RMS2 Encoding a GDSL Lipase Mediates Lipid Homeostasis in Anthers to Determine Rice Male Fertility[OPEN]

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Plant male gametogenesis is a coordinated effort involving both reproductive tissues and sporophytic tissues, in which lipid metabolism plays an essential role. Although GDSL esterases/lipases have been well known as key enzymes for many plant developmental processes and stress responses, their functions in reproductive development remain unclear. Here, we report the identification of a rice male sterile2 (rms2) mutant in rice (Oryza sativa), which is completely male sterile due to the defects in tapetum degradation, cuticle formation in sporophytic tissues, and impaired exine and central vacuole development in pollen grains. RMS2 was map-based cloned as an endoplasmic reticulum-localized GDSL lipase gene, which is predominantly transcribed during early anther development. In rms2, a three-nucleotide deletion and one base substitution (TTGT to A) occurred within the GDSL domain, which reduced the lipid hydrolase activity of the resulting protein and led to significant changes in the content of 16 lipid components and numerous other metabolites, as revealed by a comparative metabolic analysis. Furthermore, RMS2 is directly targeted by the male fertility regulators Undeveloped Tapetum1 and Persistent Tapetal Cell1 both in vitro and in vivo, suggesting that RMS2 may serve as a key node in the rice male fertility regulatory network. These findings shed light on the function of GDSLs in reproductive development and provide a promising gene resource for hybrid rice breeding.

Male fertility is essential for the sexual plant life cycle generational alternation as well as for crop production in agriculture. In grass (Poaceae) plants, male gametophytes are generated within the anther compartment of the stamen, which contains a filament and an anther. In addition to normal mature pollen grains, successful male fertility requires normal sporophytic tissues that can dehisce and pollinate at the appropriate moment. During microgametogenesis, primary sporogenous cells differentiate into microspore mother cells (MMCs) and then undergo one meiotic and two mitotic divisions to form mature pollen, while the primary parietal cell differentiates into epidermis, endothecium, middle layer, and tapetum from the outside to the inside, each of which has a unique function as well as coordinating roles in anther development (Goldberg et al., 1993; McCormick, 1993; Scott et al., 2004). The anther epidermis located on the outermost layer is composed of cutin and wax polymers, primarily providing a protective environment for the pollen inside (Yeats and Rose, 2013). The functions of endothecium and middle layer are related to the transportation of ions or other secreting materials to the innermost layer tapetum, which further transports nutrients into the pollen sac. In addition, during pollen maturation, tapetum may be degraded via a coordinated programmed cell death mechanism to provide nutrients for pollen development.

Male reproductive development is a fine-tuned process. Dysfunction of any of the genes controlling the major events of microsporogenesis could lead to abnormal pollen and male sterility. For example, the rice (Oryza sativa) MULTIPLE SPOROCYTE (MSP1) gene,
which is a close ortholog of EXCESS MICROSPOROCYTE51 in Arabidopsis (Arabidopsis thaliana), controls early sporogenic development (Zhao et al., 2002; Nonomura et al., 2003). The msp1 mutant showed excessive sporocytes and disordered anther wall layers (Nonomura et al., 2003). Numerous meiosis-related genes have been cloned and characterized for their functions in male fertility, including PAIR1 (Nonomura et al., 2004), PAIR2 (Nonomura et al., 2006), PAIR3 (Yuan et al., 2009), ZEPI (Wang et al., 2010), and PSS1 (Zhou et al., 2011) in rice and ZYP1 (Higgins et al., 2005), ASY1 (Armstrong et al., 2002), SDS (Azumi et al., 2002), and RCK (Chen et al., 2005) in Arabidopsis. Many of these genes are found to be highly conserved among various plant species (Chang et al., 2009). Recent studies reported two genes in rice, DEFECTIVE CALLOSE IN MEIOSIS1 and OsRR24/LEPTOTENEN1, which played essential roles in male meiotic cytokinesis and in establishing meiotic leptotene chromosomes, respectively (Zhang et al., 2018a; Zhao et al., 2018). In addition, abnormal anther wall development also affects male fertility. Genes influencing this regulatory cascade include ABORTED MICROSPORES (Sorensen et al., 2003), DYSFUNCTIONAL TAPETUM1 (Zhang et al., 2006), and MALE STERILITY1 (Ito et al., 2007) in Arabidopsis and other plant species. ETERNAL TAPETUM1 (EAT1), which encodes a basic helix-loop-helix (bHLH) transcription factor, regulates the programmed cell death of tapetum through promoting aspartic protease activity of OsAP25 and OsAP37 in rice (Niu et al., 2013). Two bHLH transcription factors, TDR INTERACTING PROTEIN2 and TAPETUM DEGENERATION RETARDATION (TDR), can form dimers with each other and act upstream of EAT1 (Fu et al., 2014; Ko et al., 2014; Ono et al., 2018). Recently, EAT1 was found to interact with UNDEVELOPED TAPETUM1 (UDT1), and the udt1 mutant exhibited delayed tapetum degradation and aborted microspores. However, the pathways regulated by UDT1 remain unclear (Ono et al., 2018).

GDSL esterases and lipases, which were named after conserved motif Gly-Asp-Ser-Leu, are a subfamily of hydrolytic/lipolytic enzymes widely present in all kingdoms (Akoh et al., 2004). Although the study of plant GDSL proteins has lagged behind that in animals and human, several cases have indicated the versatile roles of GDSL in various biological processes such as seed oil metabolism, stress resistance, and morphogenesis of cuticle. For example, CDEF1 in Arabidopsis is a plant cutinase belonging to the GDSL lipase/esterase family. Ectopic expression of CDEF1 led to cuticular defects. Interestingly, CDEF1 is highly expressed in mature pollen and pollen tubes, implying that CDEF1 may degrade the stigma cuticle during pollination (Takahashi et al., 2010). In rice, two GDSL lipase genes, OsGLIP1 and OsGLIP2, were identified to act as negative regulatory factors of rice disease resistance by modulating lipid metabolism (Gao et al., 2017). Genes with similar functions, such as GLIP1 (Arabidopsis), GLIP2 (Arabidopsis), GLIP3 (Arabidopsis), GLIP4 (Arabidopsis), TcGLIP (Tanacetum cinerariifolium), and CaGLIP1 (Capsicum annum), were also found in numerous other species (Oh et al., 2005; Hong et al., 2008; Lee et al., 2009; Kikuta et al., 2012; Han et al., 2019). In addition, GDSL esterases and lipases have been associated with plant tissue morphogenesis and development. GhGDSL1 and Brittle Leaf Sheath1 (BS1) were reported to play important roles in the biosynthesis of secondary cell wall in cotton (Gossypium hirsutum) fiber and rice, respectively (Yadav et al., 2017; Zhang et al., 2017). In Arabidopsis, the pollen coat extracellular lipase EXL4 was required for efficient pollen hydration, whereas EXL6 is involved in pollen exine formation (Updegraff et al., 2009; Dong et al., 2016). These results are suggestive of roles for GDSL esterases and lipases in male reproductive development in plants. However, few GDSL genes related to male fertility have been identified, and the underlying mechanism is not yet well understood.

Rice serves as one of the major food crops in the world and is a model monocotyledonous plant. Male fertility and anther development are of vital significance for hybrid rice breeding (Wilson and Zhang, 2009; Chang et al., 2016; Wu et al., 2016). A rice genome survey identified 114 GDSL esterase/lipase genes, but none of them have been characterized to function in male fertility and anther development so far (Chepyshko et al., 2012). Here, we report the cloning of the Rice Male Sterile2 gene (RMS2), which encodes a GDSL esterase/lipase protein. Mutation of RMS2 can cause shrunken anthers with abnormal pollen, resulting in complete male sterility. Cytological and genetic analyses indicated that RMS2 has lipase activity and is required for anther development and pollen fertility.

RESULTS

rms2 Is a Completely Male-Sterile Mutant

From a γ-irradiation-induced mutant population (Long et al., 2016), we identified a male-sterile mutant denoted rms2 in the background of cv 9311 (indica rice). Under natural growth conditions, rms2 showed normal vegetative growth like the wild type (Fig. 1A; Supplemental Table S1). However, during the reproductive development stages, the mutant exhibited typical male-sterile phenotypes such as slightly delayed heading date, partly sheathed panicle, and conspicuous white and shrunken anthers (Fig. 1, B and C). Iodine potassium iodide (I2-KI) staining assay detected no viable pollen grains, which consequently resulted in completely sterile plants (Fig. 1, D and E). We tested the female fertility of rms2 by reciprocal cross rms2 × cv 9311 using rms2 as the maternal parent. The maternal parent plants and F1 plants produced seeds normally, suggesting viable female organ development in rms2. The F2 population had an approximate 3:1 segregation ratio (sterility:fertility = 106:336, χ² = 0.2443 < χ²0.05 = 3.84, χ² test used), which suggested that
the male sterility in \textit{rms2} is caused by a single recessive genetic locus.

**Histological and Cytological Analyses of \textit{rms2}**

Employing histological semithin transverse sectioning, we cytologically characterized male reproductive development in \textit{rms2} and the wild type (Fig. 2). According to the previous classification of pollen growth, we tentatively characterized the sections into eight stages (Feng et al., 2001). During early development, from the early premeiosis stage to the young microspore stage, MMCs of both \textit{rms2} and the wild type went through normal meiosis and properly formed released microspores (Fig. 2, A–E and I–M). Normal anther parietal cells including epidermis, endothecium, middle layer, and band-type-shape tapetum could all be found both in the wild type (Fig. 2, A–E) and \textit{rms2} (Fig. 2, I–M). Obvious differences were observed during the start of the vacuolated pollen stage, in which wild-type pollen became large vacuolated microspores with condensed and degrading tapetal layer cells and an invisible middle layer (Fig. 2F). In contrast, the \textit{rms2} anthers still had visible middle layer cells, hilly shape, and vacuolated and lightly stained tapetum. Particularly, \textit{rms2} microspores exhibited deformed shape, possibly due to the small, shrunken central vacuole found in the cell (Fig. 2N). From the vacuolated pollen stage to the mature pollen stage, the tapetum of the wild type gradually degenerated and vacuolated microspores went through mitosis, turning to mature pollen grains with fully accumulated nutrients on the surface (Fig. 2, F–H). However, the \textit{rms2} middle layer cells were still visible with the tapetum hardly changed. Meanwhile, \textit{rms2} pollen were very shrunken and lacked starch granules (Fig. 2, N–P).

To gain deeper insight into pollen development in the wild type and \textit{rms2}, we subsequently performed scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses on the anthers and pollen from the vacuolated pollen stage to the mature pollen stage (Figs. 3 and 4). In the vacuolated pollen stage, epidermis, endothelium, and degrading tapetum cells were clearly visible in the cells of the wild-type anther wall, the middle layer was invisible, and tapetal cells formed ubisch bodies along their surface (Fig. 3, A and B). Conversely, we observed a nearly intact middle layer and tapetum cells with dense organelles and small vacuoles, but no ubisch bodies in the \textit{rms2} anther walls (Fig. 3C). In terms of microspores in this stage, the pollen exine of \textit{rms2} was found to be relatively normal (i.e. similar to the wild type), but \textit{rms2} microspores displayed an irregular shape, in which there were many small vesicles that did not fuse to form a large central vacuole (Fig. 3D). In the next mitosis stage, ubisch bodies, which are implicated in the exportation of substances secreted by tapetum, started to form in the \textit{rms2} anther wall cells (Fig. 3G). But the \textit{rms2} tapetal cells exhibited unregulated proliferation and extrusions without clear signs of breakdown, which was in contrast to the degenerated tapetal layer in wild-type anthers (Fig. 3, E and G). In comparison with the wild type, the \textit{rms2} microspores in this stage had more severely shrunken cytoplasm, and the nexine failed to form a continuous layer (Fig. 3, F and H). In the mature pollen stage, the wild-type anther wall was left with only epidermis and endothecium layers, and the pollen grains became spherical in shape with accumulated starch and lipidic materials (Fig. 3, I and J). However, the four-layer anther wall of \textit{rms2} was still visible (Fig. 3K), and the pollen grains were completely collapsed with little or no cytoplasm components (Fig. 3L).

