# CHARACTERIZATION OF RICE PHYTOHORMONE BETA-GLUCOSIDASE

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# การวิเคราะห์หน้าที่ของเอนไซม์ไฟโตฮอร์โมนเบต้า-กลูโคซิเดสในข้าว

นางสาวมนัสชนก กองดิน



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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**CHARACTERIZATION OF RICE PHYTOHORMONE**

**BETA-GLUCOSIDASE**

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

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มนัสชนก กองดิน : การวิเคราะห์หน้าที่ของเอนไซม์ไฟโตฮอร์โมนเบต้า-กลูโคซิเดสในข้าว (CHARACTERIZATION OF RICE PHYTOHORMONE BETA-GLUCOSIDASE) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.เจมส์ เกตุทัต-คาร์นส์, 106 หน้า.

เอนไซม์เบต้า-กลูโคซิเดสของพืช จัดอยู่ในกลุ่มไกลโคไซด์ไฮโดรเลส กลุ่มที่ 1 (GH1) ที่มีหน้าที่เกี่ยวข้องกับกระบวนการสำคัญ หลายกระบวนการ อาทิ การปลดปล่อยฮอร์โมนพืชจากไกลโคไซที่ถูกกักเก็บ เช่น เอนไซม์ Os4BGlu12 จากข้าวสามารถย่อยสารประกอบ salicylic acid glucoside (SAG) และ tuberonic acid glucoside (TAG) ในขณะที่ เอนไซม์ Os4BGlu13 สามารถย่อยสารประกอบ TAG SAG และ gibberellin glucose ester (GA-GE) นอกจากนี้กรดแอบไซสิก (abscisic acid, ABA) ถูกจัดเป็นฮอร์โมนพืชที่มีความสำคัญทางกระบวนการทางชีววิทยา หนึ่งในบทบาทนั้นคือ การตอบสนองต่อสภาวะเครียด เช่น สภาพดินเค็ม หรือแล้ง และสามารถยับยั้งการยืดตัวของยอดอ่อน ปฏิกิริยา ABA glucose ester (ABA-GE) เป็นกลไกผันกลับได้ที่ทำให้ ABA ของพืชหยุดการทำงาน การทดลองนี้ได้นำเอนไซม์ Os1BGlu4 Os3BGlu7 Os4BGlu12 Os4BGlu13 Os4BGlu18 Os7BGlu26 และ Os9BGlu31 ซึ่งเป็นเอนไซม์เบต้า-กลูโคซิเดสจากข้าวที่ถูกสร้างขึ้นโดย *Escherichia coli* มาทดสอบกิจกรรมของเอนไซม์ โดยการตรวจวัดปริมาณกลูโคสที่ถูกปล่อยปล่อยจาก ABA-GE จากการศึกษาจลศาสตร์ของเอนไซม์โดยใช้ ABA-GE เป็นสารตั้งต้นในการทำปฏิกิริยากับเอนไซม์ดังกล่าว พบว่า เอนไซม์ Os4BGlu13 มีกิจกรรมของเอนไซม์สูงที่สุด โดยมีค่า $K_M$ เท่ากับ 1.66 $k_{cat}$ เท่ากับ 20.59 $k_{cat}/K_M$ เท่ากับ 12.40 รองลงมาคือ เอนไซม์ Os4BGlu12 โดยมีค่า $K_M$ เท่ากับ 10.88 $k_{cat}$ เท่ากับ 7.50 และ $k_{cat}/K_M$ เท่ากับ 0.689 จากนั้นเอนไซม์ Os4BGlu9 Os4BGlu10 Os4BGlu11 Os4BGlu12 and Os4BGlu13 จากข้าว ซึ่งถูกจัดเป็นเอนไซม์เบต้า-กลูโคซิเดสกลุ่ม GH1 At/Os7 ได้ถูกนำมาเชื่อมต่อกับโปรตีนเรืองแสงสีเขียว เพื่อติดตามตำแหน่งของโปรตีนในเซลล์พืช โดยใช้ *Agrobacterium tumefaciens* ส่งถ่ายยีนเข้าสู่ใบของต้นใบยาสูบ จากการศึกษาพบว่าเอนไซม์ทั้ง 5 ตัว เคลื่อนที่ไปสะสมอยู่ระหว่างเซลล์ผนังเซลล์และเยื่อหุ้มเซลล์ของใบยาสูบ จากนั้นทำการตรวจสอบความสามารถในการย่อยซับสเตรท ABA-GE และ $GA_4GE$ ในพืช โดยทำการผลิตต้นอะราบิดอฟซิสที่มีการแสดงของยีนเบต้า-กลูโคซิเดส Os4BGlu9 Os4BGlu10 Os4BGlu11 Os4BGlu12 หรือ Os4BGlu13 จากข้าว พบว่าโปรตีนสกัดจากต้นอะราบิดอฟซิสดังกล่าวสามารถย่อยซับสเตรท ABA-GE, $GA_4$-GE และ *p*-nitrophenyl β-D-glucopyranoside (*p*NPGlc) ได้ดีกว่าโปรตีนที่สกัดจาก

ต้นอะราบิดอฟซิสที่ไม่มีการถ่ายยีน (wild type) จากนั้นนำเมล็ดของต้นอะราบิดอฟซิสที่มีการแสดงออกของเบต้า-กลูโคซิเดสจากข้าวมาเพาะในอาหารสังเคราะห์สูตร ½ MS เป็นเวลา 7 วัน แล้วทำการย้ายไปเลี้ยงต่อในอาหารสังเคราะห์สูตร ½ MS ที่มีการเติม 0.01 ไมโครโมลาร์ ABA หรือ ABA-GE และ 0.05 ไมโครโมลาร์ $GA_4$ หรือ $GA_4GE$ จากการทดลองพบว่า กลุ่มทดลองที่มีการเติม 0.01 ไมโครโมลาร์ ABA ต้นอะราบิดอฟซิสที่มีการแสดงออกของเบต้า-กลูโคซิเดสจากข้าว มีความยาวของต้นและราก ที่ยาวกว่าต้นอะราบิดอฟซิสปกติ (wild type) ส่วนกลุ่มทดลองที่มีการเติม ABA-GE พบว่า ต้นอะราบิดอฟซิสที่มีการแสดงออกของเบต้า-กลูโคซิเดส Os4BGlu13 มีความยาวของต้นและรากสั้นกว่า ต้นอะราบิดอฟซิสปกติ ซึ่งสอดคล้องกับกิจกรรมของเอนไซม์ Os4BGlu13 ที่ผลิตจาก *E.coli* ในขณะที่ต้นอะราบิดอฟซิสที่มีการแสดงออกของเบต้า-กลูโคซิเดสจากข้าวที่เลี้ยงในอาหารสังเคราะห์ที่มี $GA_4$-GE พบว่าต้นอะราบิดอฟซิสที่มีการแสดงออกของ Os4BGlu12 และ Os4BGlu13 มีความยาวของรากมากกว่า ต้นอะราบิดอฟซิสปกติ จากผลการทดลองพบว่า เอนไซม์เบต้า-กลูโคซิเดสจากข้าว กลุ่มGH1 At/Os7 เป็นโปรตีนที่อยู่ระหว่างผนังเซลล์และเยื่อหุ้มเซลล์ ซึ่งสามารถย่อย ABA-GE หรือ โฮโมนพืชฮอร์โมนพืชไกลโคไซชนิดอื่น ๆ ที่บริเวณอะโพพลาสต์



สาขาวิชาเคมี ลายมือชื่อนักศึกษา Manatcharak Konglin

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา

MANATCHANOK KONGDIN : CHARACTERIZATION OF RICE PHYTOHORMONE BETA-GLUCOSIDASE. THESIS ADVISOR : PROF. JAMES R. KETUDAT-CAIRNS, Ph.D. 106 PP.

PHYTOHORMONE/ RICE/ BETA-GLUCOSIDASE/ ABCISIC ACID GLUCOSE ESTER/ GLYCOSIDE HYDROLASE

In plants, β-glucosidases belonging to glycoside hydrolase family 1 (GH1) have been implicated in several fundamental processes, including release of bioactive phytohormones from inactive glycoside storage forms. For instance, rice Os4BGlu12 hydrolyzes salicylic acid glucoside (SAG) and tuberonic acid glucoside (TAG), while Os4BGlu13 hydrolyzes TAG, SAG and gibberellin glucose ester (GA-GE). Abscisic acid (ABA) is a phytohormone that plays critical roles in various biological processes. One of its best characterized roles is in adaptive responses to abiotic stresses, such as high salt and dehydration stress, and it also inhibits shoot elongation. ABA glucose ester (ABA-GE) is the major metabolite of reversible ABA inactivation of ABA in plants. Here, several rice β-glucosidases that have been expressed in *Escherichia coli*, including Os1BGlu4, Os3BGlu7, Os4BGlu12, Os4BGlu13, Os4BGlu18, Os7BGlu26 and Os9BGlu31, were screened for release of glucose from ABA-GE. Os4BGlu13 exhibited highest hydrolysis activity with ABA-GE followed by Os4BGlu12. The kinetic parameters of these enzymes for ABA-GE hydrolysis were $K_M$, 10.88, $k_{cat}$, 7.50, and $k_{cat}/K_M$, 0.689 for Os4BGlu12 and $K_M$, 1.66, $k_{cat}$, 20.59, $k_{cat}/K_M$ 12. 40 for Os4BGlu13. Rice β- glucosidases in a subclade of phylogenetic cluster At/Os7, including Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 were

fused to green fluorescent protein (eGFP) to see subcellular localization by Agrobacterium-mediated transformation of tobacco. All five-rice beta-glucosidase-eGFP fusion proteins appeared to be localized to the apoplast. To determine whether the enzymes in At/Os7 are able to hydrolyze ABA-GE and $GA_4$-GE *in planta*, *Arabidopsis thaliana* lines overexpressing rice Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 or Os4BGlu13 β-glucosidases were produced. The plant extracts of these lines could hydrolyze ABA-GE, $GA_4$-GE and *p*-nitrophenyl β-D-glucopyranoside (*p*NPGlc) better than wild type plant extract. The Arabidopsis overexpressing rice β-glucosidase were germinated in ½ MS for 7 days and transplanted to plates supplemented with 0.01 μM ABA or ABA-GE or 0.05 μM $GA_4$ or $GA_4$-GE. In the ABA treatment, plants overexpressing the rice β-glucosidases exhibited longer root and shoot lengths than wild type. ABA-GE treatment found Arabidopsis overexpressing rice Os4BGlu13 exhibited shorter root and shoot lengths than wild type, which is consistent with the high enzyme activity toward ABA-GE of Os4BGlu13 expressed in *E. coli*. When the Arabidopsis lines overexpressing the rice β-glucosidases were transplanted to media supplemented with $GA_4$-GE, Os4BGlu12 and Os4BGlu13 exhibited root lengths longer than wild type. Based on these observations we propose that rice β-glucosidase in this subclade of At/Os7 are cell wall proteins which hydrolyze ABA-GE and other phytohormone glucoconjugates in the apoplast.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid |
| APS | Amonium persulfate |
| bp | Base pairs |
| CV | Column volume |
| cDNA | Complementary deoxynucleic acid |
| DNaseI | Deoxyribonuclease I |
| dNTPs | Deoxyribonucleotide triphosphate |
| GH | Glycoside hydrolase |
| GH1 | Glycoside hydrolase family1 |
| Glc | Glucose |
| h | Hour |
| IMAC | Immobilized Metal Affinity Chromatography |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| kDa | Kilo Dalton |
| LB | Luria-Bertani Lysogeny broth |
| min | Minute |
| mRNA | messenger RNA |
| MW | Molecular weigh |
| MS | Murashige & Skoog media |

# LIST OF ABBREVIATIONS (Continued)

| | |
|---|---|
| 4NP | 4-nitrophenyl |
| PCR | Polymerase chain reaction |
| *p*NPGlc | 4-Nitrophenyl-β-D-glucopyranoside |
| PVDF | Polyvinylidene fluoride |
| OD | Optical density |
| RNase | Ribonuclease |
| Rpm | Revolutions per minute |
| SDS | Sodium dodecyl sulfate |
| PCR | Polymerase chain reaction |
| PMSF | Phenyl methyl sulfonyl fluoride |
| *P* value | Probability value |
| SDS-PAGE | polyacrylamide gel electrophoresis |
| TEMED | Tetramethyl ethylenediamine |
| Tris | Tris(hydroxymethyl)aminomethane |
| v/v | Volume per volume |

# CHAPTER I

# INTRODUCTION

## 1.1 General introduction

β-Glucosidases (EC. 3.2.1.21 β-D-glucosidases) are enzymes that hydrolyze glycosidic linkages to release glucose from the non-reducing termini of oligo-saccharides and aryl and alkyl glucosides (Ketudat Cairns and Esen, 2010). They have been found in wide range of living organisms, from bacteria and archaea to multicellular eukaryotes, including mammals and plants. A number of crucial biological reactions in living cell are driven by their action.

Among the broad range of their enzymatic duties, hydrolysis of plant phytohormone glycoconjugates were addressed in this study. Absicsic acid (ABA) is one crucial plant phytohormone playing a role in biological processes, especially responses to adverse stresses, such as drought, salinity, cold and pathogen attack. *In planta* ABA level is regulated by biosynthesis and catabolism. The production of ABA is provided via de novo biosynthesis pathway. First, zeaxanthin is generated in the plastid and consequently changed to xanthoxin, Xanthoxin translocates from the plastids to the cytoplasm and is converted to ABA (Vishal and Kumar, 2018). The translocation of ABA between cells, tissues and organs also plays important roles in the physiological response of the whole plant to stress conditions. ABA is a weak acid which can diffuse passively across biological membranes when it is protonated (Wilkinson and Davies, 2010). Since accumulation of ABA negatively affects the

biological function of general plant cells, much of the de novo biological synthesized ABA is metabolically glycosylated with glucose to form ABA-glucosyl ester (ABA-GE) (Piotrowska and Bajguz, 2011). In Arabidopsis ABA can be converted to ABA-GE by UDP-Glucosyltransferasen71C5 to storage at the endoplasmic reticulum and vacuoles (Liu et al., 2015; Bray and Zeevaart, 1985; Lehmann and Glund, 1986). When the level of ABA is increased in response to abiotic stress, such as cold, water-deficiency and salt stress, ABA-GE could be converted to free ABA. ABA-GE much be hydrolyzed to free ABA before it can enter to the phloem and then be translocated out of the leaf (Xu et al., 2002). β-Glucosidase was reported to be able to release abscisic acid (ABA) from the physiologically inactive ABA-GE pool in the leaf apoplast (Dietz et al., 2000). An *Arabidopsis* β-glucosidase (AtBG1) has been found to hydrolyze ABA-GE to ABA free form (Lee et al., 2006). Loss of AtBG1 affected stomata closure, and resulted in early germination and sensitivity to abiotic stress. AtBG1 is localized to the ER, and accumulation of ABA immune reactively in the cytoplasm near ER tubules has been report (Ondzighi-Assourme et al., 2016). AtBG1-overexpressing creeping bentgrass has increased ABA levels and enhanced drought tolerant ability compared with wild type (Han et al., 2012). In addition, there is another one *Arabidopsis* β-glucosidase (AtBG2), which localizes in the vacuole, which also has the ability to hydrolyze ABA-GE to produce free ABA during dehydration stress (Xu et al., 2012).

Some rice β-glucosidases have been characterized to be able to hydrolyze phytohormone conjugates. Tuberonic acid (TA) glucoside (TAG) could be hydrolyzed by a β-glucosidase from rice (OsTAGG1, Os4BGLu13) to releases active TA (Wakuta et al., 2010). In addition to TAG salicylic (SA) glucoside (SAG) could be hydrolyzed

by a β-glucosidase from rice (Os4BGlu13, OsTAGG2) expressed in a yeast system to release active SA (Wakuta et al., 2011, Hemino et al., 2012). Wakuta et al. (2010) and Hua et al. (2015) reported Os4BGlu13, which is closely related to Os4BGlu12, has activity toward with TAG, SAG, oligosaccharides and $GA_4$-GE.

Here, we report biochemical and physiological analysis of rice glycoside hydrolase family1 (GH1) β-glucosidases, including Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13, which belong to plant GH1 phylogenetic cluster At/Os7 (Opassiri et al., 2006) possess abscisic glucose ester β-glucosidase activity. We found that Os4BGlu12 and Os4BGlu13 expressed in recombinant *Escherichia coli* exhibits hydrolysis of ABA-GE better than several recombinant rice β-glucosidases. Os4BGlu12 and Os4BGlu13 hydrolyzed the ABA-GE with $k_{cat}/K_M$ 0.689 $mM^{-1}s^{-1}$ and 12.4 $mM^{-1}s^{-1}$. Os4BGlu12-GFP fusion protein was localized to the cell wall. Finally, their over expression in *Arabidopsis* resulted in a change of phenotypes when they were germinated on media containing ABA, ABA-GE, $GA_4$ and $GA_4$-GE compared to their wild type and plant extracts exhibit hydrolysis activity with ABA-GE, $GA_4$-GE and *p*NPGlc better than wild type extracts.



## 1.2 Research objectives

The objectives of this study included:

1. To clone the genes for the putative rice phytohormone β-glucosidases, and express the proteins.

2. To characterize the properties and substrate specificity of the putative rice phytohormone β- glucosidases with ABA-GE

4. To study localization of the putative phytohormone β-glucosidases in plants.

3. To overexpress the putative phytohormone β-glucosidases in plants, to see the effect of these enzymes on the phytohormones activation and effect on growth and development of the plant.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Phytohormones

Plant growth and development requires recognition and integration of many environmental and endogenous signals. Growth and development of plants is regulated by complex interactions among various hormones. Overall growth and developmental stages of plant are under strict regulation by several classes of plant hormones, also called phytohormones. Phytohormones are present at low concentrations in plants and coordinate cellular activities. It is now clear that these include more than the originally recognized five classes of phytohormones of auxin, gibberellins, ethylene, cytokinin and abscisic acid. More recently discovered natural growth-regulating substances that have phytohormone-like roles include polyamines, oligosaccharides, salicylates, jasmonates, sterols, including brassinosteroids, dehydrodiconiferyl alcohol glucosides, turgorins, systemin and other peptides and unrelated natural stimulators and inhibitors (Gaspar et al., 1996; Beligni and Lamattina, 2001). Each of these acts with its own particular properties.

Auxins were the first class of plant hormones to be identified. The primary auxin in plants is indole-3-acetic acid (IAA). The functions of auxins include stimulation of differential growth in response to gravity or light stimuli (Zhao, 2010). Many auxins,

both natural and synthetic, are now known and all have similar effects on plant growth and development.

Cytokinins are a group of hormones that promote cell division in plant roots and shoots and the growth of buds. Cytokinins can be produced chemically from nucleic acids. Naturally occurring cytokinins are $N^6$-substituted purine derivatives Iso-pentenyl adenine (iP), zeatin (Z), and dihydrozeatin (DZ) are the predominant cytokinins found in higher plants. (Werner et al., 2001). Cytokinins are known to delay senescence in leaf tissues, promote mitosis, and stimulate differentiation of the meristem in shoots and roots.

Gibberellins (GAs) are a group of about 136 known natural and synthetic compounds. They are not only found in plants (Gaoet al., 2017), but also in fungi and bacteria. The first GA isolated from a higher plant, $GA_1$, was identified 40 year ago. Some gibberellins stimulate shoot elongation, seed germination, and fruit and flower maturation. GAs are synthesized in the root and stem apical meristems, young leaves, and seed embryos. GAs break dormancy (a state of inhibited growth and development) in the seeds of plants that require exposure to cold or light to germinate (Gupta and Chakrabarty, 2013).

Abscisic acid (ABA) is a 15-carbon weak acid that was first identified in the early 1960s. It acts as a growth inhibitor that accumulates in abscising cotton fruit and leaves of sycamore trees photo periodically induced to become dormant (Wasilewska et al., 2008). ABA has been shown to regulate many aspects of plant growth and development, including embryo maturation, seed dormancy, germination, cell division and elongation, floral induction, and responses to environmental stresses, such as drought, salinity, cold, pathogen attack and UV radiation.

Ethylene is small hydrocarbon gas that has an important hormonal role integrating developmental events with stimuli (Burg, 1973). It plays an active role in inhibition of cell division and cell elongation, which extends to the stages of flower formation and fruit ripening. It is also an important plant stress hormone.

Brassinosteroids (BRs) are important plant growth regulators. Sixty-five free BRs, and five conjugates have been characterized from the plant kingdom (Bajgus and Tretyn, 2003). BRs can induce a broad spectrum of cellular responses, such as stem elongation, pollen tube growth, leaf bending, root inhibition and induction of ethylene biosynthesis (Fariduddin et al., 2014).

Two phytohormones that are thought to participate in defense responses are salicylic acid (SA) and jasmonic acid (JA). In recent years, SA has been the focus of intensive research, due to its function as an endogenous signal mediating local and systemic plant defense responses against pathogens. SA has been found to play a role during the plant response to abiotic stresses, such as drought, chilling, heavy metal toxicity, heat, and osmotic stress. Jasmonic acid (JA) and its methyl ester, methyl jasmonate (JAMe), are naturally occurring regulators of higher plant development, responses to external stimuli, and gene expression. JA was first isolated from cultures of the fungus *Lasiodiplodia theobromae*. Numerous derivatives of JA are found in plants including hydroxylated forms, such as tuberonic acid and cucurbic acid, and amino acid conjugates. The role of most of the derivatives of JA is unclear, although tuberonic acid or its glucoside has been proposed to regulate tuber formation in potato (Sarkar, 2008).

## 2.2 Conjugates of phytohormones

Regulation of plant hormones occurs in the course of the biosynthetic pathways and by deactivation via catabolic processes. All these metabolic steps are in principle irreversible, except for some processes, such as the formation of esters, glucosides and amide conjugates, where the free parent compound can be liberated by enzymatic hydrolysis. Several compounds in the biosynthetic and degradative pathway of plant hormones can exhibit biological activity, giving rise to a very complex network of signaling molecules at the cellular level (Piotrowska and Bajuz, 2011). The concept of reversible conjugation of phytohormones suggests that under changeable physiological conditions hormone conjugates can be a source of free hormones. Most conjugates of plant hormones are inactive, and some function to reduce the active hormone pool.

