Studies on the Function of Pectin Methylesterification Maintenance during Reproductive Development in Rice

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Abstract

Pectin synthesis and modification are vital for plant development, although the underlying mechanisms are still not well understood. Furthermore, reports on the function of pectin in the pistil are limited. Herein, we report the functional characterization of the *OsPMT16* gene and *OsPMT10* gene, which encode putative pectin methyltransferases (PMT) in rice. The cell walls of rice leaves contain less pectin, and chemical analysis of pectin in the flower organ had not been previously performed. Therefore, in the present study, the amount of pectin in the reproductive tissues of rice was investigated. Of the reproductive tissues, the pistil was especially rich in pectin; thus, I focused on the pistil.

The expression of *OsPMT16 and OsPMT10* was confirmed in the pistil, and effects of pectin methylesterification regulation on the reproductive stage were investigated by studying the phenotype of the T-DNA insertion mutants.

The *ospmt16* mutant showed significantly reduced fertility. When the flowers were observed, tissue morphogenesis was abnormal

in the pistil. Immunofluorescence staining by pectin-specific monoclonal antibodies of the pistil revealed that total pectin and esterified pectin were decreased among *ospmt16* mutants. These results indicate that OsPMT16 contributes significantly to pistil development during reproductive growth.

The *ospmt10* mutant did not have a significant effect on vegetative growth, but the fertility rate was reduced by approximately half. In the ospmt10 mutant, pollen tube elongation was observed in the transmitting tissue of the style, but approximately half of the pollen tubes did not extend all the way to the ovule. Tissue cross-sections of the upper ovary were prepared, and immune-histochemical staining using LM19 and LM20 showed that methylesterified pectin distribution was decreased in *ospmt10* compared with the wild-type. The decreased expression of methylesterified pectins in *ospmt10* may have resulted in loss of fluidity in the apoplast space of the transmitting tissue, rendering it difficult for the pollen tube to elongate in the transmitting tissue and thereby preventing it from reaching the ovule.

This study shows that maintenance of methylesterification of pectin by OsPMT10 and OsPMT16 plays important roles in the development of pistil, pistil transmitting tissue, and fertilization system by maintaining the soft condition for pollen tube passage.

Abbreviations

AIR	alcohol-insoluble residue
CTAB	cetyltrimethlammonium bromide
GalA	galacturonic acid
HGA	homogalacturonan
PCR	polymerase chain reaction
PME	pectin methylesterase
PMT	pectin methyltransferase
qRT-PCR	quantitative real-time polymerase chain reaction

Introduction

Structurally and functionally, pectin is the most complex polysaccharide in the plant cell wall (Mohnen, 2008). Substantial evidence indicates that pectin plays essential roles in plant growth and development, which is consistent with the requirement for large numbers of genes for pectin synthesis and modification (Ridley *et al.,* 2001; Mohnen, 2008; Atmodjo *et al.,* 2013). Biochemically, pectins are polysaccharides that are rich in galacturonic acid (GalA); they are divided into three main types: homogalacturonan (HGA), rhamnogalacturonan-I, and rhamnogalacturonan-II (Willats *et al.,* 2001). HGA biosynthesis and modification have recently been identified as key determinants of plant development (Wolf *et al.,* 2009).

The cell wall plays important roles in plant development. It imposes spatial constraints on the plant cell; in higher plants, it provides mechanical strength, determines cell shape, participates in cell–cell communication, and protects against attacks by pathogens and predators (Somerville *et al.,* 2004). Ordered deposition of cell wall

materials and their composition is important for coordinating the planes of cell division and expansion during development (Baskin, 2001).

Plant cell walls are primarily composed of polysaccharides, including cellulose microfibrils and matrix components (McNeil *et al.,* 1984; Gigli-Bisceglia *et al.,* 2019). One matrix component is pectin, which is negatively charged and tends to form gel-like structures (Jarvis, 1984). Pectin and its modifications play important roles in plant physiological processes such as plant development and growth, leaf senescence, plant–pathogen interactions, and abiotic stress responses (Wolf *et al.,* 2009; Lionetti *et al.,* 2012; Palin and Geitmann, 2012; Fang *et al.,* 2016; Qi *et al.,* 2017).

Chemically, pectin is a mixture of heterogeneously branched polysaccharides (Ridley *et al.,* 2001). The main pectic polysaccharide is linear HGA, which consists of linear α -1,4-linked d-GalA, a compound that is variably methyl esterified at C6 (Gigli-Bisceglia *et al.,* 2019). Pectic polysaccharides are synthesized in the Golgi apparatus (Driouich *et al.,* 1993; Calderan-Rodrigues *et al.,* 2019),

and a substantial portion of HGA is secreted in methyl esterified form (Li *et al.,* 1997, 2002; Lennon and Lord, 2000). The degree of pectin methylesterification is important for determining the adhesive properties of pectin. The number of free carboxyl groups in pectin is determined by the degree of pectin methylesterification, which is regulated by the coordinated activities of pectin methyltransferases (PMTs), pectin methyl esterase (PME), and PME inhibitors (Senechal *et al.,* 2014). Several PME genes have been identified and investigated (Tieman *et al.,* 1992; Gaffe *et al.,* 1997; Wen *et al.,* 1999; Hongo *et al.,* 2012). PMT activity has been described, and the enzyme properties characterized, in several plant species (Goubet *et al.,* 1998; Goubet and Mohnen, 1999; Ishikawa *et al.,* 2000; Łekawska-Andrinopoulou *et al.,* 2013). However, functions of the PMT gene are not well understood, especially during the reproductive process.

Plant reproductive processes require active intercellular communication and cell-wall changes. Reproductive tissues are particularly rich in pectin compared with other tissues, indicating that pectin plays an important role in a variety of processes from

pollination to fertilization (Lord, 2000; Mollet *et al.,* 2000). During pollination, pollen recognizes stigma calcium and begins to germinate. After pollination, pollen tubes that enter the stigma extend through the adhering cells of the transmitting tissue toward the ovary (Lord, 2000). Consequently, dicotyledonous plants have been shown to contain a large amount of pectin in the transmission tissue along the pollen path (Iwai *et al.,* 2006). However, reports on the amount of pectin and degree of methylesterification in the transmission tissues of monocotyledons are limited.

In *Arabidopsis thaliana,* defective mutations in the *TSD2* gene, which is presumed to encode a PMT, cause abnormal cell adhesion and hypocotyl differentiation during vegetative growth, and undifferentiated cells are observed. The problem is thought to arise during the PMT communication process (Krupkova *et al.,* 2007). This gene is also expressed in the reproductive organs, indicating the importance of PMT in the reproductive process. The enzymatic activity of PMT and mutants in the gene encoding these enzymes have been characterized in rice and several plant species (Goubet *et al.,*

1998, Ishikawa *et al.,* 2000, Mohnen D 2008, Xu *et al.,* 2017, Hasegawa *et al.*, 2020, Du *et al.,* 2020). However, the function of the pectin methyltransferase (PMT) gene is not well understood, especially in reproduction.

Since the discovery of pollen tubes approximately 150 years ago, significant research has been conducted to determine how pollen tubes reach the ovule for pollination. Some studies suggest that mechanochemical and pollination-inducing signals induce pollen tubes from the stigma to the ovary (Rosen 1971, Heslop-Harrison 1987, Mascarenhas 1993, Higashiyama and Takeuchi 2015). Signals are exchanged between male and female cells during pollen tube elongation (Higashiyama and Takeuchi 2015, Lu et al. 2020). There may be a combination of mechanisms ensuring the proper direction of pollen tube growth, and directional, biochemical and mechanical support cues may exist in multiple areas along the pollen tube growth pathway (Holdaway-Clarke and Hepler, 2003).

Corn silk is probably the best-described structure involved in the mechanical induction of pollen tubes (Booy *et al.,* 1992, Lu *et al.,*

2020). Corn silk hair, which corresponds to the stigma, connects the main axis of the style. The pollen germinates in the silk hair, and the pollen tube grows according to the direction of the silk hair. When the main axis of the style is reached, the pollen tube crosses multiple rows of cortical cells before entering the transmitting tissue. The tip of the pollen tube is directed toward the ovary and continues to move in that direction. Thus, the structure of the silk hairs may provide mechanical rails for the pollen tube to the ovary.

In plant reproduction, active cell-to-cell communication and cell wall changes are required. Reproductive tissues are particularly rich in pectin compared with other tissues and play important roles in various processes from pollination to fertilization (Lord 2000, Mollet *et al.,* 2000). Pollinated pollen is induced by calcium in the stigma to begin germination. After pollination, the pollen tube invading the stigma penetrates the adherent cells of the transmitting tissue and extends toward the ovary (Lord 2000). In the pistil, the calcium concentration increases approaching the embryo sac, with many calcium transporters found at the tip of the pollen tube. When calcium

is removed, an abnormality is observed in pollen tube elongation. Calcium is important for the induction of pollen tubes and is actively taken up by pollen tubes (Malhó 1998, Pierson *et al.,* 1996, Calabrese and Agathokleous 2020).

In addition, the growing pollen tube actively synthesizes pectin-rich cell walls at its tip (Li *et al.,* 1996, Calabrese and Agathokleous 2020). HGA is highly methylesterified in the cell wall of the tip immediately after synthesis and is then demethylesterified by pectin methylesterase (PME) (Lennon and Lord 2000, Lu 2020). Several PME genes have been identified and investigated (Gaffe *et al.,* 1997, Wen *et al.,* 1999, *Jeong et al.,* 2015, Leroux *et al.,* 2015). In addition, although the transmitting tissue before pollination maintains a high level of methylesterification, demethylesterification proceeds rapidly after pollination (Suárez *et al.,* 2020), and the pollen tube and transmitting tissue are demethylesterified. Pectin is required for the pollen tube and pistil transmitting tissues to adhere to each other (Mollet *et al.,* 2020). Thus, regulation of pectin homogalacturonan methylesterification and the calcium concentration are important for

pollen guidance. Although pollen mechanical guidance is an essential factor for sexual reproduction, the mechanisms and function of cell wall pectin during pollen mechanical guidance are unclear. Therefore, in this study I focused on the regulation of the pectin methylesterification in pollen tube mechanical guidance.

To date, only a few studies have examined the role of pectin in female reproductive processes, and almost no reports have examined the relationship between these processes and seed fertility. Therefore, in the present study, I focused on the pistil. To elucidate the function of maintaning pectin methylesterification in pistil development and fertilization process, I analyzed PMTs in rice and mutants of PMTs.

OsPMT16 and *OsPMT10* showed high levels of expression in pistil in the rice PMT genes according to the RiceXpro database. Hence, I focused on the *OsPMT16* genes and *OsPMT10*, and the effects of regulated pectin methylesterification on the reproductive stage were investigated by studying the phenotype. Since chemical analysis of floral pectin has not been previously performed, I also examined the amount of pectin in rice reproductive tissues. Here,

focusing on the pistil, I obtained a T-DNA insertion mutant of *OsPMT10* and *OsPMT16*, the expression of which was confirmed in the pistil, and investigated the function of pectin methylesterification in pollen tube mechanical guidance and development of pistil transmitting tissues.

Chapter 1

Rice Putative Methyltransferase Gene *OsPMT16* Is Required for Pistil Development Involving Pectin Modification .

1. Materials and Methods

1.1 Plant Material and Growth Conditions

Wild-type (WT) (cv. Dongjin) and *OsPMT16* (gene locus: Os06g0712800 (RAP-DB), AP014962 (GenBank)). Rice plants were cultivated in a greenhouse during the natural growing season.

1.2 Extraction and Analysis of Cell Wall Polysaccharides

Alcohol-insoluble residue (AIR) generation, neutral sugar composition assay, uronic acid assays, and methyl ester assays were performed as described previously (Wood and Siddiqui, 1971; Filisetti-Cozzi and Carpita, 1991) with slight modifications. Expanded leaf, glume, anther, and pistil were sampled for each of 4 to 8 independent biological replicates per treatment. Frozen samples were powdered with a mortar and pestle and washed in 80% EtOH. The

supernatant was removed after centrifugation for 5 min at 15,000 × g. The pellet was washed with a mixture of methanol and chloroform (1:1 ratio) and then with acetone. A mixture of phenol, acetic acid, and water (2:1:1 ratio) was then added to the pellet. This process was repeated three times; the sample was then dried at room temperature for more than 1 h. After being washed with acetone, the samples were air-dried for more than 12 h. The alcohol-insoluble residues were used as the cell wall material. A total of 2 mg AIR was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°Cfor 2 h. After hydrolysis, samples were centrifuged at 15,000 × g for 5 min. The supernatant was the TFA-soluble fraction. The pellets were hydrolyzed with 72% H_2SO_4 at room temperature for 2 h and then diluted to 4% H_2SO_4 and boiled for 1 h. H_2SO_4 solutions were neutralized with $Ba(OH)_2$. In this study, pectin refers to the uronic acid content of AIR. Hemicellulose and cellulose are defined as the amount of neutral sugar in the TFA-soluble and TFA-insoluble fractions of cell wall material, respectively. Uronic acid was quantified with the sulfamate/carbazole method with 0.4 mg AIR (Filisetti-Cozzi and Carpita, 1991). Neutral sugar content in the

TFA-soluble and TFA-insoluble fractions was determined with the phenol sulphuric acid method.

