OsmiR528 regulates rice-pollen intine formation by targeting an uclacyanin to influence flavonoid metabolism

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The intine, the inner layer of the pollen wall, is essential for the normal development and germination of pollen. However, the composition and developmental regulation of the intine in rice (Oryza sativa) remain largely unknown. Here, we identify a microRNA, OsmiR528, which regulates the formation of the pollen intine and thus male fertility in rice. The mir528 mutant showed aborted pollen development at the late binucleate pollen stage, significantly decreasing the seed-setting rate. We further demonstrated that OsmiR528 affects pollen development by directly targeting the uclacyanin gene OsUCL23 (encoding a member of the plant-specific blue copper protein family of phytocyanins) and regulating intine deposition. OsUCL23 overexpression phenocopied the mir528 mutant. The OsUCL23 protein localized in the prevacuolar compartments (PVCs) and multivesicular bodies (MVBs). We further revealed that OsUCL23 interacts with a member of the proton-dependent oligopeptide transport (POT) family of transporters to regulate various metabolic components, especially flavonoids. We propose a model in which OsmiR528 regulates pollen intine formation by directly targeting OsUCL23 and in which OsUCL23 interacts with the POT protein on the PVCs and MVBs to regulate the production of metabolites during pollen development. The study thus reveals the functions of OsmiR528 and an uclacyanin during pollen development.

The intine layer of pollen is essential for pollen grain maturation and pollen tube germination. Abnormal intine development causes pollen sterility and affects seed-setting; therefore, the identification of regulators of intine formation is important for elucidating the mechanisms of pollen formation and function, especially for crop breeding. Here, we report a microRNA, OsmiR528, which regulates pollen intines' formation and male fertility in rice (Oryza sativa). OsmiR528 directly targets the uclacyanin family member OsUCL23 to regulate flavonoid metabolism and pollen intine development. This study revealed the function of OsmiR528 and an uclacyanin in pollen development.

Significance

The intine layer of pollen is essential for pollen grain maturation and pollen tube germination. Abnormal intine development causes pollen sterility and affects seed-setting; therefore, the identification of regulators of intine formation is important for elucidating the mechanisms of pollen formation and function, especially for crop breeding. Here, we report a microRNA, OsmiR528, which regulates pollen intines' formation and male fertility in rice (Oryza sativa). OsmiR528 directly targets the uclacyanin family member OsUCL23 to regulate flavonoid metabolism and pollen intine development. This study revealed the function of OsmiR528 and an uclacyanin in pollen development.

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lines (OXmirR528, expression levels shown in Fig. 1C). The phenotypes of mir528 and OXmirR528 plants were then analyzed from the vegetative stage to the reproductive stage. The mir528 mutants were slightly shorter than the wild-type (WT) plants (SI Appendix, Fig. S1 A and B), and their panicles were semisterile (Fig. 1 D and E). Conversely, the OXmirR528 plants had a higher seed-setting rate (Fig. 1 D and E), suggesting that OsmiR528 might be involved in the regulation of fertility.

We next analyzed floral development in the mutant plants to identify how OsmiR528 affects fertility. The phenotypic analysis showed that both the mir528 and OXmirR528 plants developed normal pistils and anthers (SI Appendix, Fig. S1C), but iodine–potassium iodide staining revealed that mir528 plants have significantly more aborted pollen grains whereas OXmirR528 plants have fewer aborted pollen grains than WT plants (Figs. 1F and 2A). To identify the exact stage at which the mutant pollen grains began to show phenotypic defects, we compared WT, OXmirR528, and mir528 pollen in paraffin (SI Appendix, Fig. S1D) and semithin sections (SI Appendix, Fig. S2A). At the pollen mother-cell stage, meiosis stage, and the young microspore stage, the pollen grains of OXmirR528 plants and the mir528 mutant were similar to that of the WT plants; however, at the late binucleate pollen stage and mature stage, half of the mir528 pollen grains had shrunk and had no cytosolic content (SI Appendix, Fig. S2A). These results showed that OsmiR528 is essential for pollen grain maturation.

**OsmiR528 Regulates Pollen Intine Formation.** Next, we investigated which process was regulated by OsmiR528 during pollen development by examining the mature pollen grains of WT, OXmirR528, and mir528 plants. Three staining methods (Alexander’s staining, auramine O staining, and calcofluor white staining) were applied to detect pollen viability, the pollen exine, and the pollen intine, respectively. As shown in Fig. 2A, the defective pollen grains in the mir528 mutant were not viable. The pollen grains of the mutant, including aborted pollen grains, had an intact exine; however, the defective pollen from the mir528 mutants exhibited very weak calcofluor white staining relative to that in the WT pollen grains (Fig. 2A), indicating that the intine might be defective in the mutant pollen. Statistical analysis showed that mir528 plants presented more nonviable, intine-defective pollen grains, whereas OXmirR528 plants presented fewer nonviable, intine-defective pollen grains than WT plants (Fig. 2B), and the heterozygous mir528 plants showed intermediate phenotypes (SI Appendix, Fig. S2B and C). The intine was previously reported to be essential for pollen germination and pollen tube elongation on the stigma (19); therefore, we next monitored pollen tube germination in the mutant, the overexpressor line, and WT plants (SI Appendix, Fig. S2D). The pollen grains germinated on the stigma 2 h after anthesis in the WT plants and OXmirR528, whereas very few mir528 pollen grains germinated. We also performed an in vitro pollen germination experiment to investigate the effects of OsmiR528 on the germination rates (SI Appendix, Fig. S2E). As shown in Fig. 2C, mir528 plants showed a significantly lower rate than that in WT plants, whereas OXmirR528 plants had a higher rate than that in WT plants. These results show that OsmiR528 is involved in pollen intine formation, which in turn affects pollen germination.