IAA is conjugated with other molecules, such as monosaccharides and polysaccharides, by ester bonds. IAA can also be conjugated with amino acids and peptides by amide bonds (Bajguz and Piotrowska, 2009). Indole-3-butyric acid (IBA) is another kind of auxin, which can also be conjugated with other molecules via amide and ester bonds, and it has been noted that IBA conjugates may be more easily hydrolyzed than those of IAA, depending on the plant system. The formation of auxin conjugates and their degradation have been reported in several plants, including both dicots and monocots. Monocots have mainly been reported to accumulate ester conjugates, while in dicots mostly amide conjugates have been observed.

The major glucose conjugate of ABA is ABA-glucosyl ester (ABA-GE) (Piotrowska and Bajguz, 2009). As noted above, the level of ABA increases in response to abiotic stress, such as cold, water- deficiency and salt stress. After the stress is relieved, ABA is metabolized to inactive forms. Abscisic acid conjugate concentrations

increased in barley xylem sap under salinity stress (Dietz et al., 2000). The glucosyl ester of abscisic acid has also been found in Xanthium leaves, and can be isolated from spinach leaves (Boyer and Zeevaat, 1982). It was reported that ABA-GE in tomato and silverbeet (Swiss chard) was found in water stress conditions, but was not hydrolyzed (Milborrow, 1978). On the other hand, ABA-GE in Arabidopsis (*Arabidopsis thaliana*) was reported to by hydrolyzed in response to drought stress, coincident with an increase in free ABA (Lee et al., 2006).

Koshioka et al. (1983) and Schneider et al. (1992) studied plant gibberellins and their glucosyl conjugates in *Pisum sativum* L., *Malus domestica* Borkh., *Pimpinella anisum* L. and maize. They found that the glucosyl moiety can be linked to either the 2-O-, 3-O-, 11-O-,13-O- or 17-O-positions of the parent GA molecule, generating a range of isomeric forms. These include $GA_1$-3-O-, $GA_3$-3-O-, $GA_8$-2-O-, $GA_{20}$-13-O-, $GA_{26}$-2-O-, $GA_{27}$-2-O-, $GA_{29}$-2-O-, $GA_{29}$-13-O- and $GA_{35}$-11-O- glucosides.

Jasmonates (JA) were shown conjugated with a variety of amino acids, as well as with methyl, glucosyl and gentiobiosyl groups (Wasternack and Hause, 2013). JA derivatives, which are thought to be inactive, may be involved in "switching off" jasmonate signaling: 12-O-β-D-glucopyranosyljasmonic acid (12-O-Glc-JA) and 12-OH-JA are abundant metabolites in many plant species, including *Solanum tuberosum*, *A. thaliana*, and *N. tabacum* (Yoshihara et al., 1989; Helder et al., 1993; Swiatek et al., 2004; Miersch et al., 2008). Interestingly, 12-O-Glc-JA, but not JA or JA-Ile, was shown to activate leaf closure in *Samanea saman* (Nakamura et al., 2011).

Moreover, the glucoside of tuberonic acid (5-hydroxy-jasmonic acid) has been proposed to play roles in tuber formation and wounding response (Nakamura et al., 2011).

## 2.3 Abscisic Acid Biosynthesis

Since the discovery of ABA in the 1960s (Addicott et al., 1968) much effort has been devoted to understanding how ABA is synthesized. Through genetic and biochemical studies, the pathway of ABA biosynthesis in higher plants is now understood. Recently, all the major genes for the enzymes in the biosynthesis pathway have been identified. It is generally known that ABA is synthesized de novo mostly through the carotenoids pathway in higher plants (Schwartz et al., 2003). ABA in higher plants is synthesized from an "indirect" pathway through the cleavage of a C40 carotenoid precursor, as shown in Figure 2.1, followed by a two-step conversion of the intermediate xanthoxin, a kind of carotenoid. The reaction is catalyzed by zeaxanthin epoxidase (ZEP), the molecular identity of which was first revealed in tobacco (Marin et al., 1996). ZEP converts zeaxanthin into violaxanthin via antheraxanthin by a two-step epoxidation to produce violaxanthin. After the epoxidation step, all-trans-violaxanthin is converted to the 9-cis isomer prior to oxidative cleavage of the epoxycarotenoid to form xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED). The product xanthoxin is then exported to the cytosol, where it is converted to ABA through a two-step reaction via ABA-aldehyde. A short-chain alcohol dehydrogenase/ reductase (SDR), encoded by the AtABA2 gene (Rook et al., 2001; Cheng et al., 2002), catalyzes the first step of this reaction and generates ABA aldehyde. ABA aldehyde oxidase (AAO) then catalyzes the last step in the biosynthesis pathway.

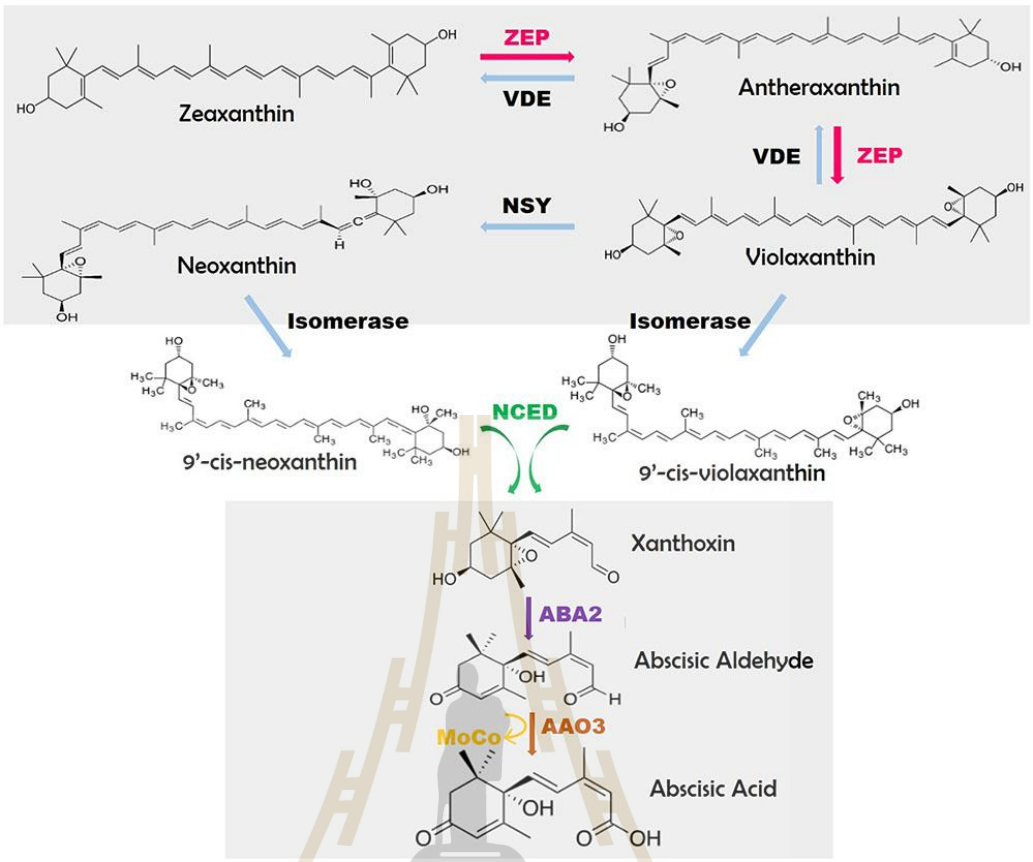


**Figure 2.1** ABA biosynthetic pathway in higher plants. ABA is derived from C40 epoxy carotenoid precursors through a pathway including an oxidative cleavage reaction in plastids (Vishwakarma et al., 2017).

In contrast to biosynthesis, there are several metabolic partway by which ABA can be removed or degraded in plants. The simplest way is the oxidation pathway through hydroxylation of the 8'-carbon atom of ABA, affording 8'-hydroxy ABA (8'-OH ABA). The oxidation product 8'-OH ABA exists in equilibrium with phaseic acid (PA; Balsevich et al., 1994; Zou et al., 1995). PA is sometimes further reduced at the 4' position to form dihydrophaseic acid (DPA) (Culter and Krochko, 1999), as shown in Figure 2.2. DPA may also be metabolized to DPA conjugates (Zeevaart, 1999). Aside

from inactivation by oxidation, free ABA can also be inactivated by conjugation to a monosaccharide like glucose. ABA-glucosyl ester (ABA-GE) is an example of such a conjugate that is inactive as a hormone. In contrast to free ABA, which is localized in the cytosol, ABA-GE is transported into vacuoles and may function as a storage form of ABA. Recent studies showed that, in addition to *de novo* ABA biosynthesis, Arabidopsis β-glucosidase1 (AtBG1) and β-glucosidase2 (AtBG2) generate ABA from ABA-GE in the endoplasmic reticulum and vacuole, respectively (Lee et al., 2006; Xu et al., 2012).

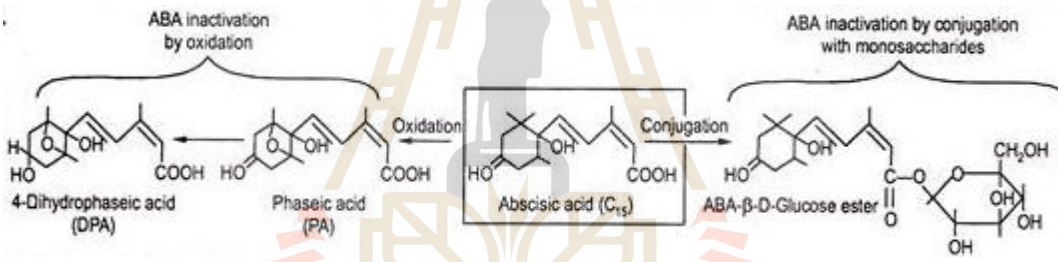


**Figure 2.2** ABA catabolism in higher plant. The pathways for ABA catabolism include conjugation with glucose to form ABA-β-D glucosyl ester or oxidation to form phaseic acid, then dihydrophaseic acid.

## 2.4 Overview of β-glucosidases

β-Glucosidases (EC. 3. 2. 1. 21 β-D-glucopyranosidases) are enzymes that hydrolyze glycosidic linkages, removing glucosyl monosaccharides from the non-reducing termini of different di- and oligosaccharides, as well as aryl and alkyl glucosides. These enzymes are found widely in living organisms, including plants,

fungi, animals, archaea and bacteria. β-Glucosidases play crucial roles, including break down of carbohydrate biomass for fungal, bacterial and archaeal nutrition, recycling of glycolipids and other glucosides in animals, plants and some fungi, release of glucose from cell-wall-derived oligosaccharides during cell wall remodeling in plants and fungi, release of defense compounds from their less toxic glycoside storage forms, activation of phytohormones and metabolic intermediates by removal of glucosyl blocking groups, release of aroma components from involatile glycosides, and release of monolignols from their glycoside storage forms in plants (Ketudat Cairn et al., 2015). Many β-glucosidases have transglucosidase activities in addition to their hydrolase activity, as shown in Figure 2.3

In mammals, there are several β-glucosidases, including the glycoside hydrolase family 1 (GH1) enzymes lactase-phloridzin hydrolase and cytoplasmic β-glucosidase, the GH family 30 (GH30) enzyme human acid β-glucosidase and the GH family 116 (GH116) bile acid β-glucosidase (Ketudat Cairns and Esen, 2010). The most studied β-glucosidase is the human lysosomal acid β-glucosidase or glucosylcerebrosidase 1 (GBA1, lysosomal glucosylceramidase, EC. 3.2.1.45, acid β-glucosidase 1, EC. 3.2.1.21), which belongs to the sequence-related glycoside hydrolases (GH) family designated GH30. This enzyme is deficient in Gaucher disease. Gaucher disease is an inherited metabolic disorder in which the glycolipid glucosylceramide (GlcCer) accumulates due to the impaired activity of lysosomal GBA1. The family GH116 nonlysosomal glucosylceramidase GBA2 was first identified as bile acid β-glucosidase. GBA2 was later found to hydrolyze the glycolipids GlcCer and glucosyllyso-sphingosine. GBA1 and GBA2 are thought to be the primary enzymes responsible for the breakdown of GlcCer. While there is extensive structural and functional knowledge

of GBA1, much less is known about GBA2, although a structural model has recently been proposed (Charoenwattanasatien et al., 2016). Human cytosolic β-glucosidase, also known as klotho-related protein (KLrP, GBA3), is an enzyme that hydrolyzes a wide variety of β-D-glucosides, such as synthetic aryl glycosides (4-nitrophenyl and 4-methylumbelliferyl monoglycosides), dietary flavonoid and isoflavone glucosides, and glucosyl ceramide (Berrin et al., 2003; Tribolo et al., 2007; Noguchi et al., 2008). Cytosolic β-glucosidase was predicted to be involved in the metabolic pathway of glucosylceramide synthesized on the cytosolic faces of the ER/Golgi membranes, although this seems unlikely to be significant given the extremely low hydrolysis rate that was reported (Hayashi et al., 2007).

β-Glucosidases have been reported in certain species of insects (Yu, 1989). These enzymes have been characterized in extracts of several insects, such as from the midgut lumen of *Tenebrio molitor* (Coleoptera) larvae; from the Coleoptera *Abracris flavolineata*, *Pheropsophus aequinoctialis*, *Tenebrio molitor*, and *Pyrearinus termitilluminans*; the Hymenoptera *Scaptotrigona bipunctata*; the Diptera, *Rhynchosciara americana*; and the Lepidoptera, *Erinnys ello, Spodoptera frugiperda*, and *Diatraea saccharalis* (Ferreira et al., 2001; Ferreira et al., 1998). Insect β-glucosidases may be divided into three classes based on their specificities. Class 1 includes the enzymes with oligosaccharide β-glucosidase and aryl (or alkyl) β-glucosidase activity. Class 2 includes the enzymes with only oligosaccharide β-glucosidase activity and, finally, class 3 is composed of enzymes with only aryl (or alkyl) β-glucosidase activity. Class 1 and 2 β-glucosidases, in spite of having different specificities, hydrolyze cellobiose and oligosaccharides derived from hemi-celluloses and carbohydrate moieties of glycoproteins. Because class 3 β-glucosidases

preferentially hydrolyze monosaccharides linked to a hydrophobic aglycone, it is possible that their natural substrates are glycolipids, such as glycosylceramides.

β-Glucosidase are most abundant in plants, where they play important roles, including functions in defense, symbiosis, cell wall catabolism and lignification, signaling, and plant secondary metabolism (Ketudat Cairns et al., 2015). Several putative β-glucosidase genes have been shown either to be induced by biotic or abiotic stress or to be necessary for successful response to the stress. When plant tissues in which they are present are disrupted, the β-glucoside phytoanticipins are activated by the action of β-glucosidases. These binary systems of two sets of components that when separated are relatively inactive provide plants with an immediate chemical defense against herbivores and pathogens. β-Glucosidases can activate four major classes of phytoanticipins: cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates (Morant et al., 2008). Plant β-glucosidases help defend against herbivores and invasive fungi by hydrolyzing relatively inert glycosides to produce toxic compounds, such as HCN, saponins, coumarins, quinones, hydroxamic acid, rotenoids, etc. (Poulton, 1990, Nisius, 1988, Duroux et al., 1998, Babcock and Esen, 1994, and Svasti et al., 1999). Monolignol glucosides give rise to monolignols that are then incorporated into the lignin polymer when activated by β-glucosidases (Wang et al., 2013). β-Glucosidase also act in the degradation of oligosaccharides, such as β-1,3- and β-1,4-linked oligosaccharides from plant cell walls (Hrmova et al., 1998). β-Glucosidases also control the biological activity of phytohormones, including auxins, cytokinins, gibberellins and abscisic acid, by hydrolyzing the glycosidic bonds in inactive glucose conjugate phytohormones to release active phytohormones (Ketudat Cairns et al., 2015). β-Glucosidases also hydrolyze secondary metabolites like

monoterpene alkaloid intermediates to allow further reaction to monoterpene alkaloids, with the final product depending on the plant. They also release plant volatiles, and act in metabolism of many other significant natural products (Ketudat Cairns and Esen, 2010).

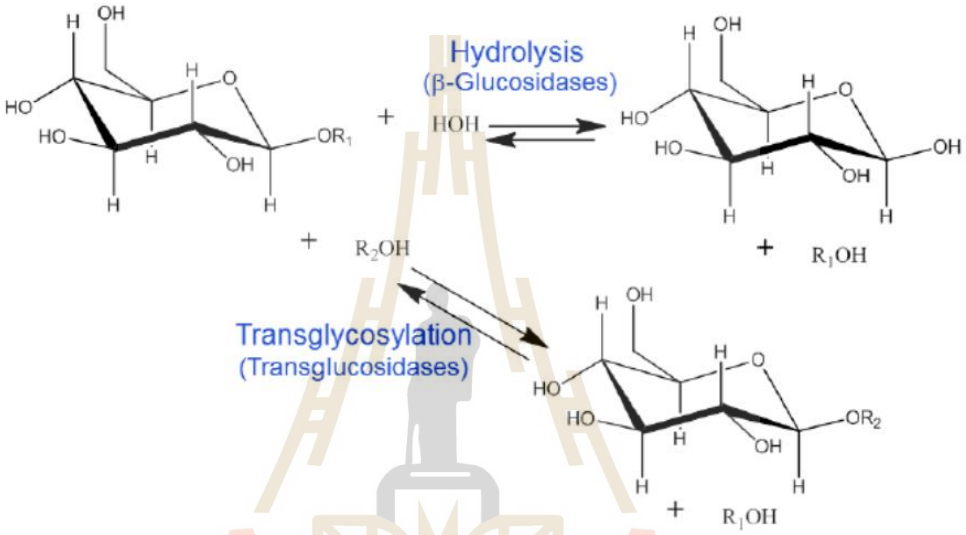


**Figure 2.3** Hydrolysis and transglycosylation reactions of β-glucosidases and transglucosidases. β-Glucosidases often have significant transglucosidase activity in addition to hydrolase activities, while transglucosidases generally have very little hydrolase activity, even at low acceptor substrate ($R_2OH$ in the figure) concentrations (Ketudat Cairn et al., 2015).

## 2.5 Plant glycoside hydrolase family 1

Glycoside hydrolases (EC 3.2.1) are enzymes that catalyze the hydrolysis of the bond at the anomeric carbon of glycosides, leading to formation of a hemiacetal or

hemiketal and the corresponding free aglycon, as shown in Figure 2.4 (Henrissat et al., 1991).

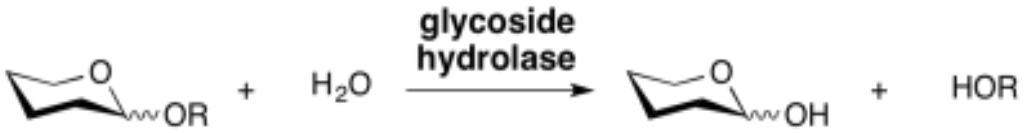


**Figure 2.4** The hydrolysis of a glycoside catalyzed by a glycoside hydrolase (GH).

β-Glucosidases are grouped into 7 of the 133 amino-acid-sequence-based glycoside hydrolase (GH) families, i.e. GH 1, 2, 3, 5, 9, 30 and 116, as classified in the CAZy database (http://www.CAZy.org/). The plant β-glucosidases that have been characterized to date fall predominantly in GH families 1 and 3, with family 1 enzymes being more numerous in plants (Ketudat Cairns and Esen, 2010). In addition to β-glucosidases, plant GH1 members include myrosinases (thio-β-glucosidases) hydrolyzing the S-glycosidic bonds of plant 1-thio-β-D-glucosides (glucosinolates), (Burmeister et al., 1997), β-mannosidases, β-galactosidases, β-glucuronidases, β-fucosidases, diglycosidases, like primeverosidase (Mizutani et al., 2002), furcatin hydrolase (Ahn et al., 2004) and isoflavone 7-O-β-apiosyl-β-1,6-glucosidase (Chuankhayan et al., 2005), hydroxyisourate hydrolase, which hydrolyzes an internal bond in a purine ring rather than a glycosidic bond (Raychaudhuri and Tipton, 2002), and tranglucosidases and trangalactosidases (Moellering et al., 2010; Matsuba et al., 2010; Luang et al., 2013).

GH1 members catalyze their reactions with a molecular mechanism leading to overall retention of the anomeric configuration, which involves the formation and breakdown of a covalent glycosyl enzyme intermediate. GH1 enzyme display a

common $(\beta/\alpha)_8$ TIM barrel structure. Apart from plant myrosinases and animal Klotho (KL) subfamily members (which lack β-glucosidase activity), all characterized GH1 enzymes contain two conserved catalytic glutamate residues located at the C-terminal ends of β-strands 4 and 7 (Jenkins et al., 1995). CsBGLU12 is a GH1 β-glucosidase from *Crocus sativus.* CsBGLU12 catalyzed the hydrolysis of flavonol β-glucosides and cello-oligosaccharides (Ahmad et al., 2017). Arabidopsis β-glucosidase BGLU15 is an apoplastic β-glucosidase that hydrolyzes flavonol *3-O-β*-glucoside-7-O-α-rhamnosides and flavonol *3-O-β*-glucosides, forming flavonol *7-O-α*-rhamnosides and flavonol aglycones (Roepke et al., 2015). AtBGLU42 in Arabidopsis is the product of a Myb72 (transcription factor) regulated gene that is required for induced systematic resistance, resulting from root colonization by beneficial microbes. Its expression is also required for secretion of phenolic compounds in response to iron deficiency (Zamioudis et al., 2014).

## 2.6 Phytohormones β-glucosidases

Among β-glucosidase functions in plants, phytohormone activation is very important. Several phytohormones can be conjugated by glucosyl conjugates. Phytohormone β-glucosidases have been reported in many plants. Schliemann (1978) reported that β-glucosidase extracts from immature fruits of *Phaseolus coccineus* can hydrolyze $GA_8$-2-O-β-D-glucopyranoside. Schliemann (1984) also reported that β-glucosidases extracted from dwarf rice (*Oryza sativa L. Cv.*) are found in soluble and particulate fractions of rice seeds and seedlings. The different fractions had different hydrolytic activities toward $GA_8$-2-O-glucoside, $GA_3$-3-O-glucoside and 1-O-$GA_3$-glucosyl ester, but purification and characterization of the β-glucosidase activities were

not performed. β-Glucosidases from different grasses, including maize, are implicated in phytohormone activation, such as the release of indole acetic acid (IAA) from its glucoconjugates (Wiese and Grambow, 1986). Brzobohatý et al. (1993) reported a beta-glucosidase encoded by a cloned maize gene cleaved the biologically inactive hormone conjugates zeotin-O-glucoside and kinetin-N3-glucoside, releasing active cytokinins.

