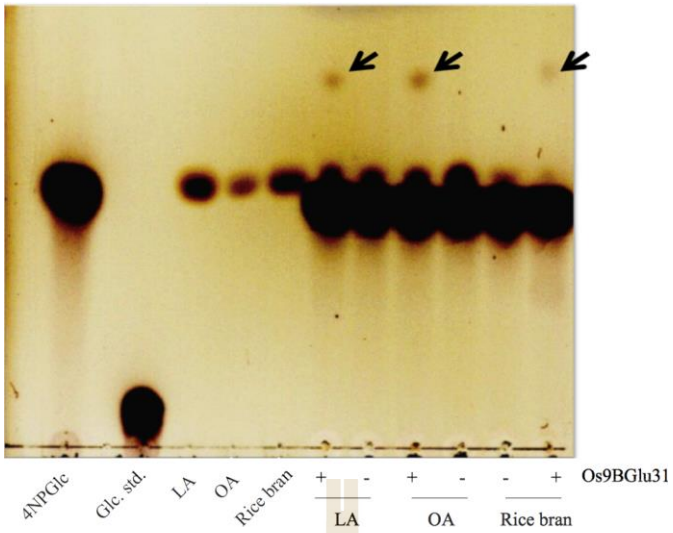

Figure 4.1 Silica gel TLC of Os9BGlu31 products with 4NPGlc donor and rice bran, oleic acid and linoleic acid acceptors. A 7:2.8:0.2 (v/v/v) chloroform/methanol/30% ammonia solvent system was used and carbohydrate or lipid containg spots detected by charring with sulfuric acid. The lanes show 4NPGlc standard, glucose standard(Glc), linoleic acid standard (LA), oleic acid standard (OA), and purified rice bran substrate (Rice bran), followed by reactions with (+) and without (−) Os9BGlu31 enzyme with linoleic acid, oleic acid, and purified rice bran substrate and 4NPGlc donor substrate.

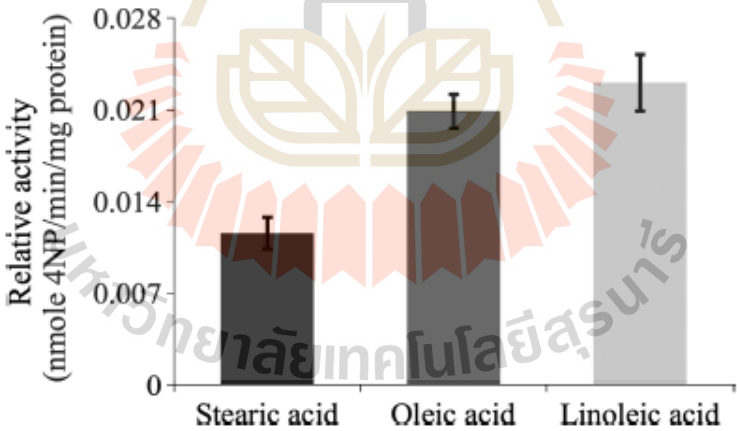

Figure 4.2 Relative activities of Os9BGlu31 with 18 carbon fatty acid acceptors. The acceptors include saturated (stearic acid, 18:0) and unsaturated (oleic acid,18:1, and linoleic acid, 18:2) fatty acids. 4NPGlc (20 mM) was used at the donor and fatty acids were tested at 0.25 mM concentration, which are nearly saturating conditions. The figure is from Komvongsa et al., (2015b).

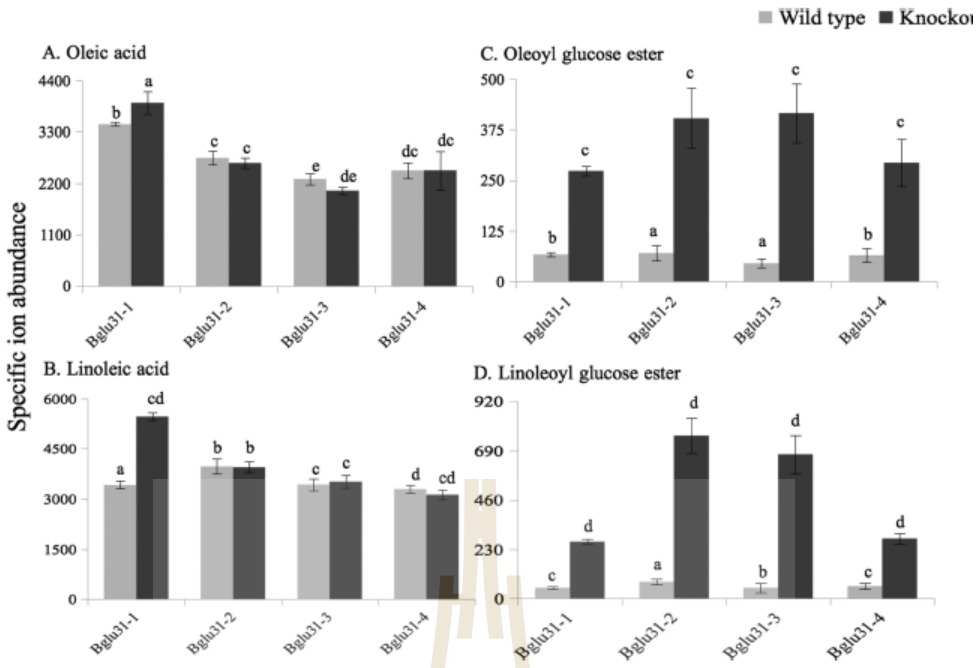

Figure 4.3 Relative levels of oleic acid, oleic acid glucose ester, linoleic acid, and linoleic acid glucose ester in lines with knock out of *Os9BGlu31* (dark bars) and segregated wild type control lines (light colored bars). The figure is from Komvongsa et al. (2015b).

4.1.2 Identificatin of feruloyl glucose as a natural substrate in rice flag leaves.

When flag leaf extracts from wild type Dongjin rice were analyzed by QToF LCMSMS, compounds were putatively identified in either positive or negative ion modes. The compounds with m/z corresponding to naringin, kaempferol 3-*O*-rutinoside, peonidine 3-*O*-β-D-galactoside, and oenin were detected only in positive ion mode, while compounds with masses corresponding to sinapoyl glucose, kaempferol 3-*O*-β-D-glucopyranosyl 7-*O*-rhamnoside, kaempferol 3-O-glucoside, tricin 7-*O*-glucoside (or tricin 5-*O*-glucoside), and gibberellin A5 were only be discovered in negative ion mode (Table 4.1). However, none of these compounds could clearly be identified as increasing or decreasing in the Os9BGlu31 mutant lines. However, difference could be seen on close inspection of the total ion chromatogram (Figure 4.4). When the mass spectrum in the region of one of these peaks was inspected, it corresponded to feruloyl glucose, which we had previously identified as a substrate and product of recombinant Os9BGlu31 *in vitro*. The extracted ion chromatogram for the mass of feruloyl glucose (355.1 a.m.u.) clearly showed one major peak that was much higher in the mutant plant flag leaf extract than in the extract from the wild type plant (Figure 4.5).

In order to quantitatively evaluate the difference between wild type and Os9BGlu31knockout lines, the MRM assay similar to that used for fatty acid glucose esters was used to measure the levels of feruloyl glucose. As shown in Table 4.2, the levels of feruloyl glucose were clearly higher in all mutant lines compared to their segregated wild type controls.

Table 4.1 Compounds tentatively identified in rice flag leaf extracts by LCMSMS

Putative compound	Negative ion mode						Positive ion mode					
	Formula	RT	UV peaks (nm)	Calc. Mass	Ext. Mass	ppm	Formula	RT	UV peaks (nm)	Calc. Mass	Ext. Mass	ppm
1. Sinapoyl glucoside	$C_{17}H_{21}O_{10}$	7.5	325	385.1140	385.1140	0	NA	NA	NA	NA	NA	NA
2.Naringin	NA	NA	NA	NA	NA	NA	$C_{26}H_{29}O_{15}$	8.5	270,350	581.1508	581.1506	-0.3
3.Rutin	$C_{27}H_{29}O_{16}$	8.7	270,348	609.1456	609.1461	0.8	$C_{27}H_{30}O_{16}$	8.7	270,350	611.1603	611.1607	0.7
4. Kaempferol-3-O-β-D-glucoparanosyl-7-O-rhamnoside	$C_{27}H_{29}O_{15}$	9.5	270, 340	593.1506	593.1512	1.0	NA	NA	NA	NA	NA	NA
5. Kaempferol-3-O-rutinoside, Kaempferol-3-glucoside-3''-rhamnoside, Kaempferol-3-O-neohesperiside	NA	NA	NA	NA	NA	NA	$C_{27}H_{31}O_{15}$	9.7	270,350	595.1679	595.1657	-3.7
6. Isorhamnetin-3-galactosyl-Isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	9.9	269, 319	623.1618	623.1618	0	$C_{18}H_{32}O_{16}$	9.9	270,320	625.1752	625.1763	1.8
7. Kaempferol-3-O-glucoside	$C_{21}H_{19}O_{4}$	10.4	270, 348	447.0933	447.0933	-1.1	NA	NA	NA	NA	NA	NA
8. Tricin-7-O-glucoside	$C_{23}H_{23}O_{12}$	10.8	348	491.1193	491.1195	0.4	NA	NA	NA	NA	NA	NA
9. Peonidine-3-O-β-galactopyranoside	NA	NA	NA	NA	NA	NA	$C_{22}H_{23}O_{11}$	10.9	268,334	463.1230	463.1235	1.1
10. Gibberalin A5	$C_{19}H_{21}O_{5}$	11.0	270, 345	329.1386	329.1394	2.4	NA	NA	NA	NA	NA	NA
11. Oenin	NA	NA	NA	NA	NA	NA	$C_{23}H_{25}O_{12}$	11.1	270,350	493.1338	493.1341	0.6
12. Cirsiliol	$C_{17}H_{13}O_{7}$	11.2	270, 348	329.0659	329.0667	2.4	$C_{17}H_{15}O_{7}$	11.2	270, 350	331.0819	331.0812	-2.1

Table 4.2 Concentrations of feruloyl glucose (FAG) determined in Os9BGlu31 mutant lines and their segregated wild type controls by MRM LCMSMS.

Line	Knockout Os9BGlu31 FAG (µM)	Wild type FAG (µM)
osbglu31-1	11.144	0.875
osbglu31-2	6.938	1.461
osbglu31-3	9.391	1.213
osbglu31-4(1)	6.139	0.393
osbglu31-4(2)	4.179	0.487
osbglu31-4(3)	5.618	0.359

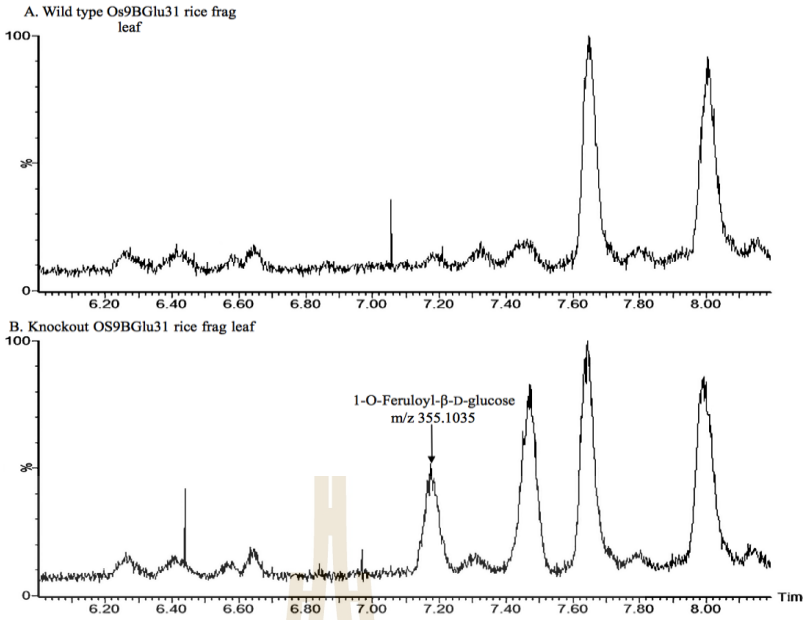

Figure 4.4 Region of the LCMS total ion chromatogram showing the different peaks between the wild type (top) and Os9BGlu31 knockout line flag leaf extracts (6.0 to 8.2 min). The peak with the mass matching 1-O-feruloyl glucose (355.1 a.m.u.) is marked in the mutant extract chromatogram.

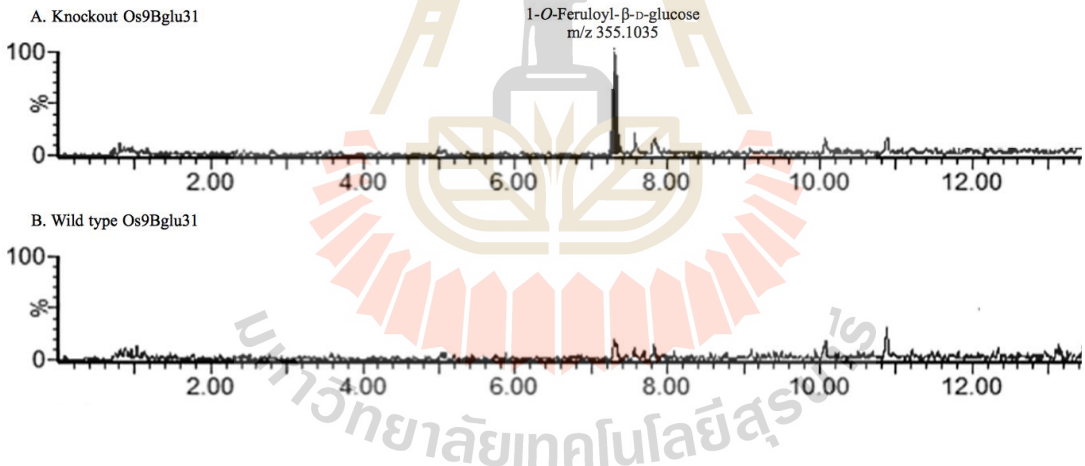

Figure 4.5 Extracted ion chromatogram comparison of the Os9BGlu31 knockout mutant (top) and segregated wild type line flag leaf ethanolic extracts. The relative abundance (percent) of the ion peaks is graphed against the chromatography time in minutes.

4.2 Activities of Os9BGlu31 active site cleft mutants

4.2.1 Identification of residues of interest

The homology model of the Os9BGlu31 structure was aligned to the structure of Os3BGlu6 covalent intermediate structure with 2-deoxy-2-fluoroglucoside, which displayed a water molecule in the position to attack and displace the glycosyl-enzyme intermediate (Firgure 4.6). It was hypothesized that the transglycosylation preference of Os9BGlu31 might be caused by a less polar environment that would be less accommodating to the attack by the water molecule compared to a less polar acceptor. Residues in close proximity with the

acceptor water molecule and were hydrophobic in Os9BGlu31 but polar in Os3BGlu6 included I172 (T181 in Os3BGlu6), L183 (Q192), and L241 (D241). W243 was also chosen for mutagenesis, since it corresponds to a critical residue for oligosaccharide binding in Os3BGlu7 (Sansenya et al., 2012).

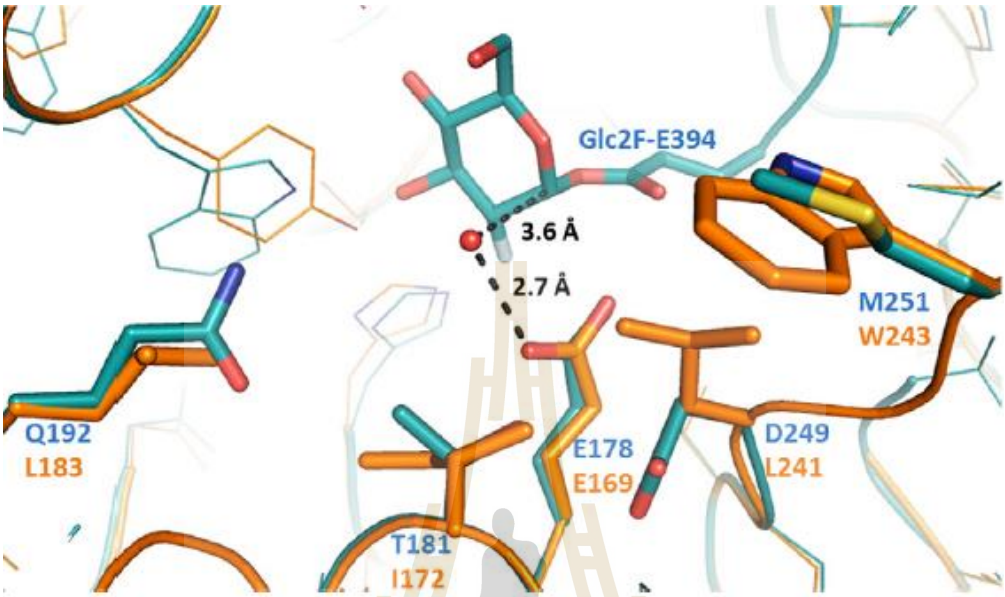

Figure 4.6 Superposition of the active sites of the homology model of Os9BGlu31 and the crystal structure of Os3BGlu6 in complex with 2-deoxy-2-fluoroglucoside. A water molecule in range to attack the anomeric center of the covalent intermediate and hydrogen bond to the catalytic base is shown with the corresponding distnaces. Sidechains identified for mutagenesis are shown in stick format and labeled with Os3BGlu6 numbers on top and Os9BGlu31 residue numbers on the bottom. (Komvongsa et al., 2015a).

3.2.2 Acceptor specificity of Os9BGlu31 mutants

The Os9BGlu31 I172T and L183Q mutatns exhibited adequate solubility and activity for purification and testing, but the L241D mutant showed low amounts of solubility and low activity, so it was not characterized further. Although both the I172T and L183Q mutants had lower activities than wild type, their relative hydrolysis versus transglycosylation ratios were not significantly different. Nonetheless, they did exhibit some differences as shown for a set of substrates in Figure 4.7.

In contrast to the other mutations, the W243N mutant (designed based on the residue at that position in the Os3BGlu7 active site showed much higher activity than wild type in transglycosylation of ferulic acid (Figure 4.7 a). The Os9BGlu31 W243N mutant also showed a higher ratio of hydrolysis to transglycosylation compared to wild type Os9BGlu31, but this difference was rather small. Because of the increased activity resulting from mutagenesis of this site, the W243 residue was further mutated to Met based on the corresponding residue in Os3BGlu6 (W243M), Ala (W243A), Asp (W243D), Phe (W243F) and Tyr (W243Y). Although none of these mutations displayed the high activity equivalent to W243N, they all showed particular relative activities toward the range of substrates tested. It was clear that W243D showed the highest ratio of hydrolysis to transglycosylation, but its activity was quite low in all assays. When the products of the reaction with ferulic acid were

analyzed by LCMS, the wild type Os9BGlu31 produced essentially only glucose ester, as reported by Luang et al. (2013). However, the W243 mutants also produced varying amounts of ferulic acid glucoside (Figure 3.8) (Komvongsa et al., 2015a). Further analysis of the products in reactions with kaempferol can be found in Komvongsa et al (2015a).

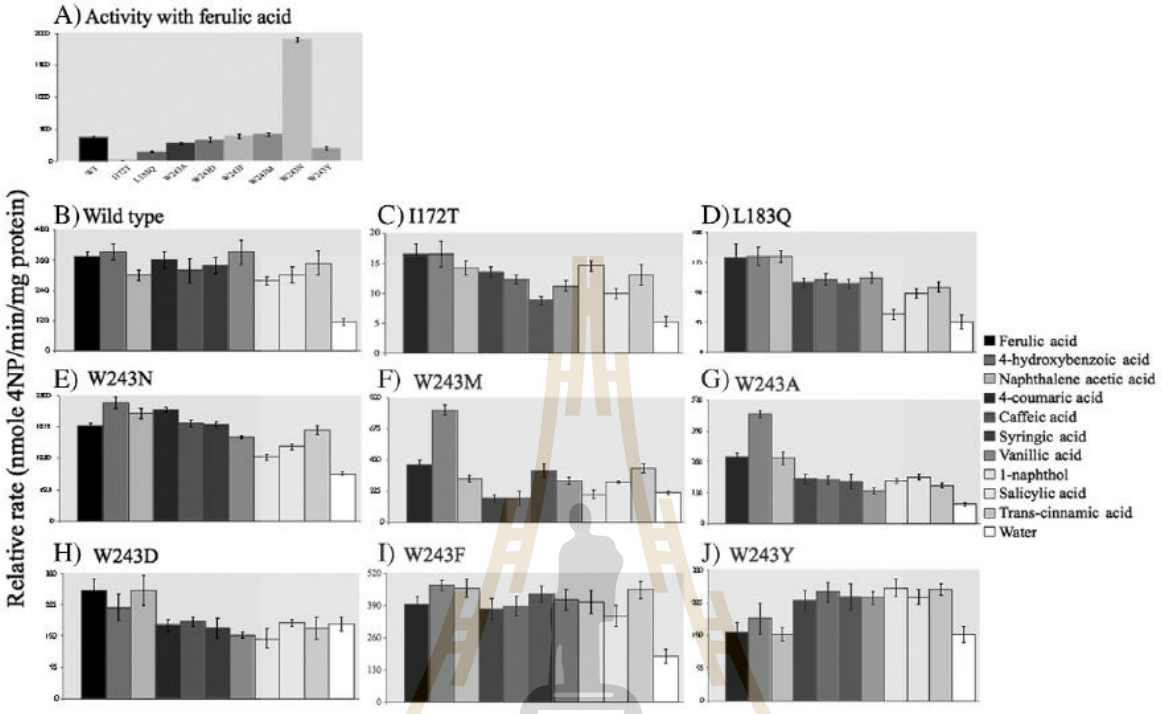

Figure 4.7 Relative activities of Os9BGlu31 wild type and mutants toward ferulic acid acceptor (a) and of each mutant toward a range of substrates. The substrates tested are defined on the right. In the case of hydrolysis, the assay was carried out in citrate buffer alone and no glucoconjugate of citrate was detected.

As one of our initial goals was to produce arbutin, the glycosylation of *para*-hydroxyquinone was also evaluated. Although wild type and mutant Os9BGlu31 enzymes could transglycosylate to make α-arbutin, evaluation of prices on the internet showed that best prices for α-arbutin were less than $100 USD per kilogram, while our donor substrate, 4NPGlc was several hundred USD per kg, so the process was not economical.

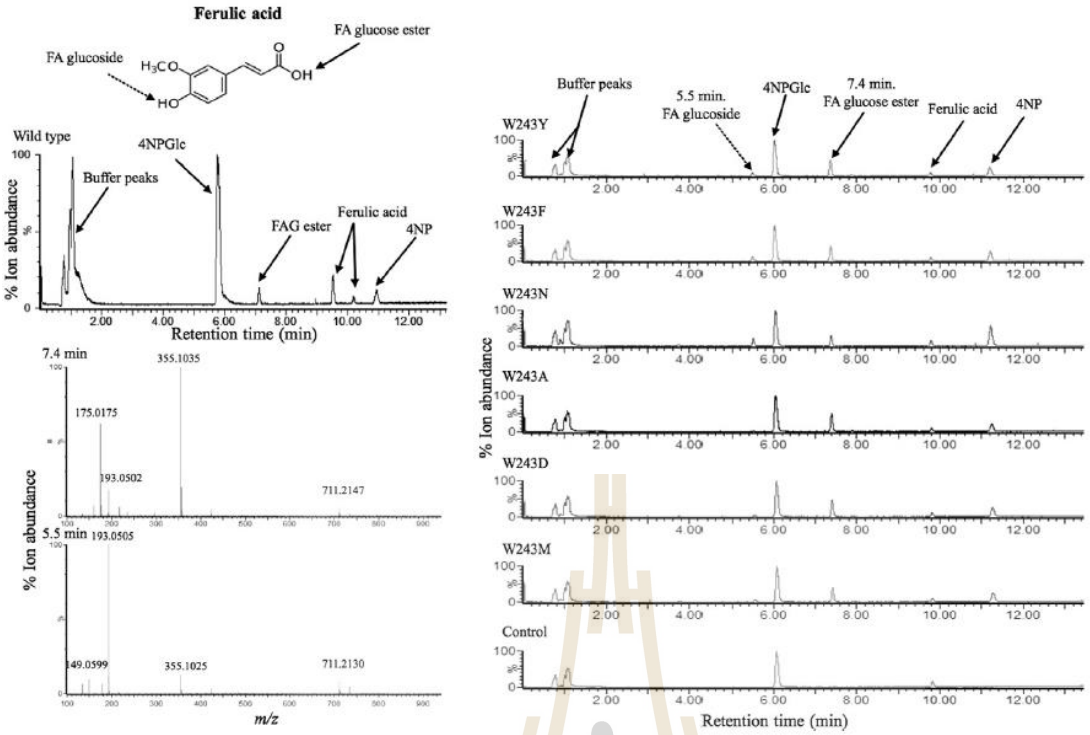

Figure 4.8 LCMS analysis of transglycosylation of ferulic acid by Os9BGlu31 and its residue 243 mutants. The structure of ferulic acid is shown to indicate the possible glycosylation positions is shown in the upper left, followed by the mass chromatogram for the wild type. The mass spectra of the peaks at 7.4 min (glucose ester) and 5.5 min (glucoside) from the W243N mutant are also shown.

4.3 Expression and characterization of new GH1 phylogenetic cluster At/Os6 proteins

When we tried to express the new rice GH1 cluster 6 genes Os1BGlu5 and Os5BGlu22, a weak band was seen in the soluble extract of the Origami B(DE3) *E. coli* strain in each case. However, the extracts gave no clear activity for these proteins, so we did not know whether they were expressed in active forms in the *E. coli* expression system. So, we concentrated on other genes from this phylogenetic cluster. Os9BGlu32 was targeted because it was found to complement an Arabidopsis BG1 knockout line by our collaborators in Korea. We ordered an Os9BGlu32 gene optimized for expression in *Pichia pastoris*, but around 50 clones were screened and activity with 4NPGlc as a substrate could not be detected. We also attempted to detect protein by an anti-His-tag antibody immune-dot blot, but did not see it.

We further attempted to clone Os5BGlu19 and Os6BGlu25 cDNA for expression of these proteins, since these are the next most highly expressed GH1 cluster 6 genes after Os1BGlu5 and Os5BGlu22. We were not able to amplify a full-length cDNA, although amplification of half cDNA was achieved. To accelerate progress on this, an Os5BGlu19-encoding gene optimized for expression in *P. pastoris* was synthesized at Genscript Corp., and cloned into pET32a for expression in *E. coli* and pPICZalphaBNH8 for expression in *P. pastoris*. On the initial attempt, soluble Os5BGlu19 protein could not be detected in *E. coli*. In the *P. pastoris*, we initially thought there was activity in the media, but it turned out to be background activity from the yeast's own proteins. When the construct with eGFP was used, the green fluorescence could be detected in the media and in the cells, but it seemed to mostly

be found in the cells, possibly in the vacuole (Figure 4.9). Attempts at IMAC purification failed, leading us to believe that the protein might have lost its N-terminal His-tag.

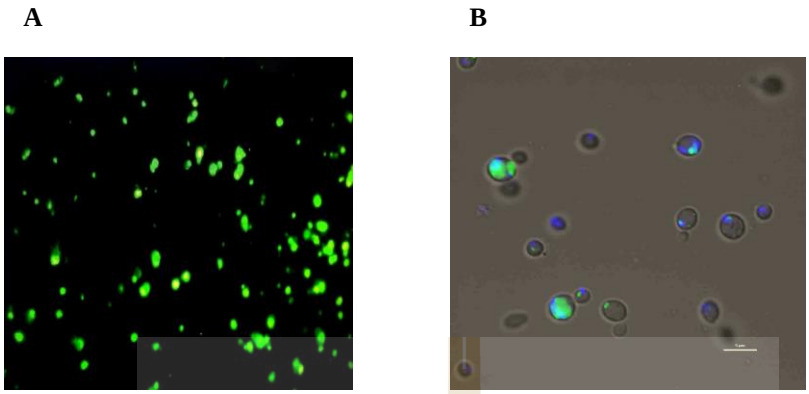

Figure 4.9 Enhanced green fluorescent protein fluorescence in *P. pastoris* cells expressing Os5BGlu19-eGFP. A. eGFP fluorescence of cells. B. Staining of cells with Hoerst stain of the DNA to show the nuclei. In a few cells to the left, the eGFP fluorescence is also apparent and is not nuclear.