1.3 DNA Extraction and Homo/Hetero Assay

Fully expanded mature leaves were sampled four independent biological replicates, frozen in liquid nitrogen and ground using a TissueLyser II instrument (Qiagen, Hilden, Germany). Total DNA was extracted using cetyltrimethylammonium bromide (CTAB) and amplified using forward (50-TTTTCAGGACAAGCCTACCG-30), reverse (50-ATTGATCGGACAAGGACGAG-30), and T-DNA (50-ACAGGACGTAAC-30) primers. The amplified DNA fragments were separated on 1% agarose gels, and bands were detected by staining with ethidium bromide.

1.4 Vector Construction and Plant Transformation for Complementation Testing

For the complementation test, a 2,031 bp upstream sequence from a genomic DNA fragment containing the entire *OsPMT16* gene was

inserted into the binary vector pSTARA-R4, and pCAMBIA1300 was transformed as a control. The binary plasmids were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation, and the calli of the *OsPMT16* mutant were transformed following the method of Hiei *et al.* (1994).

1.5 RNA Extraction and Gene Expression Analysis

Plant material was frozen in liquid nitrogen and ground with a TissueLyser II instrument (Qiagen). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and recombinant DNase I (Roche, Basel, Switzerland) according to the manufacturers' protocols. Then, cDNA was synthesized using ReverTra Ace (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. The *OsPMT16* transcript level was quantified using forward (5'-GACCCGTTGTGATGATCTCC- 3') and reverse (5'-AATCTTGTGTTGGGGAGTGC-3') primers. For the endogenous control, the 17S rRNA transcript was quantified using 17S rRNA-forward (5'- GCAAATTACCCAATCCTGAC-3') and 17S rRNA-

reverse (50-CTATTGGAGCTGGAATTACC-30) primers. The amplified DNA fragments were separated on a 1% agarose gel, and bands were detected by staining with ethidium bromide. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan (Holland *et al.,* 1991) or SYBR Green I (Qiagen) using cDNA as the template in a Model 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). To determine the role of *OsPMT16* during reproductive development, we used an *OsPMT16* promoter::GUS assay to monitor *OsPMT16* expression during reproductive development. A 2,031 bp 50-UTR fragment of *OsPMT16* was amplified from the genomic DNA. The promoter fragment was then cloned into pBI121 for transformation into *Oryza sativa* (cv. Nipponbare) via *Agrobacterium* (Shu *et al.,* 2015). GUS staining was performed on the flower as described by Shu *et al.* (2015). Briefly, samples were incubated in staining solution (20 mm X-Gluc in phosphate buffer) for 12 h at 37°C and then rinsed in 70% ethanol for microscopic observation.

1.6 Immunohistochemistry

WT anthers at the same developmental stage were fixed with 4% PFA, 0.25% glutaraldehyde, and 0.05 M phosphate buffer (pH 7.5), and embedded in 5% agar. Sections (50-mm thickness) were cut using a Leica VT1200S microtome (Leica Microsystems, Wetzlar, Germany) and subjected to immunohistochemical analysis using the TSATM Kit #12 with horseradish peroxidase (HRP)-goat anti-rabbit IgG and Alexa Fluor 488 tyramide (Micro Probes/Invitrogen, Eugene, OR, United States) according to the manufacturer's protocol. Primary LM19 and LM20 antibodies (PlantProbes, Leeds, United Kingdom) were used at a dilution level of 1:20. The HRP conjugate was used at a dilution level of 1:100. The negative control lacked the primary antibody. The sections were visualized using fluorescence microscopy (Leica Microsystems).

1.7 Phylogenetic Analysis

I conducted a search of the SALAD database to find genes with the rice PMT domain pfam03141 (Methyltransf_29, Putative S-adenosyl-

L-methionine-dependent methyltransferase). I identified 19 genes as candidates. Multiple sequence alignment was performed with ClustalX for full-length sequences of these rice candidate genes and *A. thaliana (TSD2)*, followed by manual adjustment.

1.8 Statistical Analysis

The data of Figures 1, 4–6, 9 were expressed as the mean values ± standard deviations (SD) taken from 4 to 9 biologically independent experiments. The experimental data of the samples were statistically analyzed through one-way analysis of variance (ANOVA) with Tukey's post-hoc test using Statistica 13.1 software (StatSoft, Inc., Tulsa, OK, United States). The results with $p \leq 0.05$ and $p \leq 0.01$ were considered statistically significant.

2. RESULT

2.1 Cell Wall Composition Analysis of Rice Flowers

Gramineae plants including rice have been reported to contain low amounts of pectin in vegetative tissues (Vogel, 2008). However, no detailed analysis of pectin in the reproductive tissues of rice has been reported. Therefore, to investigate the cell-wall composition of the actual reproductive tissues of rice, Nipponbare WT mature leaves, flower buds before flowering, flower buds after flowering, and the cell walls of pistils were analyzed biochemically. The cell walls of vegetative tissues, such as mature leaves comprised only ~5% pectin, as previously reported (Sumiyoshi *et al.,* 2015). However, in the reproductive tissues, the cell walls of pistils comprised ~43% pectin (Figure 1).

2.2 Selection of Homozygotes for T-DNA Insertion Mutants and Complementary Testing

I searched the SALAD rice genome database for genes with rice PMT domains and identified 19 genes as candidates. Amino acid sequences

of the candidate genes, including the conserved domain of pfam03141 (Methyltransf_29, Putative S-adenosyl-L-methionine- dependent methyltransferase), were obtained from the RAP-DB rice annotation database. The amino acid sequence of the putative PMT gene *TSD2*, which was first reported in A. thaliana, was obtained from the Arabidopsis annotation database TAIR. A phylogenetic tree based on the sequences was created using multiple sequence alignment programs and ClustalX (Figure 8). To date, few studies have examined the role of pectin in female reproductive development; therefore, we searched for rice PMT genes that were highly expressed in the pistil. According to the RiceXpro database, the *OsPMT16* gene (*Os06g0713800*) had high expression. I then obtained the T-DNA insertion *OsPMT16* mutant from the Pohang University of Science and Technology (POSTECH, Pohang, South Korea). The T1 generation was generated from ordered mutants and a homo/hetero assay was performed to select homozygous individuals using genomic PCR. In addition, complements (cPMT16) were prepared to identify the causative genes for phenotypic abnormalities observed in the

mutants. A full-length genome construct including the original promoter region was inserted into the pSTARA-R4 vector and introduced into each mutant. T-DNA was inserted as a promoter at -272 bp in *OsPMT16* to create *ospmt16* mutants (Figure 2A). I confirmed that *OsPMT16* was not expressed in the mutant and that expression was restored in the complement (Figure 2C). Nullsegregant individuals were isolated from *ospmt16* mutants and used for experiments as WT (Figure 2B).

2.3 Gene Expression and Phenotypic Analysis of *OsPMT16* Mutants During Vegetative Development

To investigate gene expression in each tissue, I examined *OsPMT16* expression levels using qRT-PCR. The gene was strongly expressed in reproductive organs, especially the pistil and anther (Figure 3A); however, almost no expression was detected in the root, stem, or leaf. Particularly high GUS signal levels were observed in pistil transmitting tissues in *OsPMT16* promoter::GUS plants (Figures 3B–D). The phenotype of the *OsPMT16* mutant during vegetative growth was

investigated. The *OsPMT16* mutant showed delayed vegetative growth compared with the WT (Figure 4C). The plant height on day 71 after sowing was short, and the number of tillers was small (Figures 4A, D). By the time of reproductive growth, the plant height was normal (Figure 4B), however, the number of tillers remained small (Figure 4).

2.4 Disordered Pistil Morphology in the *OsPMT16* Mutant

During reproductive development, the fertility rate decreased by ~12% in the *OsPMT16* mutant (Figure 5B). Flowers were sampled before heading and flowering, and their morphology was investigated. Pistils were slightly shorter in the *OsPMT16* mutant than in the WT, and the angle of the stigma opening was narrower. Stamens were slightly shorter in the *OsPMT16* mutant (Figure 5A). In cPMT16, which was very similar to the WT, these phenotypes were not observed (Figure 5A). Despite the very low fertility of the *OsPMT16* mutant, only minor differences in reproductive organ morphogenesis were observed between the *OsPMT16* mutant and WT. To identify the

stage in which abnormalities occurred during differentiation, the pistil was sampled at each stage and its morphology was evaluated. Pistils were collected from flowers at glume lengths of 3, 5, and 7 mm before heading, and at 7 mm after heading. *OsPMT16* gene expression increased gradually during pistil growth (Figure 6B). Delayed growth was observed in the stamen stigma and pistil during morphogenesis at 7 mm before heading (Figure 6A). To identify the defects responsible for low fertility, I compared pistil cross-sections at 7 mm before the heading stage from *OsPMT16* and WT plants. Toluidine blue staining indicated that the pistil of the same mutant *OsPMT16* line exhibited abnormal morphogenesis compared to the WT (Figure 7). Crosssections revealed that the WT pistil was composed of a single outer epidermis layer, transmitting tissues with high cell density, and a vascular bundle. However, in *OsPMT16*, the outer epidermal cells and transmitting tissues of the pistil were vacuolated. Cells of transmitting tissues contained less cytosol, were disordered, and had far fewer cells than the WT. Normal vascular bundles were developed even in the *OsPMT16* mutant. I examined cell wall sugar distribution

using monoclonal antibodies against pectic polysaccharide epitopes. I prepared cross-sections by staining with de-methyl esterified pectin-specific antibody (LM19) and methyl esterified pectin-specific antibody (LM20). The *OsPMT16* mutant exhibited decreased signals of both LM19 and LM20 in the pistil style. In WT, high LM20 signal levels were observed in pistil transmitting tissues; I also detected GUS signals in the transmitting tissues of p*OsPMT16*::GUS plants (Figure 3C). Slightly less uronic acid was detected in the pistils of the *OsPMT16* mutant than in the WT and cPMT16, but this difference was not significant (Figure 9).

3. DISCUSSION

3.1 In Rice Reproductive Tissues, Pistil Is Especially Rich in Pectin

Type I cell walls of dicotyledonous plants such as *A. thaliana* contain as much as 35% pectin in vegetative tissues, whereas rice type II cell walls contain approximately only 5% in the whole body (Yokoyama and Nishitani, 2004). However, an abnormal reproductive phenotype in pectin-modified rice has been reported (Jang *et al.,* 2003; Sumiyoshi *et al.,* 2015).

In the present study, the constituent sugars in different organs of WT rice were investigated. Results revealed that mature leaves contained approximately only 5% pectin, which was consistent with reports in previous studies. However, when other organs were investigated, the cell walls in reproductive tissues, especially pistils, contained ~43% pectin (Figure 1). Based on these results, even in monocotyledonous plants known for having low amounts of pectin in vegetative tissues, pectin was shown to be abundant in reproductive tissues. Therefore, pectin likely plays an important role in the

reproductive growth phase in rice as well as in dicotyledonous plants with reproductive tissues rich in pectin.

3.2 Regulation of Pectin Methylesterification by *OsPMT16* Is Essential During Rice Vegetative Growth

PMT likely plays an important role in vegetative growth based on weak cell hypocotyl adhesion and abnormal leaf bud differentiation in Arabidopsis putative PMT gene *QUA2/TSD2* deletion mutants (Mouille *et al.,* 2007; Krupkova *et al.,* 2007). In the *OsPMT16* mutant, an increase in plant height was delayed during vegetative growth and the number of tillers decreased (Figure 4). In the cell walls of rice tillers, the cellulose synthase *GNT1* may be involved (Fanata *et al.,* 2013), however, there are no reports on pectin. Based on the phenotypes observed in the present study, the degree of pectin methylesterification may control the number of tillers. However, the influence on growth is not substantial, and the plant can continue to develop to the flower stages.

To date, reports of phenotypes of reproductive tissues of

PMT-deficient mutants have not been published. Dicotyledonous plants such as *A. thaliana* are rich in pectin even in vegetative tissues. Therefore, abnormalities in the regulation of pectin methyl-esterification first affect vegetative growth, causing growth to cease and failure in flower development.