Barley primary leaves (*Hordeum vulgare* cv. Gerbel) also contain β-glucosidase activity which releases abscisic acid (ABA) from the physiologically inactive ABA-glucose conjugate pool in the leaf apoplast (Dietz et al., 2000). An *Arabidopsis* β-glucosidase (AtBG1) has been found to hydrolyze ABA-GE to form free ABA (Lee et al., 2006). AtBG1, which localizes to the endoplasmic reticulum (ER), increases ABA levels upon dehydration stress through a mechanism called polymerization-mediated activation. Loss of AtBG1 causes defective stomatal movement, early germination, abiotic stress-sensitive phenotypes, and lower ABA levels, whereas plants with ectopic AtBG1 accumulate higher ABA levels and display enhanced tolerance to abiotic stress. Xu et al. (2012) reported another *Arabidopsis* β-glucosidase (AtBG2). AtBG2, produced ABA by hydrolyzing ABA-GE and plays a role in osmotic stress response. AtBG2 localized to the vacuole as a high molecular weight complex and accumulated to high levels under dehydration stress. BG2 hydrolyzed ABA-GE to ABA *in vitro*. In addition, BG2 increased ABA levels in protoplasts upon application of exogenous ABA-GE.

Jin et al. (2011) reported that transformed tobacco (*Nicotiana tabacum*) plants expressing a fungal β-glucosidase (Bgl-1) in plastids exhibited higher GA levels than untransformed plants. They suggest that β-glucosidases can release of active GA hormones from their conjugates, although no activity on phytohormone glucosyl conjugates was demonstrated and conjugate levels were not measured.

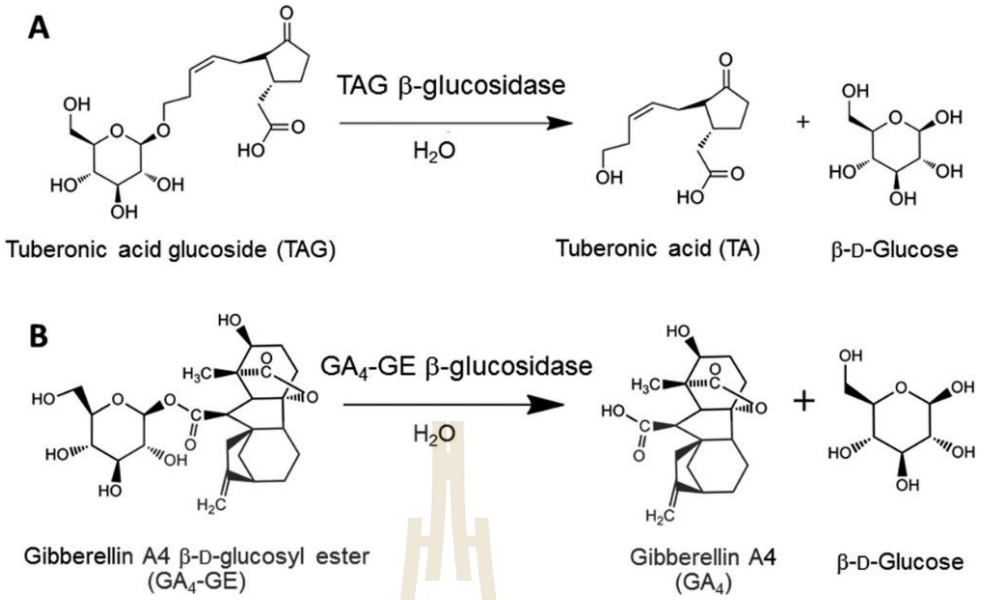


**Figure 2.5** Reactions of rice phytohormone β-glucosidases of interest. A) Tuberonic acid β-glucoside is hydrolyzed by TAG β-glucosidase 1 (TAGG1, Os4BGlu13) to release tuberonic acid and glucose. B) $GA_4$-GE is hydrolyzed by gibberellin glucose ester β-glucosidase to release $GA_4$ and glucose (Hua et al., 2013)

Another phytohormone conjugate that has been studied is tuberonic acid (TA) glucoside (TAG). OsTAGG1 (Os4BGlu13) is a tuberonic acid glucoside hydrolyzing β-glucosidase found to hydrolyze a variety of natural substrates, such as tuberonic acid glucoside (TAG), methyl tuberonic acid glucoside, jasmonoyl-1-β-glucoside, and salicylic acid glucoside, as well as p-nitrophenyl β-D-glycosides (Wakuta et al., 2010). The tuberonic acid (TA) and TAG are produced from jasmonic acid (JA) by hydroxylation and further glycosylation, respectively. Since TAG was hydrolyzed most efficiently, the authors concluded that OsTAGG1 is a specific β-glucosidase hydrolyzing tuberonic acid glucoside to release the active TA, but this TA cannot be

converted into jasmonic acid (Wakuta et al., 2010). Wakuta et al. (2011) found that the closely related enzyme Os4BGu12 can also hydrolyze phytohormones conjugates, such as SAG and TAG and designated it OsTAGG2. Hua et al. (2015) reported Os4BGlu13, which is closely related to Os4BGlu12, has activity toward $GA_4$-GE in addition to TAG, SAG, and oligosaccharides (Figure 2.5).

## 2.7 Rice β-glucosidases

Opassiri et al. (2006) identified GH1 genes from the rice (*Oryza saltiva* L.) genome, and their gene structures, predicted protein products and evidence of expression were evaluated. They found forty GH1 genes could be identified in rice databases, including 34 putative active rice β-glucosidase genes, 2 pseudo genes, 2 gene fragments and 2 likely endophyte genes. A phylogenetic tree of the predicted protein sequences of rice and Arabidopsis GH1 genes was split into 8 clusters containing both Arabidopsis and rice sequences that are clearly more closely related to each other than to other proteins from the same plants. Two Arabidopsis clusters that are more distantly diverged from the clusters containing both rice and Arabidopsis were also identified and numbered At I and At II (Figure 2.6), although these group with At/Os7 cluster in some phylogenetic analyses (Ketudat-Cairns et al., 2012).

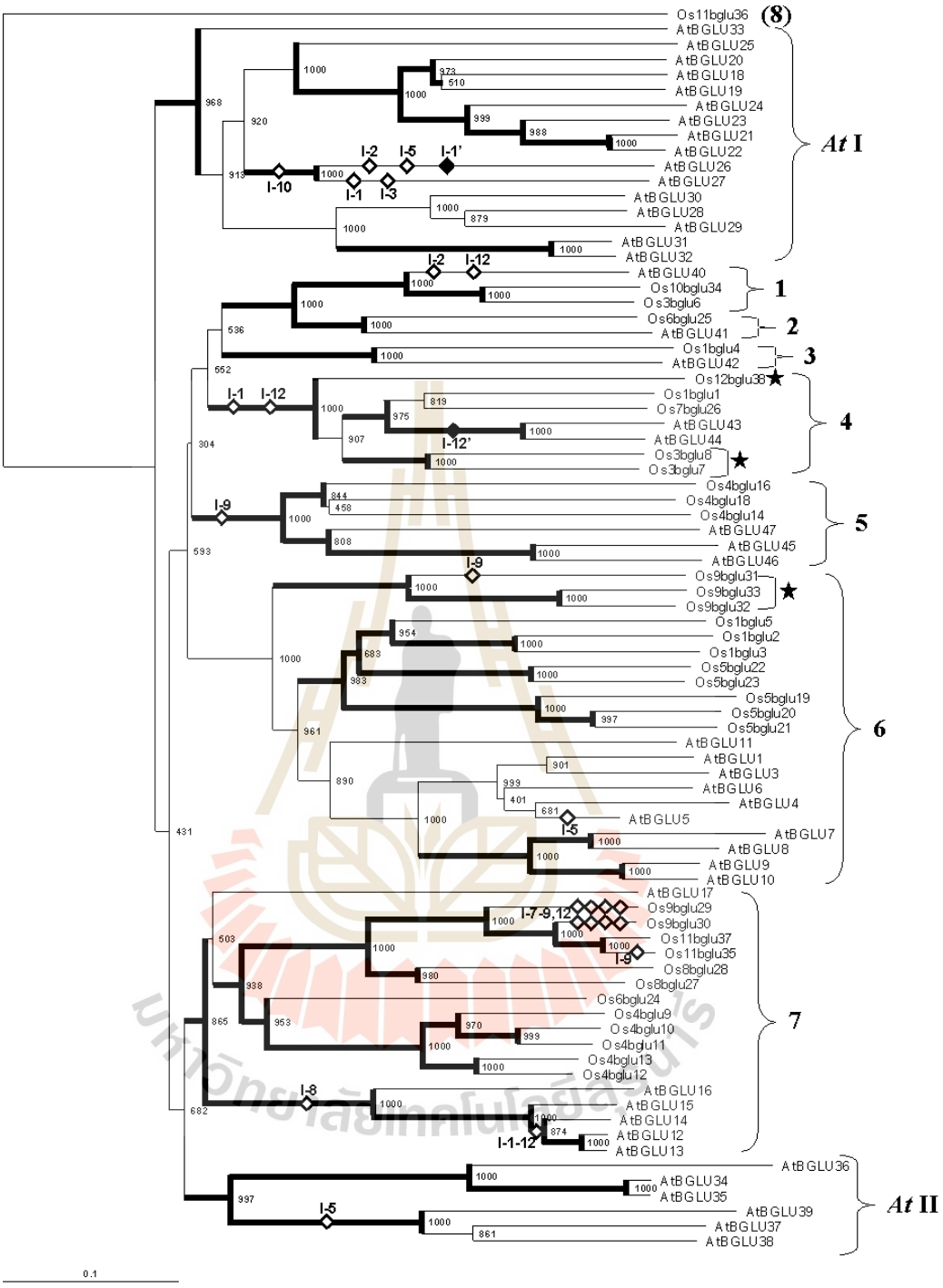


**Figure 2.6** Phylogenetic tree of the predicted protein sequences of rice and *Arabidopsis* glycoside hydrolase family 1 genes (Opassiri et al., 2006). The clusters supported by maximum parsimony analysis are shown as bold lines, and the loss and gain of introns are shown as open and closed diamonds, respectively.

Nowadays, several rice β-glucosidases have been isolated and characterized, for example, Akiyama et al. (1998), determined the N-terminal sequence of cell wall-bound β-glucosidase from germinated rice seeds, which could hydrolyze laminari-oligosaccharides. Opassiri et al. (2003) isolated and characterized rice BGlu1 (also called Os3BGlu7) and BGlu2 (Os9BGlu30). Both enzymes are highly expressed in rice shoots during germination, and BGlu1 was also highly expressed in rice flowers. They found that both enzymes can hydrolyze *p*-nitrophenol (*p*NP) β-D-glucoside, β-D-fucoside, and other *p*NP β-D-glycosides, to a lesser extent, along with some natural glucosides. Moreover, rice BGlu1 β-glucosidase can catalyze transglycosylation of short β-(1,3)- and β-(1,4)-linked gluco-oligosaccharides (Opassiri et al., 2004). Moreover, the At/Os cluster 4 contains Os3BGlu7 and Os3BGlu8, which are β-glucosidases acting on oligosaccharides, and Os7BGlu26, which is as a β-D-mannosidase (Opassiri, 2003; Kuntothom, 2009). Another rice β-glucosidase that has been characterized is Os4BGlu12, which was found to be induced by herbivore attack and salinity stress (Opassiri et al., 2006) and it has recently been reported that the transcription of Os4BGlu12 is up-regulated by wounding, methyl jasmonate and ethephon responses (Opassiri et al., 2010).

Os4BGlu14, Os4BGlu16 and Os4BGlu18 cluster with Arabidopsis BGLU45, 46 and 47 and *Pinus contorta* coniferin/syringin β-glucosidase, which act on monolignol β-glucosides. Baiya et al. (2014) reported the expression of Os4BGlu16 and Os4BGlu18 β-glucosidases in recombinant systems and they found that these isoenzymes act as monolignol β-glucosidases. The Os3BGlu6 protein was also expressed in *E. coli* and characterized they found Os3BGlu6 have efficiently hydrolyzed *p*NP-β-D-fucoside, *p*NP-β-D-glucoside, and *p*NP-β-D-galactoside, but had

little activity toward other *p*NP-glycosides (Seshadri et al., 2009). It efficiently hydrolyzed heptyl and octyl glucosides, along with β-(1,3)- and β-(1,2)-linked disaccharides, but had little activity on longer oligosaccharides and β-(1,4)-linked oligosaccharides. Hua et al. (2013) reported that Os3BGlu6 also has activity toward the gibberellin conjugate $GA_4$-1-O-acyl glucose ester, which was relatively high when compared with other rice GH1 β-glucosidases. Crystal structures of Os3BGlu6 and its complexes with 2-deoxy-2-fluoroglucoside and octyl-glucoside suggested that side chain of methionine-251 in the mouth of the active site may block the binding of extended β-(1,4)-linked oligosaccharides. Mutagenesis revealed that this residue indeed modulates β-(1,4)-linked oligosaccharide binding (Sansenya et al., 2012). A cytoplasmic rice β-glucosidase Os1BGlu4 was expressed in *Escherichia coli* (*E. coli*) and characterized Os1BGlu1 have efficiently hydrolyzed β-(1,3)-linked oligosaccharides with degree of polymerization (DP) 2-3, and β-(1,4)-linked oligosaccharides of DP 3-4, and salicin (Rouyi et al., 2014).

The GH1 family has recently been recognized to contain TGs that function in production of glucoconjugates in plants (Moellering et al., 2010; Matsuba et al., 2010; Luang et al., 2013). Arabidopsis SENTITIVE TO FREEZING 2 (SFR2; AtBGLU48) can transfer a galactose from monogalactosyl diacyl glyceride to another galactolipid to generate digalactosyl diacyl glyceride and trigalactosyl diacyl glyceride (Moellering et al., 2010). The corresponding rice enzyme Os11BGlu36 likely has the same function. Rice Os9BGlu31 is member of the GH1 At/Os6 cluster. Os9BGlu31 transglucosidase acts to transfer glucose between a broad range of phenolic acids and their 1-O-acyl β-D-glucose esters (Luang et al., 2013). A loss of function mutation of Os9BGlu31 showed a build-up of fatty acid glucose conjugates in rice leaves, which suggested that

fatty acid glucose ester act as glucosyl donor substrates for transglucosidase activity *in planta* (Komvongsa et al., 2015). Previous plant transglucosidase studies found that an acyl-glucose, 1-O-β-D-vanillyl-glucose (VG), acts as the donor molecule in the glucosyl transfer reaction at the 5 position of the anthocyanin cyanidin 3-glucoside in carnations (Matsuba et al., 2010). Similarly, AtBGLU10 is an acyl-glucose-dependent anthocyanin transglucosidase that transfers glucose to anthocyanin A9 to change it to A11 (Miyahara et al., 2013). A flavonol 3-O-glucoside; 6''-O- gluccosyltransferase (AtBGLU6) glycosylates flavonol 3-O-glucoside 7-O-rhamnoside (F3G7R), thereby converting it to flavonol 3-O-gentiobioside 7-O-rhamnoside (F3GG7R) (Ishihara et al., 2016).

## 2.8 Subcellular localization of GH1

Several reports have proposed plant GH1 family proteins are localized at the cell wall, based on the results of proteomic approaches (Jamet et al., 2008; Chen et al., 2009; Cho et al., 2015). β-Glucosidases BGLU15, BGLU22, BGLU37 and BGLU44, which are grouped into GH1, were proposed to localize at the cell wall based on proteomic data (Xu et al., 2004). Arabidopsis BGLU15 is also localized in the cell wall. BGLU15 possibly acts with of cell wall kinases in plant responses to pathogen attack (Chivasa et al. , 2002). Another Arabidopsis enzyme, BGLU23 is restricted to ER bodies (Matsushima et al., 2003). Andreasson et al. (2001) showed that myrosinase was localized in vacuoles of myrosin cells (idioblasts). Subcellular compartmentation of β-glucosidase was studied in rye, maize and wheat seedlings by immunecytochemical methods. In all three species, β-glucosidase was found in plastids, cytoplasm and cell walls (Nikus et al., 2001).

Localization of several rice GH1 β-glucosidases has been reported. Subcellular localization of Os9BGlu31-GFP in rice calli suggested that the Os9BGlu31 protein is most likely to be localized in the vacuole. Another rice β-glucosidase is Os1BGlu4 is a cytoplasmic rice β-glucosidase base on observation of an Os4BGlu4-GFP fusion in maize protoplasts and tobacco leaf (Rouyi et al., 2014). Os4BGlu16 rice monolignol β-glucosidase was localized to the cell wall (Baiya et al., 2018). This apoplastic localization and the effect of these enzymes on monolignol glucoside levels suggest monolignol glucosides from the vacuole may meet the monolignol β-glucosidases, despite their different localization. The protein product for the Os4BGlu12 gene has highest sequence similarity to the previously described cell wall bound β-glucosidase purified from rice seedlings (Akiyama et al., 1998), suggesting that it is also localized to the cell wall.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 General materials

### 3.1.1 Chemicals and reagents

The basic chemicals and reagents used in this thesis work and their primary suppliers are listed in Table 3.1.

**Table 3.1** Chemical reagents and sources**.**

| **Reagent** | **Source** |
|---|---|
| • Brilliant blue R250<br>• Dithiothreitol (DTT)<br>• Ethidium bromide<br>• 2-Mercaptoethanol<br>• Triton X-100 | Acros Organic |
| • Acetic acid<br>• Tris (hydroxymethyl)-aminomethane<br>• Ammonium sulfate<br>• Calcium chloride<br>• Citric acid<br>• Chloroform<br>• Di-sodium hydrogen phosphate | Carlo Erba |

**Table 3.1** Chemical reagents and sources (Continued).

| **Reagent** | **Source** |
|---|---|
| • Di-sodium hydrogen phosphate anhydrous | Acros Organic |
| • Ethylenediaminetetraacetic acid disodium salt (EDTA) | |
| • Ethanol | |
| • Glass beads 500-750 um | |
| • Glucose | |
| • Glycerol | |
| • Hydrochloric acid | |
| • Isopropanol | |
| • Methanol | |
| • Mannitol | |
| • Sodium acetate (anhydrous) | |
| • Sodium carbonate (anhydrous) | |
| • Sodium dodecyl sulfate (SDS) | |
| • Sucrose | |
| • Sodium chloride | |
| • Bacto-agar | Himedia |
| • CleriGel super, Plant culture | |
| • Murashinge & Skoong (MS) | |
| • Peptone | |
| • Yeast Extract | |
| • Skim Milk | |

**Table 3.1** Chemical reagents and sources (Continued).

| **Reagent** | **Source** |
|---|---|
| • HPLC grade Water<br>• Acetonitrile | Labscan |
| • *p*-Nitrophenol<br>• Sodium hydroxide | Merck |
| • Restriction enzymes: *Hind*III, *Nco*I, *Sac*I, *Sal*I, *Pst*I, *Xba*I, *Xho*I | New England and Biolabs |
| • QIAQuick Gel purification kit<br>• QIAQuick PCR purification kit | QIAGEN |
| • Silvet L-77 | PhytoTechnology Laboratories |
| • Deoxyribonucleotide triphosphate (dATP, dGTP, dCTP, and dTTP)<br>• T4-DNA ligase<br>• *Pfu* polymerase | Promega |
| • Abscisic acid<br>• Dimethyl sulfoxide (DMSO)<br>• Phenylmethanesulphonylfluoride (PMSF)<br>• *p*-Nitrophenyl β-D-glucopyranoside (4NPGlc)<br>• Tetracycline | Sigma |
| • Abscisic acid glucose ester (ABA-GE)<br>• Gibberellic acid glucoside ($GA_4$-GE) | Suranaree university of technology |

**Table 3.1** Chemical reagents and sources (Continued).

| Reagent | Source |
|---|---|
| • Agarose<br>• *Taq* DNA polymerase<br>• Protein Marker | Vivantis |
| • *EF-Taq* polymerase<br>• *Pfu* polymerase | SolGent, Daejeon |

**3.1.2 Oligonucleotides primers.**

Oligonucleotides for cloning and expression Os4BGlu9 and Os4BGlu11 were synthesized by Bio Basic Inc. (Canada) and are shown in Table 3.1. Oligonucleotides for RT-PCR overexpression of rice phytohormone β-glucosidases in Arabidopsis and rice were synthesized by Macrogen Corp. (Seoul, South Korea) and are shown in Table 3.2

**3.1.3 Plant, bacterial and yeast stains and plasmids.**

Seven-day-old rice (*Oryza saltiva* L. ssp. *Indica* cv. KDML105) seedlings were used to extract RNA for cloning of cDNA encoding the mature Os4BGlu9 and Os4BGlu11 proteins. *Arabidopsis thaliana* ecotype Columbia were used for floral dip transformation. *Escherichia coli* stain DH5α and XL1-Blue were used for cloning. Origami (DE3) and Origami B(DE3) were used for protein expression. *P. pastoris* strain SMD1168H was used to produce the Os4BGlu11 protein.

A cDNA optimized for Os4BGlu11 expression in *Pichia pastoris* was synthesized and inserted into pUC57 vector by GenScrip Corporation (Piscataway, NJ, USA). The vectors used for cloning and expression of phytohormone β-glucosidases

included pENTR™/D-TOPO (Invitrogen, CA, USA), pET32a(+) (Novagen, WI, USA), and pPICZαB(NH8) (Toonkool et al., 2006).