SEM was conducted to further investigate the abnormalities of anther cuticle and pollen exine in \textit{rms2} (Fig. 4). In the wild type, the cuticles were synthesized starting from the pollen mitosis stage, and the epidermis became fully covered by spaghetti-like cutin layers in the mature pollen stage (Fig. 4, A–1, C–1, and E–1). Interestingly, cuticle synthesis in the \textit{rms2} epidermis was much delayed, as cuticles could barely be found on the \textit{rms2} epidermis in the pollen mitosis stage (Fig. 4, B–1 and D–1). However, when it reached the mature pollen stage, the \textit{rms2} epidermis was fully covered by cutin layers, even in a denser manner than that of the wild type (Fig. 4F–1). Similarly, the formation of ubisch bodies on the inner surface of anthers (Fig. 4, A–2, B–2, C–2, D–2, E–2, and F–2) as well as the sporopollenin synthesis on the pollen exine (Fig. 4, A–3, A–4, B–3, B–4, C–3, C–4, D–3, D–4, E–3, E–4, F–3, and F–4) in \textit{rms2} were also delayed from the vacuolated pollen stage to the pollen mitosis stage, which is in accordance with the delayed tapetal cell degeneration revealed by TEM (Fig. 5). The above results suggested that the male sterility of \textit{rms2} is likely attributed to the delayed tapetum

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**Figure 1.** Phenotypes of the wild type (WT) and \textit{rms2}. A, Plant morphology of wild-type and \textit{rms2} mutant plants after heading. Bar = 10 cm. B, Comparison of the male organs of the wild type and \textit{rms2} before anthesis. Bar = 2 mm. C, Comparison of wild-type and \textit{rms2} floret morphology. Bar = 2 mm. D and E, \textit{I$_2$-KI} assay of mature pollen grains. Bars = 0.1 mm.
degradation and hysteretic cuticle and exine formation during anther development.

**RMS2 Encodes a GDSL Lipase**

To identify the gene RMS2, we adopted a positional cloning approach using the F2 population derived from the cross of rms2 and MH63 (indica rice). RMS2 was finally mapped on chromosome 2, restricted to a locus within a 125-kb region between Indel 2-1 and RM13011, and cosegregated with the marker Indel 3 (Fig. 5A). According to the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/), six predicted open reading frames (ORFs) were located in this region. We Sanger sequenced all six candidate genes and found that three nucleotide deletions and one base substitution (TTGT to A) occurred in the coding region of ORF3, which likely caused the substitution of the 230th and 231st residues from LV to H on the resulting protein (Fig. 5, B and C). It was found that the transcription of ORF3 was highly reduced in rms2 (Supplemental Fig. S1A). ORF3 (LOC_Os02g18870) is annotated as a lipase/acylhydrolase with a GDSL domain located between amino acids 56 and 377. Three-dimensional structure prediction revealed that the mutated site is located in an α-helix structure, which may be involved in the formation of a pocket structure for the binding of substrates. To the best of our knowledge, this is a gene with no functional reports available so far.

To confirm that ORF3 is RMS2, we introduced a DNA fragment containing the entire LOC_Os02g18870...
genomic sequence including its native promoter into the \textit{rms2} background. A total of seven independent rice transgenic lines (\textit{rms2COM}) were obtained, and all were completely rescued to normal pollen fertility like the wild type (Fig. 6; Supplemental Fig. S2A; Supplemental Table S2). In addition, we generated two homozygous knockout transgenic lines using the CRISPR/Cas9 technique. \textit{Cr-RMS2-4} and \textit{Cr-RMS2-7} had an ATACAAG deletion in the second exon and a C deletion in the first exon, respectively (Fig. 7, A–C), which knocked down gene transcription and likely disrupted the resulting proteins by shifting their ORFs (Supplemental Figs. S1B and S2B). Both mutants exhibited shrunken anthers and completely sterile pollen grains, which phenocopied \textit{rms2} (Fig. 7, D–I; Supplemental Table S2). Taken together, we conclude that \textit{LOC_Os02g18870} is \textit{RMS2}. We attempted to overexpress \textit{RMS2} in the cv Nipponbare background (\textit{japonica} rice). The positive \textit{Ox-RMS2} transgenic plants displayed normal anther development and seed set like the wild type (Supplemental Fig. S1B and S2B).

Using the RMS2 protein sequence as a query, a homology search in the National Center for Biotechnology Information database identified 16 putative orthologs that are functionally known in plants. RMS2 harbored four conserved blocks I to V and had conserved residues Ser-Gly-Asn-His in blocks, which is a typical feature of GDSL lipases (Supplemental Fig. S3). Phylogenetic analysis showed that RMS2 is located in a separate clade, which is genetically distinct from other known GDSL-like proteins, such as WDL1 (rice), ZmMs30 (\textit{Zea mays}), BS1 (rice), BnSSCE3 (\textit{Brassica napus}), and SFAR4 (Arabidopsis; Fig. 8A). Arabidopsis GLIP1, GLIP2, GLIP3, and GLIP4 are highly conserved in evolution and have critical functions in plant resistance (Oh et al., 2005; Lee et al., 2009). \textit{OsGLIP2} (rice), \textit{TcGLIP} (\textit{T. cinerariifolium}), \textit{CaGLIP1} (\textit{C. annuum}), and \textit{BnGLIP} (\textit{B. napus}) also have important functional roles in stress response (Hong et al., 2008; Kikuta et al., 2012; Gao et al., 2017). It was noted that ZmMs30, the first reported GDSL protein associated with pollen fertility, was also in a separate clade (An et al., 2019). The above results suggested that RMS2 is a typical GDSL lipase/acylhydrolase with functions distinct from other known plant GDSLs.
RMS2 Is Predominantly Transcribed in Anther and Its Protein Is Localized in the Endoplasmic Reticulum

To investigate the temporal and spatial expression patterns of RMS2, reverse transcription quantitative PCR (RT-qPCR) was conducted using various rice tissues. The results showed that RMS2 was weakly transcribed in most of the tested tissues such as root, seed, palea, lemma, and leaf (Fig. 8B). However, robust transcription of RMS2 was detected in developing anthers, in which RMS2 started to accumulate in the early-stage anther, gradually increased to peak levels from the meiosis stage to the vacuolization stage, and then declined during anther maturation (Fig. 8B). mRNA in situ hybridization was performed to determine more precise spatial and temporal expression patterns of RMS2. Consistent with the qPCR analysis, intense digoxigenin signal was detected in the anther wall, including endothecium, middle layer, and tapetum, at the MMC stage (Fig. 8C). Along with the degradation of the middle layer and tapetum, RMS2 expression was reduced at the vacuolated pollen stage (Fig. 8, D–F). RMS2 was also expressed in the pollen at the MMC stage (Fig. 8C), and the transcriptional level kept increasing from the MMC stage to the microspore stage, then started to decline in the vacuolated pollen stage along with the development of microspores (Fig. 8, C–F). As a negative control, the sense strand RNA probe only exhibited a background level of signal.

Figure 4. SEM images of anthers and pollen grains from the wild type (WT) and rms2. Comparisons of the SEM observation of wild-type (A, C, and E) and rms2 (B, D, and F) anthers are shown at the vacuolated pollen stage (A and B), mitosis stage (C and D), and mature pollen stage (E and F). A-1 to F-1, Anther epidermis of the wild type and rms2. A-2 to F-2, Inner surface of wild-type and rms2 anthers. A-3 to F-3, Pollen grains of the wild type and rms2. A-4 to F-4, The pollen grain outer surface of the wild type and rms2. Bars = 10 μm.
Therefore, RMS2 likely functions primarily in developing anthers from the early meiosis stage to the premature pollen stage.

To determine the subcellular localization of RMS2, we constructed a p35S:RMS2-GFP vector and transiently expressed the recombinant protein with a set of cellular compartment marker proteins in rice proplasts. It was found that the RMS2 colocalized with the endoplasmic reticulum (ER) marker in cytosolic compartments (Fig. 8H). Hence, RMS2 is localized in the ER.

**RMS2 Has Esterase Activity and Modulates Lipid Metabolism**

Given the typical GDSL domain found in RMS2, we hypothesized that lipid metabolism may be altered in the rms2 mutant. This hypothesis was first tested by a histological staining assay on the mature anthers using Sudan Red 7B, an efficient nonfluorescent lipid dye (Brundrett et al., 2009). The results showed that anther and pollen grains of the wild type were intensely stained, but the anther in rms2 showed much weaker staining.
signal compared with the wild type and the degraded 
*rms2* pollen could hardly be stained (Fig. 9A). Subse-
quently, the lipid hydrolase activity of RMS2 was tested 
using *p*-nitrophenyl butyrate as a substrate. Purified 
GST-MS2 and GST-MS2(M) (the mutated form of the 
protein as in *rms2*) recombinant proteins from *Esche-
richia coli* were first used for the assay. Unfortunately, 
neither of the proteins exhibited hydrolase activities as 
the GST tag did, possibly due to a lack of posttransla-
tional modifications on the recombinant proteins or 
other complex components in the in vitro assay 
(Fig. 9B). Alternatively, we utilized the extracts of total 
anther proteins from the wild type and *rms2* for the 
hydrolase assay. As a result, the mutant had a signifi-
cantly lower hydrolytic activity than the wild type, 
suggesting that RMS2 hydrolyzed the lipid substrate 
and therefore exhibited general lipase activities 
(Fig. 9C). To exclude the possibility that the divergent 
enzyme activities resulted from protein stability, we 
performed a cell-free degradation assay on GST-RMS2 
and GST-RMS2(M). It appeared that both proteins 
showed similar degradation curves, implying that the 
protein stability of RMS2 was not affected by the mu-
tation (Supplemental Fig. S4).

**Figure 6.** Genetic complementation test of *rms2*. A, Mature plant 
morphology of the wild type (WT; left), the *rms2* mutant (middle), and 
the transgenic plant (*rms2*COM; right). Bar = 10 cm. B, Panicles of the 
wild type (left) and *rms2*COM (right). Bar = 2 cm. C to F, The com-
plemented transgenic plant (*rms2*COM) reverted to normal anther 
morphology (C and D) and pollen fertility (E and F). Bars = 1 cm (C and 
D) and 500 μm (E and F).

RMS2 Gene Transcription Is Activated by UDT1 and PTC1

To understand the role of RMS2 in the regulatory net-
work of rice male reproductive development, yeast one-
hybrid analysis was carried out to screen for upstream
regulators of RMS2. Four key male fertility regulatory transcription factors, TDR (LOC_Os02g02820), GAMYB (LOC_Os01g59660), PTC1 (LOC_Os09g27620), and UDT1 (LOC_Os07g36460), were selected as activators in the assay. It was found that PTC1 and UDT1 could bind to the RMS2 promoter in yeast while TDR and GAMYB could not (Fig. 10A). Subsequently, we performed chromatin immunoprecipitation (ChIP)-qPCR analysis to test the PTC1 and UDT1 binding on RMS2 in vivo. UDT1 has been reported to be a bHLH transcription factor, which may activate target gene transcription by binding to the E-box (CANNTG) in the promoter region. We searched the RMS2 promoter and found two putative E-box elements. As expected, ChIP-qPCR results demonstrated that UDT1 was significantly enriched (greater than threefold) in the target region where the closest E-box is located (Fig. 10B). This protein-DNA binding relationship was further verified in vitro by electrophoresis mobility shift assay (EMSA), as UDT1 retarded the shift speed of the probe containing the E-box (CANNTG) in the promoter region. We searched the RMS2 promoter and found two putative E-box elements. As expected, ChIP-qPCR results demonstrated that UDT1 was significantly enriched (greater than threefold) in the target region where the closest E-box is located (Fig. 10B). This protein-DNA binding relationship was further verified in vitro by electrophoresis mobility shift assay (EMSA), as UDT1 retarded the shift speed of the probe containing the E-box (CANNTG) in the promoter region.