3.3 *OsPMT16* Affects Pistil Morphogenesis

QUA2/TSD2 is expressed in Arabidopsis flowers and in the double-deficient mutant of CGR2 and CGR3; the germination and elongation of pollen tubes weaken, the long-horned fruit becomes shorter, and the number of seeds decreases, indicating that PMT may play an important function during reproductive growth (Krupkova *et al.,* 2007; Mouille *et al.,* 2007; Kim *et al.,* 2015). Furthermore, highly methyl esterified pectin may be involved in the morphogenesis of the style in the olive pistil (Suárez *et al.,* 2013). In the present study, the *OsPMT16* mutant was found to have significantly reduced fertility (Figure 5) and abnormal pistil and transmitting tissue morphogenesis (Figure 7). However, significant differences in phenotype

morphogenesis were not observed in other organs (Figure 5). Therefore, pectin modification by *OsPMT16* in transmitting tissues is required for normal pistil morphogenesis. PMT was considered to have an important function during the reproductive growth phase in rice with type II cell walls as well as in A. thaliana with type I cell walls. Because levels of methyl esterified pectin, which was detected using the antibody LM20, were greatly reduced, the ospmt16 mutant was hypothesized to exhibit decreased pectin methylesterification in the pistil style (Figure 7). In pistils that exhibited severe anomalies, signals of both LM19 (indicating dimethyl esterified pectin) and LM20 were greatly reduced (Figure 7). In the hypocotyl of the double-deficient mutant of *CGR2* and *CGR3*, the total amount of uronic acid and methylesterification of uronic acid decreased (Kim *et al.,* 2015). In the present study, the amount of uronic acid, which is the main component of pectin, in pistils differed little between *OsPMT16* and WT (Figure 9). Therefore, the amount of pectin does not have noticeable effects on the *ospmat16* phenotype. In addition to controlling the degree of methylesterification, *OsPMT16* may be

involved in the biosynthesis and secretion of pectin. In the *OsPMT16* mutant, anthers were abnormal at every stage from the very beginning of morphogenesis and shorter than WT anthers (Figure 5). In addition, I observed a pollen decrease of about 20%, as well as an abnormal anther and pollen formation (Figure 1). Therefore, pectin methylesterification by *OsPMT16* is not essential for pollen maturation. In addition, the pectin originally synthesized at the tip of the pollen tube is highly methyl esterified (Lennon and Lord, 2000). Reduced amounts of methyl esterified pectin at the germination site of the tube have been reported (Kim *et al.,* 2015). Therefore, pollen tube elongation may be affected by the *OsPMT16* mutation.

QUA2/TSD2, which is presumed to be an Arabidopsis PMT, has an S-adenosyl-L-methionine binding domain (SAM domain, pfam03141: Methyltransf_29), and GUS staining has been reported in reproductive tissues such as the pistil (Krupkova *et al.,* 2007). In the present study, the amino acid sequence of *OsPMT16* was also found to have a SAM domain. The *OsPMT16* gene sequence had 51% homology to *QUA2/TSD2*. In the phylogenetic tree, *OsPMT16* and

QUA2/TSD2 formed different clusters (Figure 8). These two genes are highly likely to encode PMTs because pectin methylesterification in pistils decreased in the *OsPMT16* mutant, and the degree of pectin methylesterification was restored in the complement. When cells differentiate, they need to communicate closely with surrounding cells (Kempinski, 2006). In the *QUA2/TSD2* deletion mutant, abnormalities in cell adhesion in the hypocotyl occurred, indicating the possibility of an abnormality in intercellular communication (Qu *et al.,* 2016; Xu *et al.,* 2017). In the present study, the complement test confirmed the recovery of abnormal morphology. The results showed that pectin Methylesterification was not regulated normally in the *OsPMT16* mutant, and insufficient adhesion caused by pectin defects resulted in abnormal cell-to-cell communication and differentiation. *OsPMT16* contributes significantly to the development and maturation of the pistil for reproductive growth.

4. Figures

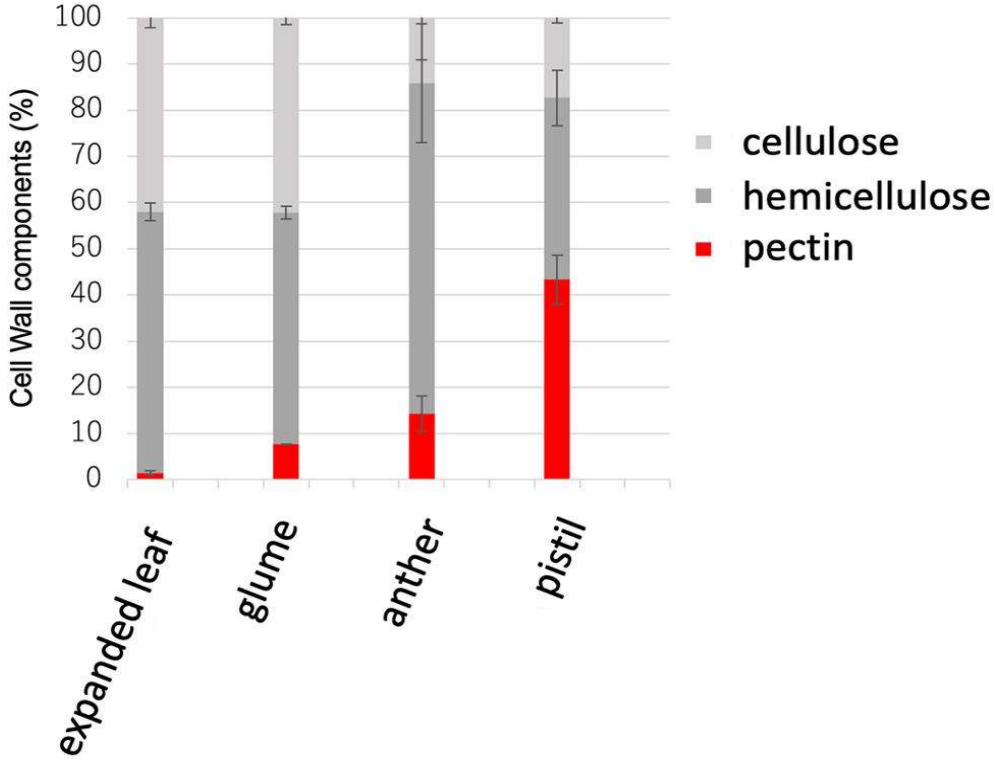


Figure 1. Cell wall sugar composition in the expanded leaf, glume, anther, and pistil. Pectin levels were measured as uronic acid content in the cell wall. Hemicellulose was measured as the amount of neutral sugar in the trifluoroacetic acid (TFA)–soluble fraction in the cell wall. Cellulose was measured as the amount of neutral sugar in the TFA-insoluble fraction in the cell wall. Data represents the means of independent biological replicates ± standard deviation (SD) for the expanded leaf (n = 7), glume (n = 8), anther (n = 4), and pistil (n = 4).

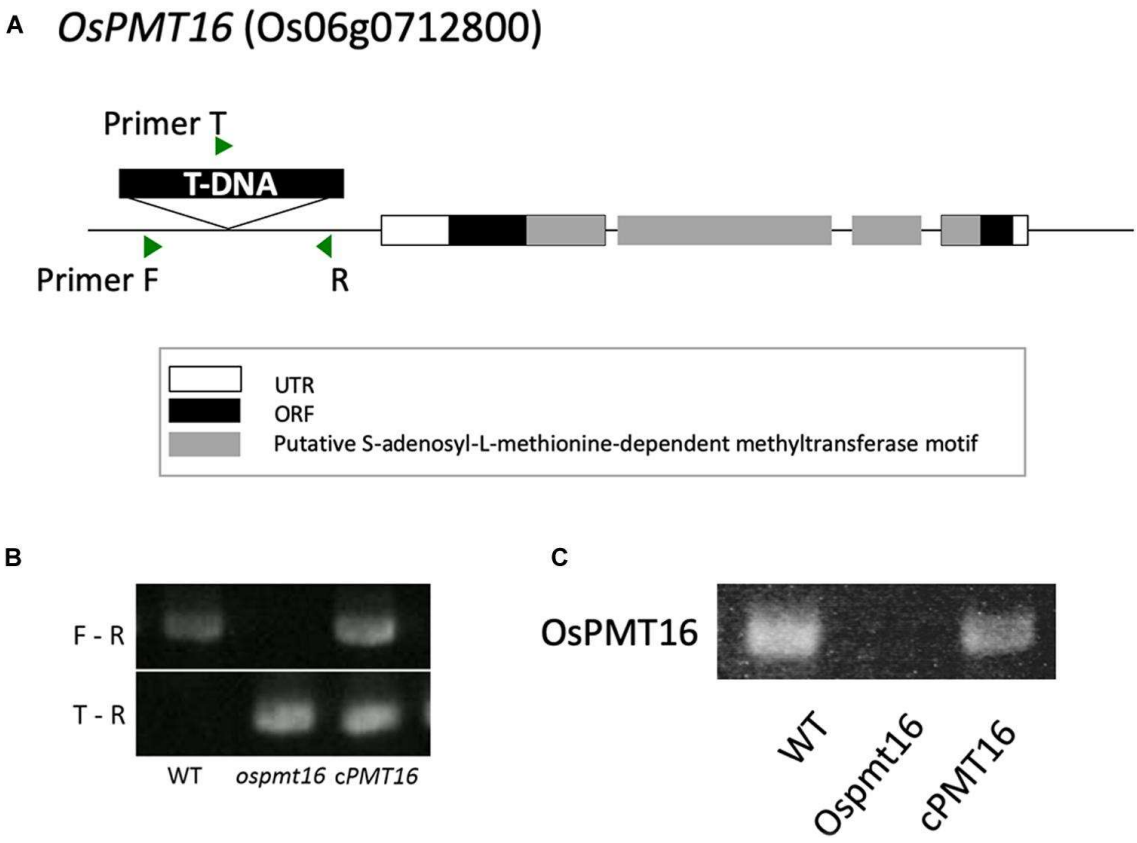
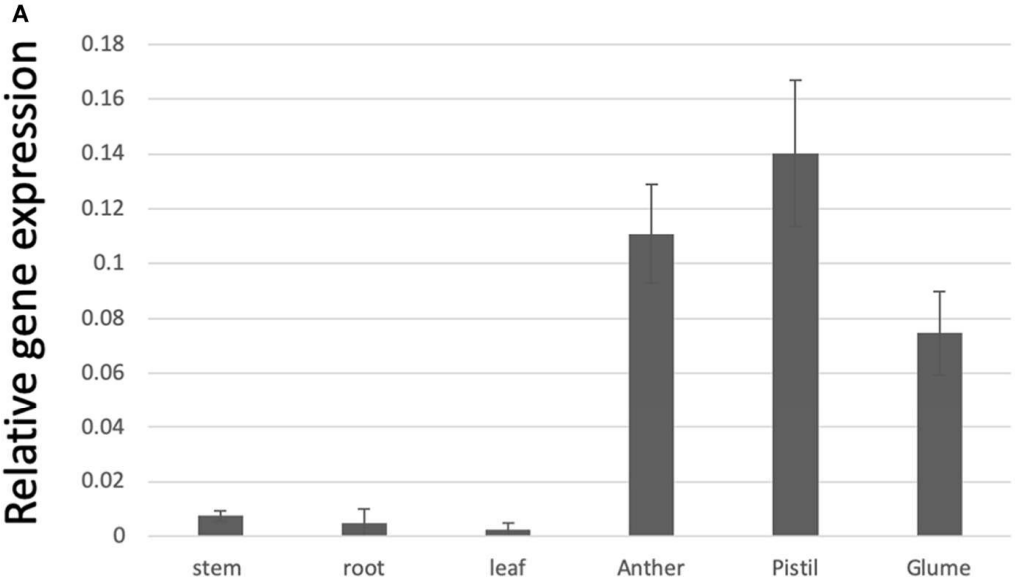
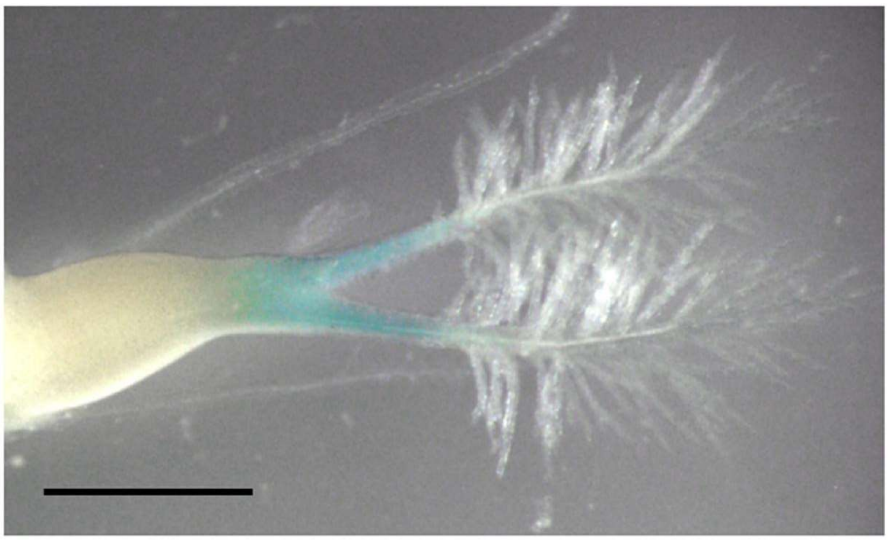


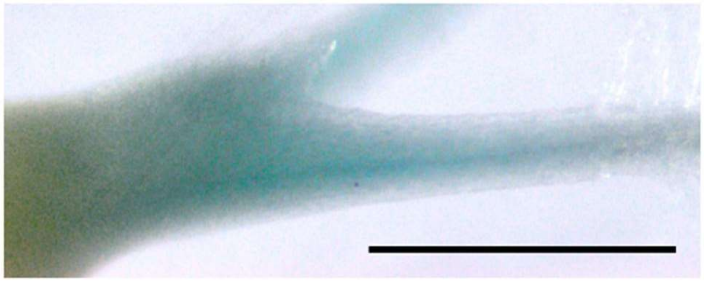
Figure 2. Structure of *OsPMT16*. The *OsPMT16* homozygous line produced a null mutant. (A)Structure of *OsPMT16*, which contains a T-DNA sequence inserted at the promoter region. (B)Homo–hetero test of T1 mutants. F, R, and T indicate primer positions. (C) Results of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of *OsPMT16* expression in flowers of the wild-type (WT), *OsPMT16* mutant, and cPMT16 (complement).