**Table 3. 2** Oligonucleotide primers used for cloning the cDNA for the phytohormone β-glucosidases Os4BGlu9 and Os4BGlu11. An extra CACC sequence was added at the 5′ end of the forward primers for directional cloning into the pENTR™/D-TOPO vector, and are shown in bold and the sequences of the restriction enzyme sites for cloning are underlined.

| **Primer name** | **Sequences (5′-3′)** |
|---|---|
| Os4BGlu9StrtF | **CACC**ATGGCGGTTGCCGGGGCAG |
| Os4BGlu9MatstrF | **CACC**ATGGGCGAGCTGCTGCCGCCGATTAG |
| Os4BGlu9StopR | CCCCTCGAGTCATCCCTGTTTCATTTCTCGGAG |
| Os4BGlu11StrtF | **CACC**ATGGCGGTTGCAGGGGCA |
| Os4BGlu11MatstrF | **CACC**ATGGCCTGCAATGGCGGCAG |
| Os4BGlu11StopR | CCCAAGCTTCGATGTTCAATTAGAAAAGGCATTGTA TGCA |



## 3.2 General methods

### 3.2.1 Preparation of competent cells of *E. coli* strains DH5α, XL1-Blue, Origami (DE3) and Origami B(DE3)

DH5α and XL1-DH5α and XL1-Blue strains were streaked on Luria Bertoli lysogeny broth agar (LB agar, 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar), while Origami (DE3) and Origami B(DE3) were streaked on an LB agar plate containing 15 µg/ml kanamycin and 12.5 µg/ml tetracycline and incubated at 37 ºC for

16-18 h. A single colony was picked and inoculated into 5 ml of LB containing antibiotic, which was then incubated with shaking at 37 ºC, 200 rpm, for 16 - 18 h, as the starter culture. Then, 1 ml of starter culture was added to 100 ml of LB broth and shaken at 37 ºC, 200 rpm until the optical density at 600 nm ($OD_{600}$) reached 0.4-0.6. The cell culture was chilled on ice for 10 min in a sterile polypropylene tube and collected at 4,000 rpm at 4 ºC for 10 min. The cell pellets were resuspended in 10 ml ice-cold sterile 0.1 M $CaCl_2$ and centrifuged to collect the cell pellets again. Finally, the pellets were resuspended in 1 ml of 0.1 M $CaCl_2$ containing 15% glycerol and 50 µl aliquots were stored at -80 ºC.

### 3.2.2 Transformation of plasmids into competent *E. coli* cells

DNA plasmids (50 –100 µg) were added into 50 µl of competent *E. coli* cells then gently mixed. The competent cell suspensions containing plasmid were incubated on ice for 30 min before transformation by heat shocking the cells at 42 ºC for 45 s and quickly chilling on ice for 5 min. Then, 200 µl LB was added to the transformed competent cells, which were then incubated at 37 ºC for 1 h. The mixtures were spread on LB agar plates containing appropriate antibiotics for each *E. coli* strain and plasmid (Table 3.3). The plates were then incubated at 37 ºC overnight.

### 3.2.3 Plasmid isolation by alkaline lysis method

A single colony on the plate containing plasmid was picked into 5 ml of LB with appropriate antibiotic and incubated at 37 ºC with shaking at 200 rpm for 16-18 h. The cultured cells were collected by centrifugation at 12,000 rpm, 1 min. The supernatant was removed and the cells were resuspended in 100 µl of lysis buffer I (50 mM glucose,

10 mM EDTA, 50 mM Tris-HCl, pH 8.0). Then, 200 µl of freshly prepared lysis buffer II (0.2 N NaOH, 1% (w/v) SDS) were added, inverted 4-6 times and chilled on ice for 3 min. After that, 150 µl of ice-cold lysis buffer III (3 M potassium acetate, pH 4.8) was added and the tube was mixed by inverting 4-6 times. The alkaline lysis reaction was incubated on ice for 5 min and the clear solution containing the plasmids was separated from the cell debris by centrifugation at 13,000 rpm 10 min. The supernatant was transferred to a new tube and one volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added into the tube and vortexed or shaken by hand thoroughly for approximately 20 s. The mixture was centrifuged at room temperature for 5 min at 13,000 rpm. The upper aqueous phase was carefully removed into a fresh tube and precipitated with 2 volumes absolute ethanol for 10 min at 4 $^{o}$C. The precipitated DNA was collected by centrifugation at 13,000 rpm for 10 min. The left over ethanol was removed by speed vacuum. Then, the DNA pellet was re-suspended in 100 µl TE buffer containing 2 µg RNase A and incubated at 37 $^{o}$C for 10 min. The RNase A-treated plasmids were further purified by adding 70 µl of ice-cold precipitation solution (20% PEG 6000, 2.5 M NaCl) and chilling on ice for 1 h. The precipitated DNA was collected by centrifugation at 13,000 rpm for 10 min. The supernatant was removed and the pellet was washed by adding 0.5 ml of 70% ethanol and inverting the tube twice, after which the ethanol solution was removed and the tube dried by speed vacuum. Finally, the DNA was re-dissolved with 30 µl of TE buffer or sterile water.

### 3.2.4 QIAGEN plasmid miniprep

The QIAprep® spin miniprep kit (QIAGEN, Hilden, Germany) was used to purify recombinant plasmid DNA according to the manufacturer' s instructions. A

single colony was picked and inoculated in 5 ml LB broth with appropriate antibiotics. The cultured cells were pelleted by centrifugation at 12,000 rpm for 1 min. The cell pellet was resuspended completely in 250 µl P1 buffer (100 mg/ml RNaseA in 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). Two hundred fifty microliters of P2 buffer (200 mM NaOH, 1% (v/v) SDS) was added to the re-suspended cells, and mixed by inverting the tube gently 4-6 times until the solution became viscous and slightly clear. After that, 350 µl of P3 buffer (3 M potassium acetate, pH 5.5) was added and mixed immediately by inverting the tube gently 4-6 times, to avoid localized precipitation. The solution was centrifuged at 12,000 rpm for 10 min to compact the white pellet. The supernatant was applied to a QIA prep column by pipetting and centrifuging at 12,000 rpm for 1 min, and then the flow through solution was discarded. To protect against nuclease activity or carbohydrate content, 0.5 ml of PB buffer (1.0 M potassium acetate, pH 5.0) was added to the column and centrifuged at 12,000 rpm for 1 min. The column was washed 2 times by applying 0.75 ml PE buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol) and centrifuging at 12,000 rpm for 1 min. The flow-through solution was discarded, and the column was centrifuged for an additional 1 min to remove residual wash buffer. Lastly, the column was placed in a new 1.5 ml micro-tube and 50 µl distilled water was added to the center of column. The column was allowed to stand for 1 min, and then centrifuged at 12,000 rpm for 1 min to elute the plasmid DNA

### 3.2.5 Preparation of *P. pastoris* strain SMD1168H competent cells

*P. pastoris* strain SMD1168H glycerol stock was streaked on a yeast extract peptone dextrose (YPD) plate without antibiotic, which was then incubated at 28 $^{o}$C for

2-3 days. A single colony was inoculated into 5 ml YPD broth and grown at 28 ºC with shaking at 220 rpm about 16 h. One milliliter of starter culture was transferred into 100 ml YPD broth and grown until the $OD_{600}$ reached 1.3-1.5. The cells were collected by centrifugation at 1,500 rpm for 5 min at 4 ºC. The pellet was washed 2 times in 100 ml then 50 ml of ice-cold sterile water and collected by centrifugation at 1,500 rpm for 5 min at 4 ºC each time. Next, the pellet was re-suspended with 10 ml of ice-cold 1 M sorbitol and centrifuged at 1,500 rpm for 5 min at 4 ºC. Finally, the pellet was re-suspended and kept in 0.5 ml of ice-cold 1 M sorbitol and 80 µl aliquots were used for transformation.

### 3.2.6 Transformation of plasmids into *P. pastoris* competent cells

The optimized pPICZαBNH8/Os4BGlu11 or pPICZαBNH8/Os4BGlu11/eGFP plasmid was linearized with *Sac*I or *Pme*I, as recommended by the supplier (New England Biolabs, Beverly, MA, USA). The restriction enzyme was inactivated by heating at 65 ºC for 10 min. Linearization of the plasmid was checked by electrophoresis on a 1% agarose gel. Then, linear DNA was precipitated by mixing with 0.1 volume of 3 M sodium acetate and 3 volumes of 100% ethanol. The DNA pellet was dissolved in 5-10 µl of sterile de-ionized water. The linearized recombinant pPICZαBNH8/Os4BGlu11 or pPICZαBNH8/Os4BGlu11/eGFP vector was transformed into SMD1168H competent cells by electroporation (MicroPulser Electroporator, Bio-Rad, CA, USA) with the parameters of 1.5 kV, 25 µF and 400 Ω (Pichia manual, Invitrogen, Agilent Corp, CA, USA). The transformed cells were selected on Yeast Extract Peptone Dextrose medium with Sorbitol (YPDS) plates containing 100 µg/ml zeocin. The YDPS plates were incubated at 28 ºC for 3-5 days. The transformed cell

colonies were selected again on a YPDS plate containing 250 µg/ml zeocin incubated in the same manner.

### 3.2.7 Transformation of plasmids into *Agrobacterium tumefaciens* (Stain GV3101)

*Agrobacterium tumefaciens* strain GV3101 was streaked on LB and incubated at 28 ºC for 2-3 days. A single colony was picked and inoculated into 5 ml of LB broth which was then incubated with shaking at 28 ºC, 200 rpm, for 16 - 18 h, as the starter culture. Then, 0.5 ml of starter culture was added to 50 ml of LB broth and shaken at 28 ºC, 200 rpm until the optical density at 600 nm ($OD_{600}$) reached 0.6-1. The cell culture was chilled on ice for 30 min in a sterile polypropylene tube and collected at 4,000 rpm at 4 ºC for 15 min. The cell pellets were resuspended in 10 ml ice-cold sterile 0.1 M $CaCl_2$ and centrifuged to collect the cell pellets again. Finally, the pellets were resuspended in 1 ml of 0.1 M $CaCl_2$ containing 15% glycerol and 50 µl aliquots were stored at -80 ºC.

A tube of competent *Agrobacterium tumefaciens* strain GV3101 cells was taken from freezer and allowed to thaw slowly on ice. Then, 1-2 µl of DNA (10-50 ng) was pipetted into competent cell suspension and mixed by tapping the tubes. The cells and DNA were transferred to pre-chilled (on ice) 2 mm gap size electroporation cuvettes and kept on ice until electroporation. The Gene Pulser unit was set to a voltage of 2.5 kV, capacitance of 25 µFD, and resistance of 400 Ohm. The cuvette was placed in the cuvette holder, engaging the electrodes and pulsed with the time constant of ~9 msec (optimum conditions are reported to be those that give time constant between 8-12 ms). LB medium (1 ml) was added directly to the cuvette immediately after the pulse and

the cuvette placed on ice. The contents of cuvette were transferred to a 15 ml Falcon tube and incubated with shaking at 28 $^{o}$C for 2 hours. Then, 100-200 µl was plated on a plate of selective media containing 50 µg of rifampicin and 50 µg/ml of spectinomycin. The plates were incubated for 2 days at 28 °C, at which time colonies should be visible.

### 3.2.8 Agarose gel electrophoresis for DNA

The purified plasmids and PCR products were checked by agarose gel electrophoresis. One percent agarose gels were prepared in TAE 1X buffer (40 mM Tris HCl, pH 8.0, 40 mM acetic acid, 1 mM EDTA, pH 8.0) or in TBE buffer (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2.5 mM EDTA, pH 8.0). The DNA samples were mixed 5:1 with 6X loading dye 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 30% (v/v) sterilized glycerol. Agarose gel electrophoresis was performed in a Gel Electrophoresis Apparatus (Bio-Rad) at a constant voltage of 120 V for 30 min. The DNA bands on the agarose gel were detected by staining with ethidium bromide (0.1 µg/ml) 45 s and de-stained in distilled water for 5 min. The DNA bands were visualized by UV irradiation on a transilluminator (Bio-Rad). The sizes of the DNA bands were estimated by comparing their migration with those of 1 kb DNA ladder (Fermentas, Ontario, Canada).

**Table 3.3** Appropriate antibiotics for each plasmid and *E. coli* strain.

| **plasmid** | **Antibiotic** |
|---|---|
| pUC57, pET32a | 50 mg/ml ampicillin |
| pPICZαBNH8, pPICZαNH8/eGFP | 25 mg/ml zeocin |
| Origami B(DE3), Origami (DE3) | 50 mg/ml ampicillin |
| | 15 mg/ml kanamycin |
| | 12.5 mg/ml tetracycline |

### 3.2.9 Purification of DNA bands from gels

The correct size DNA bands that had been separated on agarose gel electrophoresis were purified with QIAquick® Gel Extraction Kit (QIAGEN). The agarose gel containing the target DNA band was excised with a blade cutter and not more than 300 mg was transferred to a microcentrifuge tube. The agarose gel purification was done according to the manufacturer's instructions.

### 3.2.10 SDS-PAGE electrophoresis

The protein profile and the apparent molecular weights of proteins in various fractions were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 12% SDS-PAGE separating gel consisted of 12% acrylamide diluted from a 30% acrylamide stock 29% (w/v) acrylamide and 1% (w/v) N, N' methylene bis acrylamide), 375 mM Tris HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% TEMED, while the 4% stacking gel consisted of 4% (w/v) acrylamide diluted from the 30% stock, 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% TEMED. Protein samples were mixed 5:1

with 6X loading buffer (50 mM Tris-HCl, pH 6.8, 10% SDS, 0.2 mg/ml bromophenol blue, 50% glycerol, 20% β-mercaptoethanol) and boiled for 5 min to denature proteins. Protein samples were loaded into sample wells, and electrophoresed through the polymerized gel in Tris-glycine electrode buffer (50 mM Tris base, 125 mM glycine and 0.1% SDS, pH 8.3) at 120 V for 1 h or until the bromophenol blue dye front reached the bottom of the gel plate. The gels were subsequently stained in staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, and 10% (v/v) acetic acid in water for 20 min and de-stained with de-staining solution 40% (v/v) methanol and 10% (v/v) acetic acid in water] for 45 min. The molecular masses of protein bands were determined by comparison to standard low molecular weight protein markers (GE Healthcare, Uppsala, Sweden), which consist of phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and bovine α lactalbumin (14.0 kDa).

**3.2.11 Determination of protein concentration**

The protein concentration was determined by the Bio-Rad (Bradford) assay (Hercules, CA, USA). Bovine serum albumin (BSA) was used as a standard at concentrations ranging from 0.1-5 µg. Water was used as a blank (0 µg). Each assay tube contained 200 µl of Bio-Rad protein assay solution and standard or sample and was made up to 1 ml with distilled water. The mixture was incubated at room temperature for 10 min. The absorbance was measured at a wavelength of 595 nm (A595) with the protein Bradford program of a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). For purified protein, the protein concentration was also

determined by measuring the absorbance at 280 nm ($A_{280}$) spectrophotometrically and calculating with the following equation:

Protein conc. (mg/ml) = [$OD_{280}$ / extinction coefficient] x dilution-fold x 1/(path length)

The extinction coefficient for each specific variant, which was calculated with the PROTEIN PARAMETERS program on the EXPASY website (www.expasy.org).

### 3.2.12 Antibody production

We designed the peptide antigen QSPDKITDRSNGDVC from a sequence in the C-terminal part of Os4BGlu11 sequence based on its uniqueness in comparison to other GH1 sequences. Anti-peptide antibodies specific for Os4BGlu11 were raised by Genscript Corporation. The peptide antigen was designed by optimization software and chemically synthesized at Genscript with a cysteine added to the N-terminus of the peptide and used to conjugate the peptide to Keyhole limpet hemocyanin. The conjugates were mixed with Freund's complete adjuvant and injected into two New Zealand white rabbits, according to the supplier's protocol. The collected blood was clotted and the serum tested for recognition of peptides and recombinant proteins by an indirect Enzyme-Linked Immuno Sorbent Assay (ELISA) assay, in which the proteins were adsorbed to the bottom of microtiter plate wells. Further antisera (anti-Os4BGlu11) dilutions were tested once a response was detected.

### 3.2.13 Western blotting

Protein samples were mixed with 6x loading dye (50 mM Tris-HCl, pH 6.8, 10% SDS, 0.2 mg/ml bromophenol blue, 50% glycerol, 20% β-mercaptoethanol) and boiled for 5 min, cooled, and centrifuged briefly before loading on 12% SDS-PAGE gels

(section 3.2.7). After electrophoresis, the gels were rinsed with water, then assembled into a wet-blot sandwich with a nitrocellulose membrane (GE Healthcare Life Science,Little Chalfont, UK) on one side and filter paper outside the gel and membrane**.** The proteins were then blotted onto the nitrocellulose membrane by electro-blotting in a wet-blot apparatus at 100V for 90 min in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). The membrane was then blocked with 5% skim milk in TBS (150 mM NaCl, 50 mM Tris-Cl, pH 7.6,) at 4 $^{o}$C overnight and incubated with the rabbit anti-peptide anti-serum developed against Os4BGlu11 (Genscript) at a dilition of 1:1000 in 5% skim milk in TBS at 4 $^{o}$C for 3 h. After that, the membrane was washed 2 times with TBST and 1 time with TBS. It was then incubated with a 1:3000 dilution of horse-radish-peroxidase-conjugated anti-rabbit IgG antibodies (Bio-Rad) in 5% skim milk in TBS at 4 $^{o}$C for 2 h. After washing two times with TBST and 1 time with TBS, the membrane was development with the ECL western blotting analysis system (chemiluminescent HRP substrate from GE Healthcare Life Science) for 10 min before exposure to Hyper-film ECL in a film cassette in the dark room, followed by film development.



## 3.3 Cloning of putative rice phytohormones β-glucosidases

### 3.3.1 RNA extraction

Rice (*Oryza sativa* L. spp. Indica cv. KDML105) seeds were obtained from the Rice Department of Thailand, Nakhon Ratchasima province. Healthy, uniform seeds were washed with 5% Chlorox for 20 min, then washed with distilled water twice, and soaked in distilled water overnight. The rice seeds were germinated according to the rules of the International Seed Testing Association (ISTA, 1999). The shoots from the

rice seedlings were harvested and used in total RNA purification. One gram of harvested shoot was ground in a mortar and pestle to powder in liquid nitrogen. The total RNA was isolated from 100 mg of 7-day-old rice seedling shoot powder with the Spectrum™ plant total RNA kit (Sigma-Adrich, St. Louis, MO, USA), according to the manufacturer's protocol.

### 3.3.2 First-strand cDNA synthesis and amplification of rice phytohormone β-glucosidases

Total cDNA was reverse-transcribed from the total RNA template. Total RNA at a concentration of 100 ng/µl was mixed with 2.5 µM oligo$(dT)_{20}$ and 0.5 mM dNTP, adjusted to a final volume of 10 µl with DEPC-treated water and incubated at 65 ºC for 5 min and immediately placed on ice at least 1 min. After that, 1X RT buffer, 5 mM $MgCl_2$, 10 mM DTT, and 40 U RNase OUT™ were added into the reaction mixture and it was incubated at 50 ºC for 2 min. Then, 200 U Superscript™III reverse transcriptase (Invitrogen) were added into the mixture and it was incubated at 50 ºC for an additional 50 min. The reaction was stopped by incubating it at 70 ºC 15 min. The mRNA was then hydrolyzed by 1 µl of Rnase-H (Invitrogen) at 37 ºC for 20 min. The single stranded cDNA was ready for PCR.

### 3.3.3 Cloning of rice phytohormone β-glucosidases from cDNA

The gene segments encoding full-length rice Os4BGlu9 and Os4BGlu11 were amplified from the cDNA synthesis product with Os4BGlu9Startf and Os4BGlu9stop and Os4BGlu11Startf and Os4BGlu11Stop primers (Table 2.1), respectively.Then the genes encoding the predicted mature proteins were then amplified from the initial PCR

products with the Os4BGlu9MatStf and Os4BGlu9Stop primers for Os4BGlu9 and the Os4BGlu11MatStf and Os4BGlu11Stop primers for Os4BGlu11. Both reactions were carried out with *Pfu* polymerase with the temperature cycling parameters shown in Table 3.4. The PCR products (1.5 kb) were check by electrophoresis on a 1% agarose gel.

**Table 3.4** Cycling parameters for amplification of Os4BGlu9 and Os4BGlu11.

| Sagment | Cycles | Temperature (°C) | Time |
|---|---|---|---|
| 1 | 1 | 95 | 1 min |
| | | 95 | 30 s. |
| 2 | 30 | 58 | 30 s. |
| | | 72 | 3 min |
| 3 | 1 | 72 | 7 min |

The amplified cDNA encoding the predicted mature Os4BGlu9 and Os4BGlu11 β-glucosidases were recombined with pENTR-D-TOPO from a pENTR™ Directional TOPO® Cloning Kit according to the supplier' s instructions (Invitrogen). The recombination reactions were used to transform chemically competent DH5α *E. coli*. The transformed DH5α competent cells were spread onto an LB plate containing the 50µg/ml ampicillin and resistant colonies were grown in small scale culture overnight at 37 °C. The plasmids were extracted by the alkaline lysis and QIAGEN mini preparation kit protocol (QIAGEN) and their cDNA insert sequences were determined by automated DNA sequencing at Macrogen Corp. (Seoul, South Korea).

The cDNA encoding the mature Os4BGlu9 and Os4BGlu11 (1500 bp) were excised from the recombinant pENTR-D-TOPO plasmids with *Nco*I and *Xho*I for Os4BGlu9 and Os4BGlu11 with *Nco*I and *HindIII*. The Os4BGlu9 and Os4BGlu11 inserts were ligated into pET32a(+) (6099 bp) that had been cut with the same restriction enzymes by a T4 ligase reaction (Invitrogen). The mixture was incubated at 15 $^{o}$C for 18 h. The ligation reactions were transformed into *E. coli* strain DH5α, selected on a 50 µg/ml ampicillin LB agar plate. Resistant clones were grown in 5 ml LB with antibiotic and plasmids were extracted by the alkaline lysis method, and the insert sequences were determined by automated DNA sequencing at Macrogen Corp (Seoul, Korea).

### 3.3.4 Cloning of pPICZαBNH$_8$/Os4BGlu11 and pPICZαBNH8/Os4BGlu11 /eGFP

An optimized gene was designed to achieve the highest possible level of expression in *Pichia patoris*, synthesized and inserted into the pUC57 plasmid by Genscript Corporation. The optimized Os4BGlu11 cDNA was cut with *PstI* and *XbaI* and ligated into the corresponding sites in pPICZαBNH$_8$ (Toonkool et al., 2006). The ligation reaction was transformed into DH5α, selected on the 250 µg/ml zeocin antibiotic plates, and the plasmid was extracted by the alkaline lysis plasmid preparation protocol. The sequence was determined by automated DNA sequencing at Macrogen Corp.

Constructed of pPICZαBNH8/Os4BGlu11/eGFP. An optimized eGFP was synthesized and inserted into the pUC57 plasmid by Genscript Corporation. The eGFP/pUC57 was digested with *Pst*I and *Sal*I and ligated into corresponding sites in

pPICZαBNH$_8$. The ligation reaction was transform into DH5α as described above. The sequence was determined by automated DNA sequencing at Macrogen Corp. The optimized Os4BGlu11 was cut with *PstI* and *XbaI* and ligated into the corresponding sites in pPICZαBNH$_8$/eGFP. Finally, these clones were confirmed again by sequencing at Macrogen Corp.

### 3.3.5 Protein expression in *E. coli* and purification

The recombinant pET32a(+)/BGlu9 and pET32a(+)BGlu11 plasmids were transformed into Origami(DE3) and Origami B(DE3) competent cells and spread onto LB-agar containing 15 μg/ml kanamycin and 12.5 μg/ml tetracycline in addition to ampicillin for Origami B(DE3). The plate was incubated at 37 ºC overnight. Single colonies overnight growing was picked and inoculated into LB media containing appropriate antibiotics to make a starter culture. To express recombinant β-glucosidases, 1% final concentration of starter culture was added into the same type of media and cultured at 37 ºC with rotary shaking at 200 rpm. The protein expression was induced when the optical density at 600 nm ($OD_{600}$) of the culture reaches 0.4-0.6. The optimum expression conditions were determined by varying the final concentration of isopropyl β-D-thiogalactopyranoside (IPTG) from 0 to 0.4 mM, and temperature at 15 ºC, 18 ºC and 20 ºC for 18 and 24 hr. The cell pellets were collected by centrifugation at 12,000 rpm 15 min at 4 ºC. The cell pellets were kept at -80 ºC for 2 h to allow freeze-thaw breakage before extraction. The bacterial cell pellets were thawed on ice and then resuspended in freshly prepared extraction buffer (20 mM Tris-HCl buffer, pH 8.0, 200 μg/ml lysozyme, 1% Triton-X 100, 1 mM PMSF, 25 μg/ml DNase I and 0.1 mg/ml soy bean trypsin inhibitor) in a ratio of 5 ml extraction buffer per gram fresh

weight of cell pellets. The resuspended cells were incubated at room temperature for 30 min. Then, the insoluble proteins were removed by centrifugation. The supernatant fraction was loaded onto a pre- equilibrated immobilized metal ion affinity chromatography (IMAC) column (GE Healthcare, Buckinghamshire, United Kingdom) with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and charged with $Co^{2+}$. After loading the cell extract supernatant, the IMAC column were washed twice with 5 column volumes (CV) of equilibration/wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) to remove unbound protein and washed again with 5 CV each of equilibration/ wash buffer containing 5 mM and 10 mM of imidazole, respectively. Bound protein was eluted with 5 CV of elution buffer (20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole). The eluted protein was checked by assaying activity with *p*NPGlc and the presence and purity of protein of appropriate size evaluated by SDS-PAGE.

### 3.3.6 Protein expression in *Pichia pastoris* and purification

The recombinant pPICZαBNH8/ Os4BGlu11 was linearized with *PmeI* or *SacI* and transformed into competent cells of *P. pastoris* strain SMD1168H by electroporation (Section 3.2.6, *Pichia* manual, Invitrogen). After the initial screening on 100 µg/ml zeocin, transformed clones were selected on a YPD plate containing 250 µg/ml zeocin. The colonies were screened for protein production in small scale cultures, as described in the *P. pastoris* manual (Invitrogen). Zeocin- resistant colonies were picked and inoculated into 5 ml BMGY in a 50 ml conical tube and grown at 28 ºC (220 rpm) until the $OD_{600}$ reached 2. Cells were harvested at 5000 rpm. The pellet was resuspended in 2 × 500 ml of BMMY in 1-liter flasks. The culture was grown at 20 ºC with shaking (220 rpm). Protein expression was induced by adding methanol to 0.5%

(v/v) final concentration every 24 h for 7 days. The supernatant was checked each day for activity with 1mM *p*NPGlc in 50 mM sodium acetate buffer, pH 5, at 30 $^{o}$C for 30 min. The protein was purified from the culture broth after removal of the cell by centrifugation. The pH of the culture broth containing the secreted protein was adjusted to 7.5 with 1 M $K_2HPO_4$ and it was loaded onto immobilized metal ion affinity chromatography (IMAC) column (GE-Healthcare) charged with $Co^{2+}$, and the column was washed with 5 CV of 5 mM and 10 mM of imidazole in 50 mM sodium phosphate buffer, pH 7.5, then the protein was eluted with 250 mM and the pool of fractions containing the enzyme was concentrated and buffer exchanged to 20 mM tris-HCl, 150 mM NaCl pH8 by centrifugal filtration. The protein band was detected on the SDS-PAGE. The protein concentration was determined by measuring the $A_{280}$ spectrophotometrically (Walker, 2002). Protein size and purity were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by the method of Laemmli (1970) with staining by Coomassie brilliant blue (see Section 3.2.10).

### 3.3.7 Expression of pPICZαBNH8/Os4BGlu11_eGFP in *P. pastoris*

Expression of pPICZαBNH8/Os4BGlu11_eGFP in *P. pastoris* was similar to that described in Section 3.3.6. The media of each clone was collected after 3 days to measure fluorescence with excitation and emission wavelengths at 480 and 509 nm, respectively. The protein expression was assessed by measurement of eGFP by fluorescence in both media culture and cell pellets. The cell pellets were extracted in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X 100, 1 mM PMSF,

25 µg/ml DNase I) containing glass beads. The cells were mixed by vortexing and sonicated before centrifuging to collect the supernatant to run SDS-PAGE.

### 3.3.8 pPICZαBNH8/Os4BGlu11_GFP Localization

To localization of Os4BGlu11/eGFP in the cell pellet, Pichai cells were prepared in 70% glycerol in TBS buffer for confocal microscopy. Other cells were stained with 300 nM DAPI stain solution. The *P. pastoris* cell suspensions were dropped in the center of the slide and placed cover slip before observance by confocal microscopy. The confocal microscope (Nikon/Ni/E, Nikon®Nikon Instruments Inc.) was set up at 509 and 461 nm emission wavelengths to detect GFP and DAPI, respectively.

## 3.4 Hydrolysis activity of rice β-glucosidases toward ABA-GE

### 3.4.1 Production of ABA-GE with Os9BGlu31 transglucosidase

Os9BGlu31 transglucosidase was used to transfer glucose from *p*NP-β-D-glucopyranoside (*p*NPGlc) to ABA to generate ABA-GE (Patent pending Thai patent application 1801003832). Briefly, 10 µg of Os9BGlu31 enzyme catalyzed the reaction of 10 mM ABA with 10 mM *p*NPGlc in citrate buffer, pH 4.5, at 37 ºC overnight. The reaction was stopped by boiling 5 min. The ABA-GE product was purified by silica gel chromatography in 8% methanol 2% acetic acid and C18 reverse phase chromatography with increasing methanol in water. The purity of the ABA-GE was verified by Ultra High-Performance Liquid Chromatography (UPLC).

### 3.4.2 The hydrolysis activity of rice GH 1 enzymes toward ABA-GE

The hydrolysis activity of enzymes belonging to GHI family from rice including Os1BGlu4 (Rouyi et al., 2014), Os3BGlu7 (Opassiri et al., 2003), Os4BGlu12 (Opassiri et al., 2006; Sansenya et al., 2012), Os4BGlu13 (Hua et al., 2013), Os4BGlu18 (Baiya et al., 2014), Os7BGlu26 (Kuntothom et al., 2009), Os9BGlu31 (Luang et al., 2013) were analyzed towards ABA-GE. The reactions were incubated 0.5 µg of enzyme with 1 mM ABA-GE in 50 mM sodium acetate pH 5.0, at 30 ºC for 30 min. The reactions were stopped by boiling 5 min, and the glucose released was quantified by the peroxidase/glucose oxidase assay method (PGO assay, Sigma Aldrich). A control reaction was incubated without enzyme and processed in the same way as a blank.

### 3.4.3 Kinetic study of BGlu12 and BGlu13 with ABA-GE

The Os4BGlu12 and Os4BGlu13 proteins were expressed and purified by IMAC, cleavage of the N-terminal thioredoxin and $His_6$ tags with enterokinase and removal of the fusion tags by IMAC, as previously described (Sansenya et al., 2012, Hua et al., 2013). Apparent kinetic parameters, $K_M$ and $V_{max}$, were determined for the purified proteins with ABA-GE. All of kinetic parameters were determined in triplicate reactions. The optimum time point, at which the velocity of hydrolysis gives a first order rate constant, was determined with a set of time course reactions. The rates were determined over a substrate concentration range of at least 0.2 $K_M$ to 3 $K_M$. Kinetic parameters were calculated by fitting the rate of product formation and substrate concentrations by nonlinear regression of the Michaelis-Menten curves with Grafit 5.0 (Erithacus Software, Horley, Surrey, U.K). The apparent $k_{cat}$ values were calculated by dividing the $V_{max}$ by the total amount of enzyme in the reaction. The assays were done

at 30 ºC in 50 mM sodium acetate, pH 5. The glucose released was determined by glucose oxidase assay as described previously (section 3.4.2).

## 3.5 *In planta* analysis of rice phytohormones β-glucosidase

### 3.5.1 Localization of BGlu9-13-GFP in tobacco

The subcellular localization of Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 belonging to cluster At/Os7 of rice glycoside hydrolase family I (Opassiri et al., 2006) were determined by transient expression in tobacco (*Nicotiana benthamiana*) leaf epithelial cells. The cDNA containing their entire open reading frames without the stop codons were amplified with the proofreading EF-Taq polymerase (SolGent, Daejeon, Korea) and the primers shown in Table 3.5. The respective PCR products were further cloned into the pENTR™/D-TOPO® vector (Invitrogen). Afterward, those amplicons were cloned into the binary vector p2GWF7 (Karimi et al., 2002) to make recombinant vectors that encoded chimeric proteins that fused those proteins' C-termini to GFP and placed their genes under the control of a CaMV35S promoter. An additional vector that encoded chimeric RFP-Korrigan1 (GenBank: AK318891) fusion protein was used to provide a plasma membrane protein marker (Von Schaewen et al., 2015). The resulting GFP fusion construct was infiltrated into a tobacco leaf by an Agrobacterium-mediated infiltration method (Rouyi et al., 2014). Subcellular localization was monitored at day 3-5 after infiltration by observation under a confocal microscope (LSM 510 META; Carl Zeiss), which was set up at 509 and 532 nm emission wavelengths to detect GFP and RFP, respectively.

**Table 3.5** Oligonucleotides primer for Localization of BGlu9-13-GFP in tobacco.

| **Primer** | **Sequence (5'→3')** |
|---|---|
| GFPBglu9F | CACCATGGCGGTTGCCGGGGCAGTGGCGA |
| GFPBglu9R | ATGTCTGTACTGGCAAACTCT |
| GFPBglu10F | CACCATGGCGGTTGCAGGTGCAATGGTGAT |
| GFPBglu10R | TTTCCGGAGGAACTTCTTGAACCA |
| GFPBglu11F | CACCATGGCGGTTGCAGGGGCAATGGT |
| GFPBglu11R | ATTAGAAAAGGCATTGTATGCA |
| GFPBglu12F | CACCATGGCAGATGGAAGTCTGAGGGGTGG |
| GFPBglu12R | TTTCAGGAGGAACTTCTTGAACCAAT |
| GFPBglu13F | CACCATGGCAGCTGCAGGGGAAGTGGTGA |
| GFPBglu13R | TTTCTGGAGGAACTCCTTGAACC |

### 3.5.2 Construction of plant expression vectors and Arabidopsis transformation

Construction of transgenic Arabidopsis lines overexpressing the 5 protein of cluster At/Os7 was performed as described for Os4BGlu14, 16 and 18 by Baiya et al. (2018). The initial clones of full-length cDNAs for each gene were ordered from the Knowledge-based Oryza Molecular biological Encyclopedia (KOME) clone bank. Briefly, the full length cDNAs were amplified with a SolGent™ *Pfu* DNA polymerase (SolGent, Daejeon, Korea) from japonica rice (cv. Nipponbare) cDNA clones Os4BGlu9 accession number AK066908, Os4BGlu10 accession number AK065793, Os4BGlu11 accession number AK242955, Os4BGlu12 accession number AK100820 and Os4BGlu13 accession number AK070962 with the primers shown in Table 3.6.

The respective PCR products were cloned into the pENTR/D-TOPO vector (Thermo-Science Invitrogen, Carlsbad, CA, USA), and then the recombinant plasmids were verified by sequencing. The cDNA inserts were cloned via LR clonase recombination (Invitrogen, Gaithersburg, MD, USA) into the Gateway binary vector pGWB502, containing the CaMV35S sequence as a strong promoter.

### 3.5.3 Floral dip transformation

All recombinant binary vectors for expression of the five phytohormone-β-glucosidase-like genes were introduced into *Agrobacterium tumefaciens* strain GV3101 by transformation as mention in section 3.2.7. Cells were cultured at 28 ºC at 200 rpm in LB broth containing 50 µg/ml spectinomycin at 28 ºC at 200 rpm until the optical density at 600 nm ($OD_{600}$) reached 1-1.5. The cells were centrifuged at 7000 rpm for 20 min. The pellets were collected and resuspended in 5% sucrose and 0.005% silwet L-77 (final OD = 0.8). The *Arabidopsis thaliana* plants (ecotype Columbia) were inverted to dip their floral buds into the suspension. The plants were covered with a plastic dome to maintain humidity and kept in darkness at 28 ºC for 24 h. After 24 h, the plants were moved to a white light chamber. The process was repeated again after 7 days (inverting the flora bud into the suspension). To select transgenic plants, seeds from T0 plants were seeded on MS containing 50 µg/ml hygromycin. Shoots that survived hygromycin selection around 14-day old or developed to produce 3 leaves were planted in soil and grown in a growth chamber at a temperature of 25 ºC until transgenic plants matured and senesced to produce seeds.

### 3.5.4 RNA isolation and RT-PCR analysis

Around 1-month-old leaves of at least two independent lines each of transgenic Arabidopsis overexpressing the 5 genes of At/Os7 were used to validate the gene expression level. Total RNA was extracted with TRIzol™ Reagent, following the company's protocol (Invitrogen). The isolated RNA extracts were reverse-transcribed from an oligo-dT primer with a First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The first-strand cDNAs were used as templates in the RT-PCR with the Arabidopsis ubiquitin gene-specific primers used as the internal control. The primers for RT-PCR determination in each gene are shown in Table 3.6, while Arabidopsis Tubulin 1 (AtG75780) gene-specific primers were used as the internal control (Oppenheimer et al., 1988).

**Table 3.6** Oligonucleotides primer for Arabidopsis overexpress rice phytohormone β-glucosidase and RT PCR.

| **Primer** | **Sequence (5'→3')** |
|---|---|
| Os4BGlu9 cloning F | 5'-CACCGAATTGCAGGTCAAGCTTCGT-3' |
| Os4BGlu9 cloning R | 5'-ACACATTGGGATTAAGTGCCAA-3' |
| Os4BGlu10 cloning F | 5'-CACCCACTCTTTTCTGTCTATGTAGG -3' |
| Os4BGlu10 cloning R | 5'-CATGCAAGAAGAGGAGAGTGAG-3' |
| Os4BGlu11 cloning F | 5'-TCTTCTGTCTATGTAGGTCATG-3' |
| Os4BGlu11cloning R | 5'-CTTATATACTATTTCGATGTTC-3' |
| Os4BGlu12 cloning F | 5'-GTTGATGGCACCAAACATTG-3' |
| Os4BGlu12 cloning R | 5'-AAGCAATGAGTGCAATGGTGC-3' |

**Table 3. 6** Oligonucleotides primer for Arabidopsis overexpress rice phytohormone β-glucosidase and RT PCR (Continued).

| **Primer** | **Sequence (5'→3')** |
|---|---|
| Os4BGlu13 cloning F | 5'-CACCGCGGGTCACACACACATACT-3' |
| Os4BGlu13 cloning R | 5'-GGGAAGAGCAATGTGTGCAACT-3' |
| RTBGlu9F | 5'-GCACTGGAAGATAAATACAACGGATT-3' |
| RTBGlu9R | 5'-TGATATTTCTCTCTGTACAACCGAA-3' |
| RTBGlu10F | 5'-TGAAATCAATAATAAGACCATGCGAC-3' |
| RTBGlu10R | 5'-TCATTTCCGGAGGAACTTCTTGAACCA-3' |
| RTBGlu11F | 5'-CACCTCTTCTGTCTATGTAGGTCATG -3' |
| RTBGlu11R | 5'-TCAATTAGAAAAGGCATTGTATGCAAC-3' |
| RTBGlu12F | 5'-CACCGTTGATGGCACCAAACATTG-3' |
| RTBGlu12R | 5'-TCATTTCAGGAGGAACTTCTTGAACCAAT-3' |
| RTBGlu13F | 5'-TGAATTCAACAATAAGACCTTACCACT-3' |
| RTBGlu13R | 5'-TCATTTCTGGAGGAACTCCTTGAACC-3' |

### 3.5.5 Growth Conditions, and Treatments of transgenic Arabidopsis

For the Arabidopsis overexpressing rice phytohormone β-glucosidases, seeds were surface sterilized with 80% ethanol for 20 min and then sterilized with 10% Clorox 10 min, washed with water 5 times and grown on ½ MS containing 1% (w/v) sucrose and 1% (w/v) phytagel (Himedia) at 28 $^{o}$C in a culture room with 80% relative humidity and a 16-h/8-h light/dark cycle. Plants were germinated on ½ MS media for 7 days. After 7 days plant were moved to ½ MS plates containing 0.01 µM ABA or ABA-GE or 0.05 µM $GA_4$ or $GA_4$-GE or no hormone for 5. Root and shoot growth

were measured 5 days after the plants were transplanted. To quantify root and shoot lengths at the end of each treatment, three independent experiments were performed with 20 plants each.

**3.5.6 Extraction total protein from Arabidopsis overexpressing rice phytohormone β-glucosidases**

Arabidopsis that overexpressed rice BGlu9, BGlu10, BGlu11, BGlu12, or BGlu13 and control were grown in ½ MS plates at 20 $^{o}$C in a culture room with 80% relative humidity and a 16-h/8-h light/dark cycle for 7 days. Then, the seedling were moved to plates containing 0.01 µM ABA for 5 days. Total protein extracts were prepared from 10 mg of fresh Arabidopsis seedlings that were ground in liquid nitrogen. After grinding 1 ml of lysis buffer (20 mM Tris-HCl 150 mM NaCl, 1 mM PMSF) was added then vortexed 5 min and sonicated on ice for 30 min. The extracts were centrifuged at 12,000 rpm for 20 min and supernatants were used in Section 3.5.7

**3.5.7 Hydrolyzing activity assay of extracts of Arabidopsis overexpressing rice phytohormone β-glucosidases torward *p*NPGlc, ABA-GE and $GA_4$-GE**

Protein extracts (10 µg of total protein) in lysis buffer were incubated with 1 mM *p*NPGlc (final concentration) in 50 mM sodium acetate buffer, pH 5, at 30 $^{o}$C for 8 h. The reactions were stopped by adding 70 µl 2 M sodium carbonate ($Na_2CO_3$). The released of p-nitrophenol (*p*NP) was quantified by measuring the absorbance at 405 nm ($A_{405}$) with a microplate reader (Thermo Labsystems, Helsinki, Finland). In other reactions, protein extracts containing 10 µg of total protein were incubated with 1 mM ABA-GE or $GA_4$-GE in 50 mM sodium acetate buffer, pH 5.0, for 4 h at 30$^{o}$C. The

glucose released was quantified by the peroxidase/glucose oxidase-based glucose assay (PGO assay, Sigma-Aldrich Corp.). A control reaction was incubated without enzyme and processed in the same way as a blank. The protein concentrations were determined by Bradford assay (1976).

## 3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GPW7). Data are plotted as mean ± standard deviation of three biological replicates. To verify the significant differences between the overexpressing rice phytohormone β-glucosidases with control wild type lines, all determinations were performed in three biological replicates. Statistical significance was evaluated using the SPSS statistics software package with one-way ANOVA followed by post hoc Scheffe's test method at a significance level of $P < 0.05$.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 The hydrolysis activity toward ABA-GE of certain enzymes belonging to GHI

To identify which enzymes may hydrolyze ABA-GE, several rice GH1 β-glucosidases that have been expressed in *E. coli*, including Os1BGlu4, Os3BGlu7, Os4BGlu12, Os4BGlu13, Os4BGlu18, Os7BGlu26, Os9BGlu 31 and barley βII were assayed for release of glucose from ABA-GE, as shown in Table 4.1. The results showed that all of the β-glucosidases tested have hydrolytic activity toward ABA-GE except Os9BGlu19 (not shown). Os4BGlu13 exhibited highest hydrolysis activity with ABA-GE followed by Os4BGlu12. Previously, Os3BGlu7 was identified as an oligosaccharide β-glucosidase (Opassiri et al., 2004), Os7BGlu26 and barley were classified as β-D-mannosidases, although they also have activity on gluco-oligosaccharides (Kuntothom et al., 2009), Os4BGlu18 is rice monolignol β-glucosidase (Baiya et al., 2014), while. Os9BGlu31 acts as a transglucosidase, which should mainly transfer glucose to an acceptor, rather than releasing free glucose (Luang et al, 2013). Os4BGlu12 and Os4BGlu13 are closely related and fall into the protein-sequence-based phylogenetic cluster At/Os7 of GH1 (Opassiri et al., 2006). Os4BGlu12 was reported to be induced in response to herbivore attack and salinity stress (Opassiri et al., 2010). Os4BGlu12 showed higher hydrolytic activity towards SAG than TAG (Wakuta et al., 2011; Himeno et al., 2013). Another enzyme in cluster At/Os7 is

Os4BGlu13, which showed hydrolytic activity toward TAG, SAG and $GA_4$-GE (Wakuta et al., 2010; Hua et al., 2015). The results showed this data support the designation of rice members in this cluster At/Os7 as putative phytohormone β-glucosidases. It was noted that this cluster also contains Os4BGlu9, Os4BGlu10, and Os4BGlu11, which are found close to Os4BGlu12 and Os4BGlu13 on rice chromosome 4, suggesting a repetitive gene duplication, and genes that might have redundant or similar functions.

