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Short communication

Genome-wide identification and expression analysis of rice pectin methylesterases: Implication of functional roles of pectin modification in rice physiology

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ABSTRACT

Pectin, which is enriched in primary cell walls and middle lamellae, is an essential polysaccharide in all higher plants. Homogalacturonans (HGA), a major form of pectin, are synthesized and methylesterified by enzymes localized in the Golgi apparatus and transported into the cell wall. Depending on cell type, the degree and pattern of pectin methylesterification are strictly regulated by cell wall-localized pectin methylesterases (PMEs). Despite its importance in plant development and growth, little is known about the physiological functions of pectin in rice, which contains 43 different types of PME. The presence of pectin in rice cell walls has been substantiated by uronic acid quantification and immunodetection of JIM7 monoclonal antibodies. We performed PME activity assays with cell wall proteins isolated from different rice tissues. In accordance with data from *Arabidopsis*, the highest activity was observed in germinating tissues, young culm, and spikelets, where cells are actively elongating. Transcriptional profiling of *OsPMEs* by real-time PCR and meta-analysis indicates that PMEs exhibit spatial- and stress-specific expression patterns during rice development. Based on *in silico* analysis, we identified subcellular compartments, isoelectric point, and cleavage sites of OsPMEs. Our findings provide an important tool for further studies seeking to unravel the functional importance of pectin modification during plant growth and abiotic and biotic responses of grass plants.

Introduction

Pectin, one of the major cell wall components found in the primary cell wall and middle lamellae, is classified into four different domains based on backbone structure and monosaccharide compositions and glycosidic linkages of substitution: homogalacturonan (HGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and xylogalacturonan (XGA) (Harholt et al., 2010; Mohnen, 2008). Structural analyses of pectin reveal that HGA, a linear polymer of (1,4)-linked-α-galacturonic acids (GalUA), accounts for approximately 65% of pectin composition, and that the dominant monosaccharide of pectin is GalUA. GalUA in HGA is methylesterified at the C-6 carboxyl by pectin methyltransferases in the Golgi apparatus (Pelloux et al., 2007). Once synthesized, methylesterified HGA is targeted to the cell walls and undergoes further modifications depending on cell types and their positions in the pectin network.

Pectin methyltesterases (PME, EC 3.1.1.11) belonging to class 8 carbohydrate esterases (CAZy, http://www.cazy.org/fam/CE8.html) catalyze the demethylesterification of GalUA in HGA (Cantarel et al., 2009). Partially demethylesterified HGA is then degraded by polygalacturonases, resulting in changes of wall properties such as rigidity, elasticity, and permeability (Moustacas et al., 1991). Since such structural remodeling of HGA by PMEs generally leads to cell wall loosening, cell types programed for cell expansion and elongation during cell development are required to increase the activity of PMEs. Moreover, recent studies showed that the methanol produced after PME action is an important signaling molecule in plant defense against biotic and abiotic stresses (Komarova et al., 2014).

Sequence analyses of several model species showed that PMEs are a large multigene family; there are 66 PMEs in *Arabidopsis thaliana*, 89 in *Poplus trichocarpa*, 43 in *Oryza sativa*, 105 in *Linum usitatissimum*, and 79 in *Lycoperscicon esculentum* (Pelloux et al., 2007; Pinzon-Latorre and Deyholos, 2013; Vandevenne et al.,

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2009). Depending on the presence or absence of the PME inhibitor (PMEI) domain at the N-terminus (also known as the PRO region), PMEs are grouped into either Type-1 PME (with PMEI domain) or Type-2 PME (without PMEI domain) (Jolie et al., 2010). Genome analysis of bacteria, fungi, and moss *Physcomitrella*, revealed the absence of PMEI domain-containing PME proteins (Pelloux et al., 2007). Although there is no experimental evidence of the functional role of PMEI domains in Type-1 PME proteins, it is postulated to play a role in self-inhibitory activity to prevent premature demethoxylation (Bosch et al., 2005). Over the last two decades, mutational and transgenic approaches have led to significant progress in understanding the major biological roles of PMEs in plants (Francis et al., 2006; Bosch et al., 2005; Hongo et al., 2012). Representative phenotypes caused by mutations in *PME* isoforms in *Arabidopsis* include a defect in pollen tube growth and pollen tetrad formation and pendant stem growth. Transgenic plants overexpressing the *Arabidopsis PMEI* gene exhibit reduced PME activities and in increased pectin methylesterification (Müller et al., 2013). These transgenic plants also have faster germination rates than the wild type and reduced seed yields. In addition to its roles in plant development, several lines of evidence indicated that PME proteins function as a key regulator in plant-microbe interactions. Down-regulation of tobacco PME expression *via* antisense suppression resulted in delayed systemic movement of tobacco mosaic virus (Chen and Citovsky, 2003). Also, Hewezi et al. reported that a cyst nematode (*Heterodera schachtii*) secretory protein directly interacts with AtPME3 and this interaction is required for enhanced susceptibility of plants to the pathogen. Moreover, *AtPME3* is rapidly induced upon infection of *Pectobacterium carotovorum* and *Botrytis cinerea* and acts as a susceptibility factor for the initial colonization by the pathogens (Raiola et al., 2011). The importance of pectin modification by PME was further demonstrated by comparing changes in the activities of PME during cold acclimation. The increase in PME activity was observed in cold-acclimated oilseed rape plants (Solecka et al., 2008). A previous study also showed that PMEs are involved in drought stress tolerance as overexpression of a pepper PMEI protein in *Arabidopsis* caused enhanced tolerance to drought stress (An et al., 2008). PMEs are also likely to be an important determinant of salt stress response. An *Arabidopsis* mutant with T-DNA insertion within the promoter of a *PMEI* gene (*At1g62760*) exhibited reduced sensitivity to NaCl stress (Jithesh et al., 2012). Taken together, previous studies demonstrate the importance of temporal and spatial regulation of PME activities in plant physiology as well as plant-microbe interactions.