A
Relative gene expression
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
stem
root
leaf
Anther
Pistil
Glume

B



C



D

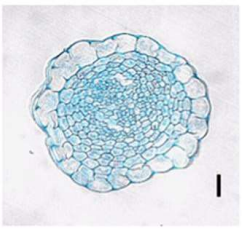


Figure 3. *OsPMT16* gene expression was high in pistil transmitting tissue. (A) Organ-specific expression patterns of *OsPMT16* in WT plants cultivated in an open field during the natural growing season. Leaves and stems at 60 days (mature plant) after seeding were used as vegetative stage organ samples. Bars represent means ± SD of four independent biological replicates. (B) Localization of p*OsPMT16*::GUS expression in the pistil. Bar, 1 mm. The experiments were performed at least twice with similar results. (C) Magnified image of a p*OsPMT16*::GUS pistil. Bar, 1 mm. (D) Cross-section of a p*OsPMT16*::GUS pistil. Bar, 10 μm.

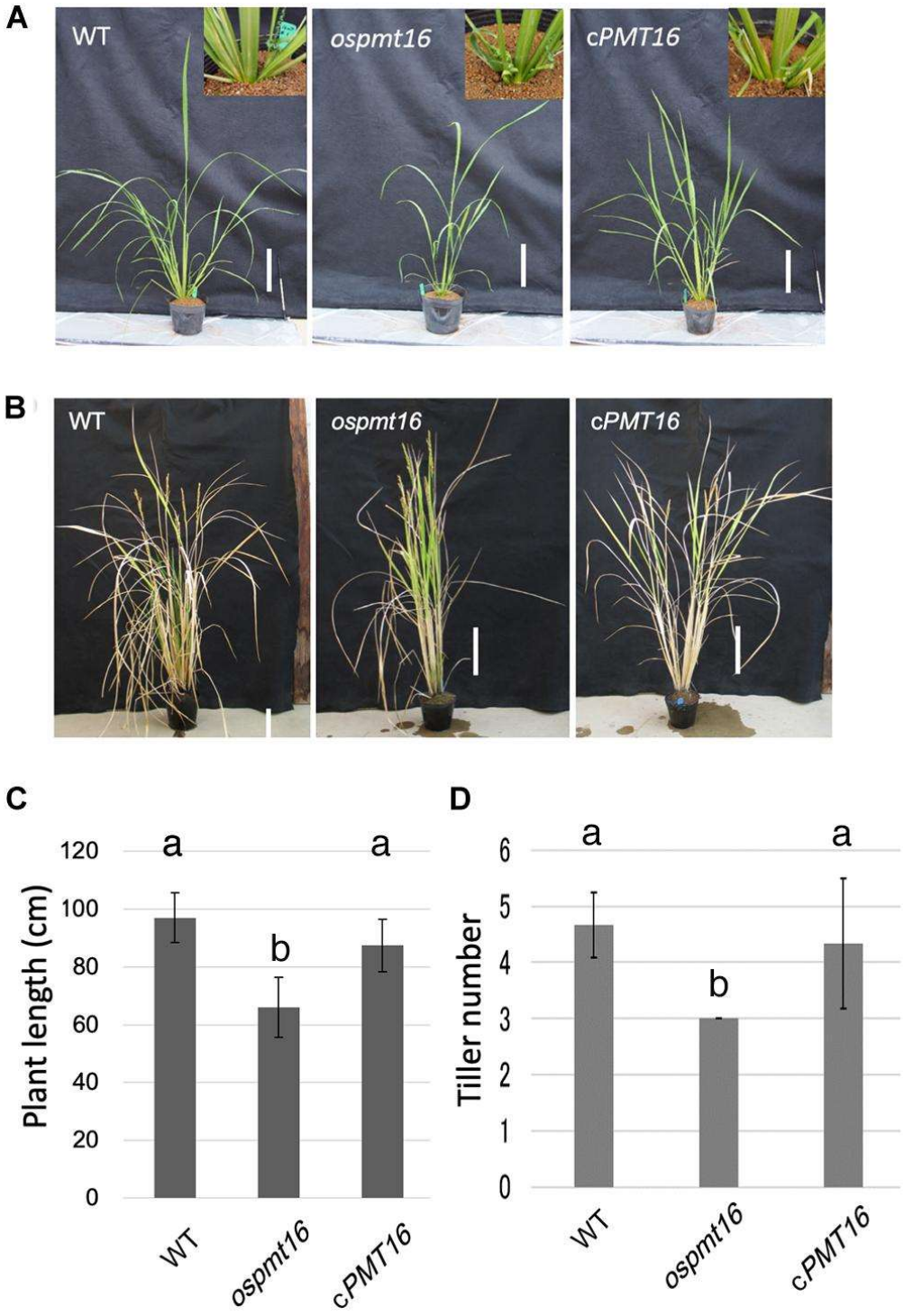


Figure 4. Phenotypes of WT, *OsPMT16*, and cPMT16 plants during the vegetative stage. WT, *OsPMT16*, and cPMT16 plant

growth (A) 71 days after seeding and (B) after flowering. Bar, 10 cm. (C) WT, *OsPMT16*, and cPMT16 plant length. Data represent the means of independent biological replicates ± SD for WT (n = 7), *OsPMT16* (n = 8), and cPMT16 (n = 4). Compared with WT, *OsPMT16* plants were shorter and (D) produced fewer tillers. Data represents the means of independent biological replicates ± SD for WT (n = 4), *OsPMT16*(n = 9), and cPMT16 (n = 4). Bars with different letters indicate a significant difference ($p \leq 0.05$).

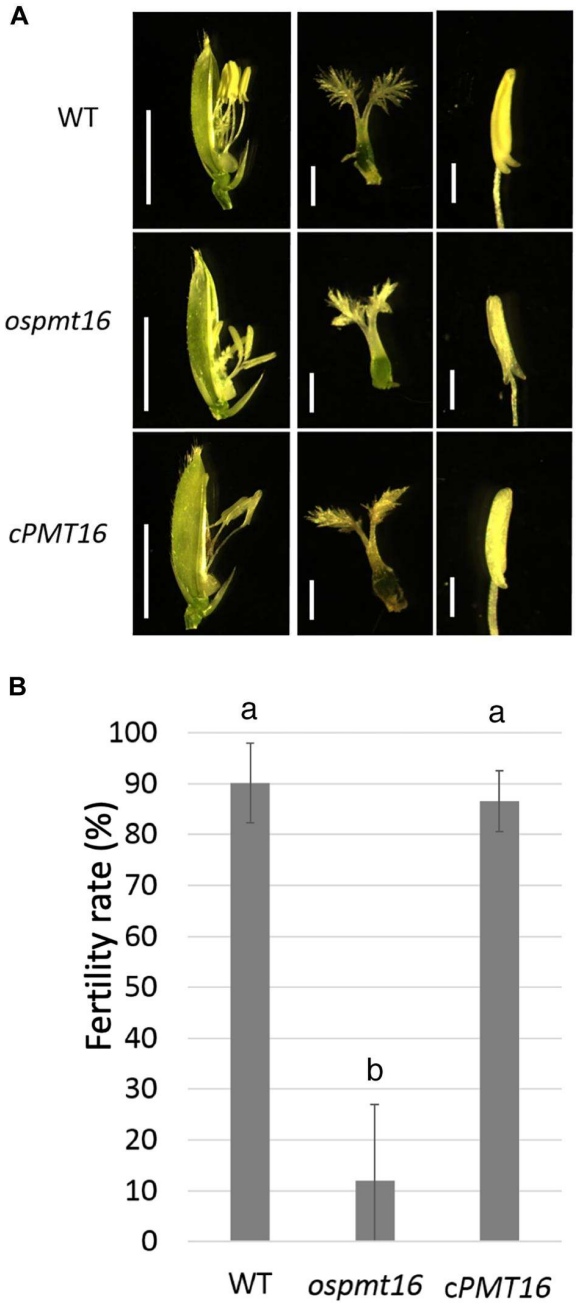


Figure 5. Phenotypic analysis of WT, *OsPMT16*, and cPMT16 flowers. (A) Stamens of the *OsPMT16* mutant had slightly smaller pistils and shorter anthers. Bars represent (left panels) 5 mm and

(middle and right panels) 1 mm. In cPMT16, which was very similar to the WT, these phenotypes were not observed. (B) Seed fertility (proportion of normal seeds in all spikelets) represents the means of independent biological replicates ± SD for WT (n = 4), *OsPMT16* (n = 9), and cPMT16 (n = 4). Despite very low fertility in the *OsPMT16* mutant, only minor differences in reproductive organ morphogenesis were observed between *OsPMT16* and WT. Bars with different letters indicate significant difference ($p \leq 0.01$).

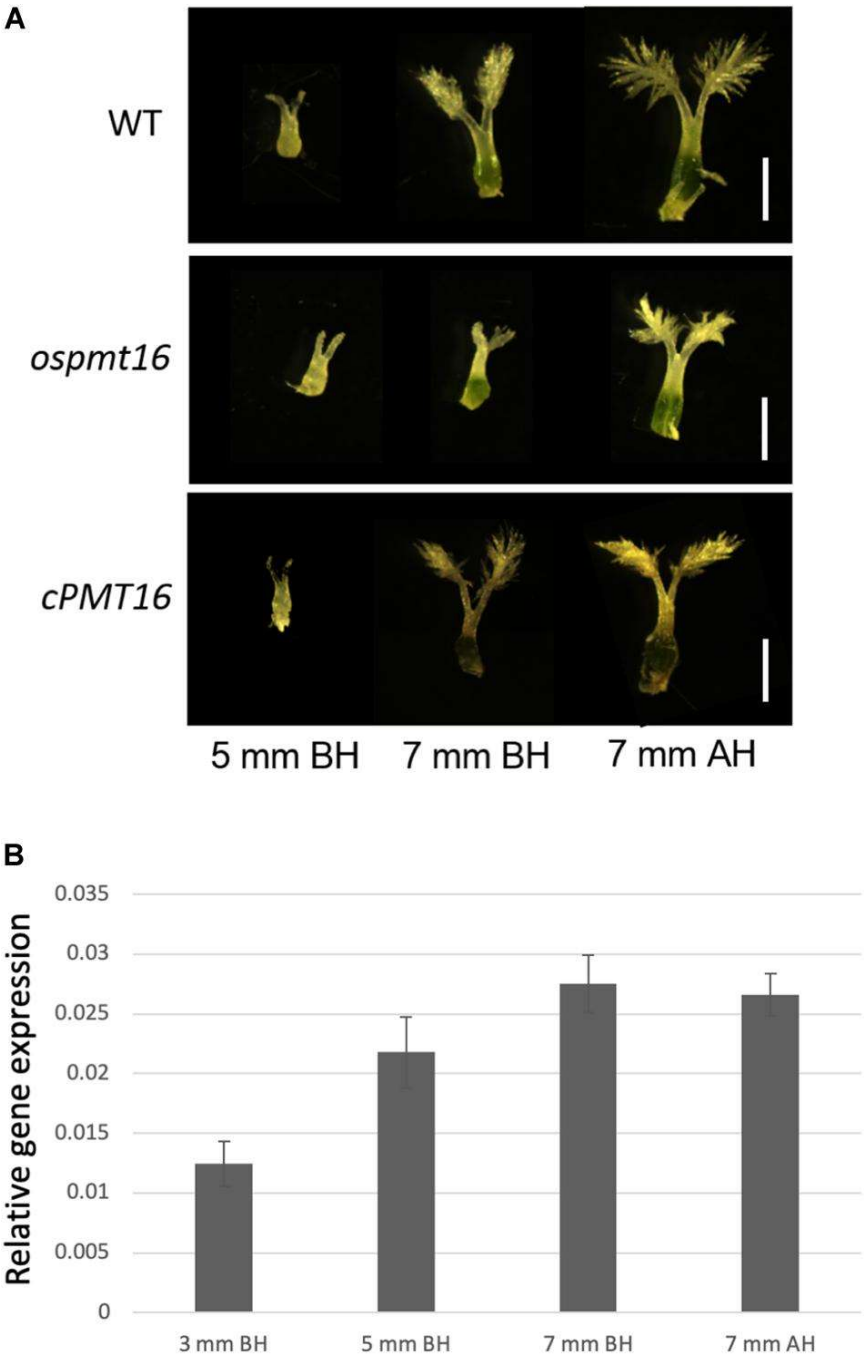


Figure 6. Phenotypic analysis of pistils of WT, *OsPMT16*, and cPMT16 plants during flower development. (A)Pistil development was observed in flowers at glume lengths of 3, 5, and 7 mm before

