รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาหน้าที่ของเอนไซม์บีตากลูโคซิเดสของข้าว ไอโซไซม์ Os4BGlu12 ในการย่อยผนังเซลล์

โดย ผศ. ดร. รจนา โอภาสศิริ และคณะ

สิงหาคม 2553

สัญญาเลขที่ MRG5280249

รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาหน้าที่ของเอนไซม์บีตากลูโคซิเดสของข้าว ไอโซไซม์ Os4BGlu12 ในการย่อยผนังเซลล์

คณะ ผู้วิจัย	สังกัด
1. ผศ. ดร. รจนา โอภาสศิริ	สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี
2. รศ. ดร. เจมส์ เกตุทัต-คาร์นส์	สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยเรื่องการศึกษาหน้าที่ของเอนไซม์บีตากลูโคซิเดสของข้าวไอโซไซม์ Os4BGlu12 ในการย่อยผนังเซลล์ ได้รับการสนับสนุนโดยทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ ประจำปี พ. ศ. 2552 จากสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย ผู้วิจัยจึงขอขอบคุณทั้งสองหน่วยงานที่ได้มอบทุนอุดหนุนการวิจัยในครั้งนี้ ขอขอบคุณมหาวิทยาลัยเทคโนโลยีสุรนารีซึ่งเป็นหน่วยงานต้นสังกัด ที่ให้การสนับสนุนด้านสถานที่ อุปกรณ์ เครื่องมือ ในห้องปฏิบัติการชีวเคมีที่จำเป็นในการทำวิจัย จนทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี

ผู้วิจัย

บทคัดย่อ

รหัสโครงการ : MRG5280249

ชื่อโครงการ : การศึกษาหน้าที่ของเอนไซม์บีตากลูโคซิเดสของข้าวไอโซไซม์ Os4BGlu12 ในการย่อยผนังเซลล์

ชื่อนักวิจัย : ผศ. ดร. รจนา โอภาสศิริ มหาวิทยาลัยเทคโนโลยีสุรนารี

E-mail Address : opassiri@hotmail.com

ระยะเวลาโครงการ : 16 มีนาคม 2552 – 15 สิงหาคม 2553

เอนไซม์บีตากลูโคซิเดสของข้าวไอโซไซม์ Os4BGlu12 ถูกจัดไว้ในไกลโคซิลไฮโดรเลสกลุ่มที่ 1 ได้มีการเพิ่มจำนวนดีเอ็นเอที่บรรจุรหัสพันธุกรรมของเอนไซม์นี้จากต้นอ่อนข้าว โปรตีนสายผสมของ Os4BGlu12 ถูกผลิตขึ้นในสภาพที่ทำงานได้ใน *Escherichia coli* และเอนไซม์สามารถย่อยโอลิโกแซคคาไรด์ที่มีพันธะแบบบีต้าได้ดี (Opassiri et al., 2006) เพื่อทราบถึงหน้าที่ของเอนไซม์นี้มากยิ่งขึ้น โดยเฉพาะบทบาทในการย่อยผนังเซลล์ โครงการนี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างการแสดงออกและการทำงานของเอนไซม์บีตากลูโคซิเดสไอโซไซม์ Os4BGlu12 กับเอนโดกลูคาเนสของข้าว จากการศึกษาด้วยวิธี Northern analysis พบว่า mRNA ของยีน *Os4bglu12* เพิ่มขึ้นอย่างมากในต้นอ่อนข้าวที่มีบาดแผลและที่ได้รับฮอร์โมนเมทิลจัสโมเนทและอีเทฟอน ตรวจพบโปรตีนสายผสมของโปรตีนติดตามชนิด GUS (กลูคิวโรนิเดส) ที่เชื่อมกับปลายคาร์บอกซีของ Os4BGlu12 ตรงช่องว่างระหว่างเซลล์หรือผนังเซลล์ของเซลล์หัวหอม (*Allium cepa*) โปรตีนสายผสมของ Os4BGlu12 ที่ผลิตได้ใน *E. coli* สามารถย่อยกลูโคโอลิโกแซคคาไรด์ที่มีพันธะ β-(1,3;1,4) ที่ได้จากการย่อยผนังเซลล์ด้วยเอนไซม์เอนโด-1,3;1,4-บีตากลูคาเนสไอโซไซม์ OsEGL1 ของข้าว ซึ่งเป็นเอนไซม์ที่มีการแสดงออกเพิ่มขึ้นเมื่อข้าวเกิดบาดแผล จึงเป็นไปได้ว่าเอนไซม์ทั้งสองอาจทำงานร่วมกันในการปรับเปลี่ยนโครงสร้างผนังเซลล์ ในการทดสอบเอนไซม์กับโอลิโกแซคคาไรด์ชนิดต่างๆ พบว่า Os4BGlu12 ย่อยกลูโคโอลิโกแซคคาไรด์ที่มีจำนวนกลูโคสต่อกัน 3-6 หน่วย ด้วยพันธะ β-(1,4) ได้ดีที่สุด เอนไซม์ย่อยไดแซคคาไรด์ของกลูโคส ที่มีพันธะ β-(1,3) ได้ดีเช่นกัน ในการทดสอบเอนไซม์กับไกลโคไซด์ที่พบในธรรมชาติพบว่า Os4BGlu12 ย่อยดีออกซีคอร์ติโคสเตอโรนกลูโคไซด์และเอพิเจนินกลูโคไซด์ได้ดีมาก จากการวิเคราะห์ชนิดกรดอะมิโนที่น่าจะอยู่ตรงบริเวณเร่งปฏิกิริยาของ Os4BGlu12 พบว่ากรดอะมิโนเหล่านี้เป็นชนิดที่คล้ายคลึงกับของเอนไซม์บีตากลูโคซิเดสกลุ่มที่ย่อยไซยาโนเจนิกกลูโคไซด์ และฟลาโวนอยด์กลูโคไซด์มากกว่ากลุ่มที่ย่อยโอลิโกแซคคาไรด์ เอนไซม์จึงอาจจะทำหน้าที่ทั้งในระบบป้องกันเซลล์ และการย่อยโอลิโกแซคคาไรด์ที่ได้จากการสลายผนังเซลล์

คำหลัก : บีตากลูโคซิเดส เอนโด-(1,3;1,4)-บีตากลูคาเนส ไกลโคไซด์ ข้าว ผนังเซลล์ บาดแผล

Abstract

Project Code : MRG5280249

Project Title : Functional characterization of rice Os4BGlu12 β-glucosidase in cell wall hydrolysis

Investigator : Asst. Prof. Dr. Rodjana Opassiri, Suranaree University of Technology

E-mail Address : opassiri@hotmail.com

Project Period : 16 March 2009 – 15 August 2010

Rice Os4BGlu12 β-glucosidase is a family 1 glycosyl hydrolase, the full-length cDNA of which was cloned from rice seedlings. The recombinant Os4BGlu12 β-glucosidase was functional expressed in *Escherichia coli* and it efficiently hydrolyzed β-linked oligosaccharides (Opassiri et al., 2006). In order to thoroughly study the biological roles of this enzyme, especially cell wall hydrolysis, this project aims to study the relationships between expression patterns and activity of rice Os4BGlu12 β-glucosidase and endoglucanase. Northern analysis revealed that mRNA transcripts of *Os4bglu12* gene significantly increased when rice seedlings were exposed to wounding, methyl jasmonate and ethephon. A GUS (glucuronidase) reporter tagged at the C-terminus of Os4BGlu12 was found to be secreted to the apoplast or cell wall when expressed in onion (*Allium cepa*) cells. Recombinant Os4BGlu12 produced in *E. coli* hydrolyzed the cell-wall derived β-(1,3;1,4)-glucooligosaccharides generated by the wounding-induced rice endo-(1,3;1,4)-β-glucanase OsEGL1, suggesting that both enzymes may act in concert in remodeling of damaged cell wall. Among oligosaccahrides tested, Os4BGlu12 hydrolyzed β-(1,4)-linked glucooligosaccharides with highest catalytic efficiency when the degree of polymerization ranged from 3 to 6. It also hydrolyzed the β-(1,3)-linked disaccharide of glucose with high catalytic efficiency. Among the natural glycosides tested, Os4BGlu12 efficiently hydrolyzed deoxycorticosterone 21-glucoside and apigenin 7-O-β-D-glucoside. The amino acid residues predicted to line the active site of Os4BGlu12 are more similar to those of cyanogenic and flavonoid β-glucosidases than oligosaccharide hydrolases, and it may function in defense, as well as in cell wall-derived oligosaccharide break-down.

Keywords: β-glucosidase, endo-(1,3;1,4)-β-glucanase, glycosides, rice, cell wall, wound

1. บทนำ (Introduction)

ผนังเซลล์พืชทุกชนิดประกอบขึ้นมาจากโพลีแซคคาไรด์หลายชนิด ซึ่งมักจะประกอบด้วย cellulose ประมาณ 15-40% hemicellulose และ pectin ประมาณ 30-40% และ lignin ประมาณ 20% (Doy and Kosugi, 2004) cellulose ทำหน้าที่เป็นโมเลกุลโครงสร้าง hemicellulose และ pectin ทำหน้าที่เป็นโครงข่ายเชื่อมโยง cellulose เข้าด้วยกัน พืชต่างชนิดกันหรือเนื้อเยื่อต่างๆ ของพืชชนิดเดียวกันมีส่วนประกอบและการจัดเรียงตัวของโพลิเมอร์ที่ผนังเซลล์แตกต่างกัน ปริมาณ cellulose ที่พบที่ผนังเซลล์มีสัดส่วนคิดเป็น 2 ใน 3 ของปริมาณคาร์โบไฮเดรตทั้งหมดของไม้เนื้อแข็ง และมีมากถึงร้อยละ 50 ของปริมาณคาร์โบไฮเดรตทั้งหมดของไม้ล้มลุก (Lynd, 1996) แหล่งของผนังเซลล์ซึ่งมีอยู่เป็นปริมาณมากที่เหลือทิ้งจากภาคเกษตรกรรม เช่น ฟางข้าว และซังข้าวโพด สามารถนำมาใช้เป็นวัตถุดิบในการแปรรูปเป็นสารผลิตภัณฑ์ของน้ำตาลได้ โครงสร้างพื้นฐานของผนังเซลล์พืชในกลุ่มธัญพืชและพืชตระกูลหญ้าแตกต่างจากพืชมีดอกตรงที่นอกจากมี cellulose แล้วยังมี 1,3-1,4-β-D-glucans ซึ่งเป็นโพลิเมอร์ที่ไม่แตกแขนงที่ประกอบไปด้วย พันธะแบบ β-(1,3) และ β-(1,4) ในโมเลกุล (Carpita and McCann, 2000) ซึ่งจะแทรกตัวและเชื่อมโยงมัด cellulose เข้าไว้ด้วยกัน ซึ่งช่วยให้ผนังเซลล์มีความแข็งแรง (Carpita, 1996)

เอนไซม์ที่เกี่ยวข้องกับการย่อยผนังเซลล์เรียกรวมกันว่า glycanohydrolases ซึ่งเป็นเอนไซม์ที่ถูกจัดไว้ใน glycosyl hydrolases family (GH) แบ่ง glycanohydrolases ได้ 3 ประเภท ได้แก่ endoglycanase, exo-glycanases และ β-glycosidases เอนไซม์ endoglycanases ย่อยพันธะไกลโคซิดิกของโพลีแซคคาไรด์ของผนังเซลล์จนได้โอลิโกแซคคาไรด์สายสั้นลง การที่เอนไซม์นี้จะย่อยสับสเตรทได้สับสเตรทจะต้องมีจำนวนกลูโคสอย่างน้อยสี่หน่วย (Hatfield and Nevins, 1987) เอนไซม์ exoglycanase ย่อยโอลิโกแซคคาไรด์ได้ทั้งจากปลายด้าน reducing หรือ non-reducing (Huber and Nevins, 1987) ส่วน β-glucosidase สามารถย่อยโอลิโกแซคคารด์จากปลาย reducing ได้เท่านั้น (Hrmova and Fincher, 2001a; Opassiri et al., 2004) มีการตั้งสมมุติฐานว่าเอนไซม์เหล่านี้ที่พบในพืชวงศ์หญ้าอาจถูกส่งออกไปที่บริเวณผนังเซลล์เพื่อย่อย cellulose และ β-glucans (Taiz, 1984; Buchanan et al., 2000) เอนไซม์ glycanase บางชนิดในพืชยังเข้าไปมีส่วนร่วมในระบบป้องกันเซลล์พืช โดยช่วยย่อยผนังเซลล์ของเชื้อราจนเป็นโอลิโกแซคคาไรด์สายสั้นๆ โอลิโกแซคคาไรด์เหล่านี้อาจทำหน้าที่เป็นตัวกระตุ้นการทำงานของยีนหลายชนิดที่ตอบสนองต่อการบุกรุกของเชื้อในระบบส่งสัญญาณของเซลล์ (Yamaguchi et al., 2002)

Hrmova and Fincher (2001a) เปรียบเทียบโครงสร้างของ glycosyl hydrolases ของพืช และอธิบายความสัมพันธ์ระหว่างโครงสร้างกับความจำเพาะต่อสับสเตรทของเอนไซม์ endohydrolase, exohydrolase และ β-glucosidase พบว่าการที่เอนไซม์ทั้งสามชนิดมีความจำเพาะต่อสับสเตรทแตกต่างกันเพราะมีรูปร่างของบริเวณจับกับสับสเตรท (binding sites) แตกต่างกัน โดยทั่วไป endohydrolases จะมีบริเวณจับกับสับสเตรทเป็นร่องยาวที่อยู่ตรงผิวนอกของโปรตีน (grooves หรือ depressions) ดังนั้น endohydrolase จึงสามารถจับกับโพลิเมอร์ของน้ำตาลแล้วจึงย่อยพันธะที่อยู่

ภายในโพลิเมอร์ด้วยกรดอะมิโนที่เป็นหมู่เร่งที่ฝังตัวอยู่ตรงบริเวณดังกล่าว บริเวณจับกับสับสเตรทของ exohydrolase มีรูปร่างเป็นแบบท่อหรือโพลงที่ลึกเข้าไปจากผิวโปรตีน (dead-end tunnel, slot, funnel) ความจำเพาะต่อสับสเตรทจึงขึ้นกับว่าสับสเตรทมีรูปร่างพอดีกับรูปร่างของบริเวณจับมากพอที่จะยื่นเข้าไปสัมผัสกับกรดอะมิโนหมู่เร่งที่อยู่ก้นของโพลงนั้นได้มากเพียงใด (Hrmova and Fincher, 2001a, 2001b) บริเวณจับของ β-glucosidase มีรูปร่างเป็นช่องแคบและตื้นที่พอจะให้กลูโคสแค่ 2 หน่วยยื่นเข้าไปได้ โดยส่วนที่เหลือจะยื่นออกมาด้านนอก (Hrmova and Fincher, 2001a)

จากข้อมูลลำดับนิวคลีโอไทด์ในจีโนมของพืชหลายชนิด เช่น Arabidopsis, ข้าว, ข้าวบาร์เลย์ และ ข้าวโพด พบว่า glucanohydrolases ที่มีอยู่เป็นจำนวนมากมีความหลากหลายทางด้านโครงสร้างและลำดับกรดอะมิโนเป็นอย่างมาก และมีบทบาทหลากหลาย ได้แก่ การสังเคราะห์ ย่อยสลาย และเปลี่ยนแปลงโครงสร้างของโอลิโกแซคคาไรด์และโพลีแซคคาไรด์ (Hoj and Fincher, 1995; Davies and Henrissat, 2002) ในการศึกษาที่ผ่านมาพบยีนของข้าวที่จัดอยู่ในกลุ่มเอนไซม์กลูคาโนไฮโดรเลสอย่างน้อย 139 ยีน (Opassiri unpublished data) เอนไซม์ endoglucanases พบมากใน glycosyl hydrolase (GH) family 9 และ 17 exoglucanases และ β-glucosidase พบมากที่ GH 1, 3, และ 5 ปัจจุบันยังไม่สามารถศึกษากลไกการสร้างและสลายผนังเซลล์พืชที่เกิดขึ้นจริงๆ ในเซลล์พืชได้เนื่องจากกระบวนนี้มีความซับซ้อนที่ต้องใช้เอนไซม์เข้าร่วมหลายชนิด ปัจจุบันมีการศึกษาถึงบทบาทหน้าที่และความจำเพาะต่อสับสเตรตของเอนไซม์ย่อยผนังเซลล์ข้าวเพียงไม่กี่ไอโซไซม์ เอนไซม์ที่ได้มีการศึกษาส่วนมากจะอยู่ในกลุ่ม GH 1, 3, 17 (Akiyama et al, 2004; Nishizawa et al, 2003; Opassiri et al, 2003; Zheng et al, 2003; Yamaguchi et al, 2002; Thomas et al, 2000; Rakwal et al, 1999)

Endo-β-glucanase ที่พบในพืชถูกจัดไว้ใน GH family 9 ในปัจจุบันมีการศึกษาพบว่าพืชแต่ละชนิดมียีนของ endo-β-glucanase จำนวนหลายยีน เช่นที่พบในมะเขือเทศ ยีนของเอนไซม์แต่ละไอโซไซม์มีการแสดงออกที่แตกต่างกันและแสดงออกมากในผลสุก บริเวณที่กำลังร่วง และที่ hypocotyls ตรงเนื้อเยื่อที่กำลังเจริญหรือโตเต็มที่แล้ว (Brummell *et al.*, 1997; Catalá *et al.*, 1997) Endo-1,4-β-glucanases ของพืชที่พบส่วนใหญ่มี 2 ชนิด ชนิดแรกเป็นเอนไซม์ที่เชื่อมอยู่กับเยื่อหุ้มเซลล์ (integral membrane proteins) ชนิดที่สองเป็นเอนไซม์ที่พบรหัสนำสัญญาณที่แสดงว่าเอนไซม์นี้จะถูกส่งเข้าไปใน endoplasmic reticulum แล้วจึงถูกส่งต่อไปนอกเซลล์ตรงบริเวณช่องว่างระหว่างเซลล์ (Mølhøj et al., 2002) ในทางทฤษฎีเอนไซม์นี้สามารถย่อย cellulose หรือ xyloglucan โดยปกติ endo-1,4-β-glucanases ที่พบในพืชไม่สามารถย่อย cellulose ตรงบริเวณที่มีการจัดเรียงโมเลกุลอยู่กันอย่างเป็นระเบียบ (crystalline cellulose) (Hayashi and Maclachlan 1984) แต่ยังไม่มีหลักฐานยืนยันชัดเจนถึงการย่อยน้ำตาลออกจาก crystalline cellulose (Maclachlan and Carrington 1991) แต่เอนไซม์นี้สามารถย่อย cellulose ตรงบริเวณที่มีการจัดเรียงตัวของโมเลกุลไม่เป็นระเบียบ (amorphous regions) endo-1,4-β-glucanases ของพืชบางชนิดรวมทั้งข้าว มีบริเวณจับกับคาร์โบไฮเดรต (carbohydrate binding domain) คล้ายกับที่พบในแบคทีเรียและราที่สามารถย่อย crystalline cellulose ได้ จากข้อมูลนี้ได้ทำให้คาดกันว่าเอนไซม์ในพืชเองน่าจะสามารถย่อย crystalline cellulose เพื่อหมุนเวียนเอาน้ำตาลกลับมาใช้ใหม่ได้ และยังพบว่า endo-1,4-β-glucanases ได้เข้าไปมีส่วนร่วมในกระบวนการย่อยสลาย

xyloglucan ซึ่งเกิดในอัตราเร็วที่สูงมากในผลสุกและในเนื้อเยื่อส่วนที่กำลังร่วง (Maclachlan and Carrington 1991, del Campillo 1999, del Campillo and Bennett 1996, Lashbrook et al. 1994)

Yoshida and Komae (2006) และ Yoshida et al. (2006) ได้แยก endo-1,4-β-glucanase ไอโซไซม์ OsCel9A ที่พบมากที่ปลายรากข้าว และโคลน cDNA ขนาดเต็มสายของเอนไซม์นี้ และพบบริเวณจับกับคารโบไฮเดรต (carbohydrate-binding domain) 2 ชุด ที่ C-terminus ของเอนไซม์ เอนไซม์นี้สามารถตัด 1,4-β-glycosidic linkages ของ carboxymethyl-cellulose, phosphoric acid-swollen cellulose, (1,3)-(1,4)-β-glucan, arabinoxylan, xylan, glucomannan, cellooligosaccharide ที่มีกลูโคสมากกว่า 3 โมเลกุล และ 1,4-β-xylohexaose ลักษณะการทำงานของเอนไซม์ที่สามารถตัดพันธะในโมเลกุล cellulose และ hemicellulose ที่หลากหลายนี้ แตกต่างจากเอนไซม์ endo-1,4-β-glucanase ในกลุ่ม GH9 ของพืชชนิดอื่นๆ ที่เคยมีการศึกษามา Yoshida et al. (2006) ได้ศึกษาการแสดงออกของยีน endo-1,4-β-glucanase จำนวน 4 ไอโซไซม์ พบว่ายีนเหล่านี้แสดงออกในเนื้อเยื่อและตอบสนองต่อฮอร์โมน auxin แตกต่างกัน และพบว่าในข้าวมีไอโซไซม์ที่มีลำดับกรดอะมิโนคล้าย KORRIGAN endo-1,4-β-glucanase ของ Arabidopsis ซึ่งเป็นไอโซไซม์ชนิดที่จับอยู่กับเยื่อหุ้มเซลล์และมีส่วนสำคัญในการสร้างผนังเซลล์ Zhou et al. (2006) พบว่าการแสดงออกของ OsGLU1 gene เพิ่มขึ้นเมื่อข้าวได้รับฮอร์โมน gibberellins และ brassinosteroids และยังพบว่าในข้าวที่มีการกลายพันธุ์ของ endo-1,4-β-glucanase ไอโซไซม์ OsGLU1 ซึ่งจับอยู่กับเยื่อหุ้มเซลล์มีการยืดขยายเซลล์ตรงจุดเชื่อมระหว่างปล้องลดลง รวมทั้งปริมาณ cellulose ที่ผนังเซลล์ก็ลดลงด้วย แต่กลับมี pectin สะสมเพิ่มขึ้น ผลการทดลองนี้แสดงให้เห็นว่าการขาดเอนไซม์นี้มีผลต่อการองค์ประกอบภายในผนังเซลล์ การที่ผนังเซลล์พืชมีปริมาณ cellulose ลดลงแต่กลับมี pectin สะสมแทนแสดงให้เห็นว่าพืชมีกลไกการควบคุมแบบย้อนกลับที่เชื่อมโยงเมทาโบลิซึมของ pectin และ cellulose (His et al. 2001) นอกจากนี้ยังพบว่า Arabidosis ที่ทำให้ยีน KORRIGAN endo-1,4-β-glucanase เกิดการกลายพันธุ์ มี non-crystalline glucan สะสมมากขึ้น glucan นี้น่าจะเป็นส่วนที่พืชไม่สามารถนำกลับไปใช้สร้าง cellulose ได้ แต่สมมติฐานนี้ไม่สามารถพิสูจน์ได้ง่ายนัก (Mølhøj et al., 2002) และยังพบว่าส่วนที่เกิดการกลายพันธุ์อยู่ตรงบริเวณที่เอนไซม์น่าจะใช้จับกับโปรตีนชนิดอื่น และไม่พบการกลายพันธุ์ที่บริเวณเร่งของเอนไซม์ (Ohmiya et al. 2000)

จากผลการทดลองดังกล่าวทำให้งานวิจัยในปัจจุบันได้ให้ความสนใจในบทบาทของ membrane-anchored endo-β-1,4-glucanases ในกระบวนการสังเคราะห์ cellulose ในพืช มีการคาดกันว่าพืชมีระบบติดตามการเปลี่ยนแปลงที่เกิดขึ้นกับโครงสร้างของผนังเซลล์ตลอดช่วงการเจริญเติบโต และมีระบบส่งสัญญาณภายในเซลล์ที่ตอบสนองต่อการเปลี่ยนแปลงดังกล่าว (Pilling and Hofte, 2003; Somerville et al., 2004) ตัวอย่างเช่น พืชกลายพันธุ์ที่ขาด cellulase synthase ทำให้ยีนที่ตอบสนองต่อสภาวะเครียดซึ่งปกติจะถูกควบคุมการแสดงออกด้วย ฮอร์โมน ethylene และ jasmonate ทำงานเพิ่มขึ้น และส่งผลให้พืชมีความต้านทานต่อเชื้อราดีขึ้น และมี lignin ผลิตสูงขึ้นด้วย (Ellis et al., 2002; Cano-Delgado et al., 2003) และงานวิจัยล่าสุดพบว่าโปรตีนที่ทำหน้าที่รับสัญญาณ คือ membrane-bound receptor kinase ซึ่งทำงานเกี่ยวข้องกับการยืดตัวของ hypocothyl มีการตอบสนองต่อภาวะที่

พืชมีการสังเคราะห์ cellulose ลดลง ซึ่งแสดงให้เห็นว่ามีกลไกเชื่อมโยงการเปลี่ยนแปลงของผนังเซลล์กับระบบการทำงานในเซลล์ (Hematy et al., 2007).