The functional role of rice PMEs is poorly understood. A previous study explored the association of aluminum toxicity with altered transcriptional expressions of eight rice *PMEs* (Yang et al., 2013). Transgenic rice plants overexpressing *OsPME14* gene displayed increased sensitivity to aluminum stress. In this report, an initial step to characterize PME functions in rice, we found that rice cell walls contain substantial quantities of pectin through chemical and immunolocalization analysis, and that cell wall proteins exhibit PME enzyme activities. Transcriptional profiling of *OsPMEs* indicates that PMEs exhibit spatial- and stress-specific expression patterns during rice development.

Materials and methods

Plant materials

Rice seeds (*Oryza sativa* cv. Dongjinbyeo) were soaked in water and pregerminated at 28 °C under a light cycle of 16 h light/8 h dark for three days and transferred to soil. Rice plants were grown in a temperature-controlled glasshouse with natural lighting conditions. For RNA and total protein extraction, various tissues from germination to reproductive stages were collected, frozen in liquid nitrogen, and stored at −80 °C until analysis. Germinating tissues (shoot and root) were collected after 4 days of pregermination and immediately used for RNA and protein extraction. Young culm indicates samples collected from the entire third and fourth internode at 14 days before heading. Old culm indicates samples collected from the bottom parts of internode at 7 days after heading.

Preparation of alcohol insoluble residues (AIRs) and measurement of uronic acid contents

AIRs were prepared as described previously (Zhong et al., 2005; Lee et al., 2007). Briefly, frozen materials of each tissue were ground under liquid nitrogen. Fine powders were suspended in 70% (v/v) ethanol using a Polytron homogenizer three times. The AIRs collected were washed sequentially in absolute ethanol and 100% acetone. The resulting residues were dried in a vacuum oven at 60 °C. In order to obtain pectin enriched fractions (PEFs), the AIRs (100 mg) were stirred to ammonium oxalate solution (50 mM) for 24 h at room temperature. Soluble fractions were collected by centrifugation and lyophilized. Uronic acid content was determined as described by Filisetti-Cozzi and Carpita (1991). Lyophilized PEFs were dissolved in 0.4 ml of 0.5 M H_2SO_4 at room temperature for 1 h 40 μl of 4 M sulfamic acid-potassium sulfamate (pH1.6) was added and mixed thoroughly. Samples were hydrolyzed in 2.4 ml of 75 mM $Na_2B_4O_7$ dissolved in concentrated H_2SO_4 at 100 °C for 20 min. After cooling, 80 μl of 0.15% (w/v) *m*-hydroxybipheneyl in 0.5% (w/v) NaOH was added. The solution was mixed and incubated at room temperature for 30 min. Absorbance at 525 nm was measured and the uronic acid content was determined by using galacturonic acid as a standard.

PME activity assay

Cell wall protein extracts were generated from germinating shoot, germinating root, 1 month old shoot, 1 month old root, 2 month old leaf, 2 month old root, young culm, spikelet, and flag leaf. Tissues were collected in liquid nitrogen and homogenized with equal volumes (w/v) of extraction buffer (100 mm Tris–HCl, pH 7.5, 500 mm NaCl containing protease inhibitor cocktail) and homogenized samples were then rotated at 4 °C for 120 min. The samples were centrifuged at 11,500 × *g* at 4 °C for 20 min and the supernatant were used immediately for all enzyme assays. A coupled enzymatic assay was performed under standard conditions as described by Grsic-Rausch and Rausch (2004) using a spectrophotometric plate reader. Heat-denatured cell wall proteins of each sample were used as a negative control.

Histology and immunodetection of recognized methyl esterified homogalacturonans

Young culm and leaves of 9-week-old plants were fixed in 2% glutaraldehyde in 1× PBS at 4 °C overnight. Tissues were dehydrated through a gradient of ethanol, embedded in LR white resin (Ted Pella Inc.). 0.5 μm thick sections were cut with a microtome and stained with 0.1% toluidine blue solution. For immunodetection, 0.5 μm thick sections were incubated with JIM 7 monoclonal antibody (Plantprobes, Leeds University, UK) and then with fluorescein isothiocyanate-conjugated secondary antibodies. The fluorescence-labeled sections were observed using confocal microscope.

Primer design and real time PCR

Using Pfam ID (PF01095), we searched Putative Function Search Tool in Rice Genome Annotation Project (rice.plantbiology.msu.edu/). Then, we used Primer-BLAST in