PTC1 encodes a PHD-finger protein that functions in tapetal cell death and pollen development in rice, but its downstream genes have not been reported yet (Li et al., 2011a). ChIP-qPCR analysis similarly showed significant enrichment (greater than threefold) in the RMS2 promoter, indicating that PTC1 may also regulate RMS2 transcription (Fig. 10B). Unfortunately, due to the challenges in purifying the recombinant PTC1 proteins from E. coli, we were not able to test the binding of PTC1 to the RMS2 promoter in vitro. Finally, a luciferase (LUC) transient transcriptional activity assay was conducted to confirm the effect of UDT1 and PTC1 on RMS2 transcription (Fig. 10C). The results showed that both UDT1 and PTC1 effectors drastically activated proRMS2::LUC reporter transcription, whereas the negative control did not, indicating that UDT1 and PTC1 are direct activators of RMS2. Given the fact that UDT1 and PTC1 shared the same target gene, RMS2, we speculated that the two transcription factors may work as a protein complex. However, as clearly indicated by the yeast two-hybrid and bimolecular fluorescence assays, no positive protein-protein interactions were detected between UDT1 and PTC1, suggesting that they may independently transactivate RMS2 expression (Supplemental Fig. S8). Interestingly, UDT1 and PTC1 displayed an obvious additive effect in activating the proRMS2::LUC reporter in the LUC assay, as cotransformation of both effectors yielded significantly more intense signals than each single effector (Fig. 10C). Finally, we examined the RMS2 mRNA abundance in the developing anthers of udt1 and ptc1 mutants and found that it was significantly down-regulated in both mutants, which further supported the conclusion that UDT1 and PTC1 are transactivators of RMS2 (Fig. 10E; Supplemental Fig. S9).

From the results above, a working model of RMS2-regulated male fertility was proposed (Fig. 11). RMS2 encodes an ER-localized GDSL lipase and is directly targeted by transcription factors UDT1 and PTC1 and, possibly, some other unknown regulators. RMS2 determines rice male fertility, including middle layer and tapetum degradation, cuticle and exine formation, and central vacuole development in pollen grains, by mediating lipid homeostasis. Overall, our work suggests that RMS2 may serve as a key node in the rice male fertility regulatory network.
DISCUSSION

**RMS2 Is Required for Anther Development by Affecting Tapetum Degradation and Cuticle and Central Vacuole Formation**

This study described the map-based cloning of a GDSL motif-encoding gene, RMS2, in which a substitution of the 230th and 231st amino acid residues from LV to H within the GDSL domain led to abnormal anthers and complete male sterility. Solid genetic evidence including genetic complementation and CRISPR/Cas9-derived allelic mutants supported this conclusion (Fig. 7), indicating that this GDSL lipase is required for rice male reproductive development. The cytological analysis revealed comprehensive effects of RMS2 on anther development, as the mutant showed obviously delayed degradation of tapetum, arrested formation of cuticle on anther epidermis, ubisch bodies, and incomplete central vacuole formation.

Figure 8. Phylogenetic tree and expression patterns of RMS2. A, A neighbor-joining phylogenetic tree showing evolutionary relationships among RMS2 and other reported GDSL-like proteins in plants. B, Expression patterns of RMS2 by RT-qPCR. Data are shown as means ± so (n = 3). C to G, In situ hybridization analysis of RMS2 in wild-type anthers using an antisense probe (C–F) and a negative control sense probe (G). C, The MMC stage. Bar = 10 μm. D, The late meiosis stage. Bar = 20 μm. E, The young microspore stage. Bar = 20 μm. F and G, The vacuolated pollen stage. Bars = 20 μm. En, Endothecium; ML, middle layer; Msp, microspore; T, tapetum. H, Subcellular localization of RMS2 protein. The fusion constructs p35s::RMS2::GFP and ER-mCherry were cotransformed into rice protoplasts and observed using a confocal laser-scanning microscope. Green fluorescence shows GFP, mCherry fluorescence shows ER marker fluorescence, and yellow fluorescence shows the merged fluorescence. The AtBIP-RFP vector was used as an ER marker. Bars = 5 μm.
on the anther inner surface, and exine on the pollen grain, as well as impaired formation of the central vacuole in the pollen grains. Anther cuticle is the protective barrier of the anther in harsh conditions. Programmed cell death of the tapetal cells at the proper development time is critical to supply enzymes and nutrients for callose dissolution, pollen wall formation, and carbohydrate metabolism (Guo and Liu, 2012). Ubisch bodies on the inner surface of anthers further transport nutrients from tapetum to anther lumen, where sporopollenin is synthesized to form exine on the pollen grains. It seems that these anther development defects are tightly associated, because these phenotypes have been commonly found in numerous rice male-sterile mutants such as udt1, defective pollen wall (dpw), dpw2, eat1, glycerol-3-phosphate acyltransferase3, apoptosis inhibitor5, and so on (Jung et al., 2005; Li et al., 2011b; Shi et al., 2011; Niu et al., 2013; Xu et al., 2017; Sun et al., 2018).

Besides the defects observed above, we also noticed that no large central vacuoles were formed during the development of rms2 microspores (Figs. 2N and 3H). Emerging evidence has shown that dynamic vacuolar changes are essential for the fertility of pollen (Clément et al., 1994; Pacini, 2000; Zhang et al., 2018b). During the transition from the tetrad release to the first pollen mitosis, large central vacuoles are formed as a storage space for the accumulation of water, polysaccharides arising from tapetum secretions, and degeneration of other organelles in the cytoplasm, which are crucial for continuing pollen development (Kjellbom et al., 1999; Aouali et al., 2001). Aquaporins in the pollen central vacuole help to control water permeability under normal or salinity conditions (Maurel, 1997). Additionally, the central vacuoles also provide mechanical support to maintain pollen shape (Úgalde et al., 2016). Therefore, the impaired formation of central vacuoles may be a major reason for the rms2 pollen sterility. Interestingly, according to the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), amino acids 27 to 379 are annotated as a zinc-finger FYVE domain, which was named after the first letter of the four FYVE proteins (Fabl, YOTB, Vac1, and EEAl; Heras and Drøbak, 2002; Kawahara et al., 2013). FYVE-containing proteins could bind to phosphatidylinositol-3-phosphate (Gaullier et al., 1998), mediating vacuole biogenesis, autophagy, intracellular trafficking, and phosphatidylinositol metabolism (Banerjee et al., 2010; Krishna et al., 2016). A case in Arabidopsis has indicated that FYVE-containing proteins affect pollen fertility by regulating vacuole morphology (Whitley et al., 2009). Given the similar observation of vacuole biogenesis in the rms2 pollen, we attempted to test the lipid-binding ability of RMS2 to various types of phospholipids. Unfortunately, no positive results were obtained, likely due to a lack of posttranslational modifications in the prokaryote-expressed GST-RMS2 used in the assay, which is also consistent with the results of the hydrolase activity assay.

Conserved Roles of GDSLs in Lipid Metabolism and Male Fertility

Lipid metabolism is critical for the building of the anther cuticle and the pollen wall. It is known that the
anther cuticle is composed of cutin and wax. Cutin polymers are built of lysophosphatidic acids, which are converted from cutin monomers derived from hydroxy- and epoxy-C16 and -C18 fatty acids by acyltransferases (Gómez et al., 2015). Wax is a mixture of alkenes, alkanes, very-long-chain fatty acids, and fatty alcohols. Exine of pollen wall is predominantly composed of sporopollenin, the components of which included fatty acid derivatives and phenolic compounds (Jiang et al., 2013). GDSLs are a large group of esterase/lipase enzymes that are widely present in prokaryotes and eukaryotes. Some GDSL-motif genes have been identified in plants, with functional implications in the development of secondary cell walls, oil synthesis and decomposition, cellulose deposition, and stress resistance through modulating lipid metabolism and homeostasis (Gao et al., 2017; Yadav et al., 2017; Zhang et al., 2017). However, only recently was the role of GDSL lipases in male fertility unraveled. ZmMs30, which encodes an anther-specific GDSL lipase, was reported to control male fertility through regulating the biosynthesis of anther cuticle and pollen exine in Z. mays (An et al., 2019). Because the mutation of ZmMs30 showed stable, full sterility in various inbred backgrounds and did not alter the major agronomic traits as well as the heterosis in production, ZmMs30 was successfully utilized as a multicontrol sterility system for hybrid seed production (An et al., 2019). Although ZmMs30 is not the closest ortholog of RMS2, both proteins showed similar spatial expression patterns, cellular compartment localization, and hydrolase activity. Mutants disrupted in both of these genes displayed quite similar phenotypes in pollen development and vegetative growth. Thus, the results indicated conserved roles of GDSL lipase in plant male fertility. Meanwhile, there is potential for RMS2 to be utilized for rice hybrid seed production, similar to ZmMs30.

Unlike the traditional Gly-X-Ser-X-Gly lipase, GDSL enzymes are conferred with flexible conformation to
A GDSL Lipase Functions in Rice Male Fertility

RMS2 Acts Downstream of Multiple Transcription Factors

In plants, many transcription factors have been reported to be involved in the reproductive development process, such as bHLH, PHD-finger, MYB, and so on (Clément et al., 1994; Li et al., 2006, 2011a; Ito et al., 2007; Aya et al., 2009; Ko et al., 2014; Yadav et al., 2017; Ono et al., 2018). These transcription factors usually act by binding to the promoters of specific downstream genes. For example, GAMYB, whose mutants exhibited defects in tapetal degradation and formation of exine and ubisch bodies, was reported to regulate two lipid metabolic genes, CYP703A3 and KAR, through the MYB-binding motif (Aya et al., 2009). A bHLH transcription factor, TDR, could regulate the transcription of OsC6 and OsCP1 to function in rice tapetum development and degeneration (Li et al., 2006).

Rice UDT1 and PTC1 belong to bHLH and PHD-finger transcription factor families, respectively, and have been long reported as major regulators of early tapetum development (Jung et al., 2005; Li et al., 2011a). Nevertheless, as transcription factors, none of their direct target genes have been reported so far, which makes their genetic regulatory network quite elusive. Interestingly, a microarray analysis once identified 12 commonly regulated downstream genes of the four mutants (ptc1, udt1, tdr, and gamyb), including several GDSL-like lipases (Li et al., 2011a). These results suggest that GDSL lipases might be common targets of these transcription factors controlling male fertility. Indeed, this study proved that both UDT1 and PTC1 can directly bind to the RMS2 promoter and activate its transcription, which is strongly supported by several pieces of in vitro and in vivo biochemical evidence (Fig. 10, B and C). Moreover, udt1, ptc1, and rms2 displayed numerous cytological similarities in male reproductive development. For example, the three mutants showed completely sterile pollen and nondegradable tapetum and middle layer; their tapetum cells were continuously vacuolized and proliferated abnormally after meiosis stage; and all showed abnormal anthers after the microspore stage with abnormal vacuolar structure of pollen exine (Jung et al., 2005; Li et al., 2011a). These results hinted that RMS2 works genetically and biochemically downstream of UDT1 and PTC1, and RMS2 is a key, converged node of the UDT1- and PTC1-mediated regulatory pathways in male reproductive development. Notably, however, the three mutants are not exactly identical in all aspects. For instance, the abortion of udt1 pollen development started before the tetrad stage, which is much earlier than in rms2, whereas ptc1 pollen showed smoother surfaces rather than the wrinkled surface of rms2 pollen. Therefore, this suggests that UDT1 and PTC1 may participate in independent regulatory pathways, but part of their functions may overlap with RMS2.

![Figure 11. Proposed working model for RMS2 regulation of male fertility in rice. RMS2 encodes an ER-localized GDSL lipase and is directly targeted by multiple transcription factors including UDT1 and PTC1. RMS2 functions in middle layer and tapetum degradation, cuticle and exine formation, and central vacuole development in pollen grains by mediating lipid homeostasis in anthers. RMS2 may serve as a key node in the rice male fertility regulatory network.](https://academic.oup.com/plphys/article/182/4/2047/6116393)

Figure 11. Proposed working model for RMS2 regulation of male fertility in rice. RMS2 encodes an ER-localized GDSL lipase and is directly targeted by multiple transcription factors including UDT1 and PTC1. RMS2 functions in middle layer and tapetum degradation, cuticle and exine formation, and central vacuole development in pollen grains by mediating lipid homeostasis in anthers. RMS2 may serve as a key node in the rice male fertility regulatory network.
Cytological Characterization of \textit{rms2}

For histological analysis, anther samples at different developmental stages of \textit{cv} 9311 and \textit{rms2} were collected and fixed in 50\% (v/v) FAA (3.7\% [v/v] formaldehyde, 3\% [v/v] acetic acid, and 50\% [v/v] ethanol) for semithin section experiments. The anatomy of pollen stages were performed as described previously (Li et al., 2011a; Xu et al., 2017). Samples were polymerized using Technovit 7100 resin (Heraeus Kulzer) and sectioned into 2-μm-thick slices using a microtome (Leica). Images were photographed using a Leica DM2500 microscope. For SEM and TEM analyses, sample tissues were sliced into 8-μm sections using a microtome (Leica). The ultrastructure of anther development was observed with a Hitachi model TM-1000 scanning electron microscope and a Hitachi H-7650 transmission electron microscope. For lipidic staining, anthers at the mature stage were soaked in a solution containing 0.1\% (w/v) Sudan red B, 50\% (v/v) polyethylene glycol-400, 45\% (v/v) glycerol, and 5\% (v/v) ethanol, and observed with a Hitachi model TM-1000 scanning electron microscope and a Hitachi H-7650 transmission electron microscope. For lipidic staining, anthers at the mature stage were soaked in a solution containing 0.1\% (w/v) Sudan red B, 50\% (v/v) polyethylene glycol-400, 45\% (v/v) glycerol, and 5\% (v/v) ethanol, and observed with a Hitachi model TM-1000 scanning electron microscope and a Hitachi H-7650 transmission electron microscope.