To understand how intine development was affected in the mutants, we used transmission electron microscopy to examine the pollen at different stages of pollen development (Fig. 2B). In the early microsporocyte stage (EMS), the WT plants developed an intine layer, which presented no intine, were not included in the statistical analysis to avoid biased results. Significant differences in intine thickness between the mutants and WT plants were observed from LBPS to MPS (Fig. 3B). OXmirR528 plants exhibited significantly thicker intines than WT pollen, whereas the normally developed mir528 pollen exhibited thinner intines than WT pollen, and the aborted pollen grains contained no starch and exhibited a severe defect in their intine layer (Fig. 3B). Previous research suggested that intine development precedes the accumulation of pollen grain inclusions such as starch (20); we therefore speculate that OsmiR528 positively regulates pollen intine formation during late gametogenesis, which affects fertility.

**OsmiR528 Targets an mRNA Encoding a Uclacyclin Protein to Regulate Pollen Grain Development.** To investigate how OsmiR528 regulates pollen grain maturation, we analyzed the potential targets of this microRNA. The mRNAs of several genes are potential targets of OsmiR528 in rice, including genes encoding an ascorbic acid oxidase (LOC_Os06g37150), a laccase (LOC_Os01g62600), a D3 protein (LOC_Os06g06050), and a member of the uclacyclin family (OsUCL23, LOC_Os08g04310) (10). The gene encoding the ascorbic acid oxidase was previously reported to be involved in the biotic- and abiotic-stress responses, but was not found to affect reproductive growth (12–14). The laccase gene (LOC_Os01g62600) was expressed at an extremely low level (SI Appendix, Fig. S4A). We excluded these 2 genes from the analysis and focused on the D3 gene and OsUCL23. The function of OsUCL23 has not yet been elucidated. D3 was known to control tillering by suppressing growth activity (21). The mRNA abundances of both OsUCL23 and D3 were down-regulated in the OXmirR528 plants and up-regulated in the mir528 mutants (SI Appendix, Fig. S4A and B), indicating that their expression levels are negatively correlated with that of OsmiR528. We then constructed overexpression lines of OsUCL23.
OsmiR528 is required for pollen grain maturation and pollen tube germination. (A) Histochemical staining of WT and mutant pollen grains showed a defect in pollen grain maturation and intines of the mir528 and OXUCL23 plants. (Scale bars, 50 μm.) (B) Ratio of pollen grains with defective intines in different transgenic plants. Values shown are the means ± SD (n = 10 plants, 200 pollen grains). Significant differences were identified using Student’s t test. (C) In vitro pollen germination rates of different transgenic plants. Values shown are the means ± SD (n = 200 pollen grains). Significant differences were identified using Student’s t test. *P < 0.05, **P < 0.01.

We also used bright blue fluorescence, the OXUCL23 pollen grains exhibited a very weak calcofluor white signal, similar to those of mir528 plants, demonstrating that the intine was defective in OXUCL23 pollen (Fig. 2 A and B). Transmission electron microscopy showed that the OXUCL23 pollen grains presented a similar exine to the WT and ucl23 pollen grains, whereas the aborted pollen grains of OXUCL23 plants could not initiate intine formation at EBPs and exhibited no intine and starch accumulation at LBPs and MPS, while the other pollen grains presented a significantly thinner intine than the WT plants at MPS (Fig. 3 A and SI Appendix, Fig. S3). The intine of the ucl23 pollen grains was thicker than the WT intine, similar to that of the OXmiR528 plants (Fig. 3 A and B). Consistent with these observations, in vivo and in vitro pollen grain germination was also repressed in OXUCL23 plants, whereas in vitro germination rates were higher in ucl23 plants than in WT plants, indicating that their defective intine inhibits the germination of the OXUCL23 pollen tubes (Fig. 2 C and SI Appendix, Fig. S2 B–E).

We further analyzed the function of the female organs of OXUCL23 and mir528 plants by crossing OXUCL23 plants with OXmiR528 plants and crossing mir528 plants with DongFeng (DJ) WT plants (OXUCL23 or mir528 plants served as the male parent or female parent) (SI Appendix, Fig. S5 A). As expected, we obtained more hybrids when using OXUCL23 or mir528 as the female parent, while fewer hybrids were obtained when using DJ as the male parent as the males were treated as the male parent or female parent (SI Appendix, Fig. S5 B and C). The hybrids obtained using OXUCL23 or mir528 as the male parent developed normal embryos (SI Appendix, Fig. S5 B), which indicated that the pistils of the OXUCL23 and mir528 plants were normally developed and that the decreased seed-setting rate in OXUCL23 and mir528 plants was caused by aborted pollen grains and a decreased pollen grain germination rate. We concluded that OsmiR528 interacts with OsUCL23 mRNA to promote its cleavage, which positively regulates pollen intine formation during late gametogenesis and consequently affects fertility.

OsUCL23 Localizes to Prevacuolar Compartments and Multivesicular Bodies. Above, we showed that OsUCL23 affects the formation of the pollen intine. Next, we investigated the mechanism that causes this effect. The OsUCL23 protein contains a 30-amino-acid (AA) signal peptide (the secretion signal) at its N terminus, a 33-aa glycosylphosