**Oryza sativa PECTIN DEFECTIVE TAPETUM1 affects anther development through a pectin-mediated signaling pathway in rice**

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Abstract

Galacturonosyltransferase (GalAT) is required for the synthesis of pectin, an important component of plant cell walls that is also involved in signal transduction. Here, we describe the rice (*Oryza sativa*) male-sterile mutant *O. sativa pectin-defective tapetum1* (*ospdt1*), in which GalAT is mutated. The *ospdt1* mutant exhibited premature programmed cell death (PCD) of the tapetum and disordered pollen walls, resulting in aborted pollen grains. Pectin distribution in the anther sac was comparable between the mutant and the wild-type, suggesting that the structural pectin was not dramatically affected in *ospdt1*. Wall-associated kinases are necessary for the signal transduction of pectin, and the intracellular distribution of *O. sativa indica* WALL-ASSOCIATED KINASE1 (*OsiWAK1*), which binds pectic polysaccharides to its extracellular domain, was affected in *ospdt1*. *OsiWAK1* RNA interference lines exhibited earlier tapetal PCD, similar to *ospdt1*. Furthermore, overexpression of *OsiWAK1* in *ospdt1* lines partially rescued the defects observed in *ospdt1*, suggesting that *OsiWAK1* plays pivotal roles in the function of *OsPD1*. These results suggest that the mutation of *OsPD1* does not dramatically affect structural pectin but affects components of the pectin-mediated signaling pathway, such as *OsiWAK1*, and causes male sterility.

Introduction

In angiosperms, sexual reproduction results in the generation of genotypically variable progeny to promote adaptability to environmental changes. Microspores are important in the reproduction and evolution of angiosperms (Hafidh et al., 2016). The tapetum, the inner cell layer of the anther sac surrounding the microspores, participates directly in microspore development via the secretion of callase, sporopollenin precursors, and assimilation products (Zhang et al., 2010; Ariizumi and Toriyama, 2011; Shi et al., 2011; Wan et al., 2011; Zhang et al., 2011a, 2011b; Xu et al., 2017). The release of these metabolites is spatiotemporally regulated...
and depends on tapetal programmed cell death (PCD) (Zhang et al., 2011a, 2011b; Shi et al., 2015a, 2015b). In rice (Oryza sativa), PCD-associated genes include ETERNAL TAPETUM (EAT1), TAPETUM DEGENERATION RETARDATION (TDR), and TDR INTERACTING PROTEIN2 (TIP2), all of which cooperatively regulate tapetal PCD (Li et al., 2006; Niu et al., 2013; Fu et al., 2014; Ko et al., 2014). PERSISTENT TAPETAL CELL1 (PTC1) encodes a PHD-finger protein that is required for the PCD of tapetal cells (Li et al., 2011). The gibberellin-dependent MYB transcription factor GAMYB regulates the expression of CYP703A3, a cytochrome P450 hydroxylase gene, to influence the PCD of tapetal cells (Aya et al., 2009). CYP704B2 is a cytochrome P450 family gene involved in the ω-hydroxylation of fatty acids, which is pivotal for sporopollenin synthesis. The cyp704b2 mutant exhibits a swollen tapetal layer, no detectable exine of microspores, and an undeveloped anther cuticle (Li et al., 2010). Mutants of O. sativa Defective in Exine Formation 1 (OsDEX1), encoding a Ca2+-binding protein, exhibit earlier PCD signals compared with the wild-type (WT), which caused earlier but incomplete degradation of the tapetum (Yu et al., 2016). However, the precise mechanism underlying tapetal PCD remains unclear.

Pectins are an important group of cell wall polysaccharides that predominantly comprise homogalacturonan (HG), rhamnogalacturonan I, and rhamnogalacturonan II. The backbones of HG are polygalacturonides, which are hydrolyzed to oligogalacturonides and D-galacturonic acid (D-GalA) by pectinase (Atmodjo et al., 2013). In Arabidopsis (Arabidopsis thaliana), GALACTURONOSYLTRANSFERASE1 (GAUT1), a galacturonosyltransferase (GalAT), catalyzes the transfer of D-GalA from UDP-D-GalA to the nonreduced end of oligogalacturonides to form HG, the most abundant pectic polysaccharide (Sterling et al., 2006; Lund et al., 2020). The mutation of GalAT genes consistently causes a dramatic decrease in the pectin content of cell walls and obvious cell wall defects, which ultimately influence cell morphology, plant height, and pollen tube growth (Bouton et al., 2002; Persson et al., 2007; Caffall et al., 2009; Kong et al., 2011; Lund et al., 2020).

Pectins are not only structural components, but they are also involved in signal transduction (Ridley et al., 2001). Wall-associated kinases (WAKs) are necessary for the signal transduction of pectin and are directly affected by pectin (Kohorn, 2016). In Arabidopsis, the extracellular domain (EXD) of CELL WAK1 binds to pectic polysaccharides in vitro, and the WAK1 fusion protein in protoplasts appears to assemble with cell wall pectin before being transported to the cell periphery (Decreux and Messiaen, 2005; Decreux et al., 2006; Kohorn et al., 2006a, 2006b). A domain-swap analysis revealed that the EXD of WAK1 recognizes oligogalacturonides in vivo and that the kinase domain triggers defense responses (Brutus et al., 2010). CELL WAK2 activates genes involved in cell expansion, pathogen resistance, stress responses, and the accumulation of reactive oxygen species (Kohorn et al., 2006a, 2006b, 2012, 2014). The pectin-mediated signaling pathway is WAK2 dependent (Kohorn et al., 2009). In addition, a WAK-like family member, OsiWAK1, is involved in anther development in rice, but its regulatory mechanism is unclear (Kanneganti and Gupta, 2011). Overall, recent studies suggest that WAKs assemble with pectin before being transported to the cell periphery. At the plasma membrane, WAKs recognize pectic polysaccharides via the EXD, and the kinase domain in the cytoplasm transmits various signals (Kanneganti and Gupta, 2008; Kohorn, 2016).

In this study, we identified the male-sterile rice mutant O. sativa pectin-defective tapetum1 (ospdt1), which exhibits premature PCD of the tapetum and disordered pollen walls, resulting in complete male sterility. Pectin deposition was not dramatically affected in the anther sac of ospdt1, and the intracellular distribution of OsiWAK1, whose EXD binds to pectic polysaccharides, was altered. OsiWAK1 RNA interference (RNAi) lines exhibited earlier tapetal PCD, similar to ospdt1. Furthermore, overexpression of OsiWAK1 in ospdt1 lines partially rescued the defects observed in ospdt1, suggesting that OsiWAK1 plays pivotal roles in the function of OsPDT1.

Taken together, our results suggest that mutation of OsPDT1 does not dramatically affect the structural pectin, but influences components of the pectin-mediated signaling pathway, such as OsiWAK1, and causes male sterility.