heading (BH) and 7 mm after heading (AH). Growth delays were observed in the stamen stigma and pistil during morphogenesis at 7 mm BH. (B) Pistil *OsPMT16* gene expression increased gradually during pistil growth.

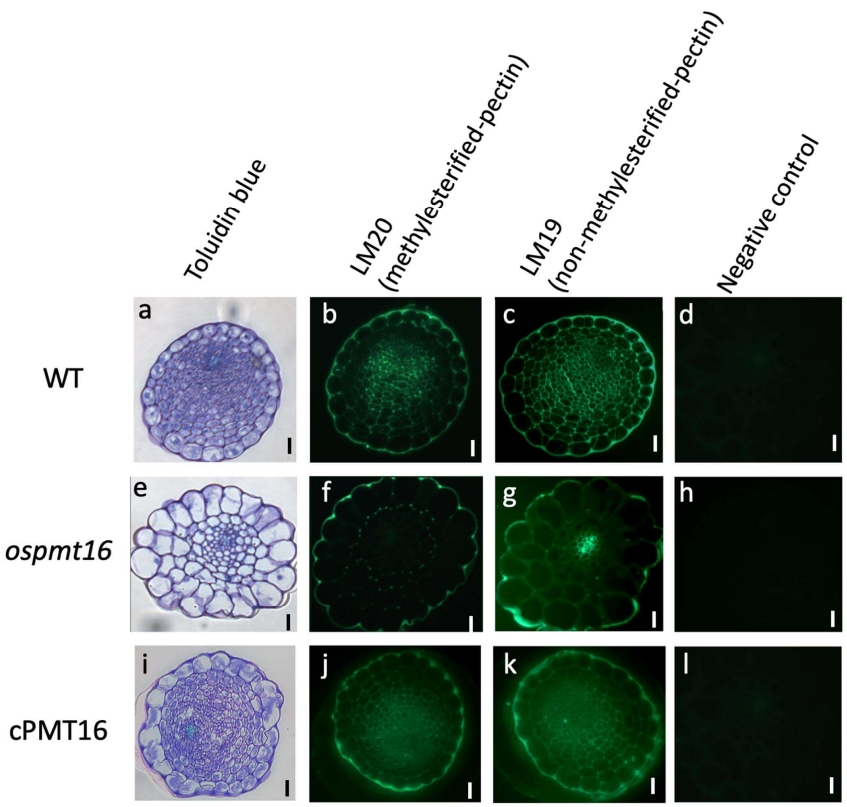


Figure 7. Immunohistochemistry of resin-embedded pistil sections at a glume length of 7 mm BH in WT, *OsPMT16*, and cPMT16 plants. Cross-sections of (a–d) WT, (e–h) *OsPMT16*, and (i–l)cPMT16 labeled with (b,f,j) anti-methyl esterified pectin (LM19) and (s,b,k) anti-esterified pectin (LM20) monoclonal anti-bodies. (a,e,i) Sections were stained by toluidine blue and observed under bright-field illumination. (d,h,i) Micrographs showing the negative control (without the first antibody step). All experiments were performed at least four times with similar results. Bars, 10 μm.

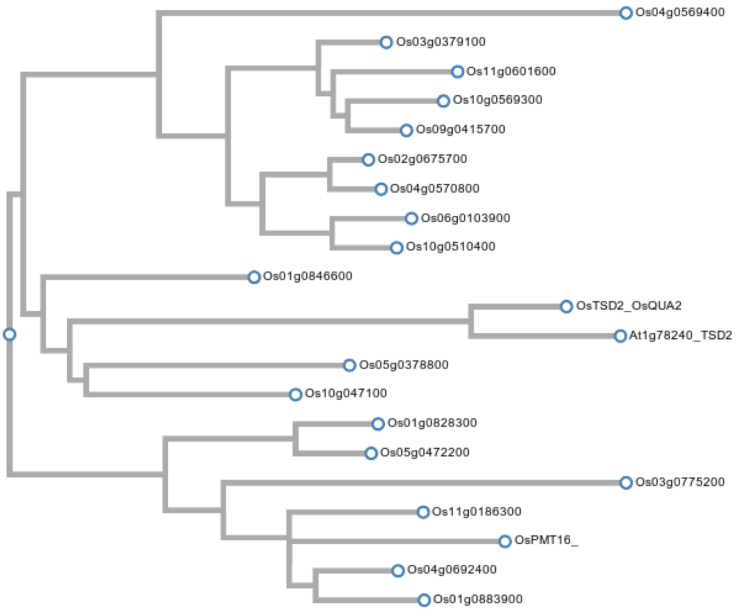


Figure 8. Phylogenetic tree of putative pectin methyltransferase (PMT) genes in rice (*Oryza sativa*), and *TSD2*, which was first reported in *A. thaliana*. Multiple sequence alignment of protein sequence was performed to construct the tree using ClustalX.

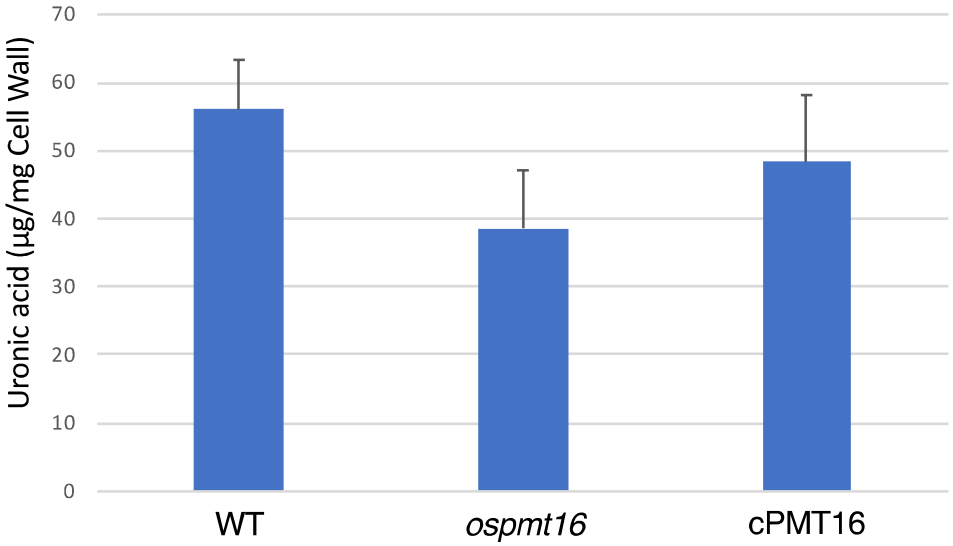


Figure 9. Amount of uronic acid in the pistil cell wall in WT, *OsPMT16*, and cPMT16 plants. Uronic acid content was measured in AIR of the pistil. Bars indicate standard deviation (SD) (n = 4)

Chapter 2

Rice Putative Pectin Methyltransferase Gene OsPMT10 Is Required for Maintaining the Cell Wall Properties of Pistil Transmitting Tissues via Pectin Modification.

1. Materials and Methods

1.1 Plant material and growth conditions

WT (cv. Hwayoung) and *OsPMT10* (gene locus: *Os10g0477100*)-mutated (*ospmt10 mutant*) rice plants were cultivated in a greenhouse during the natural growing season.

1.2 Determination of Fertility Rate

The following procedure is based on the method described by Yan et al (2017). In each treatment, 30 spikelets of each line were grown to maturity. Fertilized spikelets were determined by the degree of kernel plumpness, and both fully and partially filled kernels were considered fertilized kernels. The fertility rate for each panicle was determined by rate from number of fertilized kernels/total spikelet number.

1.3 Pollen germination and elongation

The following procedure is based on the method described by Chhun *et al* (2007). Rice flowers were artificially pollinated using hands. And then, after 30 min, the pistils were stained with 0.1% aniline blue in 2% K_3PO_4 buffer, pH 8.5 on a glass slide, and germinated pollen was detected by UV fluorescence (Leica; DMRB, Wetzlar, Gremany). Aniline blue staining was performed using the method described by Ryan *et al.* (1998) and Singh *et al.* (2002) with modifications.

1.4 DNA extraction and amplification

Fully expanded mature leaves were frozen in liquid nitrogen and ground using the TissueLyser II instrument (Qiagen, Hilden, Germany). Total DNA was extracted using cetyltrimethylammonium bromide and amplified using forward (5′- GCCGAGTCAAGTCCAGAATC -3′), reverse (5′- TACTTGGGGTTGAGGTCCTG -3′), and T-DNA-specific (5′- ACAGGACGTAAC-3′) primers and EX Taq Polymerase (Takara, Kyoto, Japan) as follows: 25 cycles of 96°C for 30 s, 60°C for 15 s, 72°C for

1min. The amplified DNA fragments were separated on 1% agarose gels, and the bands were detected by ethidium bromide staining.

1.5 Vector construction and transformation into plants for the complementation test

A genomic DNA fragment containing the entire *OsPMT10* gene along with the 2,031 bp upstream sequence was inserted into the binary vector pSTARA-R4 for the complementation test. The binary plasmids were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. Transformation of *ospmt10* mutant and selection of transgenic rice were performed as described by Sato *et al*(2011)

1.6 RNA extraction and gene expression analysis

The following procedure is based on the method described by Hasegawa *et al* (2020). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) with DNase I (Roche, Basel, Switzerland) treatment. Then, cDNA was

synthesized using the ReverTra Ace® (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. The *OsPMT10* transcript level was quantified by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using forward (5′- GCCGAGTCAAGTCCAGAATC -3′) and reverse (5′- TACTTGGGGTTGAGGTCCTG -3′) primers. For the endogenous control, the 17S rRNA transcript was also quantified, using forward (5′-GCAAATTACCCAATCCTGAC-3′) and reverse (5′-CTATTGGAGCTGGAATTACC-3′) primers. The amplified DNA fragments were separated on a 1% agarose gel, and bands were detected by staining with ethidium bromide. qRT-PCR of the cDNA was performed using TaqMan DNA polymerase or SYBR Green I (Qiagen) on the Model 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The ubiquitin gene (EU604080) was used as the normalizing reference gene.

I performed an p*OsPMT10*::GUS assay to monitor the expression of *OsPMT10* during reproductive development. A 2,031 bp 5′-UTR fragment of *OsPMT10* was amplified from the genomic DNA. A *OsPMT10* promoter fragment was amplified by PCR using rice genomic DNA and

primers (5′- ATCGTGCCAAAGAAAACTGG -3′ and 5′- CAAGGGACCGAGGATTCATA -3′) and BIOTAG™ DNA Polymerase (Bioline, Toronto, Canada) as follows: 35 cycles of 98°C for 10 s, 55°C for 10 s, 68°C for 1 min. The promoter fragment was then cloned into pBI121 for transformation into *Oryza sativa* (cv Nipponbare) via *Agrobacteria* (Shu et al., 2015). Transformed calli were selected on medium containing kanamycin (100 mg l^{-1}) as described (Konishi *et al.*, 2011). Plants were regenerated from kanamycin-resistant calli, transferred to soil and grown in a greenhouse. GUS staining was performed in the flower, as described by Shu et al. (2015). In brief, the samples were incubated in staining solution (20 mm X-Gluc in phosphate buffer) for 12 h at 37°C and then rinsed in 70% ethanol for microscopic observation.