## 4.2 Expression and purification of Os4BGlu12 and Os4BGlu13

The Os4BGlu12 and Os4BGlu13 fusion proteins with N-terminal thioredoxin, $His_6$ and S-tags were expressed in *E-coli* stain Origami B(DE3) and Rosettagami(DE3) respectively, and induced with 0.4 mM IPTG at 20 $^oC$ for 16-18 h (Opassiri et al., 2006; Hua et al., 2015). The soluble protein of recombinant Os4BGlu12 and Os4BGlu13 were purified by IMAC, cleavage of the N-terminal thioredoxin and $His_6$ tags from the enzymes with enterokinase, and removal of the fusion tag by IMAC. Os4BGlu12 and Os4BGlu13 were observed at approximately 55 kDa and approximately 95% purity, as judged by SDS-PAGE (Figure 4.1). These purified proteins were used to study their kinetic parameters for ABA-GE hydrolysis.

**Table 4.1** ABA-GE hydrolysis activity of several rice GH1 beta-glucosidases produced by recombinant expression in *E. coli*. The activities were assayed by incubating 0.25 µg of enzyme with 1 mM ABA-GE in 50 mM buffer (sodium acetate, pH 5) at 30 ºC for 30 min.

| **Rice GH1 B-Glucosidases** | **Specific Activity** (µM/mg protein/min) |
|---|---|
| Os4BGlu12 | $1.54 \times 10^{-3}$ |
| Os4BGlu13 | $1.63 \times 10^{-3}$ |
| Os1BGlu4 | $1.31 \times 10^{-3}$ |
| Os3BGlu7 | $1.17 \times 10^{-3}$ |
| Os4BGlu18 | $1.15 \times 10^{-3}$ |
| Os7BGlu26 | $0.11 \times 10^{-3}$ |
| Barley βII | $0.57 \times 10^{-3}$ |
| Os9BGlu 31 | $0.06 \times 10^{-3}$ |

## 4.3 Kinetic analysis of Os4BGlu12 and Os4BGlu13 hydrolysis of ABA-GE

The apparent kinetic parameters for hydrolysis of ABA-GE were determined for Os4BGlu12 and Os4BGlu13. Os4BGlu13 has higher catalytic efficiency with $K_M$ = 1.66 mM, $k_{cat}$ = 20.59 $s^{-1}$, and $k_{cat}/K_M$ = 12.4 $mM^{-1}s^{-1}$ for ABA-GE hydrolysis than Os4BGlu12, which has $K_M$ = 10.88 mM, $k_{cat}$ = 10.50 $s^{-1}$, and $k_{cat}/K_M$ = 0.689 $mM^{-1}s^{-1}$. The higher efficiency largely reflects the 6-fold lower $K_M$ of Os4BGlu13 compared to Os4BGlu12.

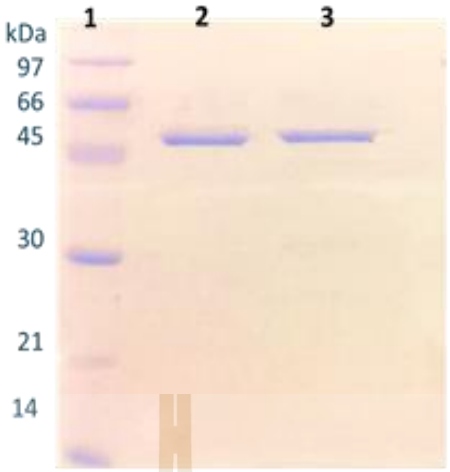


**Figure 4.1** SDS- PAGE analysis of Os4BGlu12 and Os4BGlu13 produced in *Escherichia coli*. Lane 1, standard protein marker; Lane 2, Os4BGlu12 after remove of the fusion tags; Lane 3, Os4BGlu13 after remove of the fusion tags.

## 4.4 Cloning and expression of Os4BGlu9 and Os4BGlu11

The cDNA segments encoding the mature Os4BGlu9 and Os4BGlu11 proteins were amplified from the KOME clones with the Genbank, accession number AK066908 for Os4BGlu9 and accession number AK242955 for Os4BGlu11. The PCR products with sizes around 1.5 kb (Figure 4.2) were cloned into pENTR™/D-TOPO (Figure 4.3) then excised and ligated into the pET32a expression vector (Figure 4.4). The cDNAs encoding the mature Os4BGlu9 and Os4BGlu11 were cloned into the pET32a expression vector, as was previously successfully used to express other rice β-glucosidase (Opassiri et al., 2003, 2006; Kuntothom et al., 2009; Seshadri et al., 2009). Expression of Os4BGlu9 and Os4BGlu11 was attempted with the recombinant pET32a/ Os4BGlu9 and Os4BGlu11 in *E. coli* stains Origami (DE3) and Origami B(DE3). The concentration of IPTG used for induction was varied from 0 to 0.4 mM

and temperature of induction from 15-20 ºC. SDS-PAGE analysis showed insoluble protein. No correct-size protein of about 66 kD could be observed in the soluble protein fractions (Figures 4.5 and 4.6), and no activity was detected. The insoluble protein from pET32a/Os4BGlu9 and pET32a/Os4BGlu11 expression in Origami (DE3) induced at 0-0.4 mM of IPTG exhibited a band at about the right size, as shown in Figure 4.7.

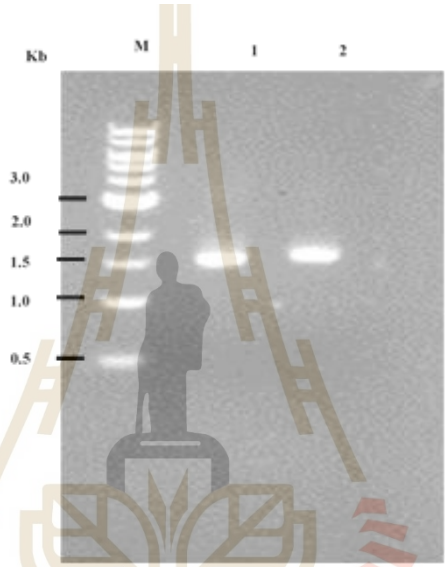


**Figure 4.2** Agarose gel electrophoresis analysis of amplification of OS4BGlu9 and Os4BGlu11 from rice cDNA. Lane M, GenRuler 1kb DNA ladder, Lane 1, Os4BGlu9 and Lane2, Os4BGlu11.

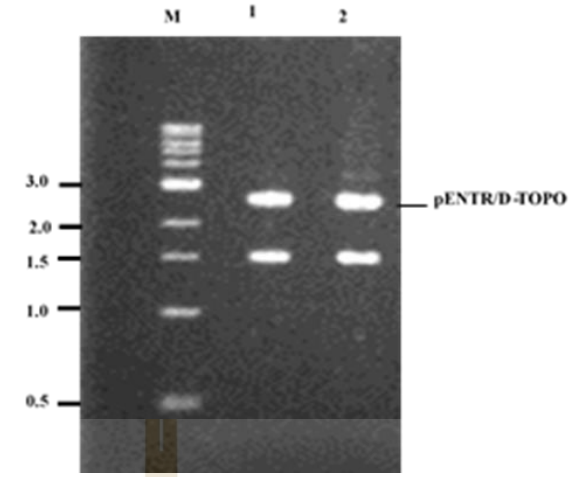


**Figure 4.3** Agarose gel electrophoresis analysis of Os4BGlu9 and Os4BGlu11 cloned into pENTR/D-TOPO. Lane M, GenRuler 1kb DNA ladder; Lane 1, the digest of vector containing Os4BGlu11, and Lane 2, Os4BGlu9, with *NcoI* and *HindIII*.

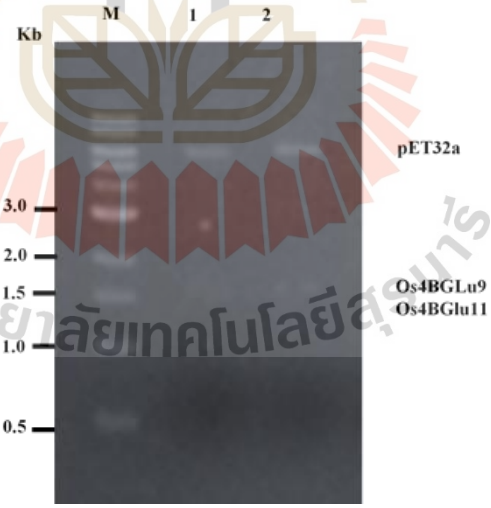


**Figure 4.4** Agarose gel electrophoresis analysis of Os4BGlu9 and Os4BGlu11 cloned into pET32a. Lane M, GenRuler 1kb DNA ladder; Lane 1 is the digest of pET32a/Os4BGlu11 and Lane 2, pET32a/Os4BGlu9, with *NcoI* and *XhoI*.

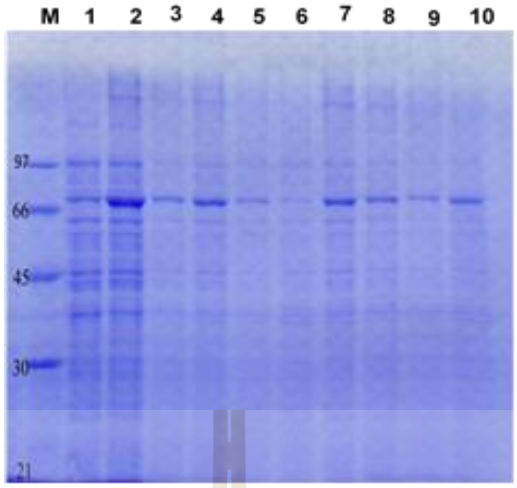


**Figure 4.5** SDS- PAGE analysis of soluble protein extract from pET32a/Os4BGlu9 expression in Origami (DE3) induced with IPTG. Cultures were induced at 15 ºC and 20 ºC for 16 h.

Lane M, Bio-Rad low molecular weight marker; Lanes 1-10 are soluble protein extracts from induced cells. Lane 1, soluble protein from induction with 0 mM IPTG at 15 ºC; Lane 2, with 0.1 mM IPTG at 15 ºC; Lane 3, 0.2 mM IPTG at 15 ºC; Lane 4, 0.3 IPTG at 15 ºC; Lane 5, 0.4 mM IPTG at 15 ºC; Lane 6, 0 mM IPTG at 20 ºC; Lane 7, 0.1 mM IPTG at 20 ºC; Lane 8, 0.2 mM IPTG at 20 ºC; Lane 9, 0.3 IPTG at 20 ºC; and Lane 10, 0.4 mM at 20 ºC.

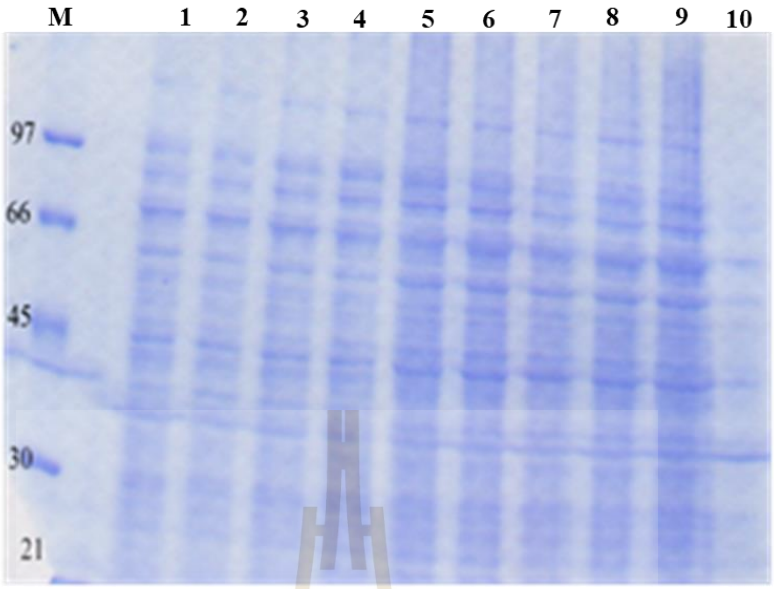


**Figure 4.6** SDS- PAGE analysis of soluble protein extracts from pET32a/Os4BGlu11 expression in Origami (DE3) induced with IPTG. Cultures were induced at 15 ºC and 20 ºC for 16 h.

Lane M, Bio-Rad low molecular weight marker; Lanes 1-10, soluble protein extracts from induction with different IPTG concentrations and temperatures. Lane 1, 0 mM IPTG at 15 ºC; Lane 2, 0.1 mM IPTG at 15 ºC; Lane 3, 0.2mM IPTG at 15 ºC; Lane 4, 0.3 IPTG at 15 ºC; Lane 5, 0.4 mM IPTG at 15 ºC; Lane 6, 0 mM IPTG at 20 ºC; Lane 7, 0.1 mM IPTG at 20 ºC; Lane 8, 0.2 mM IPTG at 20 ºC; Lane 9, 0.3 mM IPTG at 20 ºC; Lane 10, 0.4 mM IPTG at 20 ºC.

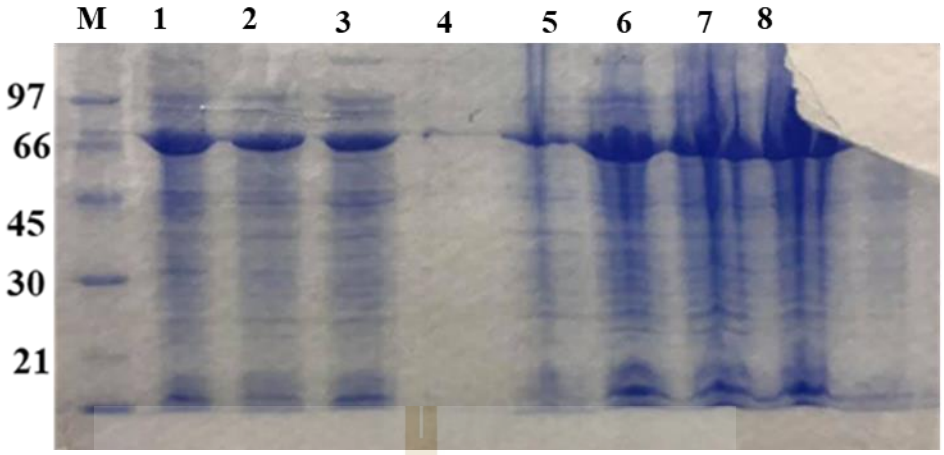


**Figure 4.7** SDS- PAGE analysis of insoluble proteins from pET32a/ Os4BGlu9 and pET32a/ Os4BGlu11 expression in Origami (DE3) induced with IPTG. Cultures were induced at 20 ºC for 16 h.

Lane M, Bio-Rad low molecular weight marker; Lane 1, pET32a/Os4BGlu9 induction with 0.1 mM IPTG; Lane 2, pET32a/Os4BGlu9 with 0.2 mM IPTG; Lane 3, pET32a/Os4BGlu9 with 0.3 mM IPTG; Lane 4, pET32a/Os4BGlu9 with 0.4 IPTG; Lane 5, pET32a/Os4BGlu11 with 0.1 mM IPTG; Lane 6, pET32a/Os4BGlu11 with 0.2 mM IPTG; Lane 7, pET32a/Os4BGlu11 with 0.3 mM IPTG; Lane 8, pET32a/Os4BGlu11 with 0.4 mM IPTG.

I attempted to express Os4BGlu9 and Os4BGlu11 from pET32a/Os4BGlu9 and pET32a/ Os4BGlu11 with N- terminal thioredoxin and $His_6$ fusion tags in *E. coli*. Unfortunately, the Os4BGlu9 and Os4BGlu11 proteins were only observed in the insoluble fraction of the cell lysates (Figure 4.6) and no activity with 4NPGlc could be detected in the soluble cell lysate, suggesting that Os4BGlu9 and Os4BGlu11 were not

expressed in soluble, active forms. Therefore, we moved on to try expression in a yeast system.

In order to construct a *P. pastoris* expression vector with Os4BGlu11 in pPICZαBNH8, an optimized Os4BGlu11 gene was synthesized. The optimized Os4BGlu11 gene was inserted into pPICZαBNH8 and agarose gel analysis showed the correct sizes for Os4BGlu11 (~1.5 kb) and Os4BGlu11/eGFP (2.3 kb) bands obtained in the digestion of pPICZαBNH8/Os4BGlu11/eGFP with *Pst*I and *Xba*I or *Pst*I and *Sal*I, respectively. Os4BGlu11 was expressed in *P. pastoris* to overcome the problem of only insoluble protein being produced in the *E. coli* system. *P. pastoris* was developed as a heterologous protein expression system using the strong and tightly regulated AOX1 promoter to allow induced production of proteins in a eukaryotic system (Cregg et al., 1985).

Unfortunately, we found that *P. pastoris* could secrete other enzymes, which could release 4-nitrophenol from 4NGlc in the media during the 7-10 days induction. The media from 7 days induction was loaded to IMAC resin, which was washed and eluted with 250 mM imidazole. No band of secreted Os4BGlu11 protein was observed in SDS-PAGE analysis of the purification fractions, as shown in Figure 4.8. The enzyme with only a His-tag could not be clearly identified when expressed in this system. Therefore, the Os4BGlu11 insert was cloned in-frame with a C-terminal eGFP fusion to facilitate expression screening. The presence of eGFP could also show whether the protein was secreted into the media of cell culture or was trapped inside the pichia cells. The pPICZαNH8/Os4BGlu11/eGFP plasmid was then transformed into *P. pastoris* SMD1168H and screened for expression.

Eighteen colonies were grown in BMMY media and protein expression induced with 0.5% methanol for 7 days at 20 $^{o}$C. Eighteen clones were examined for fluorescence in the media on the first and fourth day of expression. The Os4BGlu11-eGFP expressed in the *P. pastoris* cells was visualized by fluorescence microscopy on a confocal microscope. Fluorescence intensity was clearly observed inside the cells, proving that the measured fluorescence of the cell pellets was from intracellular eGFP from the Os4BGlu11-eGFP fusion protein. Moreover, nuclear staining with DAPI was mixed with the *pichia* cells to compare the intracellular accumulation to the location of the DNA in the nucleus, as shown in Figure 4.9. This result suggested that Os4BGlu11 may be located in the vacuole. It is possible that a part of the OsBGlu11 sequence acts as a vacuole localization signal in the yeast.

The cells were collected by centrifugation and the pellet were broken by glass beads. The cell lysate was subjected into IMAC and eluted with 250 mM imidazole. The fluorescence emission from the crude and the eluted fractions were examined as shown in Figure 4.10. No band of Os4BGlu11 protein was detected in SDS-PAGE, as shown in Figure 4.11.

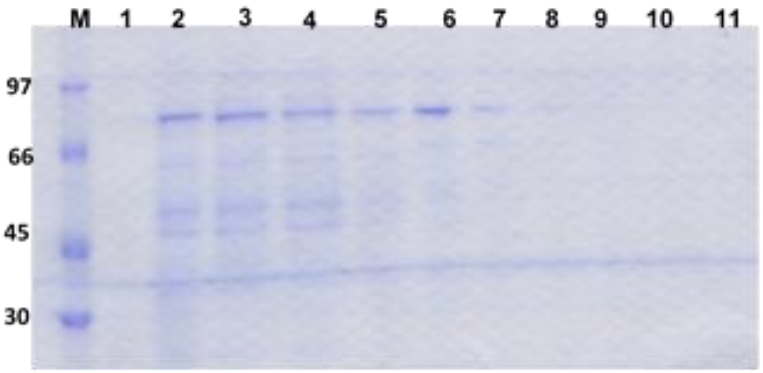


**Figure 4.8** SDS-PAGE analysis of fractions from attempted purification of Os4BGlu11 from Pichai media by IMAC.

Lane M, Bio-Rad low molecular weight marker Lane 1, media of pPICZαBNH8/Os4BGlu11 expressed in *Pichia pastoris* strain SMD1168H; Lane 2, flow-through fraction of protein that passed through the IMAC column; Lane 3, protein from wash with EQ buffer (20 mM Tris HCl,150 mM NaCl, pH 7.5); Lane 4, protein from wash with 5 mM imidazole; Lanes 4-11, fractions eluted with 250 mM imidazole.