ข้าวมีเอนไซม์ endo-1,4-β-glucanase อยู่มากถึง 22 ไอโซไซม์ อาจเนื่องจากการจัดโครงสร้างผนังเซลล์ข้าวที่ประกอบไปด้วย cellulose, hemicellulose หรือ β-glycans เชื่อมโยงกันอยู่นั้นมีโครงสร้างแตกต่างกันในเนื้อแต่ละบริเวณหรือในแต่ละช่วงของการพัฒนา (Carpita, 1996) และไอโซไซม์เหล่านี้อาจมีบทบาทที่แตกต่างกันไปในแต่ละกระบวนการ เช่น wall loosening, cell elongation, wall synthesis เอนไซม์ที่พบในข้าวอาจผ่านวิวัฒนาการการดัดแปลงโครงรูปให้เหมาะสมกับโครงสร้างของผนังเซลล์ในที่มีความซับซ้อนแตกต่างกัน ถึงแม้ส่วน amorphous cellulose fibrils ถูกย่อยได้ง่าย แต่บริเวณ crystalline cellulose กลับถูกย่อยได้ยากมาก จึงเป็นไปได้ว่าพืชน่าจะใช้เอนไซม์หลายชนิดเข้ามาร่วมกันทำงาน ซึ่งยังไม่พบข้อพิสูจน์ดังกล่าว

มีการคาดกันว่า endoglucanase น่าจะทำงานร่วมกับ β-glucosidase ในการย่อย cellulose หรือ β-glucans ชนิดต่างๆ ของผนังเซลล์ (Akiyama et al.,2004) เพื่อนำโอลิโกแซคคาไรด์ หรือน้ำตาลโมเลกุลเดี่ยวที่ย่อยได้หมุนเวียนกลับไปสังเคราะห์ cellulose, hemicellulose หรือ pectin ในช่วงที่มีพืชมีการเปลี่ยนแปลงโครงสร้างของผนังเซลล์ เช่น ในช่วงที่เกิดการแบ่งเซลล์หรือการยืดขยายของเซลล์ (Carpita, 1996) ในงานวิจัยก่อนหน้านี้ผู้วิจัยได้ศึกษา Glycosyl hydrolase family 1 (GH1) β-glucosidase ไอโซไซม์ Os4bglu12 พบว่าเอนไซม์มีความจำเพาะในการย่อย β-1,3-linked disaacharide และ β-1,4-glucooligosaccharides ซึ่งมีลักษณะการทำงานคล้ายกับ exogucanase (Opassiri et al., 2006) จากการทดลองนี้ทำให้คาดว่าเอนไซด์นี้น่าจะมีบทบาทสำคัญในการย่อยสลายผนังเซลล์ คือเอนไซม์สามารถโอลิโกแซคคาไรด์หรือไดแซคคาไรด์ที่เกิดจากการย่อยผนังเซลล์ด้วย endoglucanase มาย่อยต่อจนกระทั่งได้น้ำตาลโมเลกุลเดี่ยวเพื่อนำน้ำตาลหมุนเวียนกลับไปสังเคราะห์ผนังเซลล์ใหม่ อย่างไรก็ตามยังไม่มีงานวิจัยที่มีหลักฐานยืนยันถึงการทำงานของร่วมกันของ endoglucanase กับ β-glucosidase ดังนั้นการศึกษาการทำงานและการแสดงออกของเอนไซม์ย่อยผนังเซลล์ข้าวอย่างเป็นระบบจึงมีความจำเป็นที่จะช่วยให้เกิดความเข้าใจในเมทาโบลิซึมที่เกิดขึ้นที่ผนังเซลล์

วัตถุประสงค์ของโครงการ (Objectives)

เพื่อให้เกิดความเข้าใจในความสัมพันธ์ของการทำงานของเอนไซม์ที่เกี่ยวข้องในการย่อยผนังเซลล์ข้าว โครงการวิจัยนี้จึงได้ศึกษา รูปแบบการแสดงออกของยีนของเอนไซม์ Os4BGlu12 β-glucosidase ของข้าว การทำงานร่วมกันระหว่าง endoglucanase และ Os4BGlu12 β-glucosidase ของข้าวในการย่อยผนังเซลล์ข้าว และศึกษาจลนศาสตร์ในการย่อยผนังเซลล์ และการย่อยสับสเตรทต่างๆ และศึกษาบริเวณที่พบ Os4BGlu12 β-glucosidase ของข้าวภายในเซลล์

2. วิธีการทดลอง (Materials and Methods)

2.1 Determination of gene expression patterns in rice by northern analysis

Rice (*Oryza sativa* L. cv. Yukihikari) seeds were germinated in the dark for 4 days at 28°C and then grown in a 12 h light-12 h dark cycle from day 4 to day 10 at 28°C and moistened with sterile distilled water. Ten-day-old rice seedlings were exposed to environmental stresses for an additional 2 days, including cold stress at 5°C, heat stress at 37°C, drought stress (grown without irrigation up to approximately 50% decrease in fresh weight), salt stress (0.3 M NaCl), flooding stress (fully submerged in distilled water), high osmotic stress (0.5 M mannitol), and mechanical wounding (aerial parts, such as leaves and stems were crushed with a plastic ruler at intervals of 1 cm). Some rice seedlings were treated with phytohormones, including 10^{-6} M abscissic acid (ABA), 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), 10^{-6} M giberellin A_3 (GA), 10^{-6} M methyl jasmonate (MeJA) for an additional 2 days. The control rice plants in each experiment were grown in the dark for 4 days at 28°C and then in 12 hr light-12 hr dark cycle from day 4 to day 12 at 28°C. Some ten-day-old rice seedlings were treated with wounding, 10^{-4} M methyl jasmonate and 10^{-4} M ethephon at different time courses.

A gene-specific probe for Os4bglu12 β-glucosidase was amplified from a rice genomic DNA as the template with the 445-3'end forward (5'-ATGGAGCAAACGTGAAGGGAT-3') and 445-3'UTR reverse (5'-AACTGGATTACTT CCATCTC-3') primers derived from the coding sequence at the C-terminal part and 3'-untranslated region (3'UTR) of the gene, respectively. The amplification was performed with 30 cycles of 94°C 45 sec, 45°C 45 sec, and 72°C 1 min, using Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN).

Total RNA was isolated from rice tissues by the SDS-phenol method described by Bachem et al. (1996). Thirty micrograms of total RNA from each sample was denatured and electrophoresed on a 1.2% formaldehyde-agarose gel and transferred onto a Hybond N+ nylon membrane (GE Healthcare, Buckinghamshire, UK) by standard procedures (Sambrook et al., 1989). The probe was labeled by Rediprime II random priming with α-[32P]dCTP (GE Healthcare) and used for hybridization with RNA blots for 16 h at 42°C. The blots were then washed once in 0.1% SDS, 2 x SSC for 30 min at 65°C and washed twice in 0.1% SDS, 0.1 x SSC for 15 min at 65°C, then exposed to a Fuji film imaging plate for 16 h at room temperature. The positions of radioactive bands were visualized with a Fuji Film BAS 1000 BioImaging Analyzer (Fuji Photo Film Co., Ltd, Tokyo, Japan).

2.2 Determination of the concert action of rice endoglucanase and Os4BGlu12 β-glucosidase in the hydrolysis of rice cell wall

2.2.1 Preparation of crude cell walls from rice seedling leaves

Ten-day-old rice seedling leaves was ground to a fine powder with mortar and pestle in liquid nitrogen. The fine powder was stirred in hot 80% (v/v) ethanol (warmed at 80°C) to remove free monosaccharides and oligosaccharides from cell wall powder. This hot ethanol wash was repeated for another 3 times and then the crude cell wall pellets were dried under vacuum and kept at -20°C until use.

2.2.2 Digestion of cell walls with rice endoglucanases and purification of cell wall hydrolysis products

Recombinant rice OsEGL 1 endo-(1,3;1,4)-β-glucanase was provided by Dr. Takashi Akiyama (National Agricultural Research Center for Hokkaido Region, JAPAN). In a 10 mL reaction containing 300 mg crude cell wall powder, 100 pg of the GST-OsEGL 1 endo-(1,3;1,4)-β-glucanase in 50 mM sodium acetate, pH 5.0, was incubated at 37°C for 6 h. The reaction was stopped by boiling for 10 min, and then centrifuged at 3,000 x g for 20 min. The supernatant was desalted with AG501-X8D ion exchange resin (Bio-Rad, Richmond, CA) and then evaporated to dryness under vacuum. The concentrated fraction was dissolved in 200 μL distilled water and then separated by thin layer chromatography (TLC) on a silica gel 60 F_{254} plate developed in acetonitrile: water (3:1 by volume). The sugar spots were visualized by submerged in an orcinol-sulfuric acid solution (0.1% (w/v) orcinol and 3% (v/v) concentrated sulfuric acid in ethanol) and heated at 120°C for 10 min. To isolate each sugar product, only edge parts (1 cm in width) of both sides of the developed silica gel plate were cut off for detection of sugar spots, and the silica gel layer at the positions of the sugars was scratched off from the unstained plate and packed into a column (1.5 cm inner diameter, 9 cm in length). The sugars were eluted from a column with 5 volumes of 80% (v/v) ethanol and evaporated to dryness under vacuum.

2.2.3 Chemical characterization of cell wall hydrolysis products

Molecular weights of purified cell wall hydrolysis products were analyzed by electrospray ionization-mass spectrometry (ESI-MS). ESI–MS measurements were performed on a time-of-flight mass spectrometer JMS-T100CS (JEOL, Japan) using direct injection. The measurement conditions were as follows: scan mode, positive ion; needle voltage, 2.0 kV;

orifice voltage, 60 V; desolvation temperature, 80°C; sample injection rate, 6 μL min^{-1}; solvent, 0.05% aqueous trifluoroacetic acid.

To analyze the sugar linkages in products released from cell wall by rice OsEGL 1, partially methylated alditol acetates (PMAAs) were prepared from each product (1-2 mg) following the method of Kim et al. (2006). In this procedure, the sample was methylated in the presence of sodium hydroxide and methyl iodide, hydrolyzed with trifluoroacetic acid to release the methylated monosaccharides. The methylated monosaccharides were reduced with an excess of sodium borohydride and then acetylated with acetic anhydride. The PMAAs were separated and analyzed with a JMS-AX500 (JEOL, Japan) equipped at GC-MS and NMR Laboratory (Faculty of Agriculture, Hokkaido University). The gas liquid chromatography (GLC) peaks were identified by mass spectrometry (MS) on an electron-impact ionization mode scanning from 50 to 500 *m/z*. PMAA standards were prepared from cellotriose and laminaritriose.

2.2.4 Hydrolysis of cell wall products by rice Os4BGlu12 β-glucosidase

2.2.4.1 Recombinant protein production of rice Os4BGlu12 β-glucosidase

A full-coding cDNA of glycosyl hydrolase family 1 *Os4bglu12* β-glucosidase (*Os4bglu12*) was previously cloned and inserted into pENTR-D/TOPO Gateway entry vector and then subcloned into pET32a+/DEST Gateway expression vector and the recombinant thioredoxin-Os4BGlu12 fusion protein was functional expressed in *E. coli* as described in Opassiri et al. (2006). For recombinant protein expression being used in this study, the selected clones were grown in LB medium containing 15 μg/mL kanamycin, 12.5 μg/mL tetracycline and 100 μg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.5-0.6. Then, IPTG was added to a final concentration of 0.3 mM, and the cultures were incubated at 20°C for 8-12 h. The soluble protein fraction was purified by immobilized metal affinity chromatography (IMAC) with Co^{2+} or Ni^{2+} resin. The enzyme molarity was calculated based on the molecular weight of 69 kDa for thioredoxin-Os4BGlu12. Protein assays were performed by the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.2.4.2 Detection of hydrolysis products by TLC

The cell wall hydrolysis products were assayed with 1 μg thioredoxin-Os4Glu12 in 50 mM sodium acetate, pH 5.0, in 50 μL reaction volume at 37°C for 0, 10, 30 and 60 min. The reaction was stopped by boiling for 10 min and the products were detected by TLC as described above.

2.3 Enzyme assays and kinetic analysis

Kinetic parameters were determined for *p*NP glycosides, oligosaccharides, and commercially available natural glycosides from triplicate assays of 5-7 substrate concentrations at time points and enzyme concentrations where the reaction rate was linear and the absorbance value was in the range of 0.1 to 1.0. The assays were done at 37°C in 50 mM sodium acetate, pH 5.0, which are the optimum temperature and pH for Os4BGlu12, respectively (Opassiri et al., 2006).

To determine sugar specificity, in a 100 μL reaction, 1.45 pmol Trx-Os4BGlu12 was incubated with *p*NP glycosides. The reactions were stopped by adding 50 μL of 0.4 M sodium carbonate, and the amount of *p*-nitrophenol released was determined from its A_{405}. For the oligosaccharides and commercially available natural glycosides (listed in Table 1), 50 μL reaction volumes containing 1.81-14.5 pmol enzyme were used, the reactions were stopped by boiling, and the glucose released was quantified by the peroxidase/glucose oxidase assay method, as described in Opassiri et al. (2003).

Kinetic parameters were calculated by nonlinear regression of Michaelis-Menten plots with Grafit 5.0 (Erithacus Software), based on the amount of nonreducing terminal glucosyl residues released in each reaction. The enzyme molarity was calculated based on the molecular weight of 69 kDa for Trx-Os4BGlu12. Protein assays were performed by the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Apparent subsite affinities were calculated from the k_{cat}/K_m values by the method of Hiromi et al. (1973).

2.4 Construction of GUS-fusion plasmid and particle bombardment of onion cells for cellular localization of the Os4BGlu12 protein

In this study, the plasmid construct was designed to link the 3'end of Os4bglu12 cDNA with the 5'end of β-glucuronidase cDNA (GUS) which is a reporter gene. The recombinant plasmid was then delivered into epidermal layers of onion bulbs by particle bombardment. Finally, the location in onion cells of recombinant protein product, a GUS (glucuronidase) reporter tagged at the C-terminus of Os4BGlu12, was observed by detecting the color product derived from the hydrolysis of X-Glu by β-glucuronidase.

A forward primer 445-1full-attB1-F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGGCGGCAGCAGGGGCAAT-3'), and a reverse primer 445-1full-attB2-R (5'-GGGGACCAC TTTGTACAAGAAAGCTGGGTATTTCAGGAGGAACTTCTT GAAC-3') were used to amplify the full length coding region of *Os4bglu12* and introduce attB1 and attB2 site at the 5' end and 3' end, respectively. The amplification were performed with 5 cycles of 94°C, 30 sec, 45°C, 1 min

and 72°C, 2 min, and 25 cycles of 94°C, 30 sec, 50°C, 1 min and 72°C, 2 min using *Pfu* DNA polymerase (Promega, Madison, USA).

The PCR product was cloned into the pDONR-Zeo vector with BP clonase according to the Gateway Technology protocol (Invitrogen). The positive clones were selected with 50 μg/mL zeocin LB-agar plates in the dark. The *Os4bglu12* cDNA was subcloned from the recombinant pDONR-Zeo-*Os4bglu12* into pMDC139 (provided by Mark Curtis, University of Zürich (2003) by LR clonase recombination according to the Gateway Technology protocol (Invitrogen). Positive clones were selected with 30 μg/mL kanamycin LB-agar plates. Plasmid size determination and PCR amplification with gene specific primers were used to identify the recombinant pMDC plasmids containing the attR-full-length gene inserts and the sequences were determined to confirm the correct reading frame of the DNA insert in the plasmid.

The resulting pMDC139-*Os4bglu12* constructs were delivered into epidermal layers of onion bulbs by particle bombardment with a Gene Gun Machine PDA-1000/He (Bio-Rad, Richmond, CA). After bombardment, the transformed cells was incubated at 25°C for 48 hr in complete darkness. After that, the epidermal layers were stained overnight with 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Glu) at 37°C and the blue color product derived from the hydrolysis of X-Glu by β-glucuronidase was observed by light microscopy.

3. ผลการทดลอง (Results)

3.1 Effects of environmental stresses and plant hormones on *Os4bglu12* gene expression in rice seedlings

Northern analysis was used to determine the transcript levels of *Os4bglu12* gene compared between 10-day-old rice seedlings that had been exposed to various conditions for 2 days to rice seedlings grown at 28°C (control). Significant increase in *Os4bglu12* mRNA levels was detected when rice seedlings were exposed to methyl jasmonate (Figure 1). The mRNA transcripts were detected at levels similar or slightly decreased compared to the control when seedlings were exposed to 37°C, drought, salt, mannitol and gibberellic acid. The transcript levels decreased when the seedlings were treated with ethylene and wounding, and decreased dramatically when plants were grown under cold (5°C) and flooding conditions, and in the presence of abscissic acid.

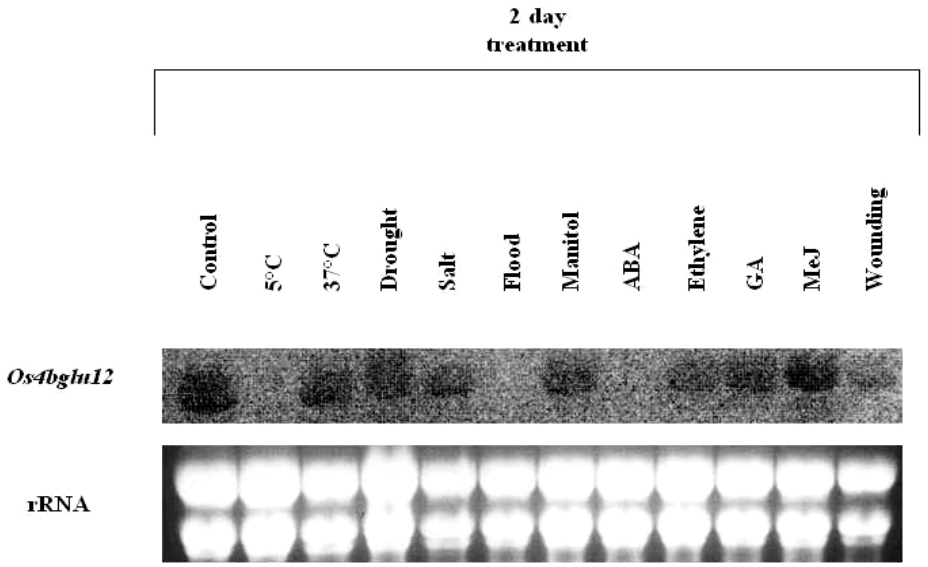


Figure 1. Northern analysis of *Os4bglu12* expression in 10-day-old rice seedlings grown under stress conditions and plant hormones for an additional 2 days. Thirty micrograms of total RNA was loaded on a 1.2% agarose formaldehyde gel and ethidium bromide-stained gel before blotting is shown under the blot. The RNA blot was hybridized with the α-^{32}P-labeled 264 bp 3'end fragment of Os4bglu12 DNA probe. Control (grown at 28°C), cold (grown at 5°C), 37°C, drought (dehydrated until losing 50% of the fresh weight was lost), salt (irrigated with 0.3 M NaCl), flood (plant were submerged in distilled water), mannitol (irrigated with 0.5 M mannitol), wounding (aerial parts such as leaves and stems were crushed by plastic ruler at intervals of 1 cm). For plant hormone treatment, seedlings were both irrigated and sprayed with 10^{-6} µM gibberellic acid A_3 (GA), 10^{-6} µM abscissic acid) (ABA), 10^{-6} M 2,4-dichlorophenoxyacetic acid (ethylene), and 10^{-6} M methyl jasmonate (MeJ).

LeÓn et al. (2001) reported that plant hormones such as methyl jasmonate, ethylene and abscissic acid are involved in diverse plant responses to abiotic stresses especially in wounding stress. In addition, Akiyama et al. (2009) reported that the transcript level of *OsEGL 1* endo-(1,3;1,4)-β-glucanase gene increased in 10-day-old rice seedlings as early as 12 h after exposeure to mechanical wounding, methyl jasmonate and ethephon. Therefore, a more detailed time-course study of expression of *Os4bglu12* gene was performed in 10-day-old rice seedlings treated with methyl jasmonate, ethephon and wounding.

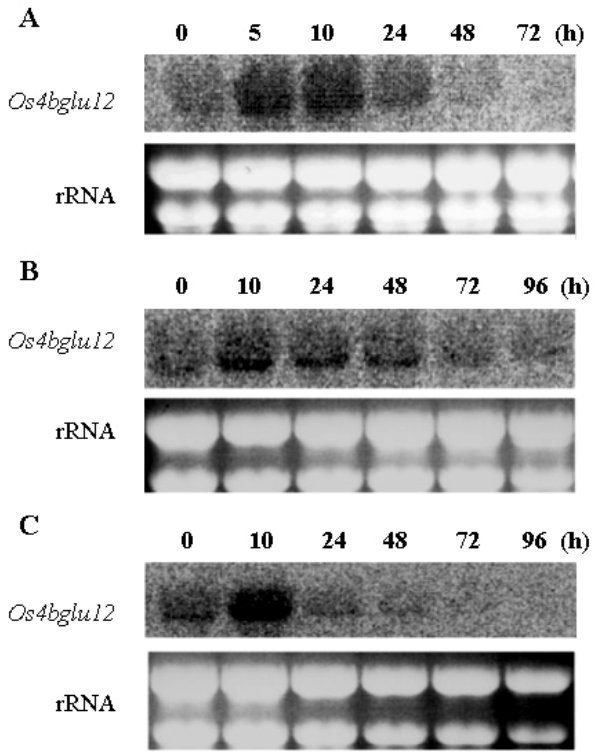


Figure 2. RNA blot analysis of *Os4bglu12* β-glucosidase expression in 10-day-old rice seedlings treated with A. wounding, B. methyl jasmonate and C. ethephon at different time-points. Thirty micrograms of total RNA were loaded on a 1.2% agarose formaldehyde gel. The RNA blot was hybridized with the α-^{32}P-labeled 3'end fragment of the *Os4bglu12* cDNA probe. The ethidium bromide-stained gel below the blots showed the RNA loading before blotting.

As seen in Figure 2A, the expression of *Os4bglu12* was induced as early as 5-10 hr after treatment with wounding and decreased gradually thereafter. Similar gene expression patterns were seen in rice seedlings treated with methyl jasmonate and ethephon. *Os4bglu12* mRNA levels significantly increased within 10 hr after treating with methyl jasmonate, after that the mRNA levels decreased slowly (Figure 2B). Treatment with ethephon induced a rapid

temporary increase in *Os4bglu12* mRNA levels within 10 hr, after which the mRNA levels decreased rapidly within 1 day and then decreased gradually (Figure 2C). The association of the induced expression patterns of *Os4bglu12* gene in response to wounding, methyl jasmonate and ethephon indicated that these treatments rapidly induced the expression of *Os4bglu12* and a high level of expression maintained within 10 hr.

3.2 Hydrolysis of rice cell wall by rice endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase

Since the wounding, methyl jasmonate and ethephon induction of expression of *Os4bglu12* gene is similar to that of rice OsEGL 1 endoglucanase, which specifically hydrolyzes (1,3;1,4)-β-glucans, so an experiment was set up to test if Os4bglu12 is able to hydrolyze oligosaccharides released from rice cell walls by OsEGL 1.

3.2.1 Hydrolysis of rice cell wall by rice endo-(1,3;1,4)-β-glucanase

The hydrolysis of crude rice cell walls by a recombinant OsEGL 1 generated three major hydrolysis products, which were denoted Os1, Os2 and Os3 (Figure 3).

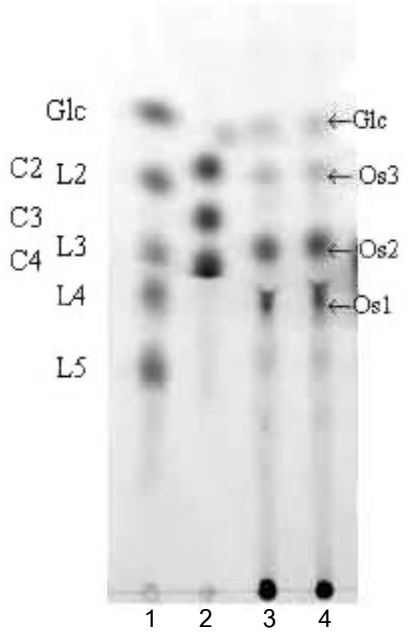


Figure 3. TLC analysis of rice cell wall hydrolysis by rice OsEGL 1 endo-(1,3;1,4)-β-glucanase Lane 1 glucose (G) and laminari-oligosaccharide standards of DP 2-5 (L2-L5); lane 2, cello-oligosaccharide standards of DP 2-4 (C2-C4); lane 3 and 4, products released from cell walls by OsEGL 1 endo-(1,3;1,4)-β-glucanase. In a 10 mL reaction containing 300 mg crude cell wall powder, 100 pg of the GST-endo-(1,3;1,4)-β-glucanase in 50 mM sodium acetate, pH 5.0, was incubated at 37°C for 6 h.

3.2.2 Chemical characterization of cell wall hydrolysis products

In the ESI-MS data, signals of oligosaccharides Os1, Os2, and Os3 were detected at *m/z* 698 ($[M+Na]^+$), 527 ($[M+Na]^+$), and 365 ($[M+Na]^+$), respectively (Figure 4). These data suggest Os1, Os2, and Os3 to be a tetrasaccharide, a trisaccharide, and a disaccharide, respectively.

The linkage of these oligosaccharides was confirmed by methylation analysis. On the basis of their retention times in GLC and their fragmentation patterns in GC-MS, three permethylated aditol acetates of Os1 and Os2 were identified as 3-O-acetyl-1,2,4,5,6-penta-O-methyl-D-glucitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. Those from Os3 were identified as 3-O-acetyl-1,2,4,5,6-penta-O-methyl-D-glucitol and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (Figure 5).

The results of the analyses confirmed that Os1 and Os2 are (1,3;1,4)-linked glucotetraose (G4G4G3Gred) and glucotriose (G4G3Gred), respectively, while Os3 is the (1,3)-linked disaccharide of glucose (G3Gred) [where G represents a β-glucosyl residue, 3 and 4 are (1,3) and (1,4) linkages, respectively; and “red” indicates the reducing terminus].

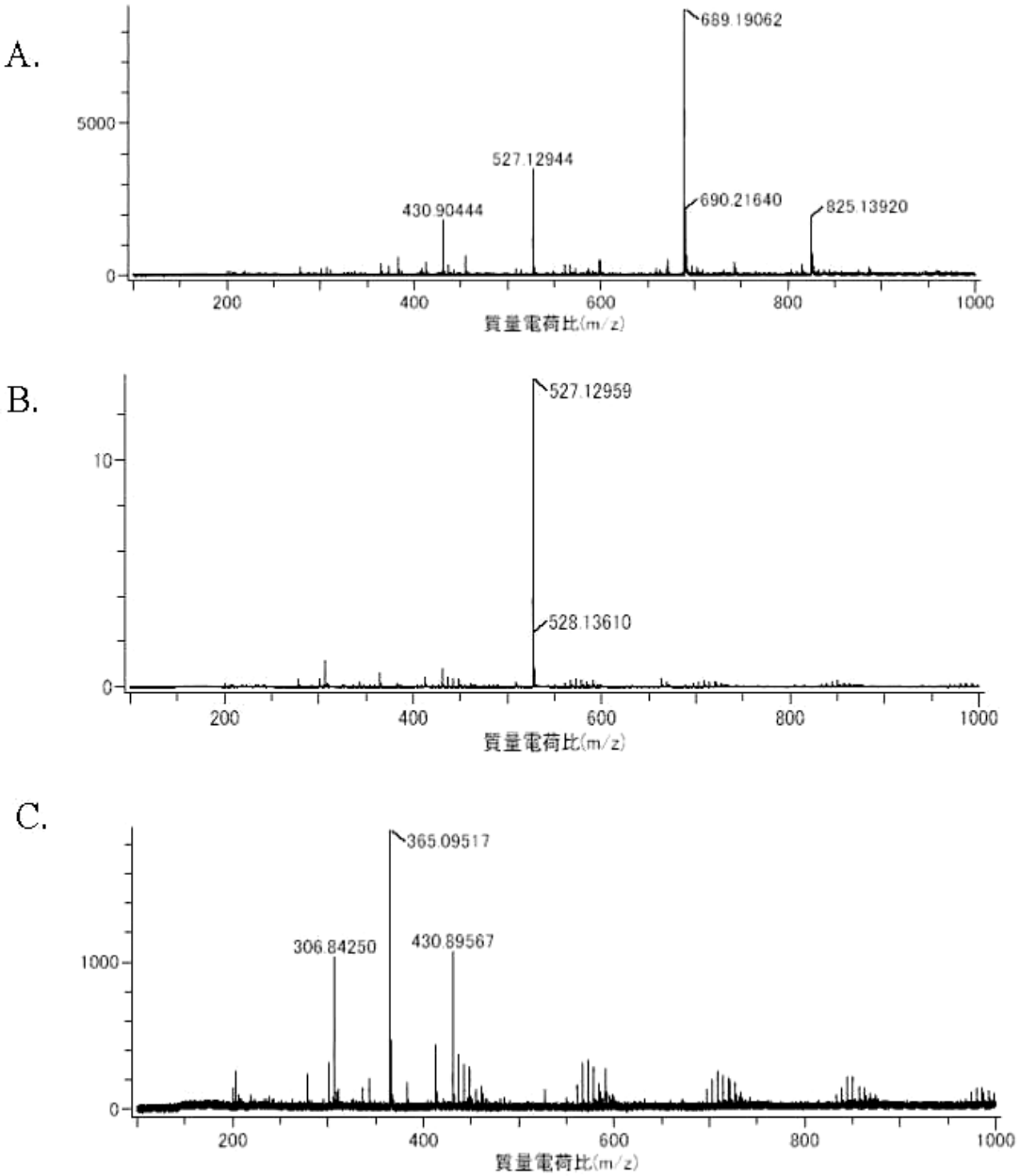
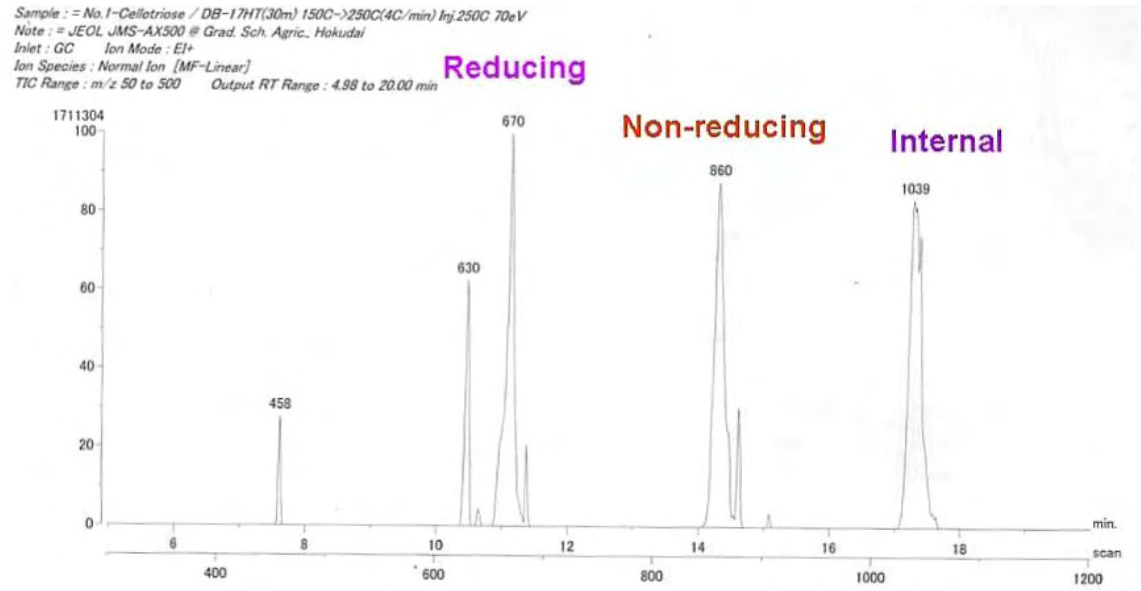
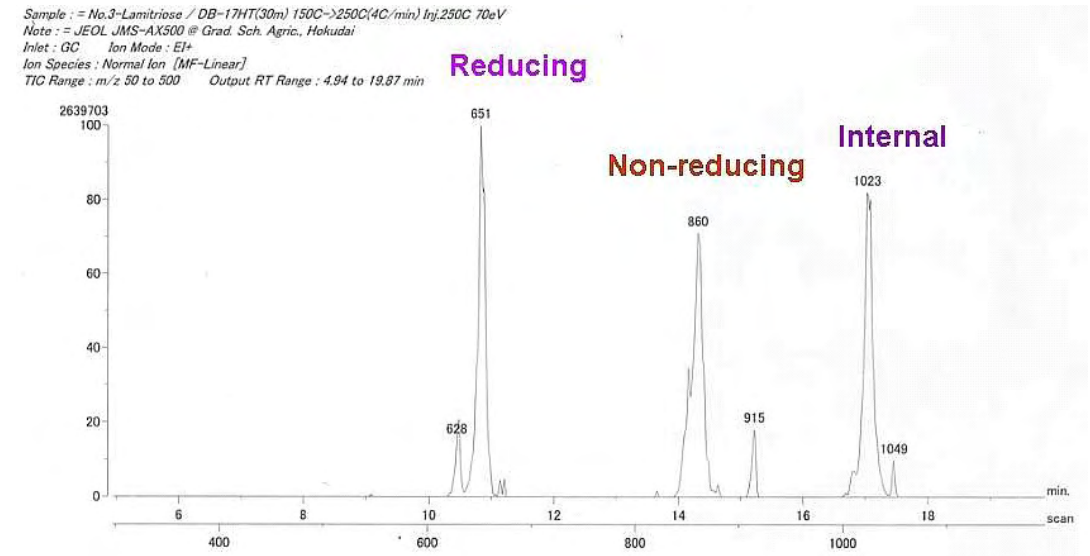


Figure 4. ESI-MS mass spectra of oligosaccharides Os1 (A), Os2 (B), and Os3 (C). ESI–MS measurements were performed on a time-of-flight mass spectrometer JMS-T100CS (JEOL, Japan) using direct injection. The measurement conditions were as follows: scan mode, positive ion; needle voltage, 2.0 kV; orifice voltage, 60 V; desolvation temperature, 80°C; sample injection rate, 6 μL min^{-1}; solvent, 0.05% aqueous trifluoroacetic acid.

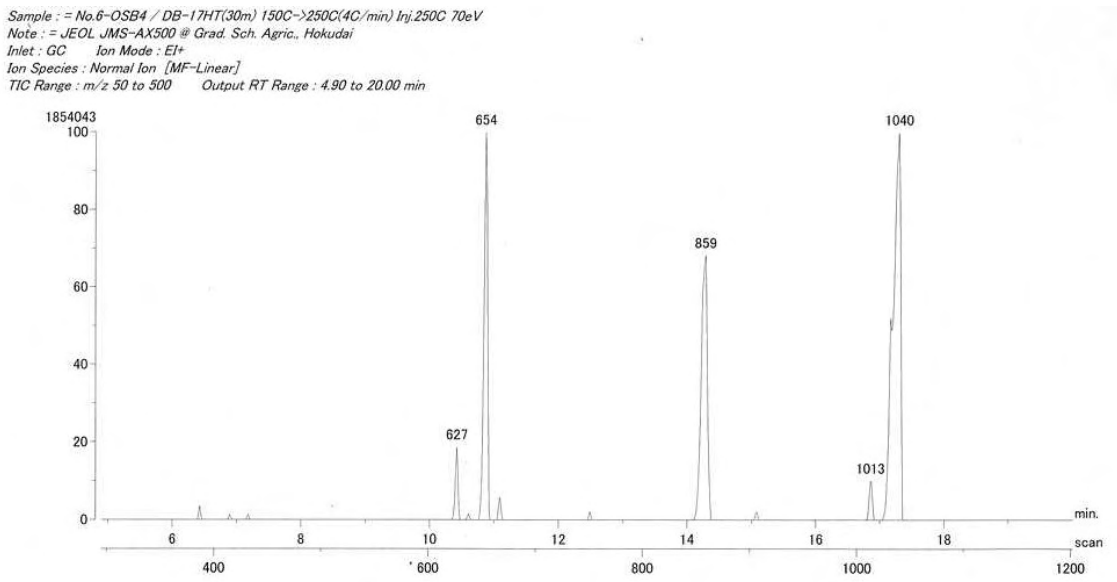
A.


Sample : = No.1-Cellotriose / DB-17HT(30m) 150C->250C(4C/min) Inj.250C 70eV
Note : = JEOL JMS-AX500 @ Grad. Sch. Agric., Hokudai
Inlet : GC Ion Mode : EI+
Ion Species : Normal Ion [MF-Linear]
TIC Range : m/z 50 to 500 Output RT Range : 4.98 to 20.00 min
Reducing
Non-reducing
Internal
1711304
458
630
670
860
1039
min.
scan

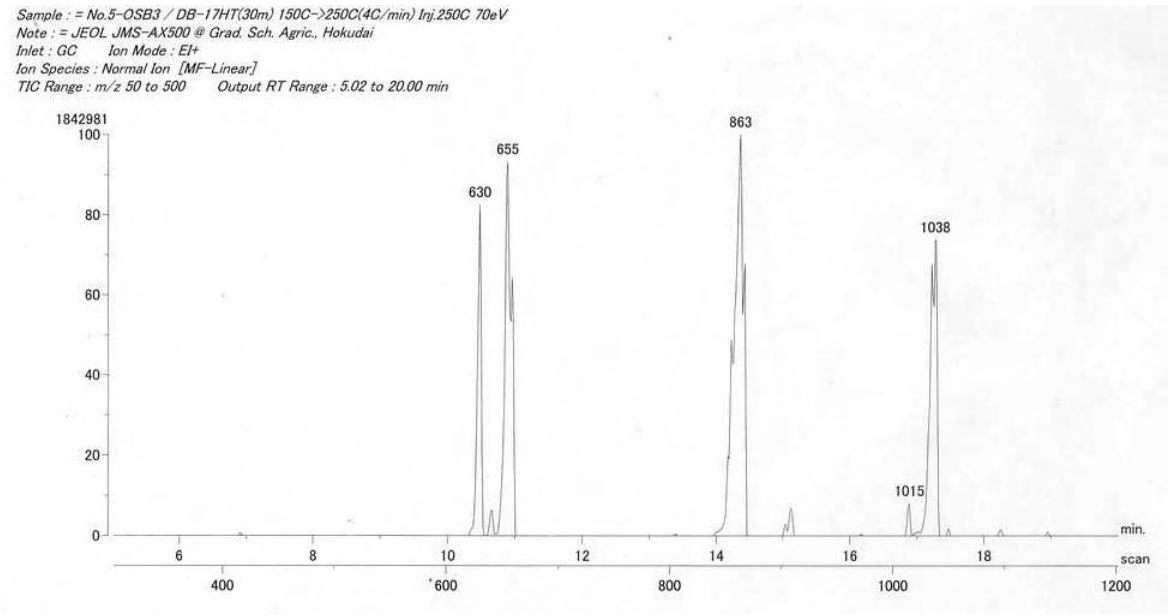
B.


Sample : = No.3-Lamitriose / DB-17HT(30m) 150C->250C(4C/min) Inj.250C 70eV
Note : = JEOL JMS-AX500 @ Grad. Sch. Agric., Hokudai
Inlet : GC Ion Mode : EI+
Ion Species : Normal Ion [MF-Linear]
TIC Range : m/z 50 to 500 Output RT Range : 4.94 to 19.87 min
Reducing
Non-reducing
Internal
2639703
628
651
860
915
1023
1049
min.
scan

C.


Sample : = No.6-OSB4 / DB-17HT(30m) 150C->250C(4C/min) Inj.250C 70eV
Note : = JEOL JMS-AX500 @ Grad. Sch. Agric., Hokudai
Inlet : GC Ion Mode : EI+
Ion Species : Normal Ion [MF-Linear]
TIC Range : m/z 50 to 500 Output RT Range : 4.90 to 20.00 min
1854043
627
654
859
1013
1040
min.
scan

D.



E.

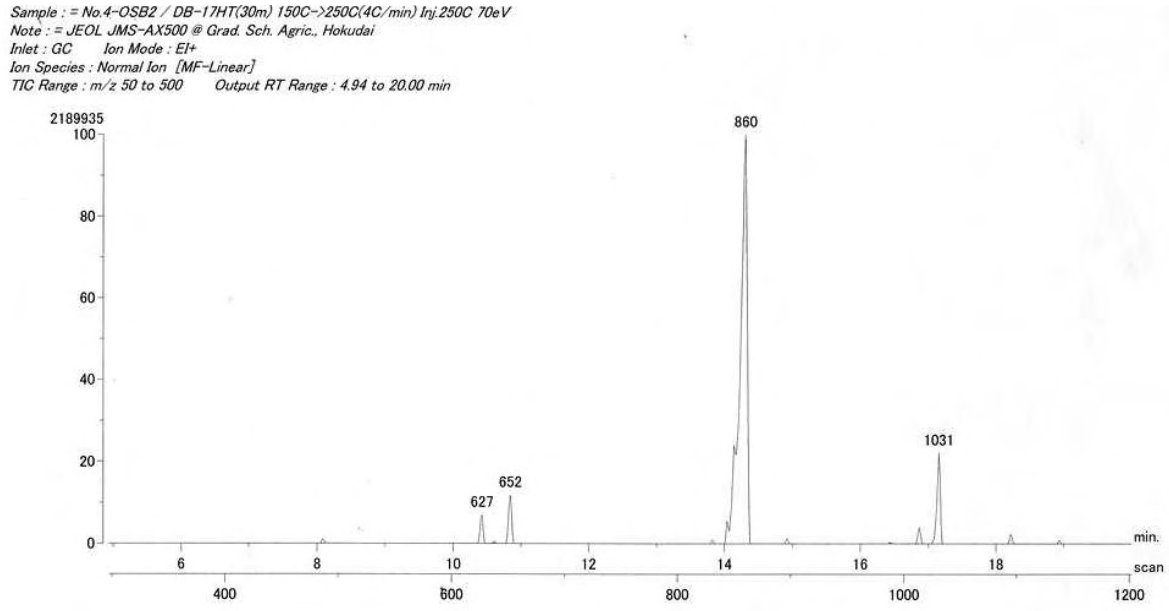


Figure 5. Gas Chromatography profiles of PMAAs generated from Os1, Os2 and Os3. A) PMAAs standard of 4-Glc prepared from cellotriose, B) PMAAs standard of 3-Glc prepared from laminaritriose, C) Os1, D), Os2, E) Os3. Reducing in A. = 4-O-acetyl-1,2,3,5,6-penta-O-methyl-D-glucitol; Reducing in B = 3-O-acetyl-1,2,4,5,6-penta-O-methyl-D-glucitol; Non-reducing in A and B = 1,5-di-O-acetyl-2,3,4,6-tetra-O-metyl-D-glucitol; Internal in A = 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol; Internal in B = 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol.