Results

Phenotypic analysis of the ospdt1 mutant

To identify genes involved in anther development in rice, we generated a mutant library of rice “Xilong 1B” via ethyl methanesulfonate (EMS) mutagenesis. The ospdt1 mutant was isolated based on its absolute male sterility phenotype. The ospdt1 mutant exhibited normal vegetative growth but produced no filled grains (Figure 1A). Compared with the WT, the ospdt1 mutant produced normal spikelets (Figure 1B) and failed to produce viable pollen grains (Figure 1, C and D). All F1 progeny from a cross between the ospdt1 mutant and the WT were fertile. In the F2 population, the phenotype segregated at a ratio of ~3:1 (fertile:sterile = 834:271, \( \chi^2 = 0.061, P > 0.05 \)), suggesting that the male sterility in ospdt1 was caused by a single recessive mutation.

Transverse sections were examined to determine the morphological defects of ospdt1 anthers. Based on the classification of anther developmental stages in rice, we classified anther development in the mutant and WT into 14 stages (Zhang et al., 2011a, 2011b). At Stages 7–10, no detectable difference was observed between WT and ospdt1 anthers (Figure 1, E–M and Q). At Stage 11, WT tapetum degenerated into cellular debris on the internal surface of the anther sac (Figure 1N), whereas the tapetum expanded abnormally in ospdt1 anthers (Figure 1R). At Stage 12, the tapetum in WT anthers was completely degenerated (Figure 1O), whereas the tapetal cells were highly vacuolated and microspores were collapsed in the ospdt1 anther sac (Figure 1S).
At Stage 14, WT anthers dehisced and released mature pollen grains (Figure 1P), whereas ospdt1 anthers showed delayed dehiscence and contained aborted microspores (Figure 1T).

We used transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to further investigate anther defects in ospdt1. At Stage 8a, microspores were normal in ospdt1 (Supplemental Figure S1, A and D). At Stage 8b, in WT anthers, the plasma membrane of microspores was undulated (Figure 2, A and B), which is pivotal for primexine formation (Ariizumi and Toriyama, 2011; Shi et al., 2015a, 2015b), and deeply stained materials were detected surrounding the microspores (Figure 2A). Undulations of the plasma membrane were reduced, and the deeply stained materials were aberrantly deposited in ospdt1 (Figure 2, F and G). At Stage

**Figure 1** Aborted pollen grains and abnormal degradation of tapetum in ospdt1 anthers. A, Plant phenotypes at the seed maturation stage. B, Spikelets without lemma before anthesis. C and D, Pollen grains at Stage 12 stained by I$_2$-KI. E–T, Transverse sections of anthers. E–H and M–P, Anthers were obtained from the WT at Stages 7, 8a, 8b, 9, 10, 11, 12, and 14, respectively. I–L and Q–T, Anthers were obtained from the ospdt1 at Stages 7, 8a, 8b, 9, 10, 11, 12, and 14, respectively. dM, degraded microspore; Dy, dyad cell; E, epidermis; En, endothecium; mP, mature pollen; MMC, microspore mother cell; Msp, microspore; T, tapetum; Tds, tetrads. Bars, 10 cm in (A), 2 mm in (B), 30 μm in (C) and (D), and 10 μm in (E–T).
9, microspores had a developed exine in WT anthers, including dense tectum and columnar bacula (Figure 2C). However, the tectum and bacula were disordered in ospdt1 (Figure 2H). At Stage 10, in WT anthers, the exine of microspores was composed of tectum, bacula, and nexine (Figure 2D). In contrast, in ospdt1 anthers, the exine was disordered (Figure 2I). At Stage 11, in WT anthers, intine formed between the microspore and exine; however, only disordered exine was retained in ospdt1 anthers (Figure 2E and J). At Stage 14, WT pollen grains were smooth and spherical (Figure 2O), whereas ospdt1 pollen grains were rough and shrunken (Figure 2T). We performed aniline blue staining to detect callose metabolism, which is pivotal for primexine formation and microspore development (Ariizumi and Toriyama, 2011; Shi et al., 2015a, 2015b). Callose was deposited around the microspore mother cells at Stage 7, and staining faded at Stage 9 in both WT and ospdt1 anthers (Supplemental Figure S2, A, D, E, and H). In WT anthers, callose was deposited at the periphery of dyad cells at Stage 8a (Supplemental Figure S2B) and was predominantly deposited in the cell compartment of tetrads at Stage 8b (Supplemental Figure S2C). However, in ospdt1 anthers, weak callose signals were detected at the periphery of dyad cells at Stage 8a (Supplemental Figure S2F), and almost no signal was detected within tetrads at Stage 8b (Supplemental Figure S2G).

The tapetal cells were not dramatically affected in ospdt1 at Stage 8a and b (Supplemental Figure S1B, C, E, and F). At Stage 9, the cytoplasm of the tapetal cells was condensed, and the Ubisch bodies were small and distributed around the inner surface of the tapetum in the WT (Figure 2K). In contrast, the Ubisch bodies were more mature in ospdt1 (Figure 2P). At Stage 10, in the WT, the Ubisch bodies were mature and nuclei were round (Figure 2, L and M). In contrast, the nuclei were shrinking in ospdt1 (Figure 2, Q and
At Stage 11, in WT anthers, the tapetum degenerated into a thin layer, and the Ubisch bodies were dispersed on the inner surface of the tapetal layer (Figure 2N). In contrast, in ospdt1, the tapetal cells contained abnormally expansive cytoplasm, were dramatically vacuolated, and exhibited plasmolysis (Figure 2S).

To determine whether tapetal PCD was abnormal in ospdt1 anthers, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. No positive signals were detected at Stage 7 in either WT or ospdt1 anthers (Figure 3, A and E). In WT anthers, no signal was observed at Stage 8a, but positive signals were first detected at Stage 8b and became intense at Stage 9 (Figure 3, B–D). In contrast, in ospdt1 anthers, strong positive signals were detected at Stage 8a, which then weakened at Stages 8b and 9 (Figure 3, F–H). These results indicate that tapetal PCD initiated earlier in tapetal cells of the ospdt1 mutant than in the WT. We also explored the expression patterns of several tapetum development-associated genes in the WT and ospdt1 at Stages 6–9. The expression of EAT1, PTC1, CYP703A3, and CYP704B2 peaked at Stages 8b–9 in the WT. The expression of these genes peaked earlier in ospdt1, that is, at Stage 8a and b (Supplemental Figure S2, I–L). The expression levels of GAMYB and TDR were reduced in ospdt1 (Supplemental Figure S2, M and N).

Molecular identification, functional complementation, and expression analysis of ospdt1
To identify the mutant gene in ospdt1, we performed map-based cloning using an F2 mapping population of 271 individuals exhibiting the ospdt1 phenotype. OsPDT1 was mapped to chromosome 9 on a 35-kb region flanked by the markers y-45 and y-51. The sequencing of candidate genes within this region revealed a transversion from “T” to “A” in the ninth exon of Os09g0531900, which resulted in a truncated protein missing the last 28 amino acid residues (Figure 4A; Supplemental Figure S3, A and B).

Functional complementation was used to test whether Os09g0531900 was OsPDT1 (Figure 4, B and C). The Os09g0531900 locus is homologous to AtGAUT1, encoding a GalAT required for HG synthesis (Sterling et al., 2006; Lund et al., 2020), and showed 84.1% identity in the potential functional domain (Supplemental Figure S3, A and B). HG is a homopolymer of D-GalA and the most abundant pectic polysaccharide (Atmodjo et al., 2013). In exploring the D-GalA content of pectin, D-GalA content in ospdt1 was 88.2% of that of the WT (Figure 4D; Supplemental Figure S4, A–C), suggesting that OsPDT1 is involved in HG synthesis.

Given that AtGAUT1 experiences posttranslational cleavage (Atmodjo et al., 2011), we constructed three OsPDT1 and GFP fusion proteins to explore the subcellular localization of OsPDT1 (Figure 4E). Transient expression of OsPDT11–141aa-GFP in protoplasts resulted in fluorescent signals in the Golgi apparatus, but not in the late endosome/prevacuolar compartment (Figure 4, F, G, and H). In contrast, no observable signals were detected in protoplasts transiently expressing OsPDT11–260aa-GFP (Figure 4I) or OsPDT1-GFP (Figure 4I). Transient expression of GFP alone was used for the positive control (Figure 4K). These results suggest that OsPDT1 is localized to the Golgi apparatus.

Reverse transcription–quantitative PCR (RT-qPCR) and in situ RNA hybridization were used to investigate the spatiotemporal expression pattern of OsPDT1 in the WT at
advanced stages of development. OsPDT1 was expressed in the root, stem, leaf, sheath, and spikelets, and its expression peaked at Stages 8b–9 in spikelets (Supplemental Figure S4D). In situ RNA hybridization provided more detailed information about OsPDT1 expression in anthers. At Stages 7–9, OsPDT1 was predominantly expressed in microspore mother cells, dyad cells, tetrads, microspores, and tapetal cells, and its expression intensity was highest at Stage 9 (Figure 4, L–O). Little expression was detected in anther locules at Stage 10 (Figure 4P). The sense probe for OsPDT1 was used in anthers at Stage 8b as a negative control (Figure 4Q).
OsiWAK1 was affected in the ospdt1 mutant

Since OsPDT1 is required for HG synthesis, we performed immunofluorescence analysis using JIM5 and JIM7 antibodies to analyze the deposition of HG in anther locules (Bouton et al., 2002; Majewska-Sawka et al., 2004; Ramirez et al., 2004; Costa et al., 2015; Amanda et al., 2016; Lund et al., 2020). No detectable difference was observed between WT and ospdt1 anthers (Supplemental Figure S5, A–P), suggesting that structural pectin, which has the highest HG content among tissues (Persson et al., 2007; Caffall et al., 2009; Kong et al., 2011; Atmodjo et al., 2013; Lund et al., 2020), was not dramatically affected in ospdt1 anther sacs. We focused our attention on the WAKs, which are necessary for the signal transduction of pectin and are directly affected by pectin (Kohorn and Kohorn, 2012; Kohorn, 2016). We investigated whether OsiWAK1, the only reported WAK family protein that participates in anther development in rice (Kanneganti and Gupta, 2011), was affected by the small defects in pectin caused by the OsPDT1 mutation.

To assess the pectin-binding ability of OsiWAK1, we performed a pectin-binding assay. An equal amount of the purified recombinant OsiWAK154–355 protein was incubated with different concentrations of polygalacturonide, a type of HG. With increasing polygalacturonide concentration, less OsiWAK154–355 appeared in the supernatant, and more appeared in the precipitate, which contains most of the polygalacturonide (Figure 5, A and B). Bovine serum albumin was used as a negative control (Supplemental Figure S6A). Consistent with the EXD of WAKs, the EXD of OsiWAK1 binds to polygalacturonide in vitro.

The assembly of WAKs and pectin is initiated before they are transported to the cell periphery, and the intracellular distribution of WAKs is affected by the pectin-binding region (Kohorn et al., 2006; Kohorn, 2016). Therefore, we constructed OsiWAK1 fusion proteins to test whether the intracellular distribution of OsiWAK1 would be affected by its pectin-binding region (Figure 5C). At 16 h after transfection, in most WT protoplasts, transient expression of OsiWAK1-eYFP resulted in the accumulation of fluorescence in ball-like structures (Figure 5D). In contrast, few ball-like structural signals were observed in WT protoplasts transiently expressing the fusion protein lacking the pectin-binding region (DEL-eYFP) (Figure 5F). The ball-like structural signals of OsiWAK1-eYFP were also decreased in ospdt1 protoplasts (Figure 5E). The percentages of WT protoplasts in which OsiWAK1-eYFP accumulated in ball-like structures, irregular features, and exclusively membrane-like structures were 58.7%, 21.6%, and 19.7%, respectively. OsiWAK1-eYFP accumulated predominantly in irregular features (43.5%) rather than ball-like structures (32.0%) in ospdt1 protoplasts ($\chi^2 = 41.074, P < 0.01$; Figure 5G). Very little of the fusion protein DEL-eYFP accumulated in ball-like structures, and in 79.1% of WT protoplasts, DEL-eYFP accumulated in irregular features (Figure 5G).

We overexpressed OsiWAK1-eYFP in the WT and ospdt1 backgrounds (Figure 5H) and examined the intracellular distribution of OsiWAK1-eYFP in anther wall cells. In the WT OsiWAK1-eYFP-overexpressing (WEO) line, fluorescent signals of OsiWAK1-eYFP accumulated in ball-like structures and the cell periphery (Figure 5I). However, in ospdt1-overexpressing OsiWAK1-eYFP (ospdt1/WEO), the fluorescent signals of OsiWAK1-eYFP mainly accumulated around the cell periphery, and in rare cases, signals were detected in ball-like structures (Figure 5J).

To explore these subcellular structures in more detail, we transiently expressed OsiWAK1-eYFP in combination with subcellular structure markers in WT protoplasts. The membrane-like and ball-like structures were detected in the endoplasmic reticulum and secretory vesicle compartment (SVC), respectively (Figure 6, A and B; Supplemental Figure S6H), and not in other subcellular structures (Supplemental Figure S5, B–G). FM4–64 is a fluorescent lipophilic dye that is absorbed by cells through endocytosis. During the early stage of treatment, FM4–64 fluorescence is present in the plasma membrane and endosome membranes of cells. Following internalization, FM4–64 is transported to the Golgi and vacuolar membranes (Bolte et al., 2004; Dettmer et al., 2006; Toyooka et al., 2009). At 16 h after transfection, we treated WT protoplasts expressing OsiWAK1-eYFP with FM4–64 for 40 min. The OsiWAK1-eYFP signals in the irregular features partially colocalized with FM4–64 fluorescence, whereas the OsiWAK1-eYFP signals from the ball-like structures were distinct from FM4–64 fluorescence (Figure 6, C and D). In the WT protoplasts, the OsiWAK1-eYFP fusion proteins in ball-like structures, but not the irregular features, were transported to the cell periphery at 72 h after transfection (Figure 6, E and F). These results suggest that the OsiWAK1-eYFP in the SVC is distinctly different from that in the irregular features, and the localization of SVC could help OsiWAK1-eYFP transport to the cell periphery.

OsiWAK1 is involved in the regulation of tapetal PCD

OsiWAK1 is a WAK family gene that participates in another development in rice (Kanneganti and Gupta, 2011), but its regulatory mechanism has remained unclear. We detected an expression peak of OsiWAK1 at Stage 8a and b in the WT and ospdt1, and OsiWAK1 expression was detected in dyad cells and the tapetum at Stage 8a (Supplemental Figure S6, I–K).

We explored another development in OsiWAK1 RNAi lines (WI; Supplemental Figure S7). WI plants exhibited severe sterility, and some pollen grains were aborted (Figure 7, A–C). We examined transverse sections of anthers. Compared with the WT (Figure 7, D–H), the microspores and tapetal cells in these sections were normal from Stages 8b–10 in WI (Figure 7, I–K), but some microspores were aborted at Stage 12, and another dehiscence was delayed at Stage 14 (Figure 7, L and M).

We then investigated another development in WEO and ospdt1/WEO. Compared with the WT, WEO exhibited severe sterility, while ospdt1/WEO exhibited absolute sterility (Figure 7, A and B). In addition, both WEO and ospdt1/
Figure 5 OsWAK1 was affected in ospdt1. A, SDS-PAGE and immunoblot analysis of the purified recombinant protein OsWAK1$_{54-355}$ in the supernatant after incubation with PGA. B, SDS–PAGE and immunoblot analysis of the purified recombinant protein in the precipitate in (A). Equal amounts of recombinant protein were incubated with different concentrations of PGA and detected in lines 1–6. C, Diagram showing the domains of OsWAK1 fusion proteins. D–F, Distribution of OsWAK1 and eYFP fusion proteins in rice protoplasts. Images were obtained from one Z-stack photograph or the maximum intensity projection at 16 h after transfection. Fluorescent signals containing clear boundaries and that appear ball-like were termed ball-like structure signals. Fluorescent signals without clear boundaries were termed irregular feature signals. D and F, OsWAK1-eYFP and DEL-eYFP transiently expressed in WT protoplasts, respectively. E, OsWAK1-eYFP transiently expressed in ospdt1 protoplasts. G, Statistical analysis of the ratios of the above protoplasts containing a ball-like structure signal, an irregular feature signal, and an exclusively membrane-like structure signal, respectively. OsWAK1-eYFP and DEL-eYFP were analyzed in 218 and 43 WT protoplasts, respectively, and OsWAK1-eYFP was analyzed in 322 ospdt1 protoplasts, chi-squared test was used, **P < 0.01. H, Expression of OsWAK1 in the WT, ospdt1, WEO, and ospdt1/WEO, respectively, **P < 0.01. The error bars represent the so of triplicate reactions. The experiment was repeated 3 times with similar results. I and J, The intracellular distribution of OsWAK1-eYFP in the anther wall cells of the WEO and ospdt1/WEO, respectively. The ball-like structure is indicated by an arrow. B, ball-like structure signal; eYFP, enhanced yellow fluorescent protein; I, irregular feature signal; M, membrane-like structure signal; MIP, maximum intensity projection; TM, transmembrane region. Bars, 5 μm in (D)–(F), and 50 μm in (I)–(J).
Weo anthers contained tetrad pollen grains at the maturation stage (Figure 7C). We then examined transverse sections of anthers. No obvious defects were observed at Stage 8b (Figure 7, N and S). However, in both Weo and ospdt1/Weo, some microspores were not released from tetrads at Stage 9 (Figure 7, O and T). At Stage 10, the tapetum expanded abnormally in Weo and ospdt1/Weo anthers (Figure 7, P and U). At Stage 12, Weo and ospdt1/Weo contained tetrad pollen grains in the anther locules (Figure 7, Q and V). At Stage 14, Weo and ospdt1/Weo anthers dehisced normally (Figure 7, R and W).

Finally, we performed a TUNEL assay to explore tapetal PCD in WI, Weo, and ospdt1/Weo anthers. Compared with WT anthers (Figure 8, A–C), PCD signals occurred earlier in WI anthers (Figure 8, D–F), and PCD was delayed in Weo anthers (Figure 8, G–I), while PCD signals in ospdt1/Weo appeared similar to those of the WT (Figure 8, J–L). However, the abnormally expanded tapetum (Figure 7U) suggests that tapetal development was not completely rescued in ospdt1/Weo.

Discussion

The tapetum and pollen walls are affected in ospdt1

In this study, we characterized the male-sterile rice mutant ospdt1 (Figure 1A), which exhibits earlier tapetal PCD than the WT (Figure 3, B and F). This suggests that earlier degradation of the tapetum might appear in ospdt1 anthers; however, the abnormal PCD of the tapetum might cause its incomplete degradation. Consistent with this, in ospdt1 anthers, the Ubisch bodies were more mature at Stage 9 (Figure 2P), and vacuolated tapetal cells were retained at Stage 11 (Figure 2S). In addition, expression peaks of the tapetum degradation-associated genes EAT1, PTC1, CYP450A3, and CYP450B2 occurred earlier in ospdt1 than in the WT (Supplemental Figure S2, I–L), and expression of GAMYB and TDR were reduced in ospdt1 (Supplemental Figure S2, M and N). This suggests that some tapetum degradation-associated genes are induced earlier in ospdt1 and that some genes fail to be induced. The reduced expression of these genes in ospdt1 may be why there is incomplete degradation of the tapetum. These results suggest that the mutation of OsPDT1 affects tapetum development.

Undulation of the plasma membrane of microspores was reduced in ospdt1 tetrads (Figure 2G), suggesting that primexine formation was abnormal in ospdt1 (Ariizumi and Toriyama, 2011; Shi et al., 2015a, 2015b). Although primexine formation is mainly controlled by sporophytic cells (Paxson-Sowders et al., 2001; Ariizumi and Toriyama, 2011; Chang et al., 2012), callose metabolism is also very important. For example, mutation of the callose synthetases GLUCAN SYNTHASE-LIKE 5 and CALLOSE SYNTHASE 5 caused simultaneously defective callose deposition and primexine formation (Dong et al., 2005; Shi et al., 2015a, 2015b), abnormal degradation of
callose affected primexine formation in defective microspore development1 and osdex1 (Yu et al., 2016; Ren et al., 2020), and primexine was deposited between the plasma membrane and callose layer (Shi et al., 2015a, 2015b). In ospdt1 anthers, we observed weak callose signals around dyad cells and tetrads (Supplemental Figure S2, F and G), and the deeply stained materials surrounding the tetrads were also aberrantly deposited (Figure 2F). Thus, we hypothesized that the aberrant deposition of callose and deeply stained materials might affect primexine formation in ospdt1. As the callose is subjected to enzymatic digestion by callase, which is mainly secreted from the tapetum (Ariizumi and Toriyama, 2011; Wan et al., 2011), abnormal tapetum development might affect callose degradation in ospdt1. Consistent with this, osdex1 also exhibited premature...
PCD of the tapetum, weak callose signals, and abnormal primexine formation (Yu et al., 2016). However, this hypothesis needs further testing.

Tapetum or primexine is essential for extine formation (Ariizumi and Toriyama, 2011; Shi et al., 2015a, 2015b), and this was disordered in ospdt1 anthers (Figure 2, H–J). Thus, we propose that mutation of OsPDT1 influences the development of tapetum and pollen wall formation, leading to absolute male sterility.

OsPDT1 affects components of the pectin-mediated signaling pathway, such as OsiWAK1

Map-based cloning revealed that OsPDT1 is homologous to GAUT1 of Arabidopsis (Figure 4, A–C, Supplemental Figure S3, A and B), which is pivotal in HG synthesis (Sterling et al., 2006; Yin et al., 2010). Consistent with this, we found a small defect in GalA content in the ospdt1 mutant (Figure 4D). However, immunofluorescence analysis using JIM5 and JIM7 antibodies revealed no difference in HG deposition between WT and ospdt1 anthers (Supplemental Figure S5, A–P). The mutation of OsPDT1 might not dramatically affect the structural of pectin, which contains most of the HG (Ridley et al., 2001; Atmodjo et al., 2013), and immunofluorescence analysis is insufficient to detect small defects in HG. In previous studies, the qua1 mutant exhibited a 25% decrease in GalA content and defective HG deposition, which ultimately affected the cell morphology and plant height (Bouton et al., 2002). In our results, the ospdt1 mutant had normal plant height and anther cell morphology (Figure 1, A, I, and J), suggesting that the structural pectin might not be

Figure 8 Overexpression of OsiWAK1 partially rescued the premature PCD in ospdt1. A–C, Anthers were obtained from the WT at Stages 8a, 8b, and 9, respectively. D–F, Anthers were obtained from the WI at Stages 8a, 8b, and 9, respectively. G–I, Anthers were obtained from the ospdt1/WEO at Stages 8a, 8b, and 9, respectively. J–L, Anthers were obtained from the ospdt1/WEO, OsiWAK1-eYFP overexpressing line in the ospdt1 background; Tds, tetrads; WEO, OsiWAK1-eYFP overexpressing line; WI, OsiWAK1 RNA-interference line. Bars, 50 μm.
dramatically affected in ospdt1. In addition to its role as a constituent of cell walls, pectin also binds to the EXD of WAKs and regulates their functions, which is necessary for pectin signal transduction (Kohorn et al., 2009; Brutus et al., 2010; Kohorn, 2016). Mutation of the gene encoding OsPDT1 might cause small pectin defects, which are insufficient to affect the structural pectin, but might affect some WAKs.

The assembly of WAKs and pectin is initiated before they are transported to the cell periphery (Kohorn et al., 2006; Kohorn and Kohorn, 2012; Kohorn, 2016). Therefore, we investigated the intracellular distribution of OsiWAK1, which is the only reported WAK family protein to participate in anther development in rice (Kanneganti and Gupta, 2011). In most WT protoplasts, OsiWAK1-eYFP was localized to the SVC (Figures 5, D, G and 6, B), which could facilitate the transport of OsiWAK1-eYFP to the cell periphery (Figure 6, E and F). Consistent with the function of the EXD of WAKs (Decreux and Messiaen, 2005), the EXD of OsiWAK1 also exhibited pectin-binding ability (Figure 5, A and B). The fusion protein lacking the pectin-binding region (DEL-eYFP) did not localize to the SVC (Figure 5, C, F, and G), suggesting that the pectin-binding region of OsiWAK1 is required for its localization to the SVC. Similar to the intracellular distribution of DEL-eYFP, the localization of OsiWAK1-eYFP in SVC was dramatically reduced in ospdt1 protoplasts (Figure 5, E and G). We overexpressed OsiWAK1-eYFP in the WT and ospdt1 backgrounds (Figure 5H). Consistent with the above results, less OsiWAK1-eYFP localized to the SVC in ospdt1/WEO than in WEO (Figure 5, I and J). Taken together, the findings that the pectin-binding region of OsiWAK1 was crucial for its localization to the SVC, that SVC localization of OsiWAK1 was dramatically reduced in ospdt1, and that OsPDT1 was a GalAT participating in pectin synthesis suggest that the ospdt1 mutant may affect OsiWAK1 through pectin.

Our results show that the ospdt1 mutant exhibited normal structural pectin in the anther sacs but suggest that the pectin-mediated signaling pathway is affected through components such as OsiWAK1. However, the mechanism of how the small pectin defects in ospdt1 affect OsiWAK1 remains unclear.

OsiWAK1 plays pivotal roles in the function of OsPDT1

OsiWAK1, which reportedly participates in anther development in rice, is affected in ospdt1 (Kanneganti and Gupta, 2011). Therefore, we constructed WI plants to explore the function of OsiWAK1. WI plants exhibited severe sterility, and some pollen grains were aborted (Figure 7, A–C). In addition, tapetal PCD occurred earlier in WI than in WT plants (Figure 8, D–F), and anther dehiscence was delayed at Stage 14 (Figure 7M), similar to ospdt1 (Figures 1T and 3F). However, in transverse sections, WI plants displayed degenerated tapetum at Stage 12, indicating that the abnormal development of tapetum in WI was less severe than that seen in ospdt1 (Figure 7L). Consistent with this, some mature pollen grains were observed in the anther sacs of WI plants at Stage 14 (Figure 7M).

The tip2 mutant exhibited delayed PCD signals, causing aborted pollen grains (Fu et al., 2014; Ko et al., 2014), while TIP2 overexpressing lines exhibited premature PCD signals, also with aborted pollen grains (Ko et al., 2017). Thus, the PCD of tapetal cells might require accurate spatiotemporal regulation, whereby both loss and enhancement of the function of tapetal PCD-associated genes would lead to aborted pollen grains. Consistent with this, the WEO lines exhibited delayed PCD signals (Figure 8, G–I), abnormal degradation of tapetum, and aborted pollen grains (Figure 7, N–R). In WEO anther sacs, some microspores failed to be released from tetrads, suggesting that polymers surrounding the tetrads, such as callose, might not be completely degraded in WEO (Figure 7, C and O; Supplementary Figure S8, A–F; Scott et al., 2004; Ariizumi and Toriyama, 2011; Wan et al., 2011).

We further explored the ospdt1/WEO lines, which exhibited similar phenotypes to WEO, including tetrad pollen grains and an expanded tapetum (Figure 7, C, T, and U). However, ospdt1/WEO did not display the ospdt1 phenotypes, including the vacuolated tapetal cells (Figure 7V) and delayed anther dehiscence (Figure 7W). Overexpression of OsiWAK1 might therefore have an epistatic effect over mutated OsPDT1; alternatively, overexpression of OsiWAK1 might rescue the defects observed in ospdt1. Further exploration of the tapetal PCD in ospdt1/WEO revealed that premature PCD was partially rescued at Stage 8a (Figure 8, J–L). In ospdt1/WEO anthers, PCD signals appeared at Stages 8b and 9, unlike in WEO and ospdt1 (Figures 3, E–H and 8, G–I). Since premature PCD in ospdt1 was considered to be a principal reason for defective microspores development, we hypothesized that overexpression of OsiWAK1 partially rescues the defects in ospdt1 instead of having an epistatic effect. It is possible that overexpressing OsiWAK1 partially rescued the premature PCD in ospdt1 at Stage 8a, but disturbed tapetum development in the subsequent stages; indeed, this might explain why ospdt1/WEO exhibited similar phenotypes to WEO rather than ospdt1. Consistent with this, ospdt1/WEO exhibited similar callose signals to WEO instead of ospdt1 in anthers (Supplemental Figures S2, F–H and 8, D–I), and the abnormal expression of EAT1, PTC1, CYP703A3, and CYP704B2 was somewhat rescued at Stages 8a and b and 8b–9 in ospdt1/WEO (Supplemental Figures S2, I–L and S9, A–D). The abnormal expression of GAMYB and TDR was not well rescued in ospdt1/WEO (Supplemental Figures S2, M and N and S9, E and F).

Taken together, RNAi of OsiWAK1 caused similar premature PCD of the tapetum to ospdt1, and overexpressing OsiWAK1 partially rescued the defects in ospdt1, suggesting that OsiWAK1 plays pivotal roles in the function of OsPDT1.

We created a model to show how OsPDT1 participates in the tapetal PCD (Figure 9). GalAT is reported to function in the Golgi apparatus and is involved in pectin.
synthesis in the Golgi lumen; then, pectin is transported to the cell wall via SVC, and OsWAK1 may assemble with pectin there; with the secretion of pectin, the OsWAK1 is also transported to the cell periphery, and delays the tapetal program cell death there. In ospdt1, the mutated OsPDT1 causes defective pectin synthesis, which may affect the integration of pectin and OsWAK1; it is possible that less OsWAK1 is transported to the cell periphery, which causes premature tapetal program cell death in the ospdt1 mutant.

**Figure 9** A proposed model for the regulatory network of OsPDT1. In WT rice, OsPDT1 is localized in the Golgi apparatus and is involved in pectin synthesis; pectin is transported to the cell wall via SVC, and OsWAK1 may assemble with pectin there; with the secretion of pectin, the OsWAK1 is also transported to the cell periphery, and delays the tapetal program cell death there. In ospdt1, the mutated OsPDT1 causes defective pectin synthesis, which may affect the integration of pectin and OsWAK1; it is possible that less OsWAK1 is transported to the cell periphery, which causes premature tapetal program cell death in the ospdt1 mutant.

**Materials and methods**

**Identification of OsPDT1 mutants**

The male-sterile rice (*O. sativa*) mutant ospdt1 was identified from a “Xilong 1B” EMS-treated mutant library and grown in the paddy fields of Southwest University, China. F2 mapping populations were generated from a cross between “Jinhui 10” and the ospdt1 mutant. Male-sterile individuals in the F2 population were selected for gene mapping. Statistical analysis was performed using IBM SPSS Statistics version 26.0 software (IBM Corporation, Armonk, NY, USA). For functional complementation, the OsPDT1 coding region (3,652 bp), upstream sequence (3,543 bp), and downstream sequence (1,430 bp) were cloned into the binary vector pCAMBIA1301. Calli induced from young panicles of the homozygous ospdt1 mutants were used for transformation with Agrobacterium (*Agrobacterium tumefaciens*, strain EHA105) carrying the pCAMBIA1301-OsPDT1 plasmid. To overexpress OsWAK1-eYFP, the coding region of OsWAK1-eYFP was cloned into the binary vector pTCK303 driven by the Ubiquitin promoter. Calli induced from heterozygous
(OsPD1/ospdt1) seeds were used for transformation with Agrobacterium (strain EHA105) carrying the pTCK303-OsiWAK1-eYFP plasmid. For RNA interference of OsiWAK1, the 207-bp OsiWAK1 coding region was amplified using primers OsiWAK1-RNAi-1F/OsiWAK1-RNAi-1R and OsiWAK1-RNAi-2F/OsiWAK1-RNAi-2R and cloned into the binary vector pTCK303. Calli induced from WT seeds were transformed with Agrobacterium (strain EHA105) carrying the pTCK303-OsiWAK1-RNAi plasmid. Individual transgenic plants were identified and confirmed by sequencing. The primers used are listed in Supplemental Table S1.

Phenotypic analysis of ospdt1
ospdt1 plants and flowers were examined under an Olympus MVX10 stereomicroscope and photographed using a Canon EOS 5D digital camera. After staining with 1% (w/v) I2-KI, mature pollen grains were examined under an Olympus BX53 microscope. Anthers at different developmental stages were fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1-M sodium phosphate buffer (pH 7.2) and postfixed in 1% (v/v) osmic acid. Following dehydration in a graded ethanol series and acetone replacement, anthers were embedded in Epon 812 resin. Transverse sections (2–4 μm thickness) were cut with a Leica RM2265 microtome, stained with 1% (w/v) toluidine blue O, and examined under an Olympus BX53 microscope. For TEM, ultra-thin sections cut using a Leica UC6 microtome were double-stained with uranyl acetate and lead citrate and examined under a Hitachi SU3500 scanning electron microscope. For SEM, mature pollen grains were examined under a Hitachi H-7500 transmission electron microscope. For TEM, ultra-thin sections cut using a Leica RM2265 microtome, stained with 1% (w/v) toluidine blue O, and examined under an Olympus BX53 microscope. Anthers at different developmental stages were selected and fixed in 1.9% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol. The fixed samples were dehydrated in a graded ethanol series, which was replaced by xylene. The samples were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) and sectioned (5-μm thickness) using a Leica RM2245 microtome. For the TUNEL assay, sections were treated using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer’s instructions and examined under a Zeiss LSM800 confocal microscope. For fluorescein isothiocyanate signal detection, excitation wavelength (488 nm) and detection wavelength (500–550 nm) were used, for propidium iodide signals detection, excitation wavelength (561 nm) and detection wavelength (600–700 nm) were used. For callose detection, the sections were incubated in 0.1% (w/v) aniline blue in phosphate buffer (4-MM K2HPO4, 0.8-M KH2PO4, pH 7.5) and examined under an Olympus BX53 fluorescence microscope. The aniline blue signals were detected under ultraviolet light. To detect pectin, the sections were incubated with JIM5 or JIM7 antibodies (PlantProbes) (1:10) in phosphate buffer (6-MM Na2HPO4, 3.5-MM KH2PO4, 2.7-MM KCl, pH 7.4) for 12 h at 4°C, washed, incubated with rabbit anti-rat antibody (Bioss Antibodies) (1:1,000) for 2 h at 37°C, washed again, and examined under a Zeiss LSM800 confocal microscope. For cy5 signals detection, excitation wavelength (640 nm) and detection wavelength (645–700 nm) were used. For the high-performance liquid chromatography, plant materials were flash frozen in liquid N2 and ground to powder, extracted with 96% (v/v) ethanol at 100°C for 20 min, and extracted with 70% (v/v) ethanol 5 times. Ninety percent (v/v) dimethylsulfoxide was added to remove starch, and the pellets were washed 5 times with distilled water, washed twice with 100% acetone, and dried overnight at 40°C. The remaining material was termed “alcohol insoluble residue” (AIR). AIR was hydrolyzed for 2 h at 105°C in 2 M H2SO4, neutralized by 4 M NaOH. The samples and GalA standard were derivatized with 1-phenyl-3-methyl-5-pyrazolone according to the method of Lin et al. (2016). The Agilent1260 Chemistry system was used to measure monosaccharide composition. Buffer A (0.05 M potassium dihydrogen phosphate buffer, pH 6.7) and buffer B (acetonitrile) were mixed to be used as eluent (83:17). The flow rate was 1 mL/min, and the column temperature was 30°C. A wavelength of 254 nm was used to detect the signal. The statistical significance based on P-values was calculated using a Student’s t test.

Expression analysis
Spikelets at different developmental stages were selected based on their length and color, and the stages were confirmed by examining transverse sections. OsPD1 cDNA (250 bp) was transcribed and labeled in vitro using a DIG RNA labeling kit (Roche, Basel, Switzerland). RNA isolation, RT-qPCR, pretreatment of sections, hybridization, and immunological detection were performed as described previously (Zhang et al., 2017). The statistical significance based on P-values were calculated using a Student’s t test. The primers used are listed in Supplemental Table S2.

TUNEL assay, callose detection, and pectin detection
Spikelets from WT and ospdt1 plants at different developmental stages were selected and fixed in 1.9% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol. The fixed samples were dehydrated in a graded ethanol series, which was replaced by xylene. The samples were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) and sectioned (5-μm thickness) using a Leica RM2245 microtome. For the TUNEL assay, sections were treated using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer’s instructions and examined under a Zeiss LSM800 confocal microscope. For fluorescein isothiocyanate signal detection, excitation wavelength (488 nm) and detection wavelength (500–550 nm) were used, for propidium iodide signals detection, excitation wavelength (561 nm) and detection wavelength (600–700 nm) were used. For callose detection, the sections were incubated in 0.1% (w/v) aniline blue in phosphate buffer (4-MM K2HPO4, 0.8–M KH2PO4, pH 7.5) and examined under an Olympus BX53 fluorescence microscope. The aniline blue signals were detected under ultraviolet light. To detect pectin, the sections were incubated with JIM5 or JIM7 antibodies (PlantProbes) (1:10) in phosphate buffer (6-MM Na2HPO4, 3.5-MM KH2PO4, 2.7-MM KCl, pH 7.4) for 12 h at 4°C, washed, incubated with rabbit anti-rat antibody (Bioss Antibodies) (1:1,000) for 2 h at 37°C, washed again, and examined under a Zeiss LSM800 confocal microscope. For cy5 signals detection, excitation wavelength (640 nm) and detection wavelength (645–700 nm) were used. For the high-performance liquid chromatography, plant materials were flash frozen in liquid N2 and ground to powder, extracted with 96% (v/v) ethanol at 100°C for 20 min, and extracted with 70% (v/v) ethanol 5 times. Ninety percent (v/v) dimethylsulfoxide was added to remove starch, and the pellets were washed 5 times with distilled water, washed twice with 100% acetone, and dried overnight at 40°C. The remaining material was termed “alcohol insoluble residue” (AIR). AIR was hydrolyzed for 2 h at 105°C in 2 M H2SO4, neutralized by 4 M NaOH. The samples and GalA standard were derivatized with 1-phenyl-3-methyl-5-pyrazolone according to the method of Lin et al. (2016). The Agilent1260 Chemistry system was used to measure monosaccharide composition. Buffer A (0.05 M potassium dihydrogen phosphate buffer, pH 6.7) and buffer B (acetonitrile) were mixed to be used as eluent (83:17). The flow rate was 1 mL/min, and the column temperature was 30°C. A wavelength of 254 nm was used to detect the signal. The statistical significance based on P-values was calculated using a Student’s t test.

Pectin-binding assay
The 905-bp PCR product of OsiWAK1 (160–1,065 bp) was ligated into the PET30a vector carrying an N-terminal poly-His tag. Escherichia coli strain BL21 (DE3) cells were transformed with this fusion vector and induced in 0.2-M IPTG at 15°C for 16 h. The cells were lysed in an ultrasonic homogenizer, and the inclusion bodies were washed with 50-M M Tris, 300-M M NaCl, 2-M M EDTA, 5-M M DTT, and 1% (v/v) Triton X-100 (pH 8.0) and dissolved in 50-M M Tris, 300-M M NaCl, 8-M M urea, and 20-M M imidazole (pH 8.0). The recombinant fusion proteins were purified on an Ni-IDA column (BBI Life Sciences, Shanghai, China) and dialyzed in 50-M M Tris, 300-M M NaCl, and 2-M M urea (pH 8.0) at 4°C overnight for renaturation. The final dialysis was performed in 50-M M Tris, 300-M M NaCl, and 10% glycerol (pH 8.0) for 8 h and the product stored at −80°C.