Map-Based Cloning

The male-sterile plants from the F2 and F3 segregating populations were used for map-based cloning. About 350 F2 and 7,832 F3 segregating individuals were used for fine mapping of \textit{rms2} on chromosome 2, respectively. Molecular marker amplification and analysis were performed following a previous report (Zhao et al., 2015). All primer sequences are provided in Supplemental Table S5.

Vector Construction and Plant Transformation

For the complementation test, the 5,604-bp \textit{rms2} genomic DNA fragment containing 1.3-kb upstream sequences and 0.8-kb downstream sequences was amplified and inserted into the pCAMBIA2300 vector (http://www.cambia.org) using T4 ligase (Takara). The recombinant vector was introduced into \textit{rms2} homozygous callus to generate transgenic plants. The \textit{rms2} homozygous callus was acquired based on the genotype of callus derived from the seeds of \textit{rms2} and \textit{rms2} heterozygous lines. The CRISPR/Cas9 system was based on a previous report (Ma et al., 2015). The annealed 19-bp genomic DNA was ligated into pYGhRNA-OX-U3 and pYGhRNA-Ox-U6a using T4 ligase (Takara). The single-guide RNA sequences and positions for \textit{rms2} and \textit{UD71} were determined in Figure 7, Supplemental Figure S9, and Supplemental Table S5. The \textit{japonica rice cv Nipponbare} was used as the transformation recipient. For overexpression, the full coding sequence (CDS) fragment of \textit{rms2} was amplified and inserted into pCAMBIA1300S under the control of a constitutive 35S promoter and transformed in the background of \textit{cv Nipponbare}. All constructs were electrotransfected into \textit{Agrobacterium tumefaciens} strain EHA105 and genetically transformed into rice following a previous report (Toki et al., 2006). Primers used in this experiment are listed in Supplemental Table S5.

Phylogenetic Analysis

The sequences used for sequence alignment and phylogenetic analysis were searched by BLAST against the National Center for Biotechnology Information database. Sequence alignment was performed using ClustalX2 and displayed using the GeneDoc utility. The phylogenetic tree was constructed using MEGA 6.06 based on the neighbor-joining method, and the statistical significance was evaluated with 1,000 bootstrap replicates (Zhao et al., 2014).

RNA Isolation and RT-qPCR

Total RNA from different tissues was extracted by TRizol according to the manufacturer’s instructions (Invitrogen). Stages of anthers were defined based on the spikelet length (Feng et al., 2001; Xu et al., 2017). For RT-qPCR analysis, RNA was reverse transcribed using a reverse transcription kit (Toyobo). Expression of \textit{rms2} in different organs was analyzed by real-time fluorescence qPCR. The reaction was performed with technical triplicates as described (Zhao et al., 2015). The transcription levels were calculated by the 2^{-ΔΔCt} values with the expression of ubiquitin gene as the internal control (Ying et al., 2017). Primers used in this experiment are listed in Supplemental Table S5.

mRNA in Situ Hybridization

The mRNA in situ hybridization was conducted as described previously (Tautz and Pfeifle, 1989). Young panicles at different developmental stages of the wild type were fixed in 50\% (v/v) FAA (3.7\% [v/v] formaldehyde, 5\% [v/v] acetic acid, and 50\% [v/v] ethanol) and embedded in paraffin. The tissues were sliced into 8-μm sections using a microtome (Leica). Primers used for mRNA in situ hybridization were labeled by digoxigenin with a DIG RNA Labeling Kit (Roche) following the manufacturer’s recommendation. Images were photographed using a Leica DM2500 microscope (Leica). Primers used in this experiment are listed in Supplemental Table S5.

Enzyme Activity Assay

The CDSs of \textit{rms2} and mutant \textit{rms2} were amplified and cloned into vector pGEX-4T-1 (GE Healthcare) using the Hieff Clone Plus One Step Cloning Kit (Yeasen Biotech). Primers used in this experiment are listed in Supplemental Table S5. The constructs were transformed into \textit{Escherichia coli} strain BL21 (DE3). Recombinant protein expression was induced by adding 1 mM isopropylthio-β-galactoside and purified using a Glutathione-Sepharose Resin Protein Purification Kit (CWBio). Total plant proteins were extracted from anthers of the wild type and \textit{rms2}. Samples were ground with liquid nitrogen and immersed in a solution containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and Roche protease inhibitor. The remaining debris was removed by centrifugation at 14,000 rpm for 4°C for 5 min, which was repeated two times to get the total plant protein.

To analyze the lipase activity, equal amounts of recombinant protein or plant total protein from wild-type and \textit{rms2} anthers were incubated with 1 mM 3-p-nitrophenyl butyrate substrate in enzyme reaction buffer (0.5 mM Hepes, pH 6.5) for 1 h at 30°C as described (Cao et al., 2017). Spectrum absorbance was measured at 405 nm at an interval of 5 min using a Tecan Infinite M200 PRO. Each experiment was conducted in triplicate.

Cell-Free Degradation Assay

About 0.2 μg of each purified recombinant protein with a GST tag was incubated in degradation buffer with or without 1 μg of total plant protein extracted from panicles of wild-type rice for 1 h at 37°C. Degradation buffer contained 25 mM Tris-HCl (pH 7.4), 10 mM NaCl, 10 mM MgCl2, 4 mM phenylmethylsulfonyl fluoride, and 10 mM ATP. The reaction volume was 20 μL. Reactions were terminated at the indicated time points, and protein degradation was evaluated by western blot using an anti-GST antibody (catalog no. CW0085, CWBio). The immune signals were quantified using Quantity Tools of Image Lab software (Bio-Rad).