3.2.3 Hydrolysis of cell wall products by rice Os4BGlu12 β-glucosidase

Os4blu12 could hydrolyze oligosaccharides released from the hydrolysis of rice cell walls by OsEGL 1. The glucose product released by Os4bglu12 trended to increase during incubation from 10 to 60 min (Figure 6). TLC analysis of Os1 hydrolysis products indicated that Os4bglu12 hydrolyzed Os1 oligosaccharide more rapidly than Os2 and Os3 and Os1 was cleaved to give glucose, Os2 and Os3 as product (Figure 6).

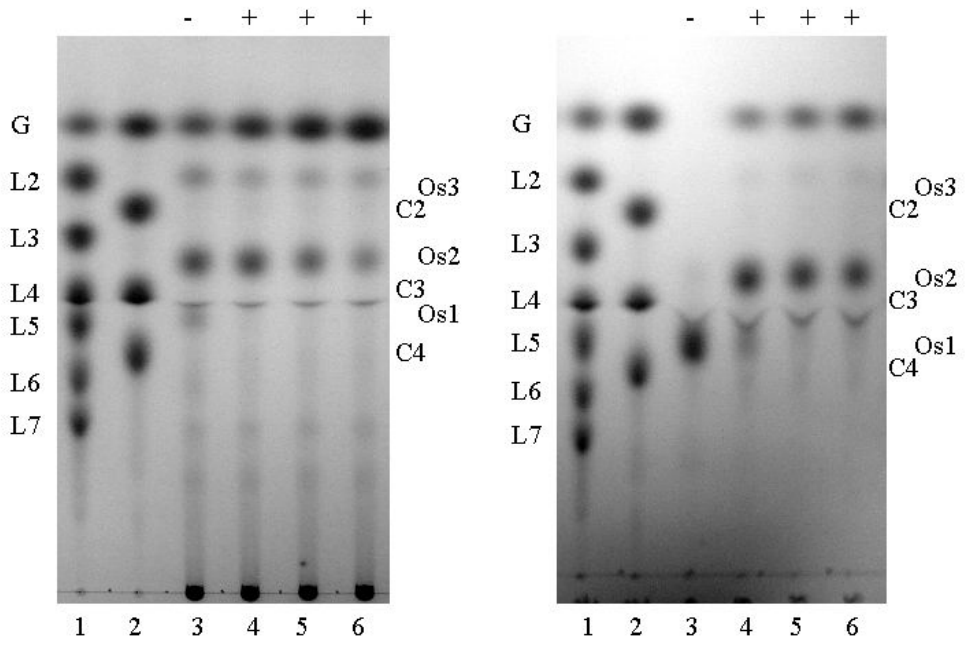


Figure 6. TLC analysis of rice cell wall hydrolysis by rice OsEGL 1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase. A, Time course of the reaction by Os4BGlu12 with crude cell wall that was pre-incubated with OsEGL 1. Lane 3, reaction without Os4BGlu12 (-); lanes 4-6, reactions incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. B, Os1 product incubated with Os4BGlu12. Lane 3, reaction without Os4BGlu12 (-); lanes 4-6, Os1 reaction incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. Lane 1 in A and B, glucose (G) and laminari-oligosaccharide standards of DP 2-7 (L2-L7); lane 2 in A and B, cello-oligosaccharide standards of DP 2-4 (C2-C4). Fifty microliter reaction mixtures composed of Os4BGlu12 (1 μg) and cell wall hydrolysis product in 50 mM sodium acetate buffer, pH 5.0, were incubated at 37°C and products analyzed by silica gel TLC with acetonitrile:water (3:1 by volume).

3.3 Kinetic analysis

The substrate specificity of Os4BGlu12 toward various kinds of disaccharides and oligosaccharides of glucose was evaluated by kinetic analysis. For disaccharides, Os4BGlu12 hydrolyzed sophorose (β-1,2), laminaribiose (β-1,3), and cellobiose (β-1,4), but not gentiobiose (β-1,6). It hydrolyzed cellooligosaccharides with DP of 3-6 and (1,3;1,4)-β-glucooligosaccharides with DP of 3-4, but not laminarioligosaccharides with DP >2. As shown in Table 1, Os4BGlu12 hydrolyzed laminaribiose with the highest catalytic efficiency (k_{cat}/K_m) value with a relatively high K_m and apparent k_{cat} values, but hydrolyzed sophorose and cellobiose with low efficiency. For cellooligosaccharides, there was a large decrease in K_m and increase in k_{cat} between DP of 2 and 3, after which the K_m remained approximately constant with increasing chain length of the substrate, while the apparent k_{cat} values increased slightly from DP of 3 to 5, but decreased at DP of 6. Catalytic efficiency (apparent k_{cat}/K_m) was within error for DP of 3 and 4, but increased 65% from DP of 4 to 5, so that the apparent k_{cat}/K_m of cellopentaose was 67-fold higher than that of cellobiose. An estimate of subsite affinities based on the assumptions of Hiromi et al. (1973), gave apparent subsite affinities of 9.3 ± 0.4 for subsite +2, 0.3± 0.4 kJ/mol for the +3 subsite, 1.3± 0.2 kJ/mol for the +4 subsite and -0.7 ± 0.2 kJ/mol for the +5 subsite. Os4BGlu12 also hydrolyzed (1,3;1,4)-glucotriose with catalytic efficiency 15-fold lower than that of cellotriose and hydrolyzed (1,3;1,4)-glucotetraose with catalytic efficiency 4-fold lower than that of cellotetraose. The K_m values for all oligosaccharides tested, except for cellobiose, were nearly equivalent.

Os4BGlu12 exhibited broad glycone specificity against *p*NP glycosides, although it did not hydrolyze *p*NP β-D-mannoside, *p*NP β-D-cellobioside, *p*NP β-D-glucoside, *p*NP β-L-fucoside and *p*NP α-L-fucoside (Table 1). The enzyme hydrolyzed *p*NP glycosides with much lower K_m and higher apparent k_{cat}/K_m values than oligosaccharides. The enzyme hydrolyzed *p*NP β-D-fucoside with the highest k_{cat}/K_m value of 47 s^{-1} mM^{-1}. It hydrolyzed *p*NP β-D-glucoside, *p*NP β-D-xyloside and *p*NP α-L-arabinoside with similar catalytic efficiency (apparent k_{cat}/K_m of 23-25 s^{-1} mM^{-1}), while *p*NP β-D-galactoside was hydrolyzed with 35% of the efficiency of *p*NP β-D-glucoside.

Several available natural glycosides could be hydrolyzed at different rates (Table 1). Deoxycorticosterone 21-glucoside, a steroid glucoside from the adrenal cortex of vertebrates, was the most efficiently hydrolyzed glycoside with a high apparent k_{cat} value (13 s^{-1}) and low K_m value (0.68 mM). Apigenin 7-*O*-β-D-glucoside, a flavone glucoside, was hydrolyzed with low apparent k_{cat} (0.83 s^{-1}) and K_m (0.12 mM) values. Those that were hydrolyzed with relatively low catalytic efficiency (below 0.67 s^{-1} mM^{-1}) included indoxyl β-D-glucoside, D-amygdalin, and linamarin. The enzyme could not hydrolyze salicin, naringin (a 7-*O*-2-α-rhamnosyl-β-glucoside),

gossypin (a flavonoid 8-*O*-glucoside), quercetin 3-β-D-glucoside (a flavonoid 3-*O*-β-glucoside) and phlorizin (phloretin 2'-*O*-β-glucoside, which is structurally similar to a flavonoid 5-*O*-β-glucoside).

Table 1 Kinetic parameters of rice Os4BGlu12 in the hydrolysis of oligosaccharides, *p*NP-glycosides, and natural glycosides.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Cellooligossaccharides (DP[b])			
2	1.8 ± 0.3	25 ± 3	0.073 ± 0.003
3	12 ± 2	4.6 ± 0.0	2.7 ± 0.3
4	17 ± 1	5.7 ± 0.5	3.0 ± 0.2
5	24 ± 3	4.8 ± 0.5	4.9 ± 0.1
6	18 ± 2	4.8 ± 0.6	3.8 ± 0.2
Laminarioligosaccharides (DP)			
2	23 ± 1	5.1 ± 0.2	4.5 ± 0.0
3	ND[a]	ND	ND
4	ND	ND	ND
Sophorose	5.2 ± 0.3	4.4 ± 0.4	1.2 ± 0.1
(1,3;1,4)-Glucotriose	0.84 ± 0.04	4.9 ± 0.9	0.18 ± 0.03
(1,3;1,4)-Glucotetraose	2.5 ± 0.2	3.3 ± 0.3	0.77 ± 0.08
*p*NP β-D-glucoside	20 ± 1	0.80 ± 0.08	24 ± 1
*p*NP β-D-fucoside	18 ± 1	0.39 ± 0.04	47 ± 2
*p*NP β-D-galactoside	12 ± 1	1.4 ± 0.2	8.5 ± 1.3
*p*NP β-D-xyloside	6.0 ± 0.3	0.24 ± 0.04	25 ± 0
*p*NP α-L-arabionoside	2.3 ± 0.1	0.12 ± 0.03	23 ± 4
Deoxycorticosterone 21-glucoside	13 ± 2	0.68 ± 0.18	20 ± 2
Indoxyl β-D-glucoside	2.9 ± 0.2	4.4 ± 0.5	0.67 ± 0.02
D-Amygdalin	0.25 ± 0.01	5.8 ± 0.2	0.042 ± 0.000
Linamarin	0.28 ± 0.04	9.4 ± 1.4	0.031 ± 0.000
Apigenin 7-*O*-β-D-glucoside	0.83 ± 0.02	0.12 ± 0.01	6.7 ± 1.6
Phlorizin	ND	ND	ND
Quercetin 3-β-D-glucoside	ND	ND	ND
Gossypin	ND	ND	ND
Naringin	ND	ND	ND
Salicin	ND	ND	ND

[a] means not detectable

[b] means degree of polymerization

3.4 Cellular localization of the Os4BGlu12 protein

In onion cells transformed with pMDC139-*Os4bglu12-GUS,* β-glucoronidase activity was observed outside the cell, while the blue color product was diffusely distributed throughout the control cells transformed with control pMDC139 plasmid (Figure 7). So, the Os4Bglu12 tagged at its C-terminus with GUS reporter (Os4BGlu12-GUS) may be secreted to the apoplast or cell wall region, which is consistent with the prediction of the PSORT program that the protein appears to be secreted outside the cell.

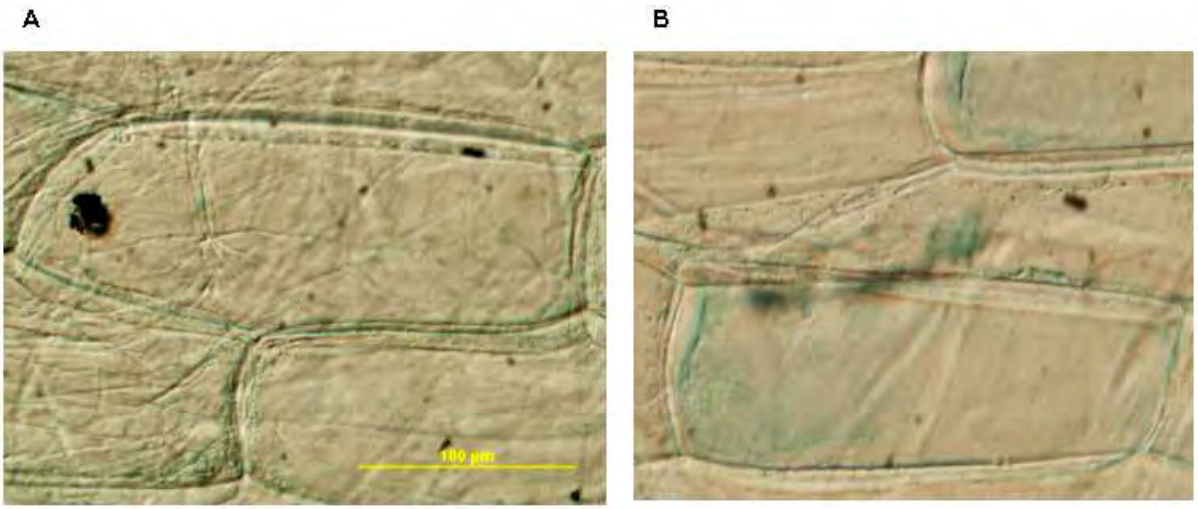


Figure 7. Extracellular localization of the Os4BGlu12 protein. The epidermal layers of onion bulbs were transformed with (A) pMDC139-Os4BGlu12 and (B) control pMDC139 plasmid without insert by particle bombardment. After 48 h incubation at 25°C in complete darkness, the transformed onion cells were stained overnight with X-Glu at 37°C, and the location of blue color product was observed by light microscopy.

4. วิจารณ์ผลการทดลอง (Discussion)

The expression of *Os4bglu12* was up-regulated in response to wounding, methyl jasmonate and ethephon treatments, with high mRNA levels observed within 10 h after treatment. Consistent with this study, Wang et al. (2005) reported that the transcript level of EST contig BHPiw028, corresponding to *Os4bglu12*, increased in response to brown planthopper attack, based on subtractive hybridization cDNA library screening. Methyl jasmonate and ethephon have been reported to be involved in wound signaling in several plant species (McCloud and Baldwin, 1997; Ziegler et al., 2001; von Dahl and Baldwin, 2007). This implies that induction of *Os4bglu12* gene expression by wounding and herbivore attack may be mediated by the methyl jasmonate and ethylene signal transduction pathway. The protein product of *Os4bglu12* gene may be involved in cell wall recycling or release of active compounds from glycosides for defense. In addition, the levels of transcripts that hybridized to the ESTs BE607353 and BG101702, which correspond to the *Os3bglu7* and *Os4bglu12* β-glucosidase genes, respectively, were induced by salt stress in salt-tolerant rice (var Pokkali), but not in the salt-sensitive cultivar IR29 (Kawasaki et al., 2001). These studies indicated that Os4BGlu12 may have functional roles in response to stresses.

One possible role of Os4BGlu12 is hydrolysis of cell-wall-derived oligosaccharides. Os4BGlu12 efficiently hydrolyzed cellooligosaccharides (β-1,4-linked) with DP of 3 to 6 and laminaribiose (β-1,3), which is more like Os3BGlu7, Os3BGlu8, and Os7BGlu26 than Os3BGlu6, which hydrolyzes laminaribiose, but not cellooligosaccharides (Opassiri et al., 2004, Khuntothom et al., 2009; Seshadri et al., 2009). The large increase in catalytic efficiency of Os4BGlu12 with increasing chain length from DP of 2 to 3 is similar to rice Os3BGlu7 (Opassiri et al., 2004), rice Os3BGlu8 and Os7BGlu26 β-glucosidases (Kuntothom et al., 2009) and different from barley β-glucosidase βII, which hydrolyzed cellobiose better than cellotriose (Hrmova et al., 1998). However, the K_m values of barley β-glucosidase βII and rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 β-glucosidases decreased with increasing DP from 3 to 6, while Os4BGlu12 was approximately constant, so Os4BGlu12 showed a much lower dependence on chain length over this range. The preference of the rice Os4BGlu12 for (1,4)-β-oligosaccharides suggested that it has an extensive subsite binding region, but primarily binds the third glycosyl residue of cellooligosaccharides with a weaker interaction at the 5^{th} residue.

The transcript level of the rice *OsEGL 1* gene, which specifically hydrolyzes (1,3;1,4)-β-glucans, increased in rice seedlings in response to wounding, methyl jasmonate and ethephon, which is similar to the expression of *Os4bglu12*. Os4BGlu12 could hydrolyze (1,3;1,4)-glucooligosaccharides released from rice cell wall by OsEGL 1 with catalytic efficiency that

increased 4-fold with increasing chain length from 3 to 4, in contrast to cellotriose and cellotetraose, which are hydrolyzed more efficiently than (1,3;1,4)-oligosaccharides, but with no significant increase in efficiency between DP of 3 and 4. This result implies that enzyme may bind different linkages in different modes, so that it can play a role in cellooligosaccharide hydrolysis and cooperate with OsEGL 1 in hydrolysis of (1,3;1,4)-β-glucans of cell walls.

The above studies suggest roles for Os4BGlu12 in release of glucose from oligosaccharides to complete the depolymerization of at least three major cell wall polysaccharides present in rice tissues, including the (β-1,4); (β-1,3) and β-(1,3;1,4)-glucans. The reorganization and alteration of these cell wall β-glucans may be required during normal developmental processes or in response to environmental stresses. There have been reports that the hydrolysis of cell wall polysaccharides also likely generates sugar signals (Rolland et al., 2006). Therefore, a role for Os4BGlu12 in degradation or modification of such signals cannot be ruled out. Together with the preference of Os4BGlu12 to hydrolyze cell wall derived-oligosaccharides, its extracellular location implies it may play a role in cell wall recycling.

Os4BGlu12 could hydrolyze deoxycorticosterone 21-glucoside and apigenin 7-*O*-β-D-glucoside with high catalytic efficiency, suggesting it could also play a role in releasing active steroids or flavonoids from their glycosides for defense. The fact the enzyme hydrolyzed apigenin 7-*O*-β-D-glucoside, but not flavonoid 3-*O*-glucoside and 8-*O*-glucoside, suggests it may have preference for the 7-*O*-linkage in flavonoid glucosides. Recently, Naoumkina et al. (2007) reported the induction of transcript levels of four flavone/isoflavone β-glucosidases in cell suspensions of a legume, *Medicago truncatula*, within 30 min to 2 h, and the accumulation of the free isoflavonoid phytoalexin medicarpin released from its glycosides in response to methyl jasmonate. Although Os4BGlu12 is thought to function in hydrolysis of oligosaccharides released from the cell walls, it is possible that this enzyme plays more than one role. Recently barley β-glucosidase βII, which is thought to help in hydrolysis of cell wall oligosaccharides during germination, has been found to hydrolyze cyanogenic glycosides from barley leaves (Nielsen et al., 2006). This gives support to the possibility of one enzyme playing roles in both the cell wall hydrolysis and defense.

The preference of the glycone moieties of Os4BGlu12 is different from the GH1 rice enzymes in that it could not hydrolyze *p*NP β-D-mannoside, but it instead hydrolyzed *p*NP β-D-xyloside and α-L-arabinoside with k_{cat}/K_m values equivalent to that for *p*NP β-D-glucoside (Opassiri et al., 2004; Kuntothom et al., 2009, Seshadri et al., 2009). The efficient hydrolysis of β-D-xyloside, is similar to white clover β-glucosidase, but is not generally found in GH1 enzymes that have been characterized (Marana, 2006).

Since the substrate specificity of Os4BGlu12 is distinct from other rice enzymes, a protein sequence alignment was performed to compare the amino acids lining the binding site of Os4BGlu12 with the residues found at the -1, +1, +2, +3 and +4 subsites in the Os3BGlu7 X-ray crystal structure (Chuenchor et al., 2008 and Protein Data Bank accession 3F5K) (Figure 8). All the residues in close contact with the sugar at the -1 subsite were conserved in all enzymes, except for V241, which is in contact with E176 catalytic acid/base in Os3BGlu7. Therefore, the differences in glycone specificities may derive from the orientation of amino acid residues at -1 subsite. As noted by Verdoucq et al. (2003), the positioning of the sugar at a glycone site might be affected by differences in aglycone binding that thereby determine the sugar specificity. Rice Os3BGlu7, Os3BGlu8, Os7BGlu26 and barley βII, were classified in a distant related group and shared 51-53% amino acid sequence identity with Os4BGlu12 (Opassiri et al., 2006). They possess almost completely different amino acids at the residues predicted to form the +1 to +4 subsites compared to Os4BGlu12, which is likely reflected in the different substrate specificities among theses enzymes. However, residue W358 at +1 and +2 subsites in Os3BGlu7, which is a conserved Trp shown to position the aglycone of other plant GH1 β-glucosidases, was conserved in all enzymes (Czjzek et al., 2000; Verdoucq et al., 2004). Os4BGlu12 is more closely related to the dicot defense enzymes white clover cyanogenic β-glucosidase (Barrett et al., 1995) and *M. truncatula* isoflavone β-glucosidase (Naoumkina et al., 2007), with which it shares 63%, and 65% protein sequence identity, respectively, and these enzymes have many identical predicted active site amino acid residues. The above mentioned differences and similarities in active site amino acid residues between Os4BGlu12 and the other enzymes may result in the unique overall binding site geometry that allows Os4BGlu12 to hydrolyze glucooligosaccharides with different linkages, *p*NP glycosides with a range of glycones and flavonoid and steroid glucosides.

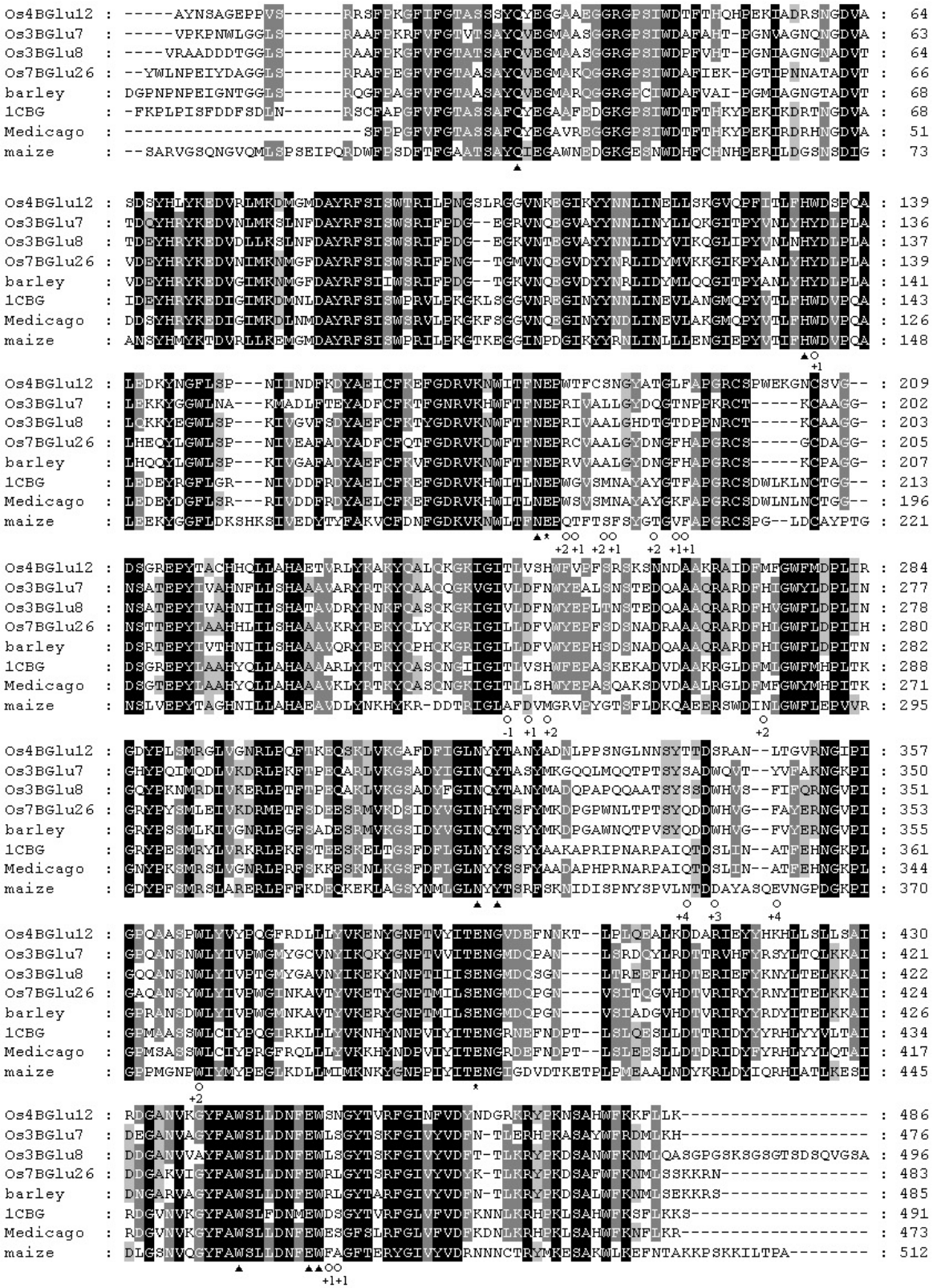

Os4BGlu12 : -----AYNSAGEPPVS------RRSFPKGFIFGTASSSYQYEGGAAEGGRGPSIWDTFTHQHPEKIADRSNGDVA : 64
Os3BGlu7 : -----VPKPNWLGGLS------RAAFPKRFVFGTVTSAYQVEGMAASGGRGPSIWDAFAHT-PGNVAGNQNGDVA : 63
Os3BGlu8 : ----VRAADDDTGGLS------RAAFPKGFVFGTATSAFQVEGMAASGGRGPSIWDPFVHT-PGNIAGNGNADVT : 64
Os7BGlu26 : --YWLNPEIYDAGGLS------RRAFPEGFVFGTAASAYQVEGMAKQGGRGPSIWDAFIEK-PGTIPNNATADVT : 66
barley : DGPNPNPEIGNTGGLS------RQGFPAGFVFGTAASAYQVEGMARQGGRGPCIWDAFVAI-PGMIAGNGTADVT : 68
1CBG : -FKPLPISFDDFSDIN------RSCFAPGFVFGTASSAFQYEGAAFEDGKGPSIWDTFTHKYPEKIRDRTNGDVA : 68
Medicago : ------------------------SFPPGFVFGTASSAFQYEGAVREGGKGPSIWDTFTHKYPEKIRDRHNGDVA : 51
maize : --SARVGSQNGVQMLSPSEIPQRDWFPSDFTFGAATSAYQIEGAWNEDGKGESNWDHFCHNHPERILDGSNSDIG : 73
Os4BGlu12 : SDSYHLYKEDVRLMRDMGMDAYRFSISWTRILPNGSLRGGVNKEGIKYYNNLINELLSKGVQPFITLFHWDSPQA : 139
Os3BGlu7 : TDQYHRYKEDVNLMRSLNFDAYRFSISWSRIFPDG--EGRVNQEGVAYYNNLINYLLQKGITPYVNLYHYDLPLA : 136
Os3BGlu8 : TDEYHRYKEDVDLLKSLNFDAYRFSISWSRIFPDG--EGKVNTEGVAYYNNLIDYVIKQGLIPYVNLNHYDLPLA : 137
Os7BGlu26 : VDEYHRYKEDVNIMRNMGFDAYRFSISWSRIFPNG--TGMVNQEGVDYYNRLIDYMVKKGIKPYANLYHYDLPLA : 139
barley : VDEYHRYKEDVGIMRNMGFDAYRFSIIWSRIFPDG--TGRVNQEGVDYYNRLIDYMLQQGITPYANLYHYDLPLA : 141
1CBG : IDEYHRYKEDIGIMRDMNLDAYRFSISWPRVLPKGKLSGGVNREGINYYNNLINEVLANGMQPYVTLFHWDVPQA : 143
Medicago : DDSYHRYKEDIGIMRDLNMDAYRFSISWSRVLPKGKFSGGVNQEGINYYNDLINEVLAKGMQPYVTLFHWDVPQA : 126
maize : ANSYHMYKTDVRLLREMGMDAYRFSISWPRILPKGTKEGGINPDGIKYYRNLINLLLENGIEPYVTIFHWDVPQA : 148
Os4BGlu12 : LEDKYMGFLSP---NIINDFKDYAEICFKEFGDRVKNWITFNEPWTFCSNGYATGLFAPGRCSPWEKGNCSVG-- : 209
Os3BGlu7 : LEKKYGGWLNA---KMADLFTEYADFCFKTFGNRVKHWFTFNEPRIVALLGYDQGTNPEKRCT-----KCAAGG- : 202
Os3BGlu8 : LQKKYEGWLSP---KIVGVFSDYAEFCFKTYGDRVKNWFTFNEPRIVAALGHDTGTDPPNRCT-----KCAAGG- : 203
Os7BGlu26 : LHEQYLGWLSP---NIVEAFADYADFCFQTFGDRVKDWFTFNEPRCVAALGYDNGFHAPGRCS-----GCDAGG- : 205
barley : LHQQYLGWLSP---KIVGAFADYAEFCFKVFGDRVKNWFTFNEPRVVAALGYDNGFHAPGRCS-----KCPAGG- : 207
1CBG : LEDEYRGFLGR---NIVDDFRDYAELCFKEFGDRVKHWITLNEPWGVSMNAYAYGTFAPGRCSDWLKLNCTGG-- : 213
Medicago : LEDEYDGFLSR---RIVDDFRDYAELCFKEFGDRVKHWITLNEPWSVSMNAYAYGKFAPGRCSDWLNLNCTGG-- : 196
maize : LEEKYGGFLDKSHKSIVEDYTYFAKVCFDNFGDKVKNWLTFNEPQTFTSFSYGTGVFAPGRCSPG--LDCAYPTG : 221
Os4BGlu12 : DSGREPYTACHHQLLAHAETVRLYKAKYQALQKGKIGITLVSHWFVEPSRSKSNNDAAKRAIDFMFGWFMDPLIR : 284
Os3BGlu7 : NSATEPYIVAHNFLLSHAAAVARYRTKYQAAQGGKVGIVLDFNWYEALSNSTEDQAAACRARDFHIGWYLDPLIN : 277
Os3BGlu8 : NSATEPYIVAHNIILSHATAVDRYRNKFQASQKGKIGIVLDFNWYEELTNSTEDQAAACRARDFHVGWFLDPLIN : 278
Os7BGlu26 : NSTTEPYLAAHHLILSHAAAVKRYREKYQLYQKGRIGILLDFVWYEPFSDSNADRAAACRARDFHIGWFLDPIIH : 280
barley : DSRTEPYIVTHNIILSHAAAVQRYREKYQPHQKGRIGILLDFVWYEPHSDSNADQAAACRARDFHIGWFLDPITN : 282
1CBG : DSGREPYLAAHYQLLAHAAAVARLYKTKYQASQNGIIGITLVSHWFEPASKEKADVDAAKRGLDFMLGWFMHPLTK : 288
Medicago : DSGTEPYLAAHYQLLAHAAAVKLYRTKYQASQNGKIGITLLSHWYEPASQAKSDVDAAIRGLDFMFGWYMHPITK : 271
maize : NSLVEPYTAGHNILLAHAEAVDLYNKHYKR-DDTRIGLAFDVMGRVEYGTSFLDKQAEERSWDINLGWFLEPVVR : 295
Os4BGlu12 : GDYPLSMRGLVGNRLPQFTKEQSKLVKGAFDFIGLNYYTANYADNLPPSNGLNNSYTTDSRAN--LTGVRNGIPI : 357
Os3BGlu7 : GHYPQIMQDLVKDRLPKFTPEQARLVKGSADYIGINQYTASYMKGQQLMQQTPTSYSADWQVT--YVFAKNGKPI : 350
Os3BGlu8 : GQYPKNMRDIVKERLPTFTPEQAKLVKGSADVFGINQYTANYMADQPAPQQAATSYSSDWHVS--FIFQRNGVPI : 351
Os7BGlu26 : GRYPYSMLEIVKDRMPTFSDEESRMVKDSIDYVGINHYTSFYMKDPGPWNLTPTSYQDDWHVG--FAYERNGVPI : 353
barley : GRYPSSMLKIVGNRLPGFSADESRMVKGSIDYVGINQYTSYYMKDPGAWNQTPVSYQDDWHVG--FVYERNGVPI : 355
1CBG : GRYPESMRYLVRKRLPKFSTEESKELTGSFDFLGLNYYSSYYAAKAPRIPNARPAIQTDSLIN--ATFEHNGKPL : 361
Medicago : GNYPKSMRSLVGNRLPRFSKKESKNLKGSFDFLGLNYYSSFYAADAPHPRNARPAIQTDSLIN--ATFEHNGKPL : 344
maize : GDYPFSMRSLARERLPFFKDEQKEKLAGSYNMLGLNYYTSRFSKNIDISPNYSPVLNTDDAYASQEVNGPDGKPI : 370
Os4BGlu12 : GPQAASPWLYVYPQGFRDLLLYVKENYGNPTVYITENGVDEFNNKT--LPLQEALKDDARIEYYHKHLLSLLSAI : 430
Os3BGlu7 : GPQANSNWLYIVPWGMYGCVNYIKQKYGNPTVYITENGMDQPAN----LSRDQYLRDTTRVHFYRSYLTQLKKAI : 421
Os3BGlu8 : GQQANSNWLYIVPTGMYGAVNYIKEKYNNPTIIISENGMDQSGN----LTREEFLHDTERIEFYKNYLTELKKAI : 422
Os7BGlu26 : GAQANSYWLYIVPWGINKAVTYVKETYGNPTMILSENGMDQPGN----VSITQGVHDTVRIRYYRNYITELKKAI : 424
barley : GPRANSDWLYIVPWGVNKAVTYVKERYGNPTMILSENGMDQPGN----VSIADGVHDTVRIRYYRDYITELKKAI : 426
1CBG : GPMQASSWLCIYPQGIRKLLLYVRNHYNNPVIYITENGRNEFNDPT--LSLQESLLDTTRIDYYYRHLYYVLTAI : 434
Medicago : GPMSASSWLCIYPRGFRQLLLYVKKHYNDPVIYITENGRDEFNDPT--LSLEESLLDTDRIDYFYRHLYYLQTAI : 417
maize : GPPMGNPWIYMYPEGLKDLLMIMKNKYGNPPIYITENGIGDVDTKETPLPMEAALNDYKRLDYIQRHIATLKESI : 445
Os4BGlu12 : RDGANVKGYFAWSLLDNFEWSNGYTVRFGIMFVDYNDGRKRYPKNSAHWFKKFLLK---------- : 486
Os3BGlu7 : DEGANVAGYFAWSLLDNFEWLSGYTSKFGIVYVDFN-TLERHPKASAYWFRDMLKH---------- : 476
Os3BGlu8 : DDGANVVAYFAWSLLDNFEWLSGYTSKFGIVYVDFT-TLKRYPKDSANWFKNMLQASGPGSKSGSGTSDSQVGSA : 496
Os7BGlu26 : DDGAKVIGYFAWSLLDNFEWRLGYTSRFGIVYVDYK-TLKRYPKDSAFWFKNMLSSKKRN---------- : 483
barley : DNGARVAGYFAWSLLDNFEWRLGYTARFGIVYVDFN-TLKRYPKDSALWFKNMLSEKKRS---------- : 485
1CBG : RDGVNVKGYFAWSLFDNMEWDSGYTVRFGLVFVDFKNNLKRHPKLSAHWFKSFLKKS---------- : 491
Medicago : RDGVNVKGYFAWSLLDNFEWESGFSLRFGLVFVDFKDNLKRHPKLSAHWFKNFLKR---------- : 473
maize : DLGSNVQGYFAWSLLDNFEWFAGFTERYGIVYVDRNNNCTRYMKESAKWLKEFNTAKKPSKKILTPA-------- : 512
Os4BGlu12 : ---------- : -
Os3BGlu7 : ---------- : -
Os3BGlu8 : TSASHPVGSAISSSHRLLLPLLLSLHFLFPSFFMFLSL : 534
Os7BGlu26 : ---------- : -
barley : ---------- : -
1CBG : ---------- : -
Medicago : ---------- : -
maize : ---------- : -

Figure 8 Amino acid sequence alignment of rice Os4BGlu12 with related β-glucosidases. The rice cDNA derived sequences are labeled as Os4BGlu12; Os3BGlu7 (AC U28047); Os3BGlu8 (AC AK120790), and Os7BGlu26 (AC EU835514). Maize is maize β-glucosidase 1 (AC U33816); barley is barley βII β-glucosidase (AC ACF07998); 1CBG is white clover cyanogenic β-glucosidase (AC ABV54745); Medicago is *Medicago truncatula* isoflavone β-glucosidase (AC ABW76288) (Naoumkina et al., 2007). Stars represent the catalytic acid/base and nucleophile residues. Open circles with the number beneath represent residues to be at the -1, +1, +2, +3 and +4 subsites based on the Os3BGlu7 3D structure (Data Bank entries 1RGM and 3F5K) as predicted by Chuenchor et al., (2008) and Kuntothom et al. (2009). Triangles mark the amino acids in close contact with the glucose residue at -1 subsite in Os3BGlu7 (Chuenchor et al., 2008). The alignment was generated using the Clustal X implementation of Clustal W (Jeanmougin et al., 1998; Thompson et al., 1994).

คำย่อ (Abbreviations)

AC, accession number; DP, degree of polymerization; ESI-MS, electrospray ionization-mass spectroscopy; GLC, gas liquid chromatography; GH1, glycosyl hydrolase family 1; GST-OsEGL1, glutathione-*S*-transferase-rice endoglucanase OsEGL1 fusion protein; MS, mass spectroscopy; PMAAs, partially methylated alditol acetates; *p*NP, *p*-nitrophenyl; TLC, thin layer chromatography; Trx-Os4BGlu12, thioredoxin-rice β-glucosidase Os4BGlu12 fusion protein.

5. สรุป (Conclusions)

In summary, this work explored the possible functions of rice Os4BGlu12, the transcript level of which was previously found to be induced in response to herbivore attack and salinity stress (Kawasaki et al., 2001; Wang et al., 2005). The expression of this gene was also up-regulated in response to wounding and wound-signaling related phytohormones. The recombinant Os4BGlu12 hydrolyzed three types of substrates, including oligosaccharides, a steroid glycoside, and a flavonol glycoside, which suggests that it might play multiple roles in rice, such as in defense and cell wall remodeling during development or wounding. The similar expression patterns of rice OsEGL1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase in wounding response and the action of Os4BGlu12 on the products of OsEGL1 support their acting in a concerted fashion. The Os4BGlu12 protein appears to be secreted outside the cell, where it may be involved in release of glucose from cell wall derived oligosaccharides in these conditions. Furthermore, the amino acid residues predicted to line the active site of Os4BGlu12 are quite distinct from those in other oligosaccharide hydrolases but close to those of cyanogenic and flavonoid β-glucosidases, supporting its possible role in hydrolysis of defensive glycosides.

เอกสารอ้างอิง (References)

Akiyama, T., Jin, S., Yoshida, M., Hoshino, T., Opassiri, R., and Ketudat Cairns, J.R. (2009). Expression of an endo-(1,3;1,4)-β-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings. J. Plant Physiol. 166: 1814-1825.

Akiyama, T., Pillai, M.A., and Sentoku, N. (2004). Cloning, characterization and expression of OsGLN2, a rice endo-1,3-β-glucanase gene regulated developmentally in flowers and hormonally in germinating seeds. Planta. 220: 80-86.

Bachem, C.W., van der Hoeven, R.S., de Bruijin, S.M., Vreugdenhil, D., Zabeau, M., and Visser, R.G. (1996). Visualization of differential gene expression using novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J. 9:745-753.

Barrett, T., Suresh, C.G., Tolley, S.P., Dodson, E.J., and Hughes, M.A. (1995). The crystal structure of a cyanogenic β-glucosidase from white clover, a family 1 glycosyl hydrolase. Biology. 3: 951-960.

Brummell, D.A., Bird, C.R., Schuch, W. and Bennett, A.B. (1997). An endo-1,4-β-glucanase expressed at high levels in rapidly expanding tissues. Plant Mol. Biol. 33: 87-95.

Buchanan, B.B., Gruissem, W. and Jones R.L. (2000). Biochemistry and Molecular Biology of Plants. John Wiley and Sons, Inc., New Jersey.

Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant J. 34: 351-362.

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

Carpita, N. and McCann, M. (2000). The cell wall. In *Biochemistry and Molecular Biology of Plants*, BB. Buchanan, W. Gruissem and RLJones (eds.). American Society of Plant Physiologists, Maryland. pp. 52-108.

Catalá, C., Rose, J.K.C. and Bennett, A.B. (1997). Auxin regulation and spatial localization of an endo-1,4-β-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls.Plant J. 12: 417-426.

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

Czjzek, M., Cicek, M., Zamboni, V., Bevan, D.R., Henrissat, B., and Esen, A. (2000). The mechanism of substrate (aglycone) specificity in β-glucosidases is revealed by crystal structures of mutant maize β-glucosidase-DIMBOA, -DIMBOAGlc, and -dhurrin complexes. Proc. Natl. Acad. Sci. USA. 97: 13555-13560.

Davies, G.J. and Henrissat, B. (2002). Plant glyco-related genomics. Biochem Soc Trans. 30: 291-297.

del Campillo, E. (1999). Curr. Top. Dev. Biol. 46: 39-61.

del Campillo, E. and Bennett, A.B. (1996). Plant Physiol. 111: 813-820.

Doy, R.H. and Kosugi, A. (2004). Cellulosomes: Plant-cell-wall-degrading enzyme complexes. Nature Reviews Microb. 2: 541-551.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002). The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. Plant Cell. 14: 1557-1566.

Hatfield, R.D., and Nevins, D.J. (1987). Hydrolytic activity and substrate specificity of an endoglucanase from *Zea mays* seedling cell wall. Plant Physiol. 83: 203-207.

Hayashi, T., and Maclachlan, G. (1984). Plant Physiol. 75: 596-604.

Hematy, K., Sado, P.E., van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J.P., and Hofte, H. (2007). A receptor-like kinase mediates the response of Arabidopsis cells to the inhibition of cellulose synthesis. Curr. Biol. 17: 922-931.

Hiromi, K., Nitta, Y., Namura, C., and Ono, S. (1973). Subsite affinities of glucoamylase: examination of the validity of the subsite theory. Biochim. Biophys. Acta. 302: 362-375.

His, I., Driouich, A., Nicol, F., Jauneau, A., and Höfte, H. (2001) Planta. 212: 348–358.

Hoj, K. R. and Fincher G.B. (1995). Molecular evolution of plant β-glucan-endohydrolases. Plant J. 7: 367-379.

Hrmova, M., and Fincher, G.B. (2001a). Plant enzyme structure: Explaining substrate specificity and the evolution of function. Plant Phyiol. 125: 54-57.

Hrmova, M., and Fincher, G.B. (2001b). Structure-function relationship of β-glucan exohydrolases from higer plants. Plant Mol. Biol. 47: 73-91.

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

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., and Gibson, T.J. (1998). Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23: 403-405.

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

Kim, J.S., Reuhs, B.L., Michon, F., Kaiser, R.E., and Arumugham, G. (2006). Addition of glycerol for improved methylation linkage analysis of polysaccharides. Carbohydr. Res. 341: 1061-1064.

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

Lashbrook, C.C., Gonzalez-Bosch, C., and Bennett, A.B. (1994). Plant Cell. 6: 1485-1493.

LeÓn, J., Rojo, E., and Sanchez-Serrano, J.J. (2001). Wounding signaling in plants. J. Exp. Bot. 52: 1-9.

Lynd, L.R. (1996).Overview and evaluation of fuel ehtanol from cellulosic biomass: technology, economics, the environment, and policy. Annu. Rev. Energy Environ. 21:403-465.

Maclachlan, G. and Carrington, S. (1991) In Biosynthesis and Biodegradation of Cellulose. Edited by Haigler, C.H. and Weimer, P.J. pp 599–621, Marcel Dekker, New York.

Marana, S.R. (2006). Molecular basis of substrate specificity in family 1 glycoside hydrolases. IUBMB Life. 58: 63-73.