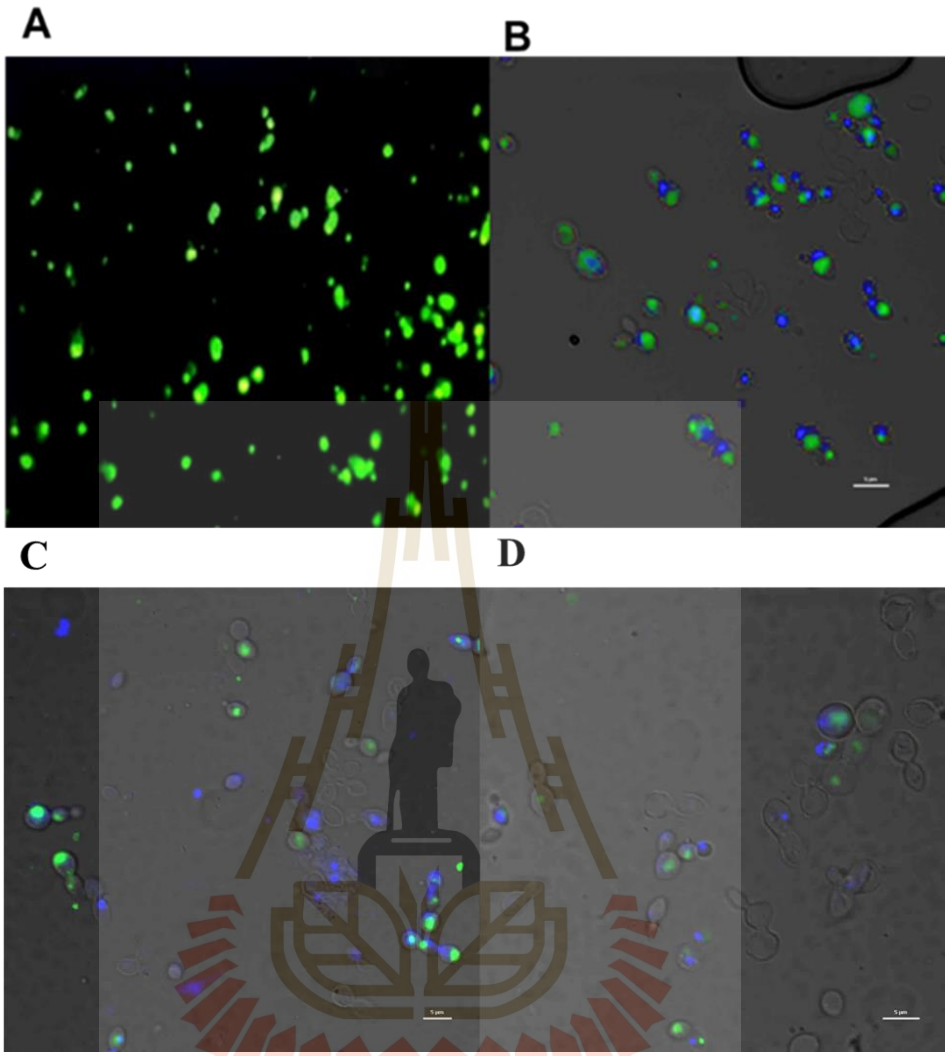


**Figure 4.9** Detection of Os4BGlu11-eGFP in *P. pastoris* cells. **A**, *P. pastoris* cells expressing Os4BGlu11-eGFP viewed under a fluorescence microscope. **B-D**, *P. pastoris* cells expressing Os5BGlu11-eGFP viewed in a confocal microscope after DAPI staining (wavelength 358-461 nm).

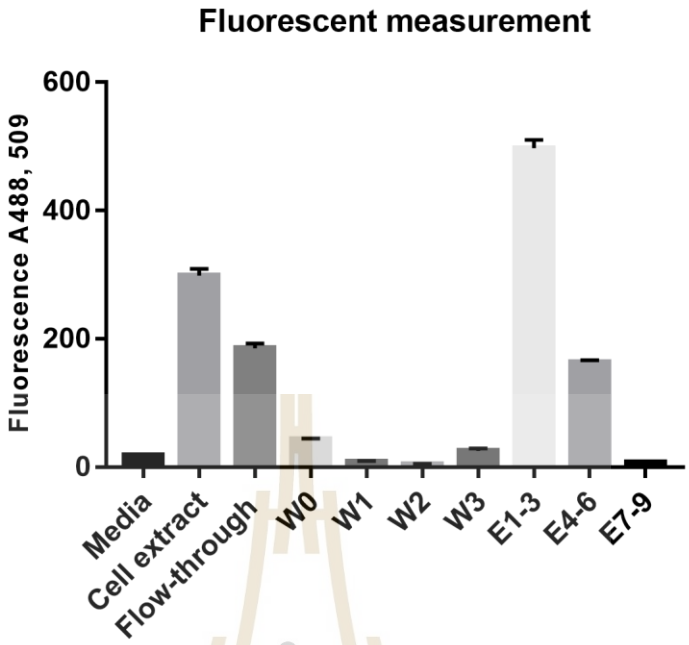


**Figure 4.10** Detection of fluorescent from pPICZαBNH$_8$-Os4BGlu11-eGFP expressed in BMMY with 0.5% methanol induction for 4 days. The fluorescent was measured with 488 nm excitation and 509 mM emission in fluorescence spectrometer. Media: secrets protein after expressed in yeast system 4 days, Cell extract: pellet of pPICZαBNH8/Os4BGlu11-eGFP after breaking with glass beads, Flow-through, Wo: 0 mM of imidazole, W1: 5 mM of imidazole, W2: 10 mM of imidazole, W3: 20 mM of imidazole, E1-E9: eluted 250 mM of imidazole.

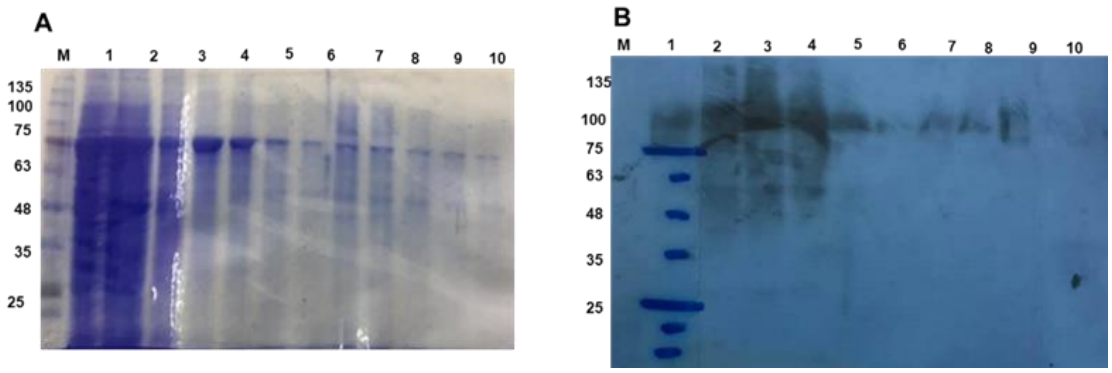


**Figure 4.11** SDS-PAGE and western blot analysis of cell extract of Pichai expressing Os4BGlu11 and fractions from purification by IMAC.

Lane M, Bio-Rad low molecular weight marker Lane 1, pellet of pPICZαBNH8/Os4BGlu11 after breaking with glass beads; Lane 2, flow-through fraction of protein that passed through the IMAC column; Lane 3, fraction of column wash with EQ buffer; Lane 4, fraction of wash with 5 mM imidazole. Lane 5, fraction of wash with 10 mM imidazole; Lane 6, fraction of wash with 20 mM imidazole; Lane 7-10, fractions eluted with 250 mM of imidazole.

## 4.5 Subcellular localization of rice phytohormone β- glucosidases Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13

Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 genes encoding proteins tagged with C-terminal GFP were transiently expressed in tobacco leaf epithelial cells to check their localization, as seen in Figure 4.13. The *N. benthamiana* leaves were co-infiltrated with either Os4BGlu9-GFP, Os4BGlu10-GFP, Os4BGlu11-GFP, Os4BGlu12-GFP or Os4BGlu13-GFP and *Arabidopsis* RFP-Korrigan (Kor. Korrigan), is an *Arabidopsis* endo-1,4-β-D-glucanase with a transmembrane domain and two putative polarized targeting signals in the cytosolic tail. It is found in

the plasma membrane and localizes to the cell plate by polarized targeting, where it is essential for cytokinesis (Zuo et al., 2000). As seen in Figure 4.12, the leaves co-infiltrated with rice BGlu-GFP and RFP-Korrigan exhibited partially overlapping signals. Most of the green signals appeared between the plasma membrane and cell wall, while the red Kor signal is expected to be anchored to the plasma membrane, thereby marking its position. Previously, the Os4BGlu12 cDNA was found to encode a protein that showed the highest similarity to the cell wall associated β-glucosidase purified from rice (Akiyama et al., 1998, Opassiri et al., 2010, Sansenya et al., 2011). This data support that Os4BGlu12 is a cell wall protein, but the sequence was not exactly the same, possibly due to the purified cell wall β-glucosidase containing a mixture of isozymes. The results for localization for Os4BGlu9, Os4BGlu10, Os4BGlu11 and Os4BGlu13 also showed that they are located near the plasma membrane and cell wall. Knowing the localization allows us to consider the natural substrate of Os4BGlu12. ABA is inactivated at the C-1 hydroxyl group by different chemicals which form different conjugates and accumulate in the vacuoles or apoplastic space (Lehmann and Glund, 1986; Dietz et al., 2000). ABA is mostly uncharged when present in the relatively acidic apoplastic compartment of plants, from which it can easily enter cells by diffusing across the plasma membrane (Finkelstein, 2013). From the results presented here, the rice chromosome 4 cluster At/Os7 β-glucosidases are also found in the apoplast, where they can hydrolyze ABA-GE in apoplast, thereby allowing ABA to diffuse into the cell.

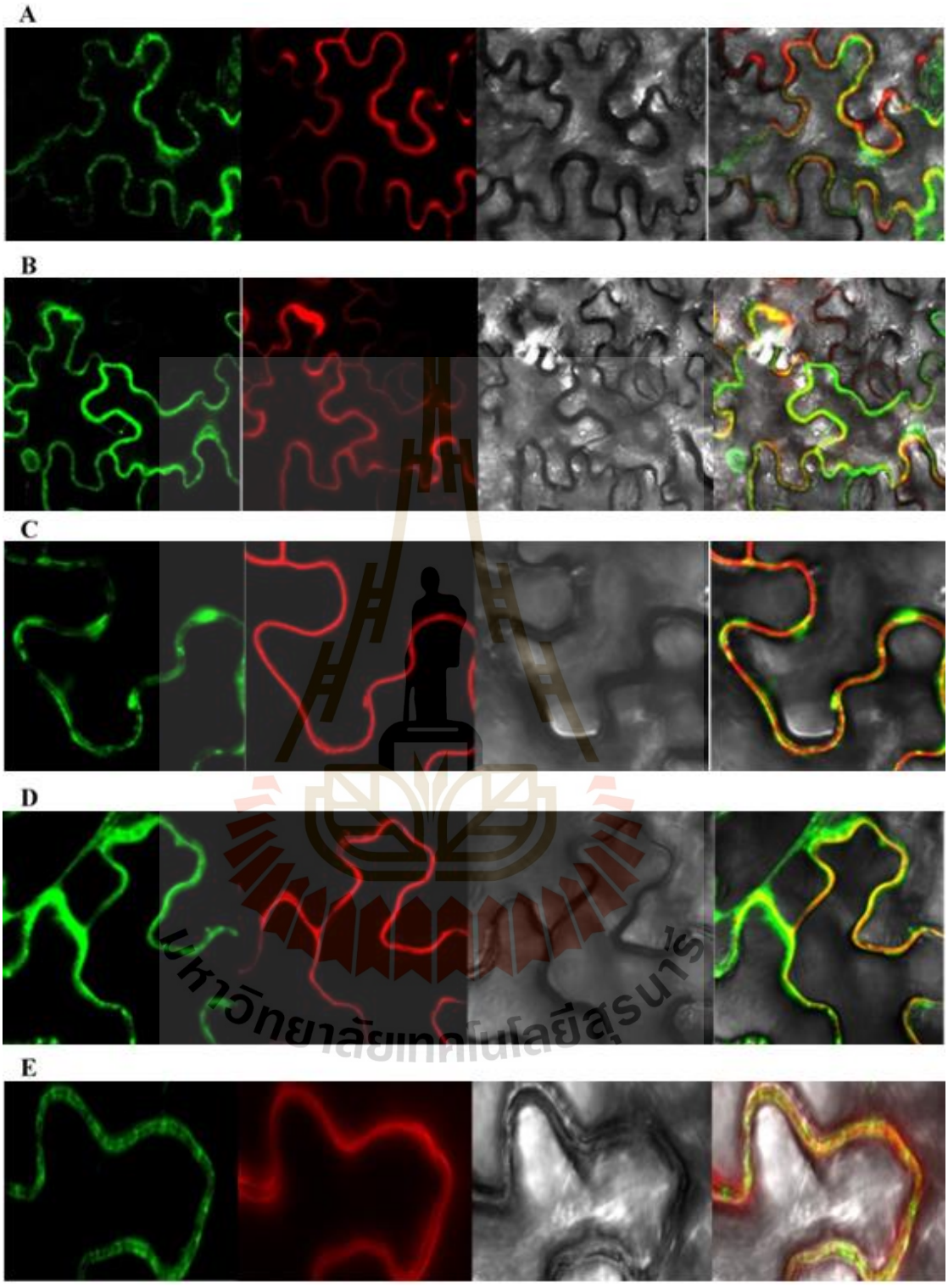


**Figure 4.12** Transient expression of *Os4BGlu9-GFP*, *Os4BGlu10-GFP*, *Os4BGlu11-GFP*, *Os4BGlu2-GFP and Os4BGlu13-GFP* co-infiltrated with *RFP-KOR1* in *N.*

*benthamiana* leaves. The *Arabidopsis* Korrigan1 (AT5G49720) gene was constructed in the *pCaMV35S: RFP-KOR1 expression plasmid* and then separately co-infiltrated into the leaves with *pCaMV35S: Os4BGlu9-GFP* (**A**), *pCaMV35S: Os4BGlu10-GFP* (**B**), *pCaMV35S: Os4BGlu11-GFP* (**C**), *pCaMV35S: Os4BGlu12-GFP* (**D**), and *pCaMV35S: Os4BGlu13-GFP* (**E**). At 3-4 days after infiltration, the epidermal cells were plasmolyzed by infiltrating 0.8 M mannitol. Microscope images were captured at 10 min after the infiltration of leaves with mannitol by confocal scanning microscopy. For each gene, the images are from left to right: GFP fluorescence, RFP fluorescence, light microscopy, and merged images.

## 4.6 Generation of transgenic Arabidopsis plants overexpressing rice phytohormone β-glucosidases.

The pH7FWG2 expression vectors containing the *Os4BGlu9*, *Os4BGlu10*, *Os4BGlu11*, *Os4BGlu12* and *Os4BGlu13* cDNAs under control of the CaMV 35S promoter (Figure 4.13) were transformed into *Arabidopsis thaliana* Col-0 by the floral dip method to obtain putative rice phytohormones β-glucosidase-overexpression lines. The expected putative rice phytohormone β-glucosidase genes were expressed in the overexpression lines, as judged by reverse transcription and PCR (RT-PCR) (Figure 4.14). The positive DNA band was amplified for each specific rice gene in the respective over- expression lines, and no bands for the rice phytohormone β-glucosidase genes were detected in control, nontransgenic rice.

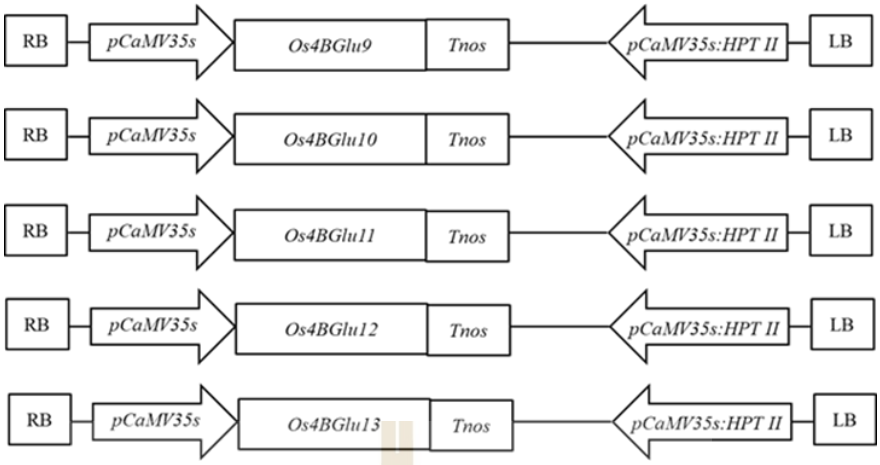


**Figure 4.13** Schematic diagram T-DNA of Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 genes. Cloned full length cDNAs including partial sequence of the untranslated regions (UTR) before start codon and after stop codon were pasted under the control of pCaMV35s promoter. LB and RB, left and right borders of T-DNA; *HPT II, hygromycin phosphotransferase II* gene.

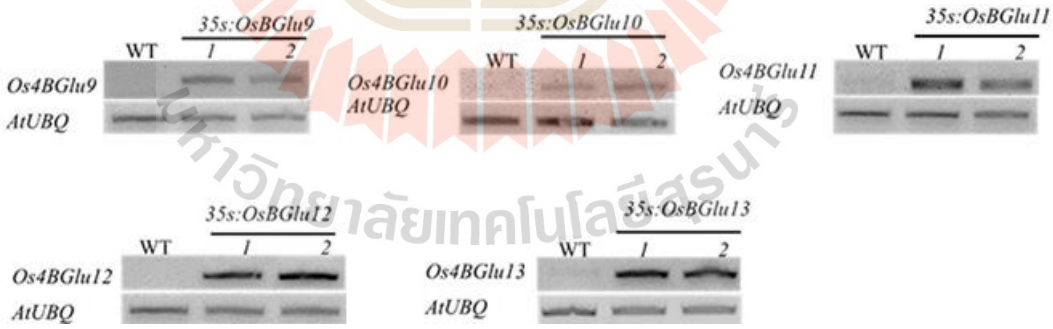


**Figure 4.14** RT-PCR analysis of rice β-glucosidases gene expression in *Arabidopsis thaliana* cultivar Columbia. Two independent lines of transgenic Arabidopsis over-expressing the designated rice phytohormones β-glucosidase were analyzed for each gene: Os4BGlu9 (A), Os4BGlu10 (B), Os4BGlu11 (C), Os4BGlu12 (D) and Os4BGlu13 (E), respectively.

## 4.7 *In vitro* hydrolysis of *p*NPGlc, ABA-GE and $GA_4$-GE by extracts of Arabidopsis overexpressing rice phytohormone β-glucosidases

The extracts of the Arabidopsis that overexpressed Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 had significantly higher hydrolysis activity with *p*NPGlc than the wild type plant extracts (Table 4.2). Among these extracts of plant overexpressing Os4BGlu11 showed the highest hydrolysis activity with *p*NPGlc, around two-fold that of wild type, while in other lines the total hydrolysis activity ranged from high to low following Os4BGlu12>Os4BGlu10>Os4BGlu13> Os4BGlu9 (Table 4.2). Hydrolysis of the phytohormone glucose esters ABA-GE and $GA_4$-GE was also evaluated. Although the line overexpressing Os4BGlu11 had the highest activity toward *p*NPGlc, the extract of the line overexpressing Os4BGlu12 had the highest hydrolysis activities for both ABA-GE and $GA_4$-GE. The activities of the Os4BGlu12 extract to the two phytohormone glucose esters were around 18-fold for ABA-GE and 13.5-fold for $GA_4$-GE that of the control Arabidopsis plant extract. Hydrolysis of the other BGlu overexpression extracts to the two phytohormone glucose esters could ranked in the order Os4BGlu13>Os4BGlu10>Os4BGlu11>Os4BGlu9 >wild type. The fact that fold increase in activities toward ABA-GE and $GA_4$-GE were higher than toward *p*NPGlc with our extracts, relative to control plants, may indicate that these substrates are natural substrates for the expressed enzymes, since endogenous Arabidopsis β-glucosidases that do not hydrolyze these well may be responsible for the background activity toward *p*NPGlc.

**Table 4.2** Hydrolysis activity of Arabidopsis over expressing rice β-glucosidases extract with *p*NPGlc, ABA-GE and $GA_4$-GE.

| substrate | Activity (µmol/mg/hr.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | control | Os4 BGlu9 | Os4 BGlu10 | Os4 BGlu11 | Os4 BGlu12 | Os4 BGlu13 | negative |
| *p*NPGlc | 51.4±1.9 | 60.7±8.6 | 86.8±3.5 | 111.4±6.4 | 101± 1.7 | 76.7±2.2 | 26±5.2 |
| ABA-GE | 55.0±1.6 | 372 ± 21 | 447 ± 30 | 403 ± 26 | 502±14 | 458 ±8.1 | 32.2±2 |
| $GA_4$-GE | 38.4±0.9 | 251 ±6.8 | 352±11 | 306 ±5.3 | 366±15 | 342±26 | 32±0.6 |

1 mM substrates were assayed for hydrolysis. The standard deviations were calculated based on three replications.

* Negative= reaction without enzyme

## 4.8 Arabidopsis overexpressing rice phytohormones β- glucosidase promoting root and shoot elongation

To compare the response to ABA and $GA_4$ of Arabidopsis overexpressing rice phytohormone β-glucosidases to that of wild type plants, the plants were grown on ½ MS plates for 7 days, then moved to plates supplemented with 0.01 µM ABA, 0.01 µM ABA-GE, 0.05 µM $GA_4$ or 0.05 $GA_4$-GE. After 5 days, root and shoot lengths were measured. The average control root length without hormone was 1.85 cm and the average control shoot length was 1.39 cm and the transgenic plants showed no significant differences from these values at p-value 0.05, as shown in Figure 4.15. In contrast, the root and shoot lengths of the plants treated with ABA showed highly significant differences between overexpression lines and control plants, which showed the shortest lengths of roots and shoots, as shown in Figure 4.16. It is worth noting that the control Arabidopsis root length did not change significantly, while the transgenic

lines roots became longer. In contrast, the average control shoot length decreased from 1.36 cm to 1.14 cm, while the shoot lengths actually increased in the transgenic β-glucosidase-overexpressing lines. The inhibition of the shoot growth by ABA as seen in the control line is the expected (Tardieu, 2010). It was surprising that the transgenic lines showed longer lengths upon treatment with ABA than with no treatment.

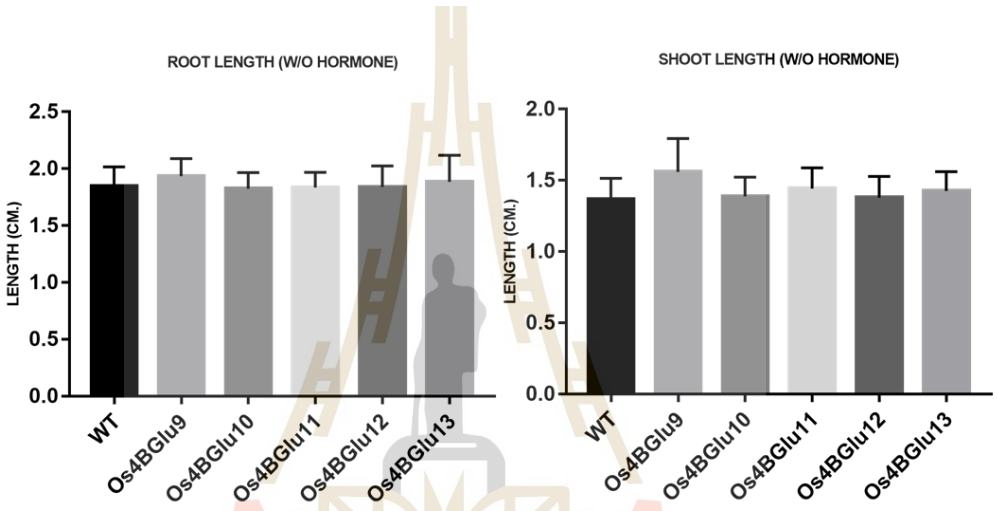


**Figure 4.15** Root and shoot lengths of control and rice β-glucosidase-overexpressing Arabidopsis seedlings. Plants overexpressing Os4BGlu9-13 were grown on ½ MS plates for 7 days and transplanted to ½ MS for 5 days. To quantify root and shoot growth inhibition, root and shoot lengths were measured in three independent experiments with 20 plants each.