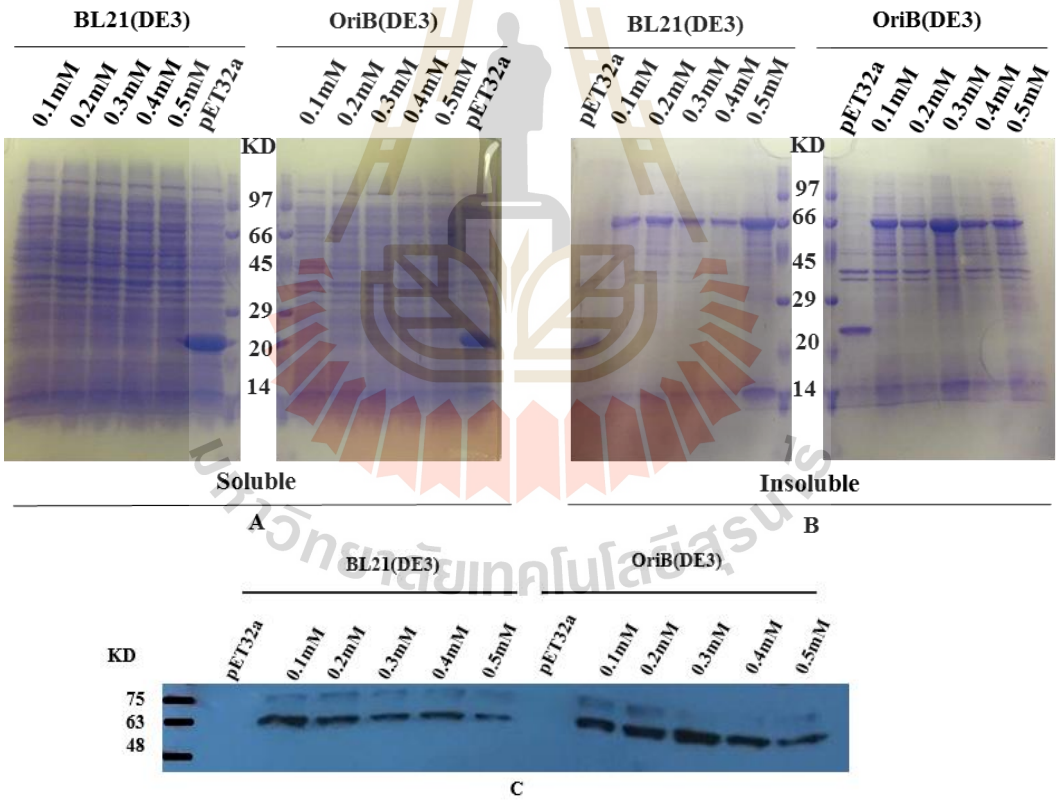

Figure 4.10 Expression of truncated Os5BGlu19 (TrOs5BGlu19). A. SDS-PAGE of the soluble fractions of *E. coli* strains BL21(DE3) and Origami B(DE3) (OriB) containing pET31a/TrOs5BGlu19 and induced with different concentrations of IPTG (concentrations given across the top). B. SDS-PAGE of the insoluble portion of the cells described in A. C. Western blot of the soluble fraction of the cells described in A with Anti-Os5BGlu19-peptide antiserum. Lanes marked pET32a, indicate cells transformed with empty pET32a plasmid and induced with 0.5 mM IPTG.

To try to eliminate any N-terminal or C-terminal protease sites of vacuole targeting sites, the Os5BGlu19 cDNA was truncated to remove the N-terminal precission protease site and C-terminal site that did not match the structure of the known proteins used for the template in homology modeling (Os3BGlu7), assuming this may be extra, unstructured region that might contain sequence that acted as a localization tag. Although this construct was intended for expression in *P. pastoris*, it was also cloned into pET32a and expression in *E. coli* resulted in a noticible soluble band (Figure 4.10). This protein could also be purified by IMAC.

When TrOs5BGlu19 was purified by IMAC with a step gradient of imidazole, the protein eluted at nearly all imidazole concentrations, with the most at 60 and 80 mM imidazole. The pooled protein was mixed with 4NPGlc in acetate buffer or with various acceptors in citrate buffer (as described for Os9BGlu31, but no reaction could be detected. Finally, the protein was mixed with 4 and 10-week old rice seedling leaf extracts as donor and FA as acceptor. The assays were then measured for FAG according to the method developed for measuring Os9BGlu31 mutant leaf extracts, and showed that FAG increased in assays containing leaf extract and FA, in the presence of recombinant Os5BGlu19 (Table 4.3). Similar assays using the extracts of cells producing Os1BGlu5, Os5BGlu22 or Os9BGlu32 did not produce any such change in FAG concentration, suggesting these proteins do not transglycosylate FA or the recombinantly expressed proteins are inactive.

Table 4.3 Production of feruloyl glucose (FAG) in reactions of Os5BGlu19 with leaf extracts and ferulic acid (FA).

Sample	Without Os5BGlu19 – FAG (µM)	With Os5BGlu19 – FAG (µM)
4 week old leaves	0.26 ± 0.04	8.9 ± 1.6
10 week old leaves	7.6 ± 1.6	20.6 ± 2.4
10 week old stem	0.12 ± 0.01	0.78 ± 0.08

Attempts to use FAG as a donor and other 4-hydroxy coumaric acids as acceptors for Os5BGlu19 did not give any activity, suggesting that either the enzyme required some cofactor from the rice extracts or the other hydroxycoumaric acid derivatives could not serve as acceptors as ferulic acid could. However, it was noted that the activity to produce FAG could only be detected in the MRM LCMSMS method.

CHAPTER 5. Analysis

5.1 Discussion

Os9BGlu31 is a unique transglucosidase from rice that can transfer glucose between a range of compounds with 4-hydroxycoumaric acids and their 1-O-glucose esters serving as some of the best substrates (Luang et al., 2013). It was fortunate that Os9BGlu31 was promiscuous enough to accept 4NPGlc as a donor substrate, so that we were able to identify that is was an active enzyme and use this commercial donor for our routine assays. This did not appear to be the case for Os5BGlu19, and it is likely not the case for Os1BGlu5, Os5BGlu22 or Os9BGlu32 for which we have not found activity, although it is difficult to know with certainty until some activity is found. The range of substrates accepted by Os9BGlu31 make it and its mutants very useful for producing glucoconjugates of interest, however, our initial expectation that we might use it for a-arbutin production appears economically unfeasible due to the low prices for a-arbutin available in the market, relative to our glucosyl donor substrate, 4NPGlc. Nonetheless, it should be useful for production of specialty chemicals where the economy of scale has not driven prices to the level of arbutin.