Subcellular Localization

To investigate the subcellular localization of \textit{RMS2}, the coding region without the stop codon was amplified from \textit{cv Nipponbare} and fused with the C terminus of \textit{GFP} in vector pCA1301-35S-S65T-GFP. The ABIP-RFP vector was used as an ER marker (Park et al., 2004). The protoplasts of rice leaves were prepared as previously described (Qu et al., 2016). Constructs were transformed into rice protoplasts according to previously reported methods (Park et al., 2010;
Ruan et al., 2019). Fluorescence signals were detected using a Zeiss LSM710 confocal laser-scanning microscope. Primers used in this experiment are listed in Supplemental Table S5.

Broadly Targeted Metabolomics Analysis

Anthers were manually collected from multiple cv 9311 and rms2 plants. Three biological replicates were prepared for each sample. All sample extracts were analyzed using ultra-performance liquid chromatography (Shim-pack UFLC SHIMADZU C8B30A) coupled to tandem mass spectrometry (Applied Biosystems 6500 Q TRAP) in the electrospray ionization mode. The analysis was done according to previous reports (Chen et al., 2013). The effluent was connected to an electrospray ionization-triple quadrupole-linear ion trap mass spectrometer. The electrospray ionization source operation was done under the following parameters: temperature = 500°C, ion spray voltage = 5,500 V, ion source gas I = 55 p.s.i., gas II = 60 p.s.i., curtain gas = 25 p.s.i., and collision gas, high. The material identification was obtained based on secondary spectrum information and annotated using a self-built Metware database and a metabolite information public database (https://metlin.scripps.edu/). Metabolite quantification was performed using multiple reaction monitoring analysis with triple quadrupole mass spectrometry (Fraga et al., 2010).

Yeast One-Hybrid Assay

The Clontech One-Hybrid System was used for the yeast one-hybrid assay. The promoter sequence (411 bp upstream of the transcriptional start site) of RMS2 was amplified and fused with the LacZ reporter in the pl.LacZI2a vector. The UDT1 or PTC1 cDNA sequence was inserted into the pB42AD vector (Takara). Plasmids were transformed into yeast strain EGY48. Transformed yeast strains were grown on synthetic drop-out/-Ura/-Trp plates containing 2% (w/v) Gal, and 80 mg L−1 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (Promega) were used as positive controls. For bimolecular fluorescence complementation, UDT1 and PTC1 CDSs without stop codons were inserted into a streamlined double ORF expression system (pDOE vector) according to a previous study (Gookin and Assmann, 2014). Then, constructs were transiently expressed in the epidermal cells of Nicotiana benthamiana by A. tumefaciens injection. Fluorescence signals were detected using a Zeiss LSM710 confocal laser-scanning microscope. Primers used in this experiment are listed in Supplemental Table S5.

Accession Numbers

Sequence data from this article for the cDNA and genomic DNA of RMS2 can be found in the GenBank/EMBL/Gramene data libraries under accession number LOC_Os02G18870.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Transcriptional levels of RMS2 in mutant and overexpression lines.

Supplemental Figure S2. The panicle morphology of complemented and knockout lines and amino acid sequences of Cr-RMS2.

Supplemental Figure S3. Sequence alignment of RMS2 and other reported GDSL lipases.

Supplemental Figure S4. Cell-free degradation assay of GST-M2 and GST-M2(M) proteins.

Supplemental Figure S5. Top 20 differential metabolites and volcano plot of differential metabolites of the wild type versus the rms2 mutant.

Supplemental Figure S6. The KEGG classification and statistics of KEGG enrichment of differential metabolites for the wild type versus the rms2 mutant.

Supplemental Figure S7. Comparison of the contents of some metabolites in the wild type and rms2.

Supplemental Figure S8. Protein-protein interaction analysis of UDT1 and PTC1.

Supplemental Figure S9. Identification of udt1 and ptc1.

Supplemental Table S1. The agronomic traits of the wild type and rms2.

Supplemental Table S2. The seed-setting rates of complemented and knockout lines.

Supplemental Table S3. Statistical table of the quantity of metabolites and the corresponding metabolite names detected in the wild type and rms2.

Supplemental Table S4. List of the differentially accumulated metabolites between the wild type and rms2.

Supplemental Table S5. Sequences of primers used in this study.

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ChIP-qPCR

The UDT1- or PTC1-specific polyclonal antibodies were generated by a commercial service (Genscript). Chromatin was isolated from 2 to 4 g of plants of cv 9311 plants according to a previous method (Bello et al., 2019). The one-twelfth volume of chromatin-containing samples without immunoprecipitation treatment was prepared for the input samples. Three replicates of each sample were performed, and the extracted DNA samples were analyzed by qPCR using region-specific primers (Supplemental Table S5). The qPCR results were calculated according to the manual of the Magna ChIP HiSens kit (Millipore).

EMSA

EMSA probes were commercially synthesized and labeled with Cy5.5 by Sunya Biological Technology. Nonlabeled DNA oligonucleotides were used as competitors. The recombinant protein GST-UDT1 was purified as described above. The DNA-binding reaction was performed in a 10-μL reaction volume and electrophoresed using 6% native polyacrylamide gels under ice and dark conditions as described (Hou et al., 2019). The fluorescence signal in the gel was visualized using an Odyssey CLX infrared fluorescence imaging system (LI-COR) at excitation wavelength 680 nm and emission wavelength 720 nm. Primers used in this experiment are listed in Supplemental Table S5.

Yeast Two-Hybrid Analysis and Bimolecular Fluorescence Complementation

For yeast two-hybrid analysis, UDT1 and PTC1 CDSs were inserted into bait vector pGBK7T and prey vector pGAD7T (Clontech). Recombinant plasmids were cotransformed into yeast strain Y2H Gold, after which the transformed yeast strains were grown on dropout medium following the manufacturer’s instructions (Clontech). pGBK7T-53 and pGAD7T-T were used as positive controls. For bimolecular fluorescence complementation, UDT1 and PTC1 CDSs without stop codons were inserted into a streamlined double ORF expression system (pDOE vector) according to a previous study (Gookin and Assmann, 2014). Then, constructs were transiently expressed in the epidermal cells of Nicotiana benthamiana by A. tumefaciens injection. Fluorescence signals were detected using a Zeiss LSM710 confocal laser-scanning microscope. Primers used in this experiment are listed in Supplemental Table S5.
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