McCloud, E.S., and Baldwin, I.T. (1997). Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. Planta. 203: 430-435.

Mølhøj, M., Pagant, S. and Höfte, H. (2002). Towards understanding the role of membrane-bound endo-β-1,4-glucanases in cellulose biosynthesis. Plant Cell Physiol. 43: 1399-1406.

Naoumkina, M., Farag, M.A., Sumner, L.W., Tang, Y., Liu, C.J., and Dixon, R.A. (2007). Different mechanisms for phytoalexin induction by pathogen and wound signals in Medicago truncatula. Proc. Natl. Acad. Sci. USA. 104:17909-17915.

Nielsen, K.A., Hrmova, M., Nielsen, J.N., Forslund, K., Ebert, S., Olsen, C.E., Fincher, G.B., Lindberg Möller, B. (2006). Reconstruction of cyanogenesis in barley (*Hordeum vulgare* L.) and its implications for resistance against the barley powdery mildew fungus. Planta. 223:1010-1023.

Nishizawa, Y., Saruta, M., Nakazono, K., Nishio, Z., Soma, M., Yoshida, T., Nakajima, E., and Hibi, T. (2003). Characterization of transgenic rice plants over-expressing the stress-inducible β-glucanase gene Gns1. Plant Mol. Biol. 51: 143-152.

Ohmiya, Y., Samejima, M., Shiroishi, M., Amano, Y., Kanda, T., Sakai, F. and Hayashi, T. (2000). Plant J. 24: 147–158.

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

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

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

Pilling, E., and Hofte, H. (2003). Feedback from the wall. Curr. Opin. Plant Biol. 6: 611-616.

Rakwal, R., Agrawal, G.K., and Yonekura, M. (1999). Separation of proteins from stressed rice (*Oryza sativa* L.) leaf tissues by two-dimensional polyacrylamide gel electrophoresis: induction of pathogenesis-related and cellular protectant proteins by jasmonic acid, UV irradiation and copper chloride. Electrophoresis. 20: 3472-3478.

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu. Rev. Plant. Biol. 57: 675-709.

Sambrook, J., Fritsch, E.F., and Maniatis, T.A. (1989). Molecular Cloning: a Laboratory Manual, second ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., and Youngs, H. (2004). Toward a systems approach to understanding plant cell walls. Science 306: 2206-2211.

Taiz, L. (1984). Plant cell expansion: regulation of cell wall mechanical properties. Annu. Rev. Plant physiol. 35: 585-657.

Thomas, B.R., Inouhe, M., Simmons, C.R., and Nevins, D.J. (2000). Endo-1,3;1,4-β-glucanase from coleoptiles of rice and maize: role in the regulation of plant growth. Int J Biol Macromol. 27: 145-149.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

Verdoucq, L., Czjzek, M., Moriniere, J., Bevan, D.R., and Esen, A. (2003). Mutational and structural analysis of aglycone specificity in maize and sorghum β-glucosidases. J. Biol. Chem. 278: 25055-25062.

Verdoucq, L., Morinière, J., Bevan, D.R.., Esen, A., Vasella, A., Henrissat, B., and Czizek, M. (2004). Structural determinants of substrate specificity in family 1 β-glucosidases. J. Biol. Chem. 279: 31796-31803.

von Dahl, C.C., and Baldwin, I.T. (2007). Deciphering the role of ethylene in plant-herbivore interactions. J. Plant Growth Regul. 26: 201-209.

Wang, X., He, R., and He, G. (2005). Construction of suppression subtractive hybridization libraries and identification of brown planthopper-induced genes. J. Plant Physiol. 162: 1254-1262.

Yamaguchi, T., Nakayama, K., Hayashi, T., Tanaka, Y., and Koike, S. (2002). Molecular cloning and characterization of a novel β-1,3-glucanase gene from rice. Biosci. Biotechnol. Biochem. 66: 1403-1406.

Yoshida, K., and Kome, K. (2006). A rice family 9 glycoside hydrolase isozyme with broad substrate specificity for hemicelluloses in type II cell walls. Plant Cell Physiol. 47: 1541-1554.

Yoshida, K., Imaizumi, N., Kaneko, S., Kawagoe, Y., Tagiri, A., Tanaka, H., Nishitani, K., and Komae, K. (2006). Carbohydrate-binding module of a rice endo-beta-1,4-glycanase, OsCel9A, expressed in auxin-induced lateral root primordia, is post-translationally truncated. Plant Cell Physiol. 47: 1555-1571.

Zheng, B.S., Yang, L., Zhang, W.P., Mao, C.Z., Wu, Y.R., Yi, K.K., Liu, F.Y., and Wu, P. (2003). Mapping QTLs and candidate genes for rice root traits under different water-supply conditions and comparative analysis across three populations. Theor. Appl. Genet. 107: 1505-1515.

Zhou, H.L., He, S.J., Cao, Y.R., Chen, T., Du, B.X., Chu, C.C., Zhang, J.S., and Chen, S.Y. (2006). OsGLU1, a putative membrane-bound endo-1,4-beta-D-glucanase from rice, affects plant internode elongation. Plant Mol. Biol. 60: 137-151.

Ziegler, J., Keinänen, M., and Baldwin, I.T. (2001). Herbivore-induced allene oxide synthese transcripts and jasmonic acid in Nicotiana attenuata. Phytochemistry. 58: 729-738.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

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

(Journal Impact Factor ในปี 2009 เท่ากับ 2.05). * corresponding author

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงสาธารณะ

มีเครือข่ายความร่วมมือทางวิชาการกับนักวิจัย ในต่างประเทศ

- Dr. Takashi Akiyama (National Agricultural Research Center for Hokkaido Region, JAPAN) ให้คำแนะนำปรึกษาและช่วยเหลือเกี่ยวกับการวิเคราะห์การย่อยสลายผนังเซลล์ข้าว และการศึกษาการแสดงออกของยีนในข้าว
- Prof. Dr. Atsuo Kimura และ Dr. Shigeki Jin (Hokkaido University, JAPAN) ให้คำแนะนำปรึกษาและช่วยเหลือเกี่ยวกับเทคนิคการวิเคราะห์โครงสร้างน้ำตาลที่ได้จากการย่อยสลายด้วยเอนไซม์

- เชิงวิชาการ

ความรู้ที่ได้ ได้นำมาใช้ในการพัฒนาการเรียนการสอน ในวิชาชีวเคมีระดับปริญญาตรี และบัณฑิตศึกษา วิชาอื่นๆ ที่เกี่ยวข้องกับชีวเคมี

3. อื่นๆ

ได้นำเสนอผลงานในที่ประชุมวิชาการระดับนานาชาติ

Opassiri, R., Maneesan, J., Akiyama, T., Ketudat-Cairns, J.R. (2009). Expression of rice cell wall hydrolysis enzyme in response to environmental stresses. The 3rd International Symposium on the Environmental Physiology of Ectotherms and Plants (ISEPEP3). 24-28 August 2009. Tsukuba Japan. (poster No. P-28)

ภาคผนวก

บทความสำหรับเผยแพร่

ผนังเซลล์พืชที่เหลือทิ้งจากภาคเกษตรกรรมสามารถนำมาใช้เป็นวัตถุดิบในการแปรรูปเป็นสารผลิตภัณฑ์น้ำตาลได้ งานวิจัยส่วนใหญ่ได้ให้ความสนใจไปที่การใช้เอนไซม์ที่ได้จากราและแบคทีเรียในการย่อยผนังเซลล์พืช แต่มีงานวิจัยเพียงเล็กน้อยที่ศึกษาเอนไซม์เหล่านี้ในพืช ผู้วิจัยจึงสนใจที่จะศึกษาเอนไซม์ข้าวที่ทำหน้าที่ย่อยสลายผนังเซลล์ข้าว ได้แก่ เอนไซม์ β-glucosidase และ endoglucanase จากข้อมูลลำดับนิวคลีโอไทด์ในจีโนมของข้าวพบว่าเอนไซม์ทั้งสองกลุ่มมีความหลากหลายทั้งโครงสร้างและลำดับกรดอะมิโนเป็นอย่างมาก ซึ่งอาจมีความสัมพันธ์กับกระบวนการสร้างและสลายผนังเซลล์ที่ต้องใช้เอนไซม์เข้าร่วมหลายชนิด อย่างไรก็ตามยังไม่มีงานวิจัยที่มีหลักฐานยืนยันถึงการทำงานของร่วมกันของเอนไซม์นี้

การศึกษานี้ได้ศึกษาการทำงานร่วมกันระหว่างเอนไซม์ β-glucosidase ไอโซไซม์ Os4BGlu12 และ endoglucanase จากการศึกษาการแสดงออกของยีนโดย พบว่า mRNA ของยีน *Os4bglu12* มีปริมาณใกล้เคียงหรือลดลงเล็กน้อยเมื่อเทียบกับกลุ่มควบคุม ในต้นอ่อนข้าวที่ปลูกในสภาวะเครียด ได้แก่ ความร้อน แห้งแล้ง ความเค็ม ค่าแรงดันน้ำสูง และที่ได้รับฮอร์โมนจิบเบอเรลลิกเอซิด และมีปริมาณลดลงอย่างมากเมื่อปลูกภายได้สภาวะเครียด ได้แก่ ความเย็น และ น้ำท่วม และเมื่อได้รับฮอร์โมนแอบซิสสิกเอซิด ปริมาณ mRNA ของยีน *Os4bglu12* เพิ่มขึ้นอย่างมากในต้นอ่อนข้าวที่มีบาดแผล และที่ได้รับฮอร์โมนเมทิลจัสโมเนทและอีเทฟอน จากการส่งถ่ายดีเอ็นเอสายผสมที่มียีน *Os4bglu12* ต่ออยู่กับยีน Gus (glucuronidase) ซึ่งเป็นยีนติดตามไปในเซลล์หัวหอม พบว่ามีโปรตีนสายผสมของโปรตีนที่ผลิตขึ้นจากดีเอ็นเอสายผสมนี้อยู่ตรงช่องว่างระหว่างเซลล์หรือผนังเซลล์ของเซลล์ เพื่อให้เข้าใจถึงหน้าที่ของเอนไซม์มากขึ้นจึงได้นำโปรตีนสายผสมของเอนไซม์ Os4BGlu12 β-glucosidase ที่ผลิตได้แบคทีเรีย *Escherichia coli* มาทดสอบกับสับสเตรทชนิดต่างๆ พบว่าเอนไซม์สามารถย่อยกลูโคโอลิโกแซคคาไรด์ที่มีพันธะ β-(1,3;1,4) ซึ่งเป็นผลิตภัณฑ์ที่ได้จากการย่อยผนังเซลล์ข้าวด้วย endo-1,3;1,4-β-glucanase ไอโซไซม์ OsEGL1 ของข้าวซึ่งเป็นเอนไซม์ที่มีการแสดงออกเพิ่มขึ้นเมื่อข้าวเกิดบาดแผล จึงเป็นไปได้ว่าเอนไซม์ทั้งสองอาจทำงานร่วมกันในการปรับเปลี่ยนโครงสร้างผนังเซลล์ ในการทดสอบเอนไซม์กับโอลิโกแซคคาไรด์ชนิดต่างๆ พบว่า Os4BGlu12 ย่อยกลูโคโอลิโกแซคคาไรด์ที่มีพันธะ β-(1,4) ที่มีจำนวนกลูโคสต่อกัน 3-6 หน่วย ได้ดีที่สุด และย่อยไดแซคคาไรด์ของกลูโคสที่มีพันธะ β-(1,3) ได้ดีเช่นกัน จากการศึกษานี้จึงคาดการว่า endoglucanase น่าจะทำงานร่วมกับ β-glucosidase ในการย่อย cellulose หรือ β-glucans ชนิดต่างๆ ของผนัง เพื่อนำโอลิโกแซคคาไรด์ หรือน้ำตาลโมเลกุลเดี่ยวที่ย่อยได้หมุนเวียนกลับไปสังเคราะห์ cellulose, hemicellulose หรือ pectin ในช่วงที่พืชมีการเปลี่ยนแปลงโครงสร้างของผนังเซลล์ นอกจากนี้เอนไซม์ Os4BGlu12 อาจจะทำหน้าที่ในระบบป้องกันเซลล์ เพราะในการทดสอบกับไกลโคไซด์ที่พบในธรรมชาติพบว่าเอนไซม์ย่อยดีออกซีคอร์ติโคสเตอโรนกลูโคไซด์และเอพิเจนินกลูโคไซด์ได้ดีมาก

ความรู้ที่ได้จากการศึกษาดังกล่าวนอกจากจะเป็นประโยชน์ต่อการศึกษาถึงบทบาทของเอนไซม์เหล่านี้ในการเจริญพัฒนาของข้าวแล้วยังอาจนำเอนไซม์ที่มีความจำเพาะกับสับสเตรตรูปแบบที่แตกต่าง

กันไปใช้ในการย่อยสลายผนังเซลล์พืชร่วมกับเอนไซม์อื่นๆ เพื่อนำน้ำตาลที่ได้มาใช้ประโยชน์ในอุตสาหกรรมการผลิตสารต่างๆ เช่น แอลกอฮอร์, และกรดอะมิโน เป็นต้น และยังเป็นข้อมูลพื้นฐานในการศึกษากลไกการทำงานของเอนไซม์ด้วยเทคนิควิศวกรรมเอนไซม์ต่อไป

Plant Science 179 (2010) 273–280

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Rice Os4BGlu12 is a wound-induced β-glucosidase that hydrolyzes cell wall-β-glucan-derived oligosaccharides and glycosides

Rodjana Opassiri[a,*], Janjira Maneesan[a], Takashi Akiyama[b], Busarakum Pomthong[c], Shigeki Jin[d], Atsuo Kimura[e], James R. Ketudat Cairns[a]

[a] School of Biochemistry, Institute of Science, Suranaree University of Technology, Muang District, Nakhon Ratchasima 30000, Thailand
[b] Department of Low-Temperature Science, National Agricultural Research Center for Hokkaido Region, Sapporo 062-8555, Japan
[c] Faculty of Sciences and Technology, Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima 30000, Thailand
[d] Graduate School of Health Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan
[e] Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

ARTICLE INFO

Article history:
Received 25 March 2010
Received in revised form 24 May 2010
Accepted 27 May 2010
Available online 4 June 2010

Keywords:
β-Glucosidase
Endo-(1,3 ;1,4)-β-glucanase
Glycosides
Rice
Cell wall
Wound

ABSTRACT

Rice Os4BGlu12 β-glucosidase is a family 1 glycoside hydrolase, the transcript levels of which have previously been found to be induced in response to herbivore attack and salinity stress. Here, high levels of *Os4bglu12* transcripts were also detected in the shoot during germination, in the leaf sheath and stem of mature rice plants under normal growth conditions. The transcripts of this gene were up-regulated in response to wounding, methyl jasmonate and ethephon in 10-day-old rice seedlings. Os4BGlu12 expressed in recombinant *Escherichia coli* hydrolyzed β-(1,3;1,4)-glucooligosaccharides generated by the wounding-induced rice endo-(1,3;1,4)-β-glucanase OsEGL1, suggesting that both enzymes may act in concert in remodeling of damaged cell wall. Among oligosaccharides tested, Os4BGlu12 hydrolyzed β-(1,4)-linked glucooligosaccharides with highest catalytic efficiency (k_{cat}/K_m = 2.7–4.9 s^{-1} mM^{-1}) when the degree of polymerization ranged from 3 to 6. It also hydrolyzed the β-(1,3)-linked disaccharide laminaribiose with high catalytic efficiency (k_{cat}/K_m = 4.5 s^{-1} mM^{-1}). Among the natural glycosides tested, Os4BGlu12 efficiently hydrolyzed deoxycorticosterone 21-glucoside (k_{cat}/K_m = 20 s^{-1} mM^{-1}) and apigenin 7-*O*-β-D-glucoside (k_{cat}/K_m = 6.7 s^{-1} mM^{-1}). The amino acid residues predicted to line the active site of Os4BGlu12 are more similar to those of cyanogenic and flavonoid β-glucosidases than oligosaccharide hydrolases, and it may function in defense, as well as in cell wall-derived oligosaccharide break-down.

1. Introduction

Plant glycosyl hydrolase family 1 (GH1) β-glucosidases (EC 3.2.1.21) hydrolyze the β-*O*-glycosidic bond at the anomeric carbon of glucose moieties at the nonreducing end of carbohydrate or glycoside molecules [1]. The glycones recognized by GH1 β-glycosidases include glucose, galactose, fucose, mannose, xylose, 6-phospho-glucose and 6-phospho-galactose. The diversity of aglycones is higher, including monosaccharides, oligosaccharides and aryl or alkyl groups. The physiological functions of these enzymes in plants based on the activities of the aglycone moieties of substrate include (1) defense against pathogens and herbivores [2–4], (2) phytohormone activation [5,6], (3) lignification [7], (4) cell wall catabolism [8,9] and (5) release of active metabolic intermediate molecules [10]. The GH1 enzymes may hydrolyze substrates with a broad range of different glycones or aglycones with different specificities, but some enzymes may be specific for only one type of glycone or aglycone. The fundamental substrate specificity of these enzymes depends on the overall dimensions and geometry of the binding site and the distribution of the active site amino acids that are important for the substrate recognition and binding, which complement the structure of the aglycone and glycone moieties of the substrate [11,12].

Forty genes homologous to GH1 β-glucosidases have been identified in rice genomic sequences, 34 of which appear to be functional in rice [13]. To date, only a few rice β-glucosidase isoenzymes have been characterized for their possible function. Partially purified β-glucosidases from rice were described that

Abbreviations: DP, degree of polymerization; ESI-MS, electrospray ionization-mass spectroscopy; GH1, glycosyl hydrolase family 1; GLC, gas liquid chromatography; GST-OsEGL1, glutathione-*S*-transferase-rice endoglucanase OsEGL1 fusion protein; MS, mass spectroscopy; PMAAs, partially methylated alditol acetates; *p*NP, *p*-nitrophenyl; TLC, thin layer chromatography; Trx-Os4BGlu12, thioredoxin-rice β-glucosidase Os4BGlu12 fusion protein.

* Corresponding author. Tel.: +66 44 224598; fax: +66 44 224185.
E-mail addresses: opassiri@sut.ac.th (R. Opassiri), jmaneesan@hotmail.com (J. Maneesan), takiyama@affrc.go.jp (T. Akiyama), busarakum_p@yahoo.com (B. Pomthong), s-jin@hs.hokudai.ac.jp (S. Jin), kimura@abs.agr.hokudai.ac.jp (A. Kimura), cairns@sut.ac.th (J.R.K. Cairns).

0168-9452/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2010.05.013

hydrolyze gibberellin glucosides [5]. Akiyama et al. [9] determined the N-terminal sequence of a cell wall-bound β-glucosidase that preferentially hydrolyzed cellooligosaccharides and laminarioligosaccharides. Opassiri et al. [14] reported the recombinant rice BGlu1, later designated Os3BGlu7, showed strong hydrolysis and glucotransferase activity with cellooligosaccharides and laminarioligosaccharides and hydrolyzed natural glycosides with low activity. Recently, Kuntothom et al. [15] characterized two rice β-glycosidases which were grouped in the same phylogenetic cluster with Os3BGlu7 β-glucosidase and plant β-mannosidases. Os3BGlu8, which has a protein sequence similar to Os3BGlu7, hydrolyzed cellooligosaccharides but not mannooligosaccharides, while Os7BGlu26, the sequence of which is more closely related to plant β-mannosidases, could hydrolyze both cellooligosaccharides and mannooligosaccharides. Seshadri et al. [16] reported that recombinant rice Os3BGlu6 preferentially hydrolyzed *n*-octyl β-D-glucoside and β-(1,3)- and β-(1,2)-linked disaccharides, and hydrolyzed apigenin 7-*O*-β-D-glucoside and several other natural glycosides, but at low catalytic rates.

Previously, the *Os4bglu12* cDNA (rice genome locus Os04g0474800) for a rice β-glucosidase, which encodes a protein with high amino acid sequence identity with the N-terminal sequence of a cell wall-bound β-glucosidase purified from rice [9], was expressed as a soluble active protein in *Escherichia coli* [13]. The preliminary analyses showed that it possessed hydrolytic activity on cellooligosaccharides and laminaribiose. While its activity appeared to be somewhat similar to that of the rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 β-glucosidases, Os4BGlu12 was classified in a distinct group on the phylogenetic tree, more closely related to dicotyledon defense β-glucosidases. Functional genomics studies have shown that the transcript level of the *Os4BGlu12* gene is induced in response to herbivore attack and salinity stress [17,18], but a thorough analysis of its expression patterns has yet to be reported.

To further investigate the physiological function of rice Os4BGlu12, we determined the expression patterns of this gene in rice tissues and in rice seedlings after wounding and treatment with phytohormones by RNA gel blot analysis. Kinetic analysis of the enzyme was also performed to evaluate the substrate specificity of Os4BGlu12 against various artificial glycosides, oligosaccharides and natural glycosides.

2. Materials and methods

2.1. Materials

The soluble active recombinant thioredoxin-Os4BGlu12 fusion protein (Trx-Os4BGlu12) was expressed in Origami B(DE3) *E. coli*, and purified as described previously [13]. The rice OsEGL1 endo-(1,3;1,4)-β-glucanase was expressed as a glutathione-*S*-transferase fusion protein, GST-OsEGL1, in DH5α *E. coli* and purified as described previously [19]. Sophorose, *p*-nitrophenyl (*p*NP) glycosides, D-amygdalin, indoxyl β-D-glucoside, salicin, phlorizin dihydrate, linamarin, naringin, deoxycorticosterone 21-glucoside, and gossypin were purchased from Sigma Chemical Co. (St. Louis, MO). Apigenin 7-*O*-β-D-glucoside and quercetin 3-β-D-glucoside were purchased from Fluka (Steiheim, Swizerland). Cellooligosaccharides with degree of polymerization (DP) of 2–6 and laminarioligosaccharides (DP 2–4) were from Seikagaku Kogyo Co. (Tokyo, Japan).

2.2. RNA gel blot analysis

Rice (*Oryza sativa* L. cv. Yukihikari) seeds were germinated in the dark for 4 days at 28 °C and then grown in a 12 h light–12 h dark cycle from day 4 to day 10 at 28 °C and moistened with sterile distilled water. Some 7-day-old seedlings were harvested and dissected into separate parts (shoot, root and endosperm). Other seedlings were transferred to soil and grown for an additional 4–5 weeks to reach the flowering stage. Rice plants were harvested and separated to six parts (flower, stem, root, node, leaf blade and leaf sheath). Ten-day-old rice seedlings were treated with wounding, 10^{-4} M methyl jasmonate and 10^{-4} M ethephon for different time courses. All plant samples were kept at −70 °C for RNA isolation.