The relatively broad substrate specificity of Os9BGlu31 makes it difficult to uncover its natural function in rice plants. Indeed, rice lines with knockouts of Os9BGlu31 expression look essentially like wild type. To discover what is different, we had to look into leaf extracts. Treatment of leaf extracts and their fractions with Os9BGlu31 and a glucose donor (4NPGlc) identified the unsaturated fatty acids oleic acid and linoleic acid as possible acceptors for Os9BGlu31 transglucosidase activity that are found in rice extracts. When an MRM LCMSMS method was developed to measure these compounds and their glucose esters, it was found that the oleoyl glucose and linoleoyl glucose esters were elevated in the Os9BGlu31 mutant plants rather than be depleted, suggesting that in the plant they mainly serve as donor substrates for Os9BGlu31. What is the acceptor for the glucose is unclear.

The biological significance of these fatty acyl glucose esters is also unclear. They are relatively unstable and might serve as short-lived messenger molecules. Indeed, they were first identified in plants during the isolation of brassinosteroids and were initially thought to have this activity (Mitchell et al., 1970; Mandava, 1988). Although Tanaka and colleagues (1979) did not find bioactivity for 1-O-oleoyl glucose when they synthesized it, they tested it in only a limited assay. So, these compounds may in fact be signaling compounds that are modulated by Os9BGlu31. We showed that Os9BGlu31 also appears to use FAG as a donor substrate, in that it is also elevated in Os9BGlu31 knockout plants. Indeed other, as yet unidentified Os9BGlu31 compounds were found to be elevated in the rice leaf extracts of *Os9BGlu31* knockout lines, although it is not know whether they are donor or acceptor substrates. Nonetheless, the complex function of Os9BGlu31 in the plant appears to involve multiple donor (and acceptor) substrates.

Surprisingly, mutations that made the Os9BGlu31 active site near the acceptor binding site for water more polar did not increase the hydrolysis vs. transglucosidation activity significantly, but mutations at the W243 residue, corresponding to the +2 subsite in celloligosaccharide-hydrolyzing β-glucosidases like Os3BGlu7 did have a significant effect on acceptor specificity. The Os9BGlu31 W243N mutant, which changed the residue to be be the same as that in Os3BGlu7 resulted in a roughly 5-fold increase in activity with ferulic acid and an increase in the hydrolysis versus transglycosylation ratio, although the mutant still catalyzed transglycosylation more rapidly than hydrolysis. The enzyme also transglycosylated naphthalene acetic acid better than ferulic acid, thereby changing the relative activities toward these two substrates, but was otherwise similar to wild type in its

relative activities toward other substrates in Figure 4.7, though with higher activity toward all these substrates. While none of the other mutations showed similarly high activity as W243N, W243D showed high hydrolysis activity relative to transglycosylation, as was originally expected for the other mutations. However, some of the other mutatations caused greater changes inspecificity, such as the Os9BGlu31 N243A and N243M mutants having much higher preference for 4-hydroxybenzoic acid than wild type and the other mutants, while the W243F and W243Y mutations, despite their similarity to wild type, showed higher relative activities toward naphthol and salicylic acid than the wild type. It should be noted that, none the less, none of these other mutations had higher activity toward any substrate relative to W243N.

Of note, all of the mutants seemed to have higher activity toward phenolic alcohols compared to the wild type. All of the W243 mutants could produce ferulic acid glucoside, whereas the wild type did not make this product in significant amounts in reaction with ferulic acid, but only produced detectable amounts of FAG. It is notable that these mutants could have higher activity and broader acceptor substrate specificity, since it suggests that they could be used to make glycosides of interest, such as glucosylating drugs to make them more soluble. Thus, the application of these mutants was the goal of our next project.

We had little success in expressing and characterizing GH1 At/Os cluster 6 enzymes other than Os9BGlu31. Most attempts to produce such proteins resulted in apparently insoluble proteins and no activity in soluble fractions. Only Os5BGlu31 could be produced in an active form, and then only after truncation of the N- and C-termini to produce a more readily soluble form. Although FA could be seen to be an acceptor substrate when mixed with rice extract in the assay with Os5BGlu19, the exact nature of the donor substrate(s) in the rice extract are still not known. No substrates could be determined for Os1BGlu5, Os5BGlu22 and Os9BGlu32, despite the use of rice extracts and FA as a donor like with BGlu19 or other substrate mixtues as with Os9BGlu32, so we do not know whether the enzymes expressed in the recombinant systems have narrower substrate specificity or are simply inactive.

Overall, the mutational analysis of Os9BGlu31 appeared to be the most successful aspect of this project. The high activity W243N and other mutants can be explored for activity against substrates of interest, such as antibiotics in the future.

4.2 Conclusions and Comments

In this project, we were able to complete the first paper on Os9BGlu31 and generate two more, on exploring the changes in activity and increase substrate and product range upon mutation of the Os9BGlu31, and one determining natural substrates found in rice extracts. Although other GH1 cluster At/Os6 enzymes were expressed in recombinant systems, only a single substrate of Os5BGlu19 could be identified, while the none were found for Os1BGlu5, Os5BGlu22 and Os9BGlu31. In the future, we anticipate exploitation of Os9BGlu31 and its mutants will allow us to synthesize many glucoconjugates, including drugs and phytohormone, which will be useful for research and clinical application.

REFERENCES (เอกสารอ้างอิง ของโครงการวิจัย)

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

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

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

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. J.Struct. Biol. 173, 169-179.

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

Eklöf JM, Brumer H. (2010) The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. Plant Physiol. 153: 456-466.

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

Gantt RW, Peltier-Pain P, and Thorson JS. (2011) Enzymatic methods for glycol (diversification/randomization) of drugs and small molecules. Nat. Prod. Rep. 28: 1811-1853.

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

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

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

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

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

Ketudat Cairns JR, Esen A. (2010) β-Glucosidases. Cell. Mol. Life Sci. Epub ahead of print.

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

Kojima M, Kamada-Nobusada T, Komatsu H, Takei K, Kuroha T, Mizutani M, Ashikari M, Ueguchi-Tanaka M, Matsuoka M, Suzuki K, Sakakibara H. (2009) Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography–tandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. Plant Cell Physiol. 50: 1201–1214

Komvongsa J, Luang S, Marques JV, Phasai K, Davin LB, Lewis NG, Ketudat Cairns JR. 2015. Active site cleft mutants of Os9BGlu31 transglucosidase modify acceptor substrate specificity and allow production of multiple kaempferol glycosides. Biochim. Biophys. Acta 1850: 1405–1414

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

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

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

Lee SS, Hong SY, Errey JC, Izumi A, Davies GJ, Davis BG. (2011) Mechanistic evidence for a front-side, SNi-type reaction in a retaining glycosyltransferase. Nat. Chem. Biol. 7: 631-638.

Lirdprapamongkol K, Svasti J. (2000) Alkyl glucoside snthesis using Thai rosewood β-glucosidase. Biotechnol. Lett. 22: 1889-1894.

Luang S, Hrmova M, Ketudat Cairns JR. (2010) High-level expression of barley β-D-glucan exohydrolase HvExoI from a codon-optimized cDNA in *Pichia pastoris*. Protein Express. Purific. 73, 90-98.

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.

Mandava NB. (1988) Plant growth-promoting brassinosteroids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 23−52.

Marti-Renom MA, Stuart A, Fiser A, Sanchez R, Melo F, Sali A. (2000) Comparative protein structure modeling of genes and genomes, Annu. Rev. Biophys. Biomol. Struct. 29: 291–325.

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

Mitchell JW, Mandava N, Worley JF, Plimmer JR, Smith MV. Brassins-a new family of plant hormones from rape pollen. Nature 1970, 225, 1065-1066.

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

Newman DJ, Cragg, GM. (2007) Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70: 461-477.

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

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

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

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

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

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

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

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

Shaik FA, Withers SG. (2008) Teaching old enzymes new tricks: engineering and evolution of glycosidases and glycosyltransferases for improved glycoside synthesis. Biochem. Cell Biol. 86: 169-177.

Svasti J, Phongsak T, Sarnthima R. (2003) Transglycosylation of tertiary alcohols using cassava β-glucosidase. Biochem. Biophys. Res. Comm. 305: 470-475.

Tanaka AK, Kobayashi A, Yamashita K. (1979) Synthesis and biological activities of 1-O-glucopyranosyl fatty acid esters. Agric. Biol. Chem. 43, 2537−2542.

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 Biol. 8, 84.

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

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

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

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

RESEARCHER CURRICULUM VITA (ประวัติผู้วิจัย)

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Biochemistry, rice functional genomics, bioinformatics, structural biology

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

7.1 Journal Publications 85 publications (82 in Scopus), H-index (Scopus): 22

7.2 Projects as Head of Project (หัวหน้าโครงการวิจัย): ชื่อโครงการวิจัย

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

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

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

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

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

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

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

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

7.2.9 Structural Basis for Substrate-Specificity in Glucooligosaccharide Hydrolyzing β-Glucosidases from Glycosyl Hydrolase Families 1 and 3, 2553-2556, Thailand Research Fund Basic Research Grant BRG5380017.

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

7.2.11 Characterization of a glycoside hydrolase family 1 group 6 hydrolase, 2554. SUT/NRCT, 2556/09. Head of project.

7.2.12 Structure, function and application of plant β-glucosidases and related enzymes, 2556-2559, Thailand Research Fund Basic Research Grant BRG5380012. Head of project.

7.2.13 Identification and Characterization of Rice Gibberellin Beta- Glucosidase, SUT1-102-55-36-01. Head of project.

7.2.14 Structure, Function and Application of β-Glycosidases, BRG5980017; 2559-2562, The Thailand Research Fund.

7.3. Projects in progress (งานวิจัยที่กำลังทำ : ชื่อแผนงานวิจัย และ/หรือโครงการวิจัย แหล่งทุน และสถานภาพในการทำวิจัยว่าได้ทำการวิจัยคุล่วงแล้วประมาณร้อยละเท่าใด)

7.3.1 Characterization of Rice Phytohormone β-Glucoses, 2558-2560, SUT, Budget Bureau, NRCT.

7.3.2 Detection and metabolism of glucosylceramide and related sphingolipids, SUT, Budget Bureau, NRCT.

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- Molecular Biology & Genetic Engineering (Plant & Animal)
- Recombinant Protein Production
- Gene Expression in cloned animals and stem cells

6. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ : ระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอโครงการวิจัย เป็นต้น

6.1 ผู้อำนวยการแผนงานวิจัย : โครงการการผลิตกรดซัคซินิคโดยใช้เชื้อแบคทีเรียในกระบวนการหมักแบบกะ ส่งรายงาน

6.2 ผู้ประสานงานชุดโครงการ การวิจัยโปรตีนแห่ง มหาวิทยาลัยเทคโนโลยีสุรนารี ส่งรายงานการวิจัยฉบับสมบูรณ์

6.3 หัวหน้าโครงการวิจัย : มีชื่อโครงการวิจัย ดังต่อไปนี้

- การพัฒนาเทคนิคเพื่อตรวจเชื้อก่อโรคปนเปื้อนในเนื้อไก่สด ส่งรายงานการวิจัยฉบับสมบูรณ์ 2559
- การโคลนและศึกษาการทำงานของ Os1BGlu4 เบตากลูโคไซเดสจากข้าว ส่งรายงานการวิจัยฉบับสมบูรณ์ 2557
- การศึกษาความยาวของเทลโลเมียร์ในลูกแมวที่เกิดจากการโคลนนิ่ง ส่งรายงานการวิจัยฉบับสมบูรณ์ 2557
- การหาเครื่องหมายโมเลกุลบ่งชี้ทางพันธุกรรมที่สัมพันธ์กับความต้านทานโรคแคงเกอร์ในมะนาวสายพันธุ์พิจิตร (M33) ส่งรายงานการวิจัยฉบับสมบูรณ์ 2556
- โมโนโคลนัลแอนติบอดีต่ออสุจิ Y ของวัว ส่งรายงานการวิจัยฉบับสมบูรณ์ 2557
- การโคลนและผลิตโปรตีนไฟโคไซยานินจากไซยาโนแบคทีเรีย ส่งรายงานการวิจัยฉบับสมบูรณ์ 2555
- การพัฒนาเครื่องหมายการตรวจสอบย้อนกลับปลานิลจากมทส ส่งรายงานการวิจัยฉบับสมบูรณ์ 2553
- การค้นหาและการแสดงออกของกลุ่มยีน Glycosyl Hydrolases ในจีโนมของข้าวหอมมะลิ ส่งรายงานการวิจัยฉบับสมบูรณ์ 2554
- การโคลนและผลิตเอ็นไซม์เอนเทอโรไคเนสสายสั้น ส่งรายงานการวิจัยฉบับสมบูรณ์ 2551
- การผลิตรีคอมบิแนนท์ทรานสกลูตามิเสนจากปลานิล ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550
- การผลิตรีคอมบิแนนท์เอนไซม์ Thermostable DNA polymerase จาก *Pyrococcus furiosus* ในแบคทีเรีย *Escherichia coli* ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550
- การศึกษาสภาวะที่เหมาะสมในการผลิตรีคอมบิแนนท์โปรตีนในถังหมัก ส่งรายงานการวิจัยฉบับสมบูรณ์ 2549
- การแสดงออกและการผลิตเบต้ากลูโคสิเดส โดย *Pichia pastoris* ส่งรายงานการวิจัยฉบับสมบูรณ์ 2548
- การจำแนกลักษณะทางพันธุกรรม สรีระวิทยา และพฤติกรรมของไก่พื้นเมืองไทย ส่งรายงานการวิจัยฉบับสมบูรณ์ 2547
- การหาแผนที่ทางพันธุกรรมของไผ่ตง ส่งรายงานการวิจัยฉบับสมบูรณ์ 2546
- การพัฒนาวิธีการตรวจหาชนิดของโครโมโซมเพศปลานิล ส่งรายงานการวิจัยฉบับสมบูรณ์ 2543
- การผลิตรีคอมบิแนนท์เอนไซม์ Taq DNA polymerase ส่งรายงานการวิจัยฉบับสมบูรณ์ 2541

ผู้ร่วมวิจัยในโครงการวิจัย ดังต่อไปนี้

- การโคลนนิ่งตัวอ่อนโค กระบือ แมว และสัตว์ป่าใกล้สูญพันธ์ โดยใช้เซลล์ร่างกายเป็นเซลล์ต้นแบบ

Investigation of Rice Beta-Glycosidase Gene Functions. (National Science and Technology

Development Agency National Center for Genetic Engineering and Biotechnology) ส่งรายงานการวิจัยฉบับสมบูรณ์ 2550

- Clonal Selection of Sweet Bamboo for Commercial and Industrial Uses ส่งรายงานการวิจัยฉบับสมบูรณ์ 2545
- Functional Analysis of the Maize bZIP Protein Opaque (NIH, USA) แล้วเสร็จ 2537

6.4 งานวิจัยที่ทำเสร็จแล้ว : ดูหัวข้อ 7.3

6.5 งานวิจัยที่กำลังทำ :

- การดัดแปลงพันธุกรรมของ *Sacchromyces cerevisiae* เพื่อให้สามารถผลิตเอธานอลจากแป้งมันสำปะหลังได้โดยตรง
  สถานภาพในการทำวิจัย : เริ่มโครงการในปีงบประมาณ 2557 และได้ดำเนินการไปแล้ว 80%
- การผลิต *Pichia pastoris* ที่มีโอเมก้า 3 สูง สำหรับใช้เป็นอาหารปลา
  สถานภาพในการทำวิจัย : เริ่มโครงการในปีงบประมาณ 2556 และได้ดำเนินการไปแล้ว 95%

7. Publications:

Kupradit, C., Innok, S., Woraratphoka, J. **Ketudat-Cairns, M.** (2016) Novel multiplex PCR assay for rapid detection of five bacterial foodborne pathogens. Suranaree J. Sci. Technol. 24(1):41-50

Parnpai, R., Ling, Y-Y., **Ketudat-Cairns, M**., Somfai, T., and Nagai, T. (2016) Vitrification of Buffalo Oocytes and Embryos Theriogenology. 86(1) 214-220

Wendong, D. Gerdthai, T. Huang , Z., **Ketudat-Cairns, M.**, Tang, R. Wang, S. (2015) Genetic analysis for anthocyanin and chlorophyll contents in rapeseed (Brassica napus L.) Ciência Rural 46 (5) 790-795

Imsoonthornruksa, S., Pruksananonda, K., Parnpai, R., Rungsiwiwut, R., **Ketudat-Cairns, M**. (2015) Expression and purification of recombinant human basic fibroblast growth factor fusion proteins and their uses in human stem cell culture J Mol Microbiol Biotechnol. 25(6):372-380

Sripunya, N., Ling, Y-Y., Panyawai, K., Srirattana, K., Ngernsoungnern, A. Ngernsoungnern, P., **Ketudat-Cairns, M**., and Parnpai, R. (2014) Cytochalasin B efficiency in the cryopreservation of immature bovine oocytes by Cryotop and solid surface vitrification methods. Cryobiology 2014 Dec 16;69(3):496-9.

Rouyi, C., Changxiang Z., Jiang C., Minna P., **Ketudat- Cairns, M**. (2014) Optimization of Quantitative Real-time PCR System on Amplification of Os1bglu4 gene. Agricultural Science and Technology 15(7):1096-1100.

Rouyi, C., Changxiang Z., Jiang C., Minna P., **Ketudat- Cairns, M**. (2014) Evaluation on Inducible Effect of pOp6 Promoter in Transgenic Rice. Agricultural Science and Technology 15(5):742-744

Srirattana, K., **M. Ketudat-Cairns**, T. Nakai, kaneda, M., R. Parnpai (2014) Effects of Trichostatin a on *in vitro* development and DNA methylation level of the Satellite I Region of Swamp Buffalo (*Bubalus bubalis*) Cloned Embryos. J Reprod Dev 60(5) 336-341

Pakping, S., **Ketudat-Cairns, M.,** Boontawan, A. (2014) Extractive Fermentation of Ethanol from Fresh Casava Roots using Vacuum Fractionation Techniques. Adv. Mat. Res 931: 1096-1100

Rouyi C, Baiya S, Lee S-K, Mahong B, Jeon J-S, Ketudat-Cairns, J. and **Ketudat-Cairns, M**.. (2014) Recombinant Expression and Characterization of the Cytoplasmic Rice β-Glucosidase Os1BGlu4. PLoS ONE 9(5): e96712. doi:10.1371/journal.pone.0096712

Parnpai, R., Liang, Y-Y., Paul A. K., Ngernsoungnern, A., Ngernsoungnern, P., and **Ketudat-Cairns, M.** (2014) Cryopreservation of Buffalo Oocytes. Thai J. Vet. Med. 44(1) 119-123

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., K. Srirattana, W. Phewsoi, W. Tunwattana, R. Parnpai, **M. Ketudat-Cairns** (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**, 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., A. Sangmalee, K. Srirattana, , R. Parnpai, **M. Ketudat-Cairns** (2011) 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(4) 1-7

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)

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

Srirattana K, Lorthongpanich C, Laowtammathron C, Imsoonthornruksa S, **Ketudat-Cairns M**, Phermthai T, Nagai T, 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 2010 Feb; 56(1):49-54.

Rattanasuk, S., and **Ketudat-Cairns, M**. (2009) Genetic Diversity of Felids' Cytochrome B Suranaree J. Sci Technol 16 (4) 283-290

Ruamkusol, 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

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

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.

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

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

Lorthongpanich, C., K. Srirattana, S. Imsoonthornruksa, N. Sripunya, C. Laowtammathron, O. Kumpong, **M. Ketudat-Cairns** and R. Parnpai (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

Muenthaisong S, Laowtammathron C, **Ketudat-Cairns, M.**, Parnpai R, 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

Toonkool, P., Metheenukul, P., Sujiwattanarat, P., Paiboon, P., Tongtubtim, N., **Ketudat-Cairns, M**., Ketudat-Cairns, J., 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

Charoenrat, T., **Ketudat-Cairns, M.**, Jahic M., Veide, A., and Enfors, S.-O., (2006) Increase total air pressure versus oxygen limitation for enhance 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., 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

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

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.

Kanchanatawee, S., Wanapu, C. and **Ketudat-Cairns, M**. (2000) Biotechnology Graduate Education in Thailand. Thai J. of Biot 2 (1): 55-62

Carlini, L.E., **M. Ketudat**, R.L. Parsons, S. Prabhakar, R. J. Schmidt and M. J. Guiltinan (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)

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

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 RJ, Messing J. (1992) Mutations of the 22- and 27-kD zein promoters affect transactivation by the Opaque-2 protein. *Plant Cell* 4:701-709

Paper Presented at National and International Conferences (since 1998 > 80 titles)

Major advisor to 6 Ph.D. and 15 M.Sc. gratuates (since 2000)

Currently major advisor to 5 Ph.D. students