1.7 Immunohistochemistry

The following procedure is based on the method described by Hasegawa *et al* (2020). WT, *ospmt10*, and cPMT10 pistils were fixed in 4% PFA, 0.25% glutaraldehyde, and 0.05 M phosphate buffer (pH 7.5). Tissue sections were subjected to immunohistochemical analysis using TSA kit #12 (Invitrogen,

Eugene, OR, USA). Primary antibodies for LM19 and LM20 (PlantProbes, Leeds, UK) were used at a dilution of 1:20. Negative controls lacked the primary antibody. Sections were visualized with fluorescence microscopy (Leica; DMRB).

1.8 Statistical Analysis

The data in Figures 1, 4, 5, and 6 are expressed as mean ± standard deviation (SD) obtained from four to nine independent biological experiments. Analysis of variance (ANOVA) and Tukey's test was performed to identify significant differences using Statistica 13.1 software (StatSoft, Inc, Tulsa, OK, USA). p-values ≤ 0.05 and p-values ≤ 0.01 were considered statistically significant. The results with p-value ≤ 0.05 and p-value ≤ 0.01 were considered statistically significant.

1.9 Data Availability

Sequence data of *OsPMT10* from this article can be found in the National Center for Biotechnology Information GenBank and Medicago truncatula Genome Database of National Agriculture and Food Research Organization

(https://rapdb.dna.affrc.go.jp/index.html) following the accession numbers. GenBank accession numbers for the genes are Os10g0477100. The authors declare that all other data supporting the findings of this study are available upon request.

2. Results

2.1 Selection of homozygotes for T-DNA insertion mutants and the complementation test

I used the rice genome database SALAD DB to search for genes containing rice PMT domains; 19 genes were identified as candidates. The amino acid sequences of the candidate genes were obtained from the rice annotation database RAP DB, and the amino acid sequence of the putative PMT gene *TSD2*, which was first reported in *A. thaliana*, was obtained from the *Arabidopsis* annotation database TAIR. Among plants producing pistils with a high pectin content, an *OsPMT10* T-DNA mutant (Os10g0477100), which had high expression in reproductive tissues in the RiceXpro database, was obtained from Postech University.

The T1 generation and homozygous individuals were selected by PCR of genomic DNA. In addition, the complement (cPMT10) was prepared to identify the genes responsible for the phenotypic abnormalities seen in the mutants. A construct in which the full-length gene including the original promoter region was inserted into the

pSTARA-R4 vector was constructed by introducing it into *ospmt10* mutant (Figure 1). In *ospmt10*, T-DNA was inserted into the third exon at nucleotide 1822 (Figure 1). I confirmed that *OsPMT10* is not expressed in the mutant but is expressed in the complement (Figure 1). Null segregants were isolated from among the *ospmt10* mutants and used as wild-type (WT) plants for comparison.

2.2 Gene expression and phenotypic analysis during the vegetative development of *ospmt10* mutants

The expression patterns of *OsPMT10* during reproductive organ development and in each organ were examined by qRT-PCR. *OsPMT10* was strongly expressed in the reproductive organs, especially in the pistil (Figure 2A), as well as in the other organs. In addition, a high GUS staining signal was observed especially in the pistil of the p*OsPMT10*::GUS plant (Figure 2B, C). The expression patterns of GUS in the transmitting tissues of pistils were very different before and after pollination. Before pollination, stigma in

pistil showed strong GUS signals, whereas, after pollination, the GUS signal patterns were not observed.

2.3 Fertility was reduced but pollen growth was similar to WT in the *ospmt10* mutant

The phenotype of *ospmt10* during vegetative growth was examined, and no abnormal traits were observed compared with the WT (Figure 7). The characteristics of the flowers, pistil, and anther were very similar to those of the WT, the mutant develops normal flowers with normal pistils and stamens (Figure 3). Furthermore, pollen isolated from the WT, *ospmt10*, and cPMT10 plants was clearly stained with iodine, indicating mature and healthy pollen (Figure 3). I examined the germination and elongation of pollen by staining pollinated stigmas with aniline blue (Figure 4). At 30 min after artificial self-pollination, ~80 and 50% of the WT pollen grains had germinated and elongated (Figure 3). And germination and elongation rate of *ospmt10* mutant and cPMT10 pollen was very similar to WT (Figure

3). However, this mutant exhibited approximately half the fertility of the WT and its complement (Figure 4).

2.4 Observations of pistil transmitting tissue and pollen tube growth in *ospmt10*

Although no abnormalities in the morphological features described above were observed in *ospmt10*, *OsPMT10* was highly expressed in the pistil transmitting tissues, and its expression level and distribution changed from before to after pollination. Therefore, the pistil transmitting tissue, which makes up the passage of the pollen tube, was observed under a microscope. Pistil development in *ospmt10* was very similar to that of the WT, and there was no difference in the total pectin signal (Figure 5A). However, differences in the localization of methylesterified and dimethyl-esterified pectins, as examined by immunohistochemistry, were apparent (Figure 5B). The signal from LM19, a demethylesterified pectin antibody, was relatively weak, and the signal from LM20, a methylesterified pectin antibody, was strong in the pistil transmitting tissue of the WT. On

the other hand, in *ospmt10*, LM19 signal was observed in the corner of the cells when they are poorly or not labeled in the WT and complemented line. And the LM20 signal and uronic acid contents were significantly decreased to approximately 28%, compared with the WT (Figure 5B, C). However, the decreased methylesterified pectin level and uronic acid contents in the pistil transmitting tissue of *ospmt10* were not apparent in cPMT10.

2.5 Pollen tube progression through the pistil transmitting tissue was halted in *ospmt10*

In WT plants, the pollen tube reached the embryo sac after 100% pollination (Figure 6A, D), but in *ospmt10*, 47.8% of the pollen tubes did not reach the embryo sac through the pistil (Figure 6 B, D). In the *ospmt10* mutant, the pollen tube was stuck in the pistil transmitting tissue and could not elongate toward the ovule (Figure 6C, D). However, elongating pollen tubes were sometimes observed to reach the ovule in the mutant ovary. This indicates that the *ospmt10* mutation does not completely abolish pollen elongation but does

severely disturb these processes. In cPMT10, the pollen tube also reached the embryo sac after 100% pollination.

3. Discussion

Solid-style transmitting tissues have a relatively extensive extracellular matrix, rich in secreted substances, which include polysaccharides, glycoproteins, and lipids (Bell and Hicks 1976). The hollow-style transmitting canal is also filled with mucilage secreted by the epidermal cells of the transmitting tract (Rosen and Thomas 1970, Heslop-Harrison J 1987). The structural features of the rice stigma are similar to those of many other types of grass, such as pearl millet (Heslop-Harrison and Heslop-Harrison 1981) Secale, Hordeum (Heslop-Harrison *et al.,* 1997).

The *ospmt10* mutant showed no difference in morphogenesis compared with the WT during vegetative and reproductive growth (Figure 3 and Figure 7). And, the ability of pollen germination and elongation were also very similar to WT (Figure 4). However, the fertility rate of *ospmt10* mutant was reduced by approximately half. I have found that the pollen grains germinate normally, whereas the pollen tube abnormally elongates in the style - transmitting tissue in

ospmt10 mutant. A rate of pollen tube elongation arrest of 47.8% was observed in transmitting tissues of the pistil (Figure 6).

Furthermore, elongation of the pollen tube may have ceased in the upper part of the ovary of *ospmt10*. As these abnormal phenotypes in *ospmt10* were restored in cPMT10, the causative mechanism is thought to be *OsPMT10* deficiency. The rate of pollen tube elongation arrest in *ospmt10* (47.8%; Figure 6D) was very similar to the fertility rate (50.1%, Figure 4). Therefore, these results suggest that the decreased fertility rate of *ospmt10* is due to the rate of pollen tube reached the embryo sac in *ospmt10*. Based on these results, I conclude that *ospmt10* is a novel type of female sterile mutation in rice, which causes the arrest of the elongation of the pollen tube. The *ospmt10* phenotype has similarity with the phenotype of the Arabidopsis *katanin1-5 (ktn1-5)* mutant (Riglet *et al.,* 2020). They reported that this phenotype is associated with specific mechanical properties of the cell walls that provide less resistance to pollen tube growth. *ktn1-5* has a similar phenotype as *ospmt10*, rendering it difficult for the pollen tube to elongate in the transmitting tissue

(Figure 6B and D). OsPMT10 may also have roles for pollen tube guidance of the pistil transmitting tissue by ensuring mechanical anisotropy.

The Arabidopsis genome contains 29 genes encoding putative methyltransferases. *QUA2/TUMOROUS SHOOT DEVELOPMENT2 (TSD2)* is included in the gene family (Krupkova et al., 2007, Mouille et al., 2007, Kim et al., 2015). The Arabidopsis PMT, *QUA2/TSD2,* contains an S-adenosyl-L-methionine dependent methyltransferase motif (SAM domain, pfam03141: Methyltransf_29), and the enzymatic activity of PMT has been characterized (Du et al. 2020), and GUS staining has been reported in reproductive tissues such as pistils (Krupkova et al. 2007). The *OsPMT10* gene sequence had 53% homology to QUA2/TSD2 and the SAM domain of PMT motif is also present in the amino acid sequence of OsPMT10. *OsPMT10* gene is highly likely to encode PMTs because pectin methylesterification in pistils decreased in the *ospmt10* mutant, and the degree of pectin methylesterification was restored in the complement.

Cotton Golgi-related 3 (CGR3) in Arabidopsis shares conserved residues with S-adenosylmethionine methyltransferases. CGR3 also plays a role in the methylesterification of homogalacturonan in Arabidopsis. In CGR2 and CGR3 double-deficient mutants, pollen tube germination and elongation are weakened, the silique is shortened, and the number of seeds is reduced, suggesting that PMTs play an important role during reproductive development (Mouille *et al.*, 2007, Kim *et al.*, 2015). *QUA2/TSD2* was also expressed in *Arabidopsis* flowers. And highly methylesterified pectin may be involved in the morphogenesis of the style in the olive pistil (Suarez *et al.*, 2013, Hasegawa *et al.*, 2020).

Because *OsPMT10* gene was also strongly expressed in the pistil (Figure 2), I focused on the transmitting tissue, through which the pollen tube passes before pollination; pollen tube arrest was observed in the *ospmt10* mutant. Although there was a difference in the localization patterns of immunohistochemical signal of demethylesterified pectins at the cell corner between the *ospmt10* and WT, these signals were weak in *ospmt10* and WT, whereas that of

methylesterified pectins were strong, in the transmitting tissue of the WT (Figure 5). This result was consistent with the distribution of each pectin type in the transmitting tissue of petunia (Lenartowska *et al.*, 2001). Methylesterified pectin has a weaker binding capacity for calcium ions, and its viscoelasticity changes from gel-like to liquid-like as its mechanical strength decreases. Therefore, pectin of the transmitting tissue may be rich in methylesterified pectin and highly fluid. On the other hand, it was found that the immunohistochemical signal of methylesterified pectins and uronic acid contents were decreased in the mutant compared with the WT (Figure 5B, C). No abnormal morphogenesis in the pistil transmitting tissue of *ospmt10* was observed (Figure 5A).

Therefore, in the mutant, the methylesterified pectin level and uronic acid content were decreased due to deletion of *OsPMT10*, suggesting that the cell wall has little bound calcium. After pollination, the pollen tube invading the stigma enters the transmitting tissue, formed of specialized cells, and moves toward the ovary and embryo sac. Stigma-style cysteine-rich adhesion is also involved in the process,

and it works as adheres and elongates (Lord 2000). Elongating pollen tubes have been shown to actively synthesize pectin-rich cell walls and membranes at the tip (Li *et al.*, 1997). Pectins are highly methylesterified in the apical cell wall immediately after synthesis and then demethylesterified by PME (Lu *et al.*, 2020). The distributions of methylesterified and demethylesterified pectins are important from the very beginning of pollen tube elongation (Heslop-Harrison 1987). In addition, although the level of methylesterification in the transmitting tissue before pollination is maintained, demethylesterification proceeds rapidly after pollination (Suárez *et al.*, 2013), and demethylesterified pectins are derived from the pollen tube and pistil transmitting tissue. The pectins in the pistil transmitting tissue before pollination are inherently highly methylesterified, and the number of calcium bridges is considered to be small; thus, the pistil transmitting tissue is more fluid, thereby allowing easy passage of the pollen tube. As there is only one ovule in the self-pollinated rice pistil, rapidly reducing pectin methylesterification and increasing elongation of the pollen tube leads to the potential selection of more efficient

sperm cells with a fast rate of pollen tube elongation. *OsPMT10* gene expression pattern in pistil transmitting tissues was also decreased after fertilization (Figure 2). In this study, the LM20 immunohistochemical signal was high in the pistil transmitting tissue after heading and before flowering in WT rice (Figure 5B). In this study, OsPMT10 was found to be essential for the regulation of pectin methylesterification in pistil transmitting tissue, and pectin methylesterification showed localized reductions in the mutant (Figure 5B). In the mutant, the fluidity of the transmitting tissue decreased with decreasing levels of pectin methyl esterification, preventing pollen tube passage.

In addition, cross-linking between pectins and calcium may have affected the dynamics of calcium. Many calcium transporters are localized at the tip of the pollen tube, and they actively take up calcium into the pollen tube, suggesting that signal transduction induced by the calcium within the pollen tube is important for pollen tube elongation (Malho *et al.*, 1994, Pierson *et al.*, 1996). In the pistil, the concentration of calcium increases approaching the embryo sac

(Higashiyama and Takeuchi 2015), and abnormal pollen tube elongation was observed by removing calcium (Malhó and Trewavas 1996, Lu et al. 2020). Thus, it has been suggested that calcium is involved in pollen tube guidance. In this study, the level of methylesterification in the transmitting tissue was decreased in the upper ovary (Figure 5). It has been suggested that regulation of calcium cross-linking by controlling methylesterification is important for growing pollen tubes (Li *et al.*, 2002). Calcium required for pollen tube elongation may not have been supplied to the cell wall and cells in *ospmt10* because of the reduced level of pectin methylation. As a result, calcium uptake required for pollen tube elongation was not sufficient in the pistil transmitting tissue of *ospmt10*, and its arrival at the embryo sac may have been delayed.

In *ospmt10*, the rate at which the pollen tube reached the embryo sac coincided with the rate of seed fertilization because the pollen tube could not proceed through the pistil transmitting tissue. The pollen tube “becoming stuck” was due to abnormal cell wall properties as a result of the decreased level of pectin

methylesterification. Based on these results, regulation of pectin methyl esterification in the transmitting tissue of the pistil may be due to the maintenance of proper cell adhesion to allow pollen tubes to pass easily through the pistil. *OsPMT10* may control pollen tube mechanical guidance by maintaining the cell wall properties in pistil transmitting tissues via pectin methylesterification.

4. Figure

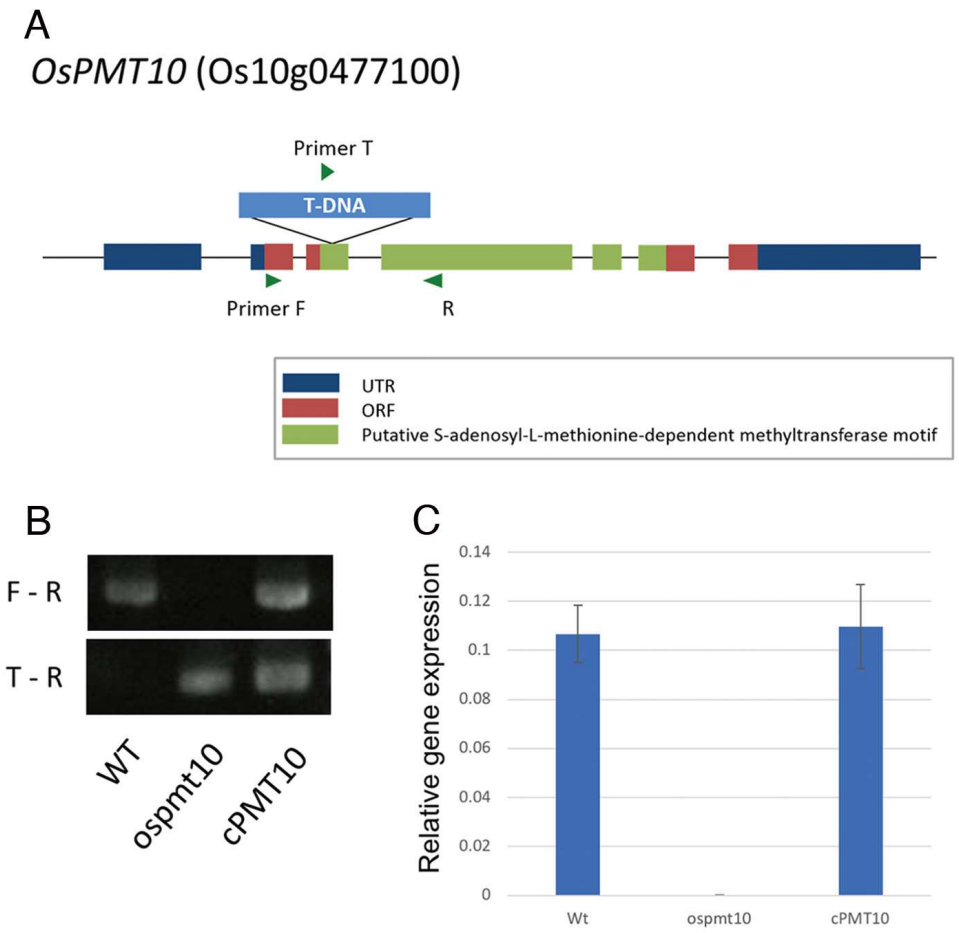


Figure 1. Gene structure of *OsPMT10* and selection of homozygous *ospmt10* mutants. (A) Structure of the *OsPMT10* gene. *OsPMT10* contains a T-DNA sequence inserted within the region of S-adenosyl-L-methionine dependent methyltransferase motif. (B) Homo-hetero test of T1 mutants. F, R, and T indicate the primer positions for the homo-hetero test. (C) *OsPMT10* expression in the flowers of WT, *ospmt10* mutant, and cPMT10 (complement) according to qRT-PCR.

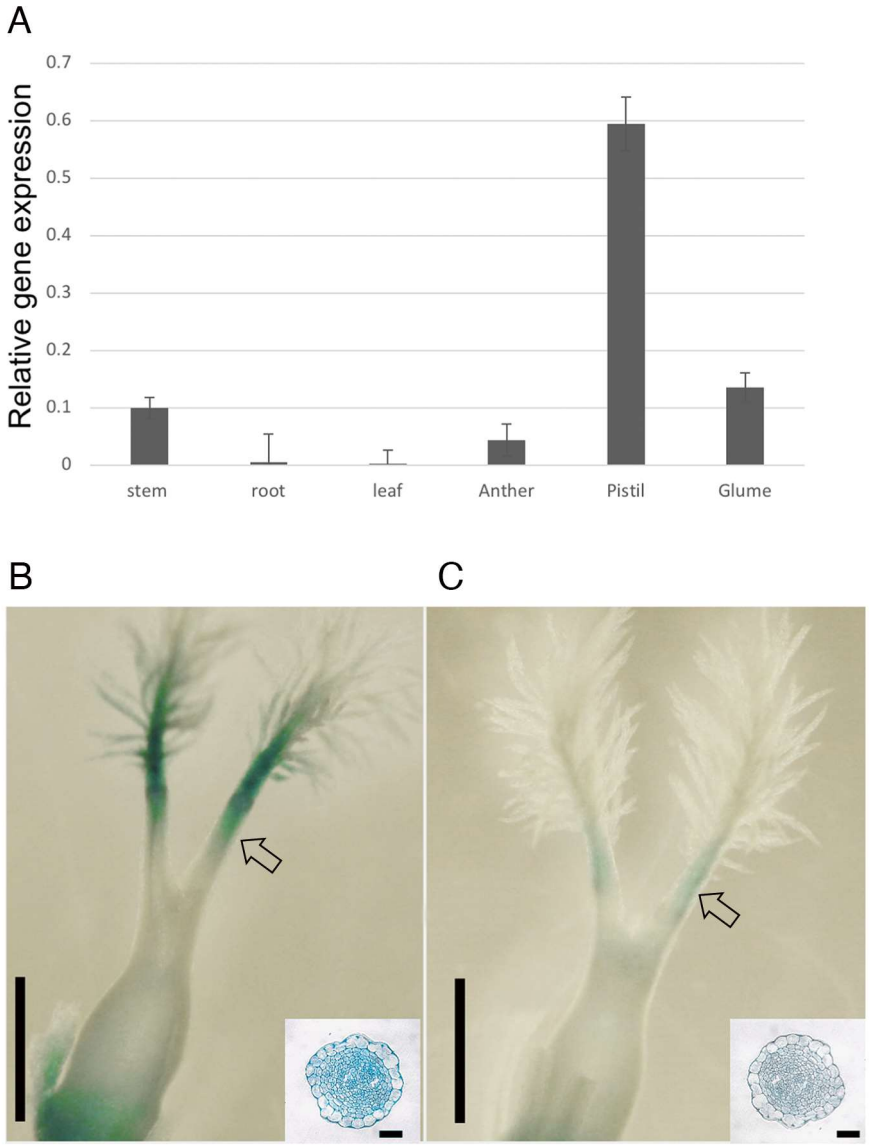

A
Relative gene expression
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
stem
root
leaf
Anther
Pistil
Glume
B
C

Figure 2. *OsPMT10* expression in pistil transmitting tissue. (A) The organ-specific expression patterns of *OsPMT10* in WT plants cultivated in an open field during the natural growing season. The mature leaves and stems at 60 days after seeding were used as vegetative-stage organ samples. Flowers were sampled after flowering. (B) Localization of p*OsPMT10*::GUS expression in the pistil before fertilization. (C) Localization of p*OsPMT10*::GUS expression in the pistil after fertilization Scale bar, 1 mm. Cross-section of the pistil in p*OsPMT10*::GUS (inset). Arrow indicates the position of sections. Scale bar, 10 μm.

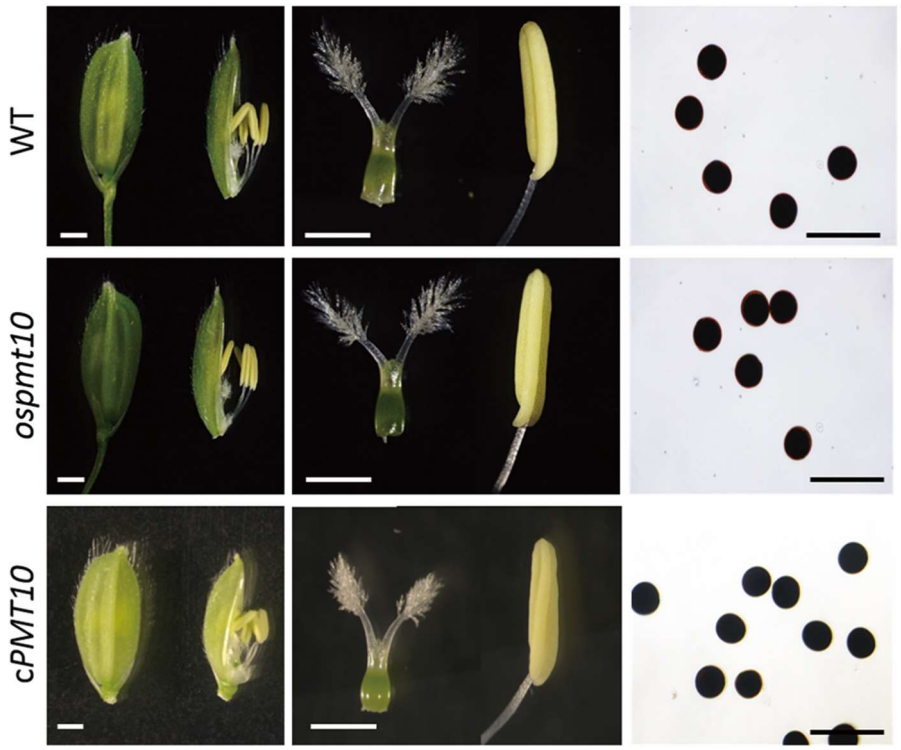


Figure 3. Phenotypic analysis of the WT, *ospmt10*, and cPMT10 in the flower. The phenotypes of WT, *ospmt10*, and cPMT10 rice were very similar. (Left Panel) Mature spikelets of WT, *ospmt10*, and cPMT10. (Middle panel) Mature pistils and anthers of WT, *ospmt10*, and cPMT10. (Right panel) Mature pollen grains of WT, *ospmt10*, and cPMT10 stained with 1% KI–I_2 solution. Scale bars, 5 mm ((left panel) and 1 mm (middle and right panels).

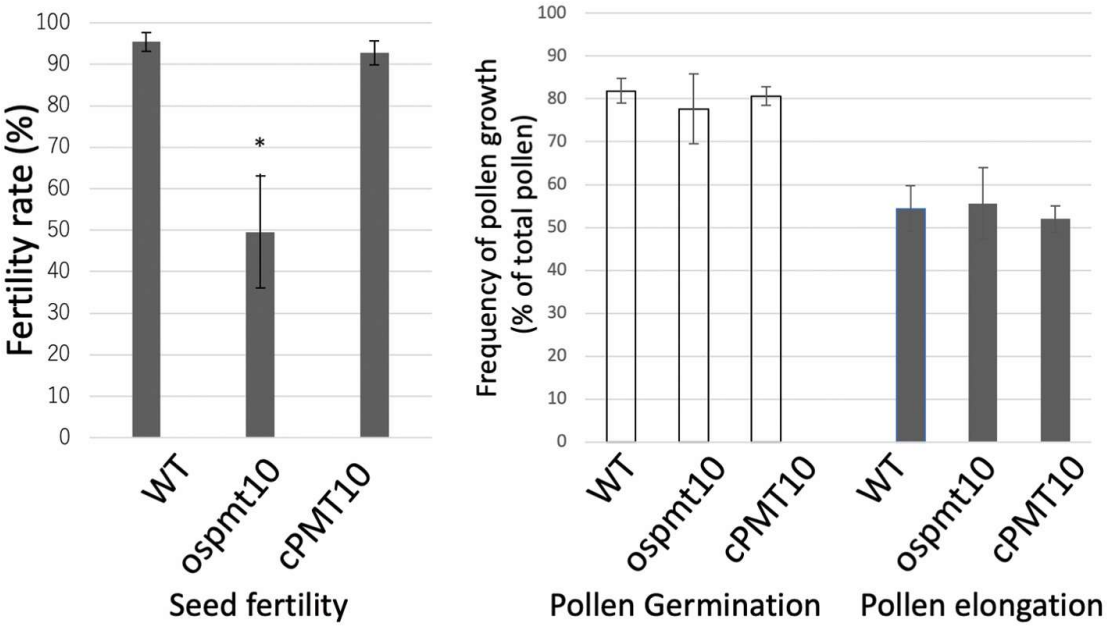

Fertility rate (%)
100
90
80
70
60
50
40
30
20
10
0
*
WT
ospmt10
cPMT10
Seed fertility
Frequency of pollen growth (% of total pollen)
100
90
80
70
60
50
40
30
20
10
0
WT
ospmt10
cPMT10
WT
ospmt10
cPMT10
Pollen Germination
Pollen elongation

Figure 4. Seed fertility and pollen germination & elongation of WT, *ospmt10*, and cPMT10 rice. Seed fertility was calculated as the proportion of normal seeds among all spikelets. Despite that fertility was reduced by approximately half in the *ospmt10* mutant compared with WT, no differences in reproductive organ morphogenesis were observed between WT, *ospmt10* and cPMT10 rice. Germination and elongation frequencies of WT, *ospmt10* and cPMT10 pollen. Open and closed bars represent the frequencies of WT, *ospmt10* and cPMT10 pollen germination or elongation, respectively. Data represents the means of independent biological replicates ± SD for WT (n = 4), *ospmt10* (n = 9), cPMT10 (n = 4).

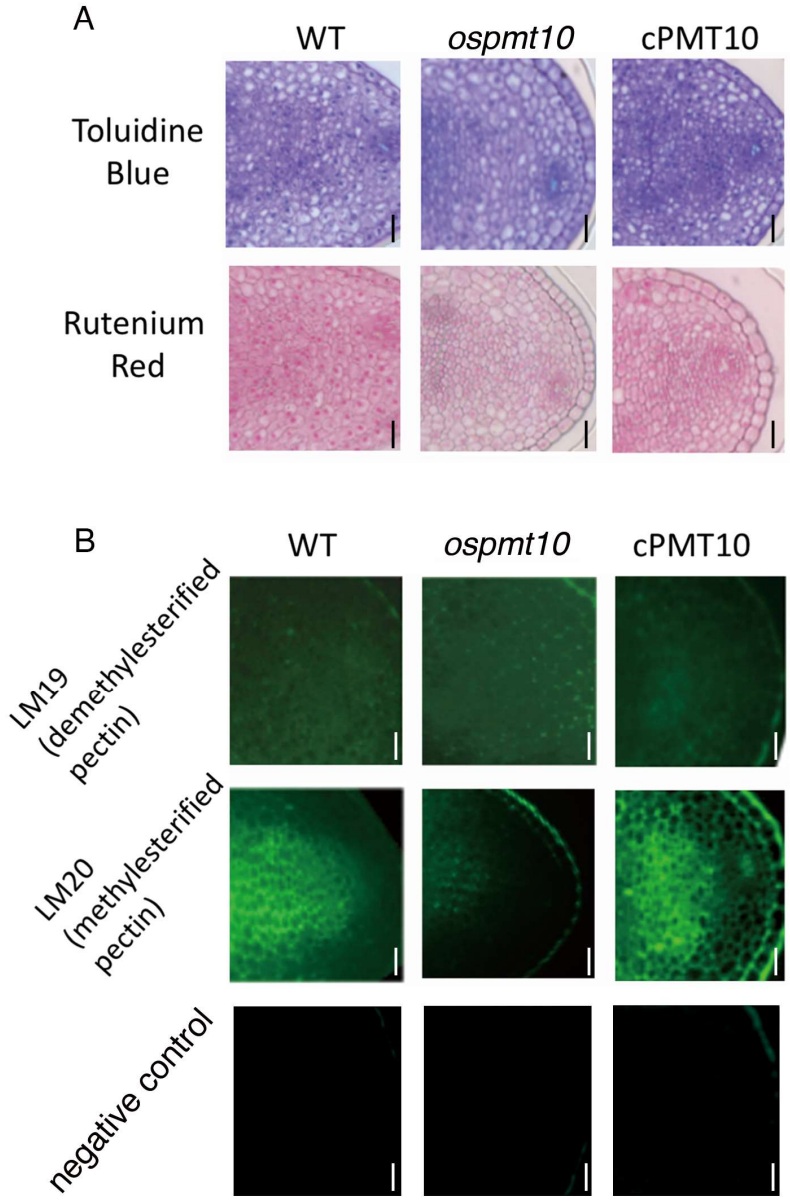

A
WT
ospmt10
cPMT10
Toluidine Blue
Rutenium Red
B
WT
ospmt10
cPMT10
LM19 (demethylesterified pectin)
LM20 (methylesterified pectin)
negative control
C
Relative uronic acid contents (%)
150
100
50
0
**
*
WT
ospmt10
cPMT10

Figure 5. Immunohistochemistry of resin-embedded pistil sections before heading in WT, *ospmt10*, and cPMT10. Tissue cross-sections from the wild-type (WT), *ospmt10*, and cPMT10 were observed under bright-field illumination stained with toluidine blue and pectin staining by ruthenium red (A). Sections were stained with LM19 (anti-demethylesterified pectin) and LM20 (anti-methylesterified pectin) monoclonal antibodies (B) The negative control, in which the first antibody step was omitted. These sections were prepared in the position where the elongation of pollen tubes is stopped in *ospmt10* mutant of the pistil (Fig 7b arrow). All experiments were performed at least four times with similar results. Scale bars, 10 μm. Amount of uronic acid in the pistil cell wall in wild-type (WT), *ospmt10*, and cPMT10 plants (C). Uronic acid content was measured in alcohol-insoluble residue of the pistil. Bars indicate standard deviation (SD) (n = 4).

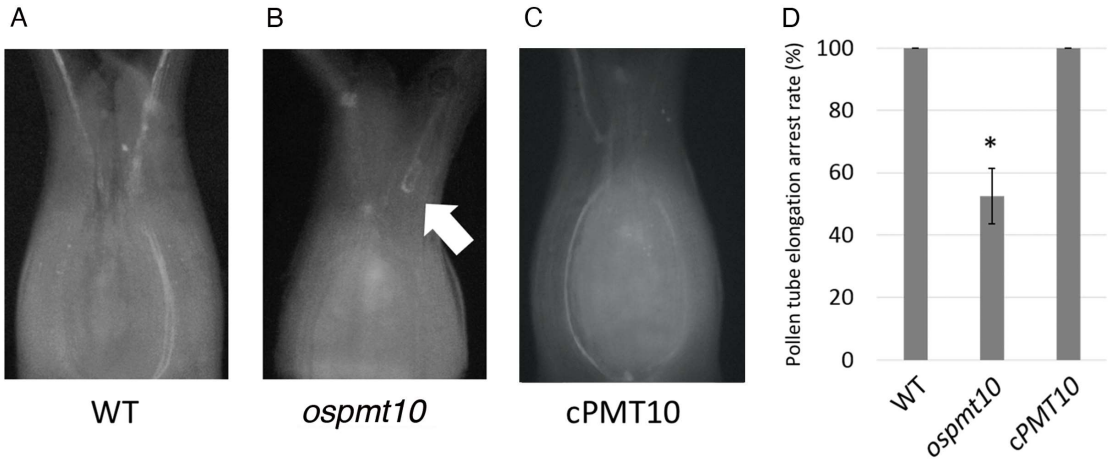


Figure 6. Pollen tube elongation in pistil transmitting tissue. (A) In Wt, the pollen tube reached the embryo sac. (B) Pollen tubes with halted progression in the middle of the pistil transmitting tissue of the *ospmt10* mutant. Arrow indicates the position of the pollen tube elongation arrest. (C) In cPMT10, the pollen tube also reached the embryo sac. (D) Pollen tube elongation arrest rate. In WT and cPMT10, the pollen tube reached the embryo sac after 100% pollination (WT: n=15, cPMT10: n=8), but in *ospmt10*, 47.8% of the pollen tubes did not reach the embryo sac (*ospmt10*: n=16).

Figure 7. Phenotype of WT, *ospmt10*, and cPMT10 rice during the vegetative stage. Plant growth at 71 days after seedling in WT, *ospmt10,* and cPMT10 rice.

General Discussion

1. Maintenance of Methylesterification of Pectin has important roles in reproductive tissues in Rice

Type II cell walls of monocotyledonous plants such as rice contain less pectin overall than type I cell walls of dicotyledonous plants (Yokoyama and Nishitani, 2004). The results of this study showed that mature leaves contained only approximately 5% pectin, which is consistent with previous reports. However, when organs other than leaves were analyzed, the reproductive tissues, especially the pistil cell wall, contained ~43% pectin (Figure 1 in Chapter 1). By analyzing the rice genome database, I picked up 19 genes as PMTs and prepared the phylogenetic tree (Figure 8 in Chapter 1). In contrast, 27 genes were reported in Arabidopsis. The *ospmt16* and *ospmt10* mutants were mutants in which different genes were independently disrupted, but similarly, both mutants were less fertilety and the distribution patterns of methylesterified and demethylesterified pectin in the pistil were different from the wild type (Figure 5,7 in Chapter 1 and Figure 4,5 in Chapter 2). These results suggest that the maintenance of methyl

esterification of pectin functions in the development of reproductive tissues in rice as in dicotyledonous plants and plays an important role in the establishment of fertility.

2. *OsPMT16* and *OsPMT10* play distinct roles in reproductive processes, including fertilization.

OsPMT16 and *OsPMT10* have a PMT domain pfam03141 (Methyltransf_29, Putative S-adenosyl-L-methionine-dependent methyltransferase) (Figure 8 in Chapter 1). These genes were expressed in the pistil but showed different phenotypes during reproduction (Figure 3-7 in Chapter 1 and Figure 2-7 in Chapter 2). *ospmt16* mutants showed abnormalities in the pistil before heading (Figure 6 in Chapter 1), *ospmt10* mutants had normal pistils but less pectin levels than WT (Figure 3, 5 in Chapter 2), *ospmt10* mutant had less pectin than WT. These findings suggest that *OsPMT16* and *OsPMT10* work at different timing stages and play different roles during reproduction. *OsPMT16* is expressed at the stage of pistil development and affects morphogenesis during organogenesis by

maintaining pectin methyl esterification, while *OsPMT10* may have a function in the late stage of pistil development by affecting the regulation of the softness of the transmitting tissues required for pollination.

From this study, even though pectin is scarce in vegetative tissues, it is abundant in reproductive tissues in rice, indicating that pectin has an important function in the process of reproductive development, and the *OsPMT16* plays an important role in the morphogenesis of the pistil transmitting tissues, which the pathway of the pollen tube. I also showed that *OsPMT10* functions in pollen tube guidance through the regulation of cell wall softness in the pistil. In this study, I showed that regulation of pectin methylesterification by *OsPMT10* and *OsPMT16* is important for the development of the pistil, the pistil transmitting tissue, and the fertilization system by maintaining a soft state for pollen tube passage.

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