A gene-specific probe for *Os4bglu12* was amplified from a rice genomic DNA as the template with the 445-3′end forward (5′-ATGGAGCAAACGTGAAGGGAT-3′) and 445-3′UTR reverse (5′-AACTGGATTACTTCCATCTC-3′) primers derived from the coding sequence at the C-terminal part and 3′-untranslated region of the gene, respectively. The amplification was performed with 30 cycles at 94 °C 45 s, 45 °C 45 s, and 72 °C 1 min, using *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN).

Total RNA was isolated from rice tissues by the SDS–phenol method described by Bachem et al. [20]. Thirty micrograms of total RNA from each sample was denatured and electrophoresed on a 1.2% formaldehyde–agarose gel and transferred onto a Hybond N+ nylon membrane (GE Healthcare, Buckinghamshire, UK) by standard procedures [21]. The probe was labeled by Rediprime II random priming with α-[^{32}P]dCTP (GE Healthcare) and used for hybridization with RNA blots for 16 h at 42 °C. The blots were then washed once with 0.1% SDS, 2× SSC for 30 min at 65 °C and washed twice with 0.1% SDS, 0.1× SSC for 15 min at 65 °C, then exposed to a Fuji film imaging plate for 16 h at room temperature. The positions of radioactive bands were visualized with a Fuji Film BAS 1000 BioImaging Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.3. Hydrolysis of crude cell walls by recombinant rice OsEGL1 and Os4BGlu12

Crude cell wall was prepared by grinding 10-day-old rice seedling leaves to a fine powder and then washing the powder 4 times with 2 vol. of 80 °C 90% (v/v) ethanol, and the cell wall pellets were dried under vacuum. In a 10 mL reaction containing 300 mg crude cell wall powder, 100 pg of the GST-OsEGL1 in 50 mM sodium acetate, pH 5.0, was incubated at 37 °C for 6 h. The reaction was stopped by boiling for 10 min, and then centrifuged at 7000 × *g* for 20 min. The supernatant was desalted with AG501-X8D ion exchange resin (Bio-Rad, Richmond, CA) and then evaporated to dryness under vacuum. The concentrated fraction was dissolved in 200 μL distilled water and then separated by thin layer chromatography (TLC) as described previously [22]. To isolate each sugar product, only edge parts (1 cm in width) of both sides of the developed silica gel plate were cut off for detection of sugar spots, and the silica gel layer at the positions of the sugars was scratched off from the unstained plate and packed into a column (1.5 cm inner diameter, 9 cm in length). The sugars were eluted from a column with 5 vol. of 80% (v/v) ethanol and evaporated to dryness under vacuum.

The cell wall hydrolysis products were assayed with 1 μg Trx-Os4Glu12 in 50 mM sodium acetate, pH 5.0, in 50 μL reaction volume at 37 °C for 0, 10, 30 and 60 min. The reaction was stopped by boiling for 10 min and the products were detected by TLC as described above.

2.4. Chemical characterization of cell wall hydrolysis products

Molecular weights of purified cell wall hydrolysis products were analyzed by electrospray ionization-mass spectrometry (ESI-MS). ESI-MS measurements were performed on a time-of-flight mass spectrometer JMS-T100CS (JEOL, Japan) using direct injection. The measurement conditions were as follows: scan mode, positive

Table 1
Kinetic parameters of rice Os4BGlu12 in the hydrolysis of oligosaccharides, *p*NP glycosides and natural glycosides.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Cellooligossaccharides (DP[a])			
2	1.8 ± 0.3	25 ± 3	0.073 ± 0.003
3	12 ± 2	4.6 ± 0.0	2.7 ± 0.3
4	17 ± 1	5.7 ± 0.5	3.0 ± 0.2
5	24 ± 3	4.8 ± 0.5	4.9 ± 0.1
6	18 ± 2	4.8 ± 0.6	3.8 ± 0.2
Laminarioligosaccharides (DP)			
2	23 ± 1	5.1 ± 0.2	4.5 ± 0.0
3	ND[b]	ND	ND
4	ND	ND	ND
Sophorose	5.2 ± 0.3	4.4 ± 0.4	1.2 ± 0.1
(1,3;1,4)-Glucotriose	0.84 ± 0.04	4.9 ± 0.9	0.18 ± 0.03
(1,3;1,4)-Glucotetraose	2.5 ± 0.2	3.3 ± 0.3	0.77 ± 0.08
*p*NP β-D-glucoside	20 ± 1	0.80 ± 0.08	24 ± 1
*p*NP β-D-fucoside	18 ± 1	0.39 ± 0.04	47 ± 2
*p*NP β-D-galactoside	12 ± 1	1.4 ± 0.2	8.5 ± 1.3
*p*NP β-D-xyloside	6.0 ± 0.3	0.24 ± 0.04	25 ± 0
*p*NP α-L-arabionoside	2.3 ± 0.1	0.12 ± 0.03	23 ± 4
Deoxycorticosterone 21-glucoside	13 ± 2	0.68 ± 0.18	20 ± 2
Indoxyl β-D-glucoside	2.9 ± 0.2	4.4 ± 0.5	0.67 ± 0.02
D-Amygdalin	0.25 ± 0.01	5.8 ± 0.2	0.042 ± 0.000
Linamarin	0.28 ± 0.04	9.4 ± 1.4	0.031 ± 0.000
Apigenin 7-*O*-β-D-glucoside	0.83 ± 0.02	0.12 ± 0.01	6.7 ± 1.6
Phlorizin	ND	ND	ND
Quercetin 3-β-D-glucoside	ND	ND	ND
Gossypin	ND	ND	ND
Naringin	ND	ND	ND
Salicin	ND	ND	ND

[a] Means degree of polymerization.
[b] Means not detectable.

ion; needle voltage, 2.0 kV; orifice voltage, 60 V; desolvation temperature, 80 °C; sample injection rate, 6 μL min^{-1}; solvent, 0.05% aqueous trifluoroacetic acid.

To analyze the sugar linkages in products released from cell wall by rice OsEGL1, partially methylated alditol acetates (PMAAs) were prepared from each product (1–2 mg) following the method of Kim et al. [23]. The PMAAs were separated and analyzed with a JMS-AX500 (JEOL, Japan). The gas liquid chromatography (GLC) peaks were identified by mass spectrometry (MS) on an electron-impact ionization mode scanning from 50 to 500 *m/z*. PMAA standards were prepared from cellotriose and laminaritriose.

2.5. Enzyme assays and kinetic analysis

Kinetic parameters were determined for *p*NP glycosides, oligosaccharides, and commercially available natural glycosides from triplicate assays of 5–7 substrate concentrations at time points and enzyme concentrations where the reaction rate was linear and the absorbance value was in the range of 0.1–1.0. The assays were done at 37 °C in 50 mM sodium acetate, pH 5.0, which are the optimum temperature and pH for Os4BGlu12 [13]. To determine sugar specificity, in a 100 μL reaction, 1.45 pmol Trx-Os4BGlu12 was incubated with *p*NP glycosides. The reactions were stopped by adding 50 μL of 0.4 M sodium carbonate, and the amount of *p*-nitrophenol released was determined from its A_{405}. Hydrolysis of oligosaccharides and commercially available natural glycosides (listed in Table 1) was assayed in 50 μL reaction volumes containing 1.81–14.5 pmol enzyme, the reactions were stopped by boiling, and the glucose released was quantified by the peroxidase/glucose oxidase assay method, as described previously [14]. Kinetic parameters were calculated by nonlinear regression of Michaelis–Menten plots with Grafit 5.0 (Erithacus Software), based on the amount of nonreducing terminal glucosyl residues released in each reaction. The enzyme molarity was calculated based on the molecular weight of 69 kDa for Trx-Os4BGlu12. Protein assays were performed by the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Apparent subsite affinities were calculated from the k_{cat}/K_m values by the method of Hiromi et al. [24].

3. Results

3.1. Expression patterns of Os4bglu12 gene in rice tissues

RNA gel blot analysis detected *Os4bglu12* transcripts in high abundance in the shoot, and at low levels in the root and endosperm in 7-day-old rice seedlings (Fig. 1). In 6-week-old mature rice plants at flowering stage, *Os4bglu12* mRNA was highly expressed in leaf

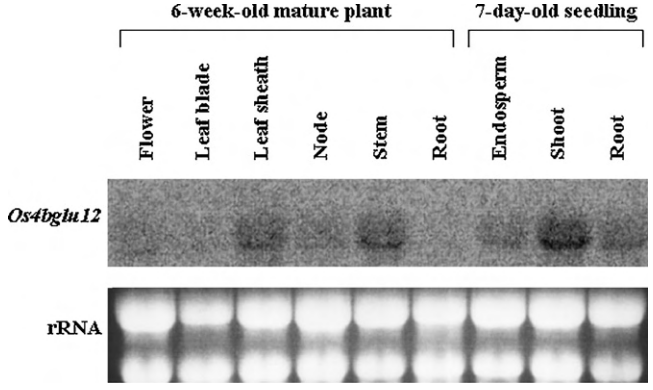


Fig. 1. RNA gel blot analysis of *Os4bglu12* expression levels in various parts of 7-day-old rice seedling and 6-week-old mature plant at flowering stage. The RNA blot was hybridized with the α-^{32}P-labeled 264 bp 3′end fragment of the *Os4bglu12* cDNA probe. Thirty micrograms of total RNA was loaded in each lane on a 1.2% agarose formaldehyde gel. The ethidium bromide-stained gel below the blot indicates the equal RNA loading before blotting.

sheaths and stems. The *Os4bglu12* transcripts were detected with moderate levels in node, flower, leaf blade and very low or no signal was seen in root.

3.2. Effects of wounding and plant hormones on expression of *Os4bglu12* gene in rice seedlings

The expression of *Os4bglu12* was induced as early as 5–10 h after wounding of 10-day-old seedlings and decreased gradually thereafter (Fig. 2A). *Os4bglu12* mRNA levels significantly increased within 10 h after treating with methyl jasmonate, after which the mRNA levels decreased slowly (Fig. 2B). Treatment with ethephon induced a rapid temporary increase in *Os4bglu12* mRNA levels within 10 h, after which the mRNA levels decreased rapidly within 1 day and then decreased gradually (Fig. 2C).

3.3. Hydrolysis of rice cell wall by rice OsEGL1 and Os4BGlu12

The wounding, methyl jasmonate and ethephon induction of expression of *Os4bglu12* gene is similar to that of rice OsEGL1 endo-(1,3;1,4)-β-glucanase, which specifically hydrolyzes (1,3;1,4)-β-glucans [19]. Os4BGlu12 was assayed with oligosaccharides released from rice cell walls by OsEGL1. As seen on the TLC, the hydrolysis of crude rice cell walls by a recombinant OsEGL1 generated three major hydrolysis products, which were denoted as Os1, Os2 and Os3 (Fig. 3). Os4BGlu12 could hydrolyze Os1, which migrated between spots of cellotetraose and cellotriose standards, and the glucose product released by the enzyme increased during incubation from 10 to 60 min (Fig. 3).

3.4. Chemical characterization of cell wall hydrolysis products

In the ESI-MS data, signals of oligosaccharides Os1, Os2, and Os3 were detected at *m*/*z* 698 ([M+Na]$^+$), 527 ([M+Na]$^+$), and 365 ([M+Na]$^+$), respectively. These data suggest Os1, Os2, and Os3 to be a tetrasaccharide, a trisaccharide, and a disaccharide, respectively. The linkage of these oligosaccharides was confirmed by methylation analysis. On the basis of their retention times in GLC and their fragmentation patterns in GC–MS, three permethylated aditol acetates of Os1 and Os2 were identified as 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Those from Os3 were identified as 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (data not shown). The results of the analyses confirmed

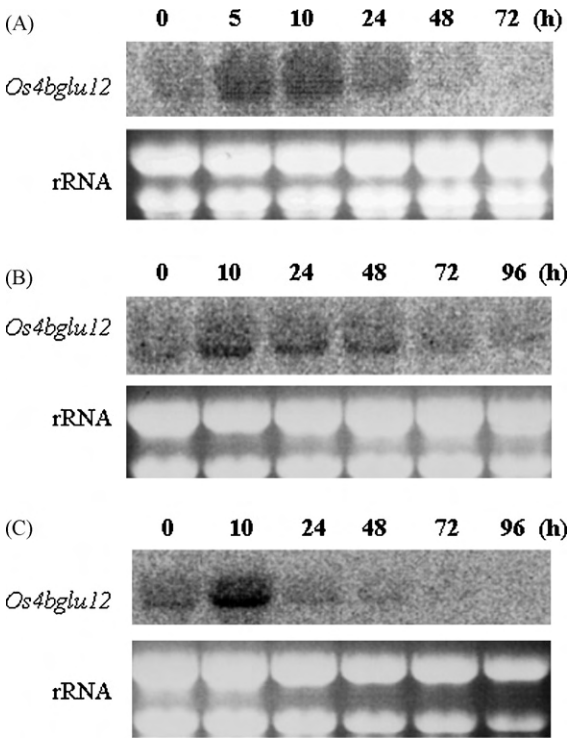


Fig. 2. RNA blot analysis of *Os4bglu12* expression in 10-day-old rice seedlings treated with (A) wounding, (B) methyl jasmonate and (C) ethephon at different time points. Thirty micrograms of total RNA was loaded on a 1.2% agarose formaldehyde gel. The RNA blot was hybridized with the α-^{32}P-labeled 264 bp 3′end fragment of the *Os4bglu12* cDNA probe. The ethidium bromide-stained gel below the blots showed the RNA loading before blotting.

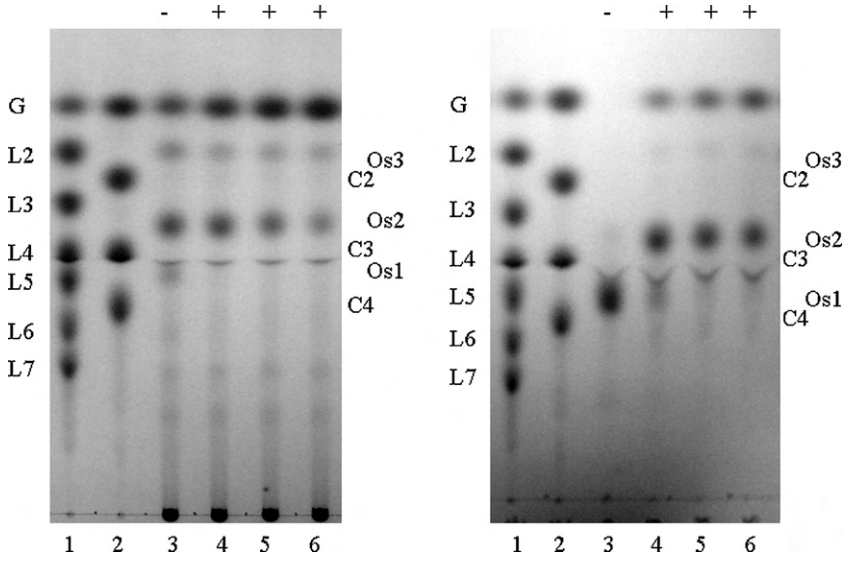


Fig. 3. TLC analysis of rice cell wall hydrolysis by rice OsEGL1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase. (A) Time course of the reaction of Os4BGlu12 with crude cell wall that was pre-incubated with OsEGL1. Lane 3, reaction without Os4BGlu12 (−); lanes 4–6, reactions incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. (B) Os1 product incubated with Os4BGlu12. Lane 3, reaction without Os4BGlu12 (−); lanes 4–6, Os1 reaction incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. Lane 1 in (A) and (B), glucose (G) and laminarioligosaccharide standards of DP 2–7 (L2–L7); lane 2 in (A) and (B), cellooligosaccharide standards of DP 2–4 (C2–C4). Fifty microliters of reaction mixtures composed of Os4BGlu12 (1 μg) and cell wall hydrolysis products in 50 mM sodium acetate buffer, pH 5.0, were incubated at 37 °C and products analyzed by silica gel TLC with acetonitrile/water (3:1 by volume).

that Os1 and Os2 are (1,3;1,4)-linked glucotetraose ($G4G4G3G_{red}$) and glucotriose ($G4G3G_{red}$), respectively, while Os3 is the (1,3)-linked disaccharide of glucose ($G3G_{red}$) [where G represents a β-glucosyl residue, 3 and 4 are (1,3) and (1,4) linkages, respectively; and "red" indicates the reducing terminus].

3.5. Kinetic analysis

The substrate specificity of Os4BGlu12 toward various kinds of disaccharides and oligosaccharides of glucose was evaluated by kinetic analysis. For disaccharides, Os4BGlu12 hydrolyzed sophorose (β-1,2), laminaribiose (β-1,3), and cellobiose (β-1,4), but not gentiobiose (β-1,6). It hydrolyzed cellooligosaccharides with DP of 3–6 and (1,3;1,4)-β-glucooligosaccharides with DP of 3–4, but not laminarioligosaccharides with DP > 2. As shown in Table 1, Os4BGlu12 hydrolyzed laminaribiose with the highest catalytic efficiency (k_{cat}/K_m) value with a relatively high K_m and apparent k_{cat} values, but hydrolyzed sophorose and cellobiose with low efficiency. For cellooligosaccharides, there was a large decrease in K_m and increase in k_{cat} between DP of 2 and 3, after which the K_m remained approximately constant with increasing chain length of the substrate, while the apparent k_{cat} values increased slightly from DP of 3 to 5, but decreased at DP of 6. Catalytic efficiency (apparent k_{cat}/K_m) was within error for DP of 3 and 4, but increased 65% from DP of 4 to 5, so that the apparent k_{cat}/K_m of cellopentaose was 67-fold higher than that of cellobiose. An estimate of subsite affinities based on the assumptions of Hiromi et al. [24], gave apparent subsite affinities of 9.3 ± 0.4 kJ/mol for subsite +2, 0.3 ± 0.4 kJ/mol for the +3 subsite, 1.3 ± 0.2 kJ/mol for the +4 subsite and -0.7 ± 0.2 kJ/mol for the +5 subsite. Os4BGlu12 also hydrolyzed (1,3;1,4)-glucotriose with catalytic efficiency 15-fold lower than that of cellotriose and hydrolyzed (1,3;1,4)-glucotetraose with catalytic efficiency 4-fold lower than that of cellotetraose. The K_m values for all oligosaccharides tested, except for cellobiose, were nearly equivalent.

Os4BGlu12 exhibited broad glycone specificity against *p*NP glycosides, although it did not hydrolyze *p*NP β-D-mannoside, *p*NP β-D-cellobioside, *p*NP α-D-glucoside, *p*NP β-L-fucoside and *p*NP α-L-fucoside (Table 1). The enzyme hydrolyzed *p*NP glycosides with much lower K_m and higher apparent k_{cat}/K_m values than oligosaccharides. The enzyme hydrolyzed *p*NP β-D-fucoside with the highest k_{cat}/K_m value of 47 s^{-1} mM^{-1}. It hydrolyzed *p*NP β-D-glucoside, *p*NP β-D-xyloside and *p*NP α-L-arabinoside with similar catalytic efficiency (apparent k_{cat}/K_m of 23–25 s^{-1} mM^{-1}), while *p*NP β-D-galactoside was hydrolyzed with 35% of the efficiency of *p*NP β-D-glucoside.

Several available natural glycosides could be hydrolyzed at different rates (Table 1). Deoxycorticosterone 21-glucoside, a steroid glucoside from the adrenal cortex of vertebrates, was the most efficiently hydrolyzed glycoside with a high apparent k_{cat} value (13 s^{-1}) and low K_m value (0.68 mM). Apigenin 7-*O*-β-D-glucoside, a flavone glucoside, was hydrolyzed with low apparent k_{cat} (0.83 s^{-1}) and K_m (0.12 mM) values. Those that were hydrolyzed with relatively low catalytic efficiency (below 0.67 s^{-1} mM^{-1}) included indoxyl β-D-glucoside, D-amygdalin, and linamarin. The enzyme could not hydrolyze salicin, naringin (a 7-*O*-2-α-rhamnosyl-β-glucoside), gossypin (a flavonoid 8-*O*-glucoside), quercetin 3-β-D-glucoside (a flavonoid 3-*O*-β-glucoside) and phlorizin (phloretin 2′-*O*-β-glucoside, which is structurally similar to a flavonoid 5-*O*-β-glucoside).

4. Discussion

The high abundance of *Os4bglu12* transcripts in rice seedling shoots is similar to the rice *bglu1* and *bglu2* β-glucosidase genes [14]. However, differences were seen in 6-week-old mature rice plants in which *Os4bglu12* gene was highly expressed in leaf sheaths and stems, while *bglu1* is highly expressed in flowers and *bglu2* in the node. The high expression of *Os4bglu12*, *bglu1* and *bglu2* in different tissues of mature rice plants might indicate functional differences between different β-glucosidase isozymes, although they may play similar roles in different tissues.

The expression of *Os4bglu12* was up-regulated in response to wounding, methyl jasmonate and ethephon treatments, with high mRNA levels observed within 10 h after treatment. Consistent with this study, Wang et al. [18] reported that the transcript level of EST contig BHPiw028, corresponding to *Os4bglu12*, increased in response to brown planthopper attack, based on subtractive hybridization cDNA library screening. Methyl jasmonate and ethephon have been reported to be involved in wound signaling in several plant species [25–27]. This implies that induction of *Os4bglu12* gene expression by wounding and herbivore attack may be mediated by the methyl jasmonate and ethylene signal transduction pathway. The protein product of *Os4bglu12* gene may be involved in cell wall recycling or release of active compounds from glycosides for defense. In addition, the levels of transcripts that hybridized to the ESTs BE607353 and BG101702, which correspond to the *Os3bglu7* and *Os4bglu12* β-glucosidase genes, respectively, were induced by salt stress in salt-tolerant rice (var Pokkali), but not in the salt-sensitive cultivar IR29 [17]. These studies indicated that Os4BGlu12 may have functional roles in response to stresses.