The pectin-binding assay was performed as described previously (Carpin et al., 2001; Decreux and Messiaen, 2005), with some modifications. One microgram of OsiWAK1

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was incubated with 0, 1, 10, 100, 200, or 400 μg PGA (Sigma, St Louis, MO, USA) in 20-mM MES, 150-mM NaCl, and 0.5-mM CaCl2 (pH 6.0) at 25°C for 1 h. Following centrifugation at 8,500g for 5 min, the precipitate (which was resuspended in 20-mM Tris, 2-mM EGTA, and 0.1% Tween-20, pH 8.2) and the supernatant were analyzed using SDS-PAGE. The proteins were transferred to a blotting membrane using an iBind 2 Dry Blotting System (Thermo Fisher Scientific, Waltham, MA, USA), followed by incubation with rabbit anti-His-Tag (Cell Signaling Technology, Danvers, MA, USA) and goat anti-Rabbit IgG-HRP (Abmart, Shanghai, China) antibodies using the iBind Automated Western System (Thermo Fisher Scientific) according to the manufacturer’s instructions. Finally, the blotting membrane was visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and photographed using ChemiDoc XR+ (Bio-Rad, Hercules, CA, USA). As a negative control, 1 μg OsiWAK1 (45–355) and 1 μg BSA (BBI Life Sciences, Shanghai, China) were incubated in the same samples, and the other conditions were identical to those described above, except that the gels were stained with Coomassie Brilliant Blue R-250 (Diamond). The primers used are listed in Supplemental Table S1.

Protoplast isolation and transfection
Homozygous ospdt1 individuals were grown from heterozygous rice seeds and confirmed by sequencing. Protoplast isolation and transfection were performed as described previously (Zhang et al., 2011a, 2011b; Lin et al., 2018). WT or ospdt1 plants were cultured in nutrient soil at 28°C under a 12-h/12-h (light/dark) photoperiod with an illumination of 12,000 lx for 10 d, washed with distilled water, and soaked in 0.6-M mannitol to induce plasmolysis. After putting the sample on ice for 30 min, the stem and sheath tissues were cut into 0.5-mm sections, and an equal volume of W5 buffer (154-mM NaCl, 125-mM CaCl2, 5-mM KCl, 2-mM MES, and 5-mM glucose, pH 5.7) was added to the solution. The solution was centrifuged at 200g for 2 min and washed once with W5 buffer. After putting the sample on ice for 30 min, the pellet was resuspended in MMG buffer (0.6-M mannitol, 15-mM MgCl2, and 4-mM MES, pH 5.7). The final cell concentration was almost 1 × 10⁶ cells mL⁻¹, as measured using a hemocytometer. For transfection, 10-μL plasmid, 100-μL protoplasts, and 110-μL PEG solution (0.2-M mannitol, 100-mM CaCl2, and 40% PEG 4000, pH 7.5) were added to the centrifuge tubes, mixed immediately, and incubated for 20 min at room temperature in the dark. To terminate transfection, 440 μL W5 buffer was added to each tube and mixed. Finally, the samples were centrifuged at 240g for 3 min, and the pellets were resuspended and cultured in W5 buffer.

OsPDT1-GFP, OsPDT1-1–141aa-GFP, and OsPDT1-1–260aa-GFP were constructed as shown in the diagrams (Figure 4E), and the native vector was PANS80-GFP. An equal amount of each construct was transiently co-expressed with the Golgi marker (OsNST1-mCherry) in WT protoplasts, and OsPDT1-1–141aa-GFP was also co-repressed with Golgi marker (OsCYP21-4-mCherry) and the late endosome/prevacuolar compartment marker (mCherry-AtSYP21), and fluorescence was detected under a Zeiss LSM800 confocal microscope. To examine the intracellular distribution of OsiWAK1, fusion proteins were constructed in accordance with the diagrams (Figure 5C), and the native vector was PANS80-eYFP. An equal amount of each construct was transiently expressed in WT or ospdt1 protoplasts, and the protoplasts were photographed under a Zeiss LSM800 confocal microscope. Images were obtained from one Z-stack photograph or the maximum intensity projection. Statistical analysis was performed using IBM SPSS Statistics version 26.0 software. OsiWAK1-eYFP was also transiently co-expressed with an endoplasmic reticulum marker (AtWAK2-1–29aa-mCherry-HDEL), a Golgi apparatus marker (OsNST1-mCherry or OsCYP21-4-mCherry), a vacuolar marker (mCherry-AtVAMP721), a late endosome/prevacuolar compartment marker (mCherry-AtSYP21), a peroxisome marker (mCherry-PTS1), a trans-Golgi network/prevacuolar compartment marker (mCherry-AtSYP52), or a trans-Golgi network/early endosome marker (mCherry-AtSYP42) in WT protoplasts. WT protoplasts that expressed OsiWAK1-eYFP were treated with FM4–64 (16 μM; Thermo Fisher Scientific) for 40 min in PEM buffer (50-mM PIPES, 5-mM EGTA, and 0.3-M mannitol, pH 6.9) at room temperature and detected under a Zeiss LSM800 confocal microscope. For GFP signals detection, excitation wavelength (488 nm) and detection wavelength (490–540 nm) were used, for mCherry signals detection, excitation wavelength (561 nm), and detection wavelength (590–650 nm) were used, for eYFP signals detection, excitation wavelength (488 nm), and detection wavelength (480–580 nm) were used, for FM4–64 signals detection, excitation wavelength (488 nm) and detection wavelength (620–700 nm) were used. The primers used are listed in Supplemental Table S2.

Accession numbers
Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: OsPDT1 (LOC_Os09g36190), OsiWAK1 (AF353091), GAUT1 (AT3G61130), EAT1 (LOC_Os04g51070), PTC1 (LOC_Os09g27620), CYP703A3 (LOC_Os08g03682), CYP704B2 (LOC_Os03g07250), GAMYB (LOC_Os01g59660), and TDR (LOC_Os02g02820).

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. TEM analysis.
Supplemental Figure S2. Aniline blue staining and expression analysis.
Supplemental Figure S3. Bioinformatic analysis of OsPDT1.
Supplemental Figure S4. High-performance liquid chromatography and expression analysis.

Supplemental Figure S5. Analysis of pectin deposition in anthers.

Supplemental Figure S6. Pectin-binding assay of the EXD of OsiWAK1, subcellular localization of OsiWAK1 fusion proteins, and expression analysis of OsiWAK1.

Supplemental Figure S7. Expression of OsiWAK1 in the OsiWAK1 RNA-interference line (WI).

Supplemental Figure S8. Aniline blue staining of the WT, the WT OsiWAK1-eYFP-overexpressing line (WEO), and the ospdt1/WEO.

Supplemental Figure S9. Expression analysis of the WT, the WT OsiWAK1-eYFP-overexpressing line (WEO), and the ospdt1/WEO.

Supplemental Table S1. Primers used for map-based cloning, functional complementation, overexpression, RNA interference, transgenic plant identification, RNA in situ hybridization, and recombinant protein generation.

Supplemental Table S2. Primers used for RT-qPCR, subcellular localization of OsPDT1, and analysis of the intracellular distribution of OsiWAK1.

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