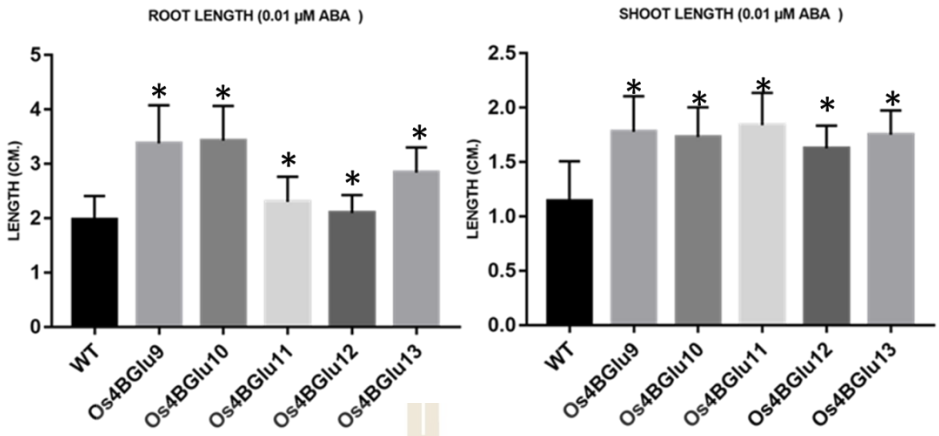


**Figure 4.16** Effect of ABA on control and rice β-glucosidase-overexpressing Arabidopsis seedling roots and shoots. Control plants and plants overexpressing Os4BGlu9-13 were grown on ½ MS plates for 7 days, transplanted to ½ MS containing 0.01 µM ABA, and grown for 5 days. To quantify root and shoot growth inhibition, root and shoot lengths were measure in three independent experiments with 20 plants each.

In another condition, Arabidopsis that over expressed the rice β-glucosidases were supplemented with 0.01 µM ABA- GE, and the root and shoot lengths were compared, as shown in Figure 4.17. In this condition, root length of the Os4BGlu13 overexpression line was shorter than the control line and the other BGlu-overexpression lines. This correlates with *in vitro* result that Os4BGlu13 has the highest hydrolysis activity with ABA- GE among β-glucosidases tested, although we could not test Os4BGlu9, Os4BGlu10, and Os4BGlu11 with recombinantly expressed enzymes. Several studies have found ABA synthesized in root tissue is released to xylem vessels and transported to shoots (Kuromori et al., 2018). Moreover, several studies have shown that ABA accumulates at much higher concentrations in leaves than in roots during

water deficiency and that ABA accumulation in roots is sometimes dependent on basipetal ABA transport from aerial organs (Qin and Zeevaart., 1999). Based on the localization of Os4BGlu13 between the cell wall and plasma membrane, it may hydrolyze ABA-GE in the apoplast to release free ABA from root to shoot, which inhibits shoot growth of plants overexpressing Os4BGlu13, as shown in Figure 4.18. These results suggest that exogenously applied ABA-GE may be absorbed by roots and hydrolyzed by ABA-β-D-glucosidase and liberated free ABA may inhibit growth in Arabidopsis shoots (Noguchi and Tanaka, 2008).

Arabidopsis over expressing rice β-glucosidases were also treated with $GA_4$ and its glucose ester (0.05 µM), with the results illustrated in Figures 4.18 and 4.19. As described before, seed germination, stem elongation, meristmetic tissue development and differentiation of floral organs are highly dependent on GA signaling (Achard and Genschik, 2009). The plants supplemented with $GA_4$ had longer root and shoot lengths than those germinated without hormone. On the other hand, plants supplemented with $GA_4$-GE had shorter root and shoot length than those supplemented with $GA_4$ and similar to those with no supplementation. In the treatment with $GA_4$-GE, Os4BGlu12 and Os4BGlu13 overexpression lines exhibited longer shoot lengths than the other overexpression lines and control. The root and shoot lengths are consistent with the previous report that Os4BGlu13 isolated from rice seedlings and recombinant Os4BGlu13 could hydrolyze $GA_4$-GE *in vitro* (Hua et al., 2015).

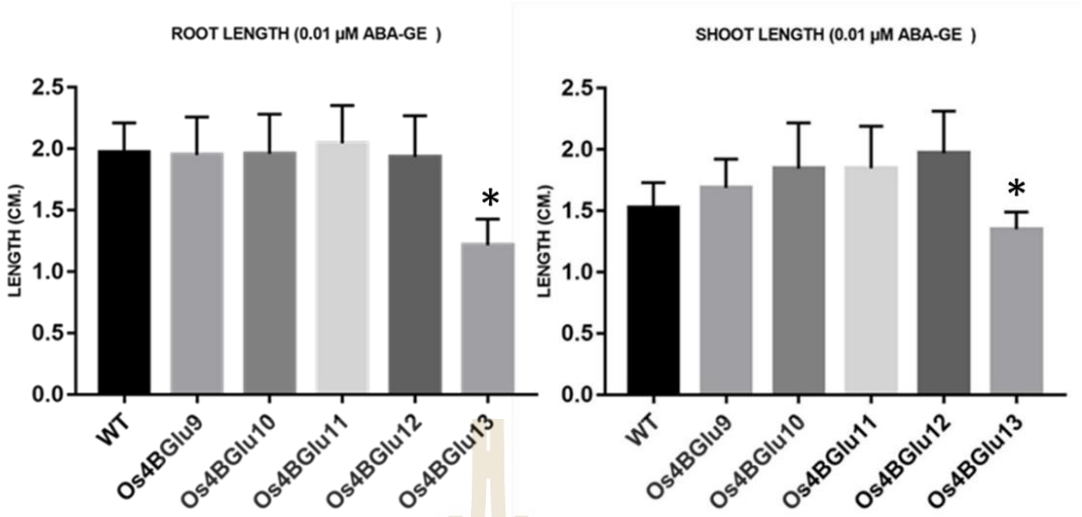


**Figure 4.17** Effect of rice β-glucosidase overexpression on response to ABA-GE. Plants overexpressing Os4BGlu9-13 were grown on ½ MS plates for 7 days and transplanted to ½ MS containing 0.01 µM ABA-GE for 5 days. To quantify root and shoot growth inhibition, root and shoot lengths were measured in three independent experiments with 20 plants each.

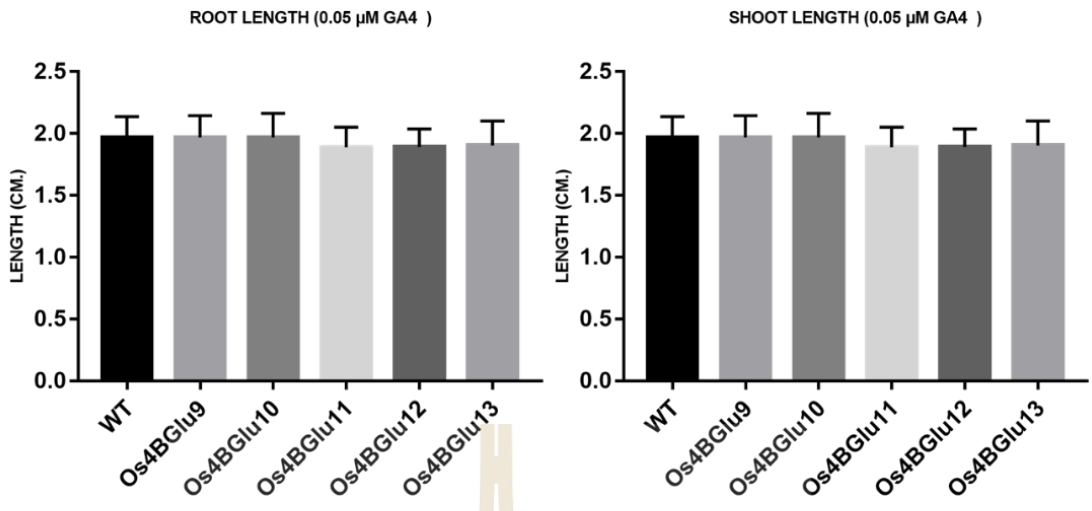


**Figure 4.18** Effect of rice β-glucosidase overexpression on response to $GA_4$. Plants overexpressing Os4BGlu9-13 were grown on ½ MS plates for 7 days and transplanted to ½ MS containing 0.05 µM $GA_4$ for 5 days. To quantify root and shoot growth response, root and shoot lengths were measured in three independent experiments with 20 plants each.

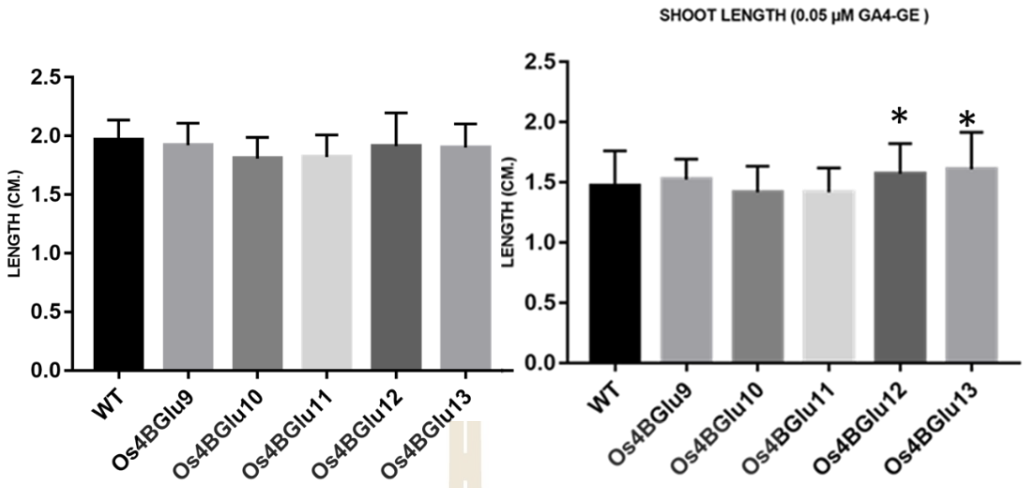


**Figure 4.19** Effect of $GA_4$-GE on wild type and rice β-glucosidase overexpressing Arabidopsis roots and shoots. Plants overexpressing Os4BGlu9-13 were grown on ½ MS plates for 7 days and transplanted to ½ MS containing 0.05 µM $GA_4$-GE for 5 days. To quantify root and shoot growth response, root and shoot lengths were measured in three independent experiments with 20 plants each.

ABA or its metabolites can also be inactivated by conjugation to another molecule; the most common conjugate is the glucosyl ester (ABA-GE). Although this was initially considered a permanent inactivation, more recent studies indicate that ABA-GE is a storage or transport form of ABA. ABA-GE accumulates in vacuoles and the apoplast, but is localized to the endoplasmic reticulum in response to dehydration. In the ER in Arabidopsis, it may be cleaved by the BG1 β-glucosidase (BG1: AT1G52400), which is rapidly activated by dehydration-induced polymerization (Lee et al., 2006). In addition, a second Arabidopsis β-glucosidase isoform (BG2/BGLU33: At2g32860) is present in vacuoles, and appears to be protected from degradation under dehydration stress conditions (Xu et al., 2012). In addition to hydrolysis activity, many

β-glucosidases have high transglycosylation activity (Opassiri et al., 2003, 2004), and related enzymes have recently been found to act as transglucosidases (TGs), which transfer glucose from one glucoconjugate to another with little hydrolysis (Matsuba et al., 2010; Luang et al., 2013). Since in this experiment the plants were grown in ABA, this high phytohormone concentration maybe toxic to the cell. The plant may eliminate the active ABA level by conjugating with it glucose to formed ABA-GE or else convert it to the other compounds and store it inside the cell (i.e. in the vacuole).

The overexpressed rice β-glucosidases may contribute to this metabolism by either hydrolyzing the glucose ester or producing glucose ester from free ABA by transglycosylation. The longer roots and shoots in ABA-treated β-glucosidase overexpressing plants suggests that these β-glucosidases may transglucosylate the ABA, perhaps releasing a growth inducer like GA from its glucoconjugate (GA-GE) in the process. However, only Os4BGlu13 seemed to release ABA from ABA-GE that was supplemented to the plant media, thereby shortening the roots and shoots (Figure 4.16). Measuring the levels of ABA and ABA-GE in the plants would help elucidate what the enzymes are doing more precisely, but unfortunately an LC-MSMS assay that we tried to develop for this purpose was not sensitive enough to measure the levels in the plants.

# CHAPTER V

# CONCLUSIONS

Based on protein sequence similarity Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 are closely related and fall into a rice chromosome 4-specific subclade of the protein-sequence-based phylogenetic cluster At/Os7 (Opassiri et al., 2006). Among these, Os4BGlu12 and Os4BGlu13 are two of the rice β-glucosidases that have been produced by recombinant expression in *Escherichia coli*. Both of these enzymes have been shown to hydrolyze the glucosides of the phytohormones salicylic acid (SA) and tuberonic acid (TA), in addition to cell wall derived oligosaccharides (Opassiri et al., 2010; Wakuta et al., 2010, 2011; Himeno et al., 2013). Os4BGlu13 was also isolated an enzyme with high activity toward $GA_4$-GE (Hua et al., 2015).

Although two β-glucosidase homologues have been identified to act on ABA-GE in Arabidopsis (Lee et al., 2006; Xu et al., 2011), no clear orthologues of these enzymes have been identified in rice. Several rice β-glucosidases that have been successfully expressed in *Escherichia coli*, including Os1BGlu4, Os3BGlu7, Os4BGlu12, Os4BGlu13, Os4BGlu18, Os7BGlu26 and Os9BGlu31 (Opassiri et al., 2003, 2006; Kuntothom et al., 2009; Luang et al., 2013; Rouyi et al., 2014; Baiya et al., 2015), were screen for release of glucose from ABA-GE. Os4BGlu13 exhibited highest hydrolysis activity with ABA-GE followed by Os4BGlu12. These results supplement the previous investigations showing Os4BGlu12 and Os4BGlu13 hydrolysis activity

toward phytohormones glucoconjugates. This data supports a role for rice Cluster At/Os7 enzymes as phytohormone β- glucosidase, particularly those in the closely related subclade of genes on rice chromosome 4 (Os4BGlu9-13).

Kinetic parameters were determined for hydrolysis of ABA-GE by Os4BGlu12 and Os4BGlu13. Os4BGlu13 had higher catalytic efficiency ($K_M$=1.66, $k_{cat}$ = 20.6 $s^{-1}$, $k_{cat}/K_M$ = 12.4 $mM^{-1}s^{-1}$) with ABA- GE than Os4BGlu12 ($K_M$=10.88, $k_{cat}$10.50 $s^{-1}$, $k_{cat}/K_M$ = 0.689 $mM^{-1}s^{-1}$). This largely reflects the 6- fold lower $K_M$ of Os4BGlu13 compared to Os4BGlu12, although the Os9BGlu31 $k_{cat}$ is also nearly 2-fold higher.

To characterize the closely related genes, attempts were made to express these enzymes in microorganisms. Rice cDNA were used to amplify the genes encoding the mature proteins of Os4BGlu9 and Os4BGlu11, which were then cloned into the pET32a expression vector to express N-terminally thioredoxin and $His_6$-tagged Os4BGlu9 and Os4BGlu11 fusion proteins. Unfortunately, the Os4BGlu9 and Os4BGlu11 proteins could not be expressed in *E. coli* as soluble, active β-glucosidases.

A synthetic gene optimized for Os4BGlu11 expression in *P. pastoris* was inserted in the recombinant pPICZαB(NH8)/Os4BGlu11 plasmid, which was used to express Os4BGlu11 as a secreted protein in *P. pastoris* strain SMD1168H at 20 °C for 4 days in methanol-supplemented medium. Os4BGlu11 enzyme apparently could not be efficiently secreted. The pPICZαB(NH8)/Os4BGlu11 was tagged with GFP to make the pPICZαB(NH8)/Os4BGlu11-GFP vector, which was used to express the Os4BGlu11-eGFP fusion protein in *P. Pastoris*, as described above. The pellet of the expression cells was observed to have green fluorescence, suggesting that the protein was located inside the cells. Inspection of the individual cells was consistent with the localization of the protein in the vacuole.

Os4BGlu12 was anticipated to be a cell wall β-glucosidase but the experiment evidence was unclear. Among the proteins encoded in the rice genome, the sequence of Os4BGlu12 was the most similar to that of a cell-wall β-glucosidase determined by protein sequencing by Akiyama et al. (1998). This study clarified the localization by making plant expression plasmids encoding chimeric proteins of Os4BGlu9-GFP, Os4BGlu10-GFP, Os4BGlu11-GFP, Os4BGlu12-GFP and Os4BGlu13-GFP and RFP-KOR1, introducing these in Agrobacterium, which was put by co-infiltration of *N. benthamiana* leaves. The fluorescence observed in confocal microscopy support the localization of Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu12 and Os4BGlu13 in the apoplast between the cell wall and plasma membrane.

Another set of plant expression vectors were produced for overexpression of the At/ Os7 cluster rice enzymes without a GFP-tag in Arabidopsis. Arabidopsis lines overexpressing rice At/Os7 cluster β-glucosidases were generate by flora dip transformation. The $T_0$ seeds were screened for hygromycin resistance. The T1 seedlings were checked for the expression of the rice β-glucosidases in Arabidopsis by RT-PCR. The Arabidopsis overexpressing the rice β-glucosidases were extracted for total protein and the extracts of these plants were found to have higher hydrolysis activity toward ABA-GE, $GA_4$-GE and pNPGlc than wild type plant extract.

Arabidopsis overexpressing rice β-glucosidases were germinated in ½ MS for 7 days seedling were moved to ½ MS containing 0.01 µM ABA or ABA-GE and 0.05 µM $GA_4$ or $GA_4$-GE or no additives. The lines overexpressing Os4BGlu9-13 had longer root and shoot lengths than wild type when grown in ABA-containing medium, but only Os4BGlu9 while the shoot and root lengths of these lines were similar to wild type Arabidopsis when grown in ½ MS without added phytohormones. At first, we had

expected the overexpressing plants to have shorter shoots and stems due to release of ABA from ABA-GE, when it had been conjugated to glucose by endogenous glucosyltransferases. The opposite effect suggests that when the ABA in the media is absorbed via root hairs into the transgenic Arabidopsis plants, the over-expressed rice At/Os7 genes may help neutralize ABA by of transglucosylation with glucose to ABA-GE. They may even transfer the glucose to ABA from another phytohormone glucosyl conjugate, such as GBA-GE, to produce active growth inducer, since the shoot and root lengths were actually longer than untreated plants for the β-glucosidase-overexpressing lines, while for wild type shoots were shorter. In the ABA-GE treatment, Os4BGlu13 exhibited root and shoot lengths shorter than wild type, while the wild type and other transgenic lines had lengths similar to untreated plants. This result is consistent with the high activity of Os4BGlu13 expressed in *E. coli* to ABA-GE. Although the wild-type and β-glucosidase-overexpressing lines had similar growth in media supplemented with $GA_4$, with $GA_4$-GE treatment, Os4BGlu12 and Os4BGlu13 exhibited shoot lengths longer than wild type, which suggested that Os4BGlu12 and Os4BGlu13 could hydrolyze $GA_4$-GE *in planta.*

In summary, rice GH1 cluster At/Os7 members Os4BGlu9, Os4BGlu10, Os4BGlu11, Os4BGlu 12 and Os4BGlu13 are β-glucosidase located extracellularly around the cell wall. Based on their activities in plant extracts, these enzymes may hydrolyze ABA-GE and $GA_4$-GE in apoplast to release free phytohormones that may enter into the cell, but their effect on ABA-treated plants suggests that they may also transglycosylate and thereby inactivate the free phytohormones, which may then be kept in the apoplast or vacuolar storage compartment.

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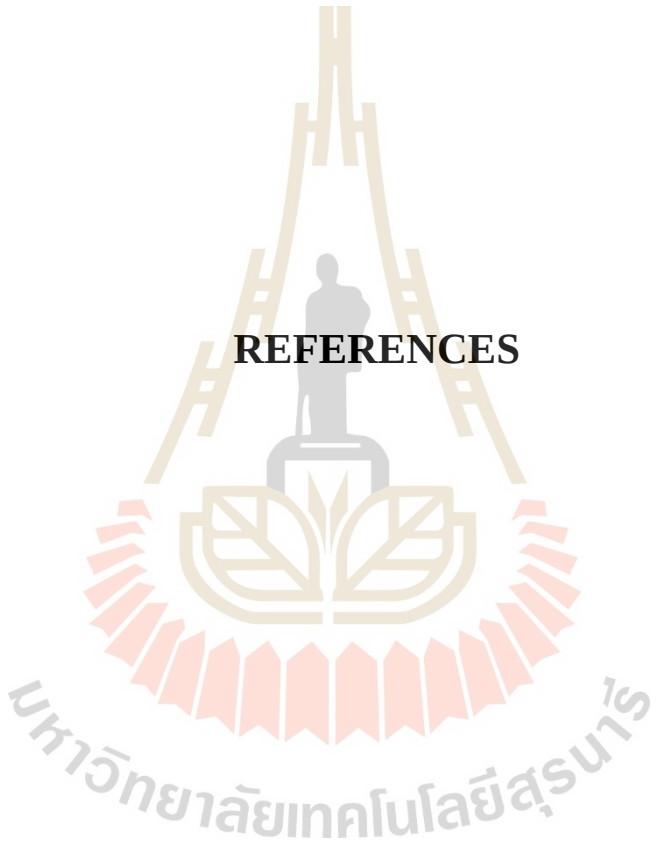

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