One possible role of Os4BGlu12 is hydrolysis of cell wall-derived oligosaccharides. Os4BGlu12 efficiently hydrolyzed cellooligosaccharides (β-1,4-linked) with DP of 3–6 and laminaribiose (β-1,3), which is more like Os3BGlu7, Os3BGlu8, and Os7BGlu26 than Os3BGlu6, which hydrolyzes laminaribiose, but not cellooligosaccharides [28,15,16]. The large increase in catalytic efficiency of Os4BGlu12 with increasing chain length from DP of 2 to 3 is similar to rice Os3BGlu7 [28], rice Os3BGlu8 and Os7BGlu26 β-glucosidases [15] and different from barley β-glucosidase βII, which hydrolyzed cellobiose better than cellotriose [29]. However, the K_m values of barley β-glucosidase βII and rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 β-glucosidases decreased with increasing DP from 3 to 6, while Os4BGlu12 was approximately constant, so Os4BGlu12 showed a much lower dependence on chain length over this range. The preference of the rice Os4BGlu12 for (1,4)-β-oligosaccharides suggested that it has an extensive subsite binding region, but primarily binds the third glucosyl residue of cellooligosaccharides at subsite +2 with a weaker interaction at the 5th residue at subsite +4.

Akiyama et al. [19] reported the transcript level of the rice *OsEGL1* gene, which specifically hydrolyzes (1,3;1,4)-β-glucans, increased in rice seedlings in response to wounding, methyl jasmonate and ethephon, which is similar to the expression of *Os4bglu12*. Os4BGlu12 could hydrolyze (1,3;1,4)-glucooligosaccharides released from rice cell wall by OsEGL1 with catalytic efficiency that increased 4-fold with increasing chain length from 3 to 4, in contrast to cellotriose and cellotetraose, which are hydrolyzed more efficiently than (1,3;1,4)-oligosaccharides, but with no significant increase in efficiency between DP of 3 and 4. This result implies that enzyme may bind different linkages in different modes, so that it can play a role in cellooligosaccharide hydrolysis and may cooperate with OsEGL1 in hydrolysis of (1,3;1,4)-β-glucans of cell walls.

The above studies suggest roles for Os4BGlu12 in release of glucose from oligosaccharides to complete the depolymerization of at least three major cell wall polysaccharides present in rice tissues, including the (β-1,4); (β-1,3) and β-(1,3;1,4)-glucans. The reorganization and alteration of these cell wall β-glucans may be required during normal developmental processes or in response to environmental stresses. There have been reports that the hydrolysis of cell

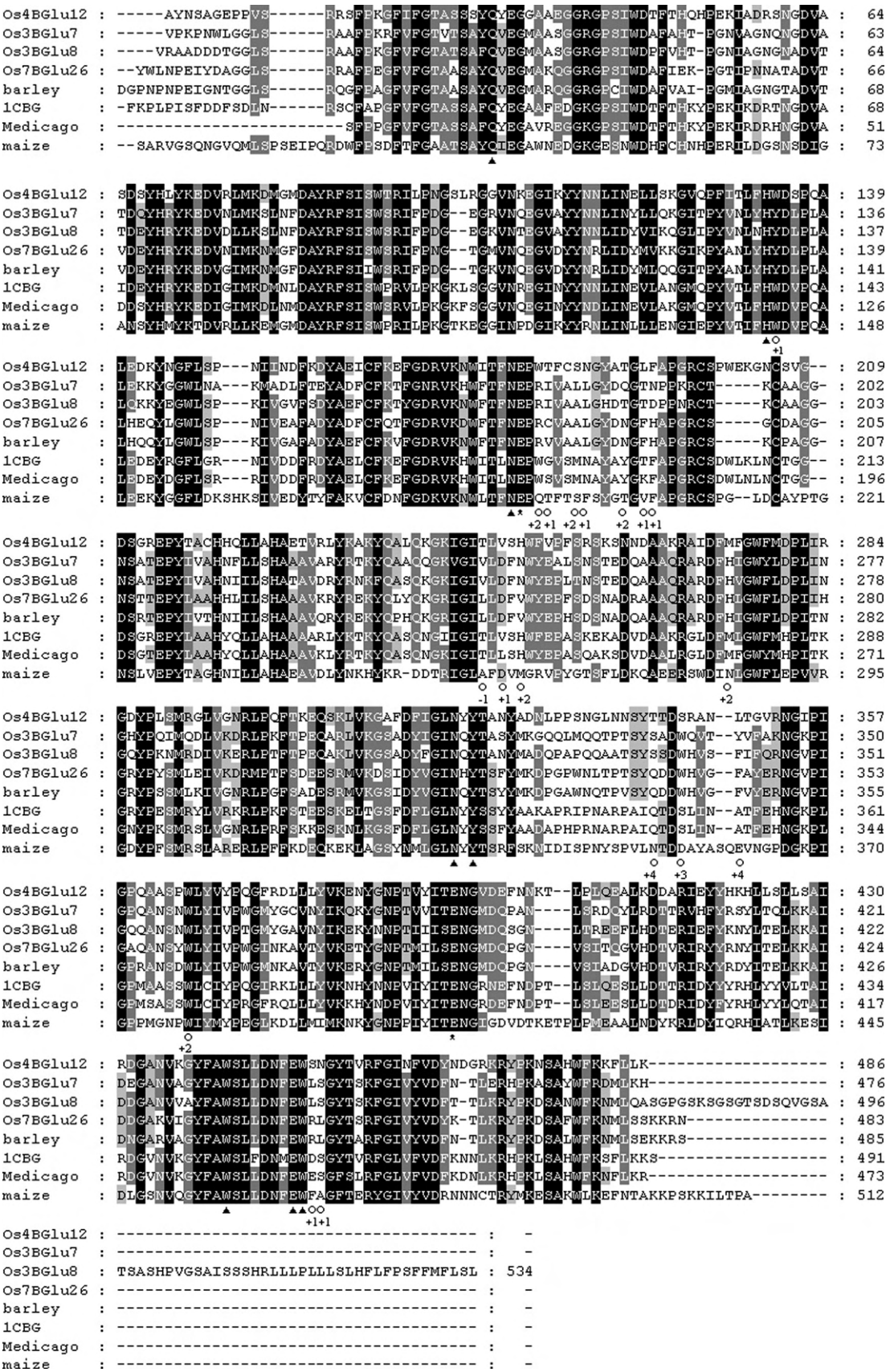


Fig. 4. Amino acid sequence alignment of rice Os4BGlu12 with related β-glucosidases. The rice cDNA derived sequences are labeled as Os4BGlu12, Os3BGlu7 (GenBank ID U28047), Os3BGlu8 (GenBank ID AK120790), and Os7BGlu26 (GenBank ID EU835514). Maize is maize β-glucosidase 1 (GenBank ID U33816); barley is barley βII β-glucosidase (GenBank ID ACF07998); 1CBG is white clover cyanogenic β-glucosidase (GenBank ID ABV54745); Medicago is *Medicago truncatula* isoflavone β-glucosidase (GenBank ID ABW76288) [31]. Stars represent the catalytic acid/base and nucleophile residues. Open circles with the number beneath represent residues to be at the −1, +1, +2, +3 and +4 subsites based on the Os3BGlu7 3D structure (PDB ID 1RGM and 3F5K) as predicted by Chuenchor et al. [35] and Kuntothom et al. [15]. Triangles mark the amino acids in close contact with the glucose residue at −1 subsite in Os3BGlu7 [35]. The alignment was generated using the Clustal X implementation of Clustal W [38,39].

wall polysaccharides also likely generates sugar signals [30]. Therefore, a role for Os4BGlu12 in degradation or modification of such signals cannot be ruled out.

Os4BGlu12 could hydrolyze deoxycorticosterone 21-glucoside and apigenin 7-*O*-β-D-glucoside with high catalytic efficiency, suggesting it could also play a role in releasing active steroids or flavonoids from their glycosides for defense. The fact the enzyme hydrolyzed apigenin 7-*O*-β-D-glucoside, but not flavonoid 3-*O*-glucoside and 8-*O*-glucoside, suggests it may have preference for the 7-*O*-linkage in flavonoid glucosides. Recently, Naoumkina et al. [31] reported that methyl jasmonate induced the transcript levels of four flavone/isoflavone β-glucosidases in cell suspensions of the legume *Medicago truncatula* within 30 min to 2 h, and the accumulation of the free isoflavonoid phytoalexin medicarpin released from its glycosides. Although Os4BGlu12 is thought to function in hydrolysis of oligosaccharides released from the cell walls, it is possible that this enzyme plays more than one role. Recently barley β-glucosidase βII, which is thought to help in hydrolysis of cell wall oligosaccharides during germination, has been found to hydrolyze cyanogenic glycosides from barley leaves [32]. This gives support to the possibility of one enzyme playing roles in both cell wall hydrolysis and defense.

The preference of the glycone moieties of Os4BGlu12 is different from the GH1 rice enzymes in that it could not hydrolyze *p*NP β-D-mannoside, but it instead hydrolyzed *p*NP β-D-xyloside and α-L-arabinoside with k_{cat}/K_m values equivalent to that for *p*NP β-D-glucoside [28,15,16]. The efficient hydrolysis of β-D-xyloside, is similar to white clover β-glucosidase, but is not generally found in GH1 enzymes that have been characterized [33]. The high activity upon *p*NP β-D-xyloside might result from a different positioning of subsite −1 residues, especially E449 and Q29 which correspond to residues that interact with O-6 of sugar residue, E451 and Q39, in *Spodoptera frugiperda* β-glycosidase [34]. This equivalent glutamate residue (Glu464) in maize ZmGlu1 β-glucosidase also specifically binds to O-6 and displayed a different conformational state in the crystal structure of ZmBGlu1 bound to the transition-state mimic glucotetrazole when compared the apo enzyme of ZmGlu1 [12]. Verdoucq et al. [12], stated that this conformational change might guide the movement of the glucosyl moiety into the correct distorted position required for nucleophilic attack to occur. In the case of rice Os3BGlu6 β-glucosidase, two conformations were observed for the corresponding glutamate (Glu451) in the apo enzyme structure, while it was locked in a single conformation in its covalent complex with 2-deoxy-2-fluoroglucoside [16]. The entropy loss upon glucoside binding was used to explain the high relative activity of Os3BGlu6 toward *p*NP β-D-fucoside. However, Os3BGlu6 has low activity toward *p*NP-β-D-xyloside, so the presence of carbon 6 of the pyranose ring must be more critical for Os3BGlu6 than for Os4BGlu12.

Since the substrate specificity of Os4BGlu12 is distinct from other rice enzymes, a protein sequence alignment was performed to compare the amino acids lining the binding site of Os4BGlu12 with the residues found at the −1, +1, +2, +3 and +4 subsites in the Os3BGlu7 X-ray crystal structure [35] and PDB ID 3F5K (Fig. 4). All the residues in close contact with the sugar at the −1 subsite were conserved in all enzymes, except for V241, which is in contact with E176 catalytic acid/base in Os3BGlu7. Therefore, differences in glycone specificities might derive from a different positioning of subsite −1 residues [12,36]. Rice Os3BGlu7, Os3BGlu8, Os7BGlu26 and barley βII, were classified in a distantly related group and shared 51–53% amino acid sequence identity with Os4BGlu12 [13]. They possess almost completely different amino acids at the residues predicted to form the +1 to +4 subsites compared to Os4BGlu12. However, residue W358 at subsites +1 and +2 in Os3BGlu7, which is a conserved Trp shown to position the aglycone of other plant GH1 β-glucosidases, was conserved in all enzymes [11,12]. Os4BGlu12 is more closely related to the dicot defense enzymes white clover cyanogenic β-glucosidase [37] and *M. truncatula* isoflavone β-glucosidase [31], with which it shares 63 and 65% protein sequence identity, respectively, and these enzymes have many identical predicted active site amino acid residues. The above-mentioned differences and similarities in active site amino acid residues between Os4BGlu12 and the other enzymes may result in the unique overall binding site geometry that allows Os4BGlu12 to hydrolyze glucooligosaccharides with different linkages, *p*NP glycosides with a range of glycones and flavonoid and steroid glucosides. However, the overall sequence difference or similarity of Os4BGlu12 with other enzymes may not reflect the aglycone specificity of the enzyme, and the molecular basis of the specificity based on the interactions with the aglycone remains to be studied.

5. Conclusions

In summary, this work explored the possible functions of rice Os4BGlu12, the transcript level of which was previously found to be induced in response to herbivore attack and salinity stress [17,18]. The expression of this gene was also up-regulated in response to wounding and wound-signaling related phytohormones. Under normal growth conditions, high transcript levels were detected in the shoot of rice seedlings and leaf sheaths and stems of 6-week-old mature rice plants. The recombinant Os4BGlu12 hydrolyzed three types of substrates, including oligosaccharides, a steroid glycoside, and a flavonol glycoside, which suggests that it might play multiple roles in rice, such as in defense and cell wall remodeling during development or wounding. The similar expression patterns of rice OsEGL1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase in wounding response and the action of Os4BGlu12 on the products of OsEGL1 implies that they may act in a concerted fashion. Furthermore, the amino acid residues predicted to line the active site of Os4BGlu12 are quite distinct from those in other oligosaccharide hydrolases, but similar to those of cyanogenic and flavonoid β-glucosidases, consistent with an additional possible role in hydrolysis of defensive glycosides. However, an additional defensive role remains to be confirmed with natural substrates isolated from rice.

Acknowledgements

This work was supported by the grant MRG5280249 from the Commission on Higher Education and the Thailand Research Fund. Additional support was provided by Suranaree University of Technology and the National Research Council of Thailand. T.A. was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

[1] J.R. Ketudat Cairns, A. Esen, β-Glucosidases, Cell. Mol. Life Sci. (2010), doi:10.1007/s00018-010r-r0399-2.
[2] J.E. Poulton, Cyanogenesis in plants, J. Plant Physiol. 94 (1990) 401–405.
[3] F.D. Dakora, D.A. Phillips, Diverse functions of isoflavonoids in legumes transcend antimicrobial definitions of phytoalexins, Physiol. Mol. Plant Pathol. 49 (1996) 1–20.
[4] H. Suzuki, S. Takahashi, R. Watanabe, Y. Fukushima, N. Fujita, A. Noguchi, R. Yokoyama, K. Nishitani, T. Nishino, T. Nakayama, An isoflavone conjugate-hydrolyzing β-glucosidase from the roots of soybean (*Glycine max*) seedlings. Purification, gene cloning, phylogenetics, and cellular localization, J. Biol. Chem. 281 (2006) 30251–30259.
[5] W. Schliemann, Hydrolysis of conjugated gibberellins by β-glucosidases from dwarf rice (*Oryza sativa* L. cv. Tan-ginbozu), J. Plant Physiol. 116 (1984) 123–132.
[6] J. Zouhar, E. Nanak, B. Brzobohaty, Expression, single-step purification, and matrix-assisted refolding of a maize cytokinin glucoside-specific β-glucosidase, Protein Exp. Purif. 17 (1999) 153–162.

[7] D.P. Dharmawardhana, B.E. Ellis, J.E. Carlson, A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin, Plant Physiol. 107 (1995) 331–339.
[8] R. Leah, J. Kigel, I. Svendsen, J. Mundy, Biochemical and molecular characterization of barley seed β-glucosidases, J. Biol. Chem. 270 (1995) 15789–15797.
[9] T. Akiyama, H. Kaku, N. Shibuya, A cell wall-bound β-glucosidase from germinated rice: purification and properties, Phytochemistry 48 (1998) 49–54.
[10] L. Barleben, X. Ma, J. Koepke, G. Peng, H. Michel, J. Stöckigt, Expression, purification, crystallization and preliminary X-ray analysis of strictosidine glucosidase, an enzyme initiating biosynthetic pathways to a unique diversity of indole alkaloid skeletons, Biochim. Biophys. Acta 1747 (2005) 89–92.
[11] M. Czjzek, M. Cicek, V. Zamboni, D.R. Bevan, B. Henrissat, A. Esen, The mechanism of substrate (aglycone) specificity in β-glucosidases is revealed by crystal structures of mutant maize β-glucosidase–DIMBOA, –DIMBOAGlc, and –dhurrin complexes, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 13555–13560.
[12] L. Verdoucq, J. Morinière, D.R. Bevan, A. Esen, A. Vasella, B. Henrissat, M. Czizek, Structural determinants of substrate specificity in family 1 β-glucosidases, J. Biol. Chem. 279 (2004) 31796–31803.
[13] R. Opassiri, B. Pomthong, T. Onkoksoong, T. Akiyama, A. Esen, J.R. Ketudat Cairns, Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β-glucosidase, BMC Plant Biol. 6 (2006) 33.
[14] R. Opassiri, J.R. Ketudat Cairns, T. Akiyama, O. Wara-Aswapati, J. Svasti, A. Esen, Characterization of a rice β-glucosidase highly expressed in flower and germinating shoot, Plant Sci. 165 (2003) 627–638.
[15] T. Kuntothom, S. Luang, A.J. Harvey, J.B. Fincher, R. Opassiri, M. Hrmova, J.R. Ketudat Carins, Rice family GH1 glycoside hydrolases with β-D-glucosidase and β-D-mannosidase activities, Arch. Biochem. Biophys. 491 (2009) 85–95.
[16] S. Seshadri, T. Akiyama, R. Opassiri, B. Kuaprasert, J. Ketudat Cairns, Structural and enzymatic characterization of Os3BGlu6, a rice β-glucosidase hydrolyzing hydrophobic glycosides and (1 → 3)- and (1 → 2)-linked disaccharides, Plant Physiol. 151 (2009) 47–58.
[17] S. Kawasaki, C. Borchert, M. Deyholos, H. Wang, S. Brazille, K. Kawai, D. Galbraith, H.J. Bohnert, Gene expression profiles during the initial phase of salt stress in rice, Plant Cell 13 (2001) 889–905.
[18] X. Wang, R. He, G. He, Construction of suppression subtractive hybridization libraries and identification of brown planthopper-induced genes, J. Plant Physiol. 162 (2005) 1254–1262.
[19] T. Akiyama, S. Jin, M. Yoshida, T. Hoshino, R. Opassiri, J.R. Ketudat Cairns, Expression of an endo-(1,3;1,4)-β-glucanase in response to wounding, methyl jasmonate, abscisic acid and ethephon in rice seedlings, J. Plant Physiol. 166 (2009) 1814–1825.
[20] C.W. Bachem, R.S. van der Hoeven, S.M. de Bruijin, D. Vreugdenhil, M. Zabeau, R.G. Visser, Visualization of differential gene expression using novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development, Plant J. 9 (1996) 745–753.
[21] J. Sambrook, E.F. Fritsch, T.A. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[22] T. Akiyama, N. Shibuya, M. Hrmova, G.B. Fincher, Purification and characterization of a (1 → 3)-β-D-glucan endohydrolase from rice (*Oryza sativa*) bran, Carbohydr. Res. 29 (1997) 365–374.
[23] J.S. Kim, B.L. Reuhs, F. Michon, R.E. Kaiser, G. Arumugham, Addition of glycerol for improved methylation linkage analysis of polysaccharides, Carbohydr. Res. 341 (2006) 1061–1064.
[24] K. Hiromi, Y. Nitta, C. Namura, S. Ono, Subsite affinities of glucoamylase: examination of the validity of the subsite theory, Biochim. Biophys. Acta 302 (1973) 362–375.
[25] E.S. McCloud, I.T. Baldwin, Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*, Planta 203 (1997) 430–435.
[26] J. Ziegler, M. Keinänen, I.T. Baldwin, Herbivore-induced allene oxide synthese transcripts and jasmonic acid in *Nicotiana attenuata*, Phytochemistry 58 (2001) 729–738.
[27] C.C. von Dahl, I.T. Baldwin, Deciphering the role of ethylene in plant–herbivore interactions, J. Plant Growth Regul. 26 (2007) 201–209.
[28] R. Opassiri, Y. Hua, O. Wara-Aswapati, T. Akiyama, J. Svasti, A. Esen, J.R. Ketudat Cairns, β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1, Biochem. J. 379 (2004) 125–131.
[29] M. Hrmova, E.A. MacGregor, P. Biely, R.J. Stewart, G.B. Fincher, Substrate binding and catalytic mechanism of a barley β-D-glucosidase/(1,4)-β-D-glucan exohydrolase, J. Biol. Chem. 273 (1998) 11134–11143.
[30] F. Rolland, E. Baena-Gonzalez, J. Sheen, Sugar sensing and signaling in plants: conserved and novel mechanisms, Annu. Rev. Plant Biol. 57 (2006) 675–709.
[31] M. Naoumkina, M.A. Farag, L.W. Sumner, Y. Tang, C.J. Liu, R.A. Dixon, Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 17909–17915.
[32] K.A. Nielsen, M. Hrmova, J.N. Nielsen, K. Forslund, S. Ebert, C.E. Olsen, G.B. Fincher, B. Lindberg Möller, Reconstruction of cyanogenesis in barley (*Hordeum vulgare* L.) and its implications for resistance against the barley powdery mildew fungus, Planta 223 (2006) 1010–1023.
[33] S.R. Marana, Molecular basis of substrate specificity in family 1 glycoside hydrolases, IUBMB Life 58 (2006) 63–73.
[34] S.R. Marana, W.R. Terra, C. Ferreira, The role of amino-acid residues Q39 and E451 in the determination of substrate specificity of the *Spodoptera frugiperda* β-glycosidase, Eur. J. Biochem. 269 (2002) 3705–3714.
[35] W. Chuenchor, S. Pengthaisong, R.C. Robinson, J. Yuvaniyama, W. Oonanant, D.R. Bevan, A. Esen, C.J. Chen, R. Opassiri, J. Svasti, J.R. Ketudat Cairns, Structural insights into rice BGlu1 β-glucosidase oligosaccharide hydrolysis and transglycosylation, J. Mol. Biol. 377 (2008) 1200–1215.
[36] L. Verdoucq, M. Czjzek, J. Moriniere, D.R. Bevan, A. Esen, Mutational and structural analysis of aglycone specificity in maize and sorghum β-glucosidases, J. Biol. Chem. 278 (2003) 25055–25062.
[37] T. Barrett, C.G. Suresh, S.P. Tolley, E.J. Dodson, M.A. Hughes, The crystal structure of a cyanogenic β-glucosidase from white clover, a family 1 glycosyl hydrolase, Biology 3 (1995) 951–960.
[38] F. Jeanmougin, J.D. Thompson, M. Gouy, D.G. Higgins, T.J. Gibson, Multiple sequence alignment with Clustal X, Trends Biochem. Sci. 23 (1998) 403–405.
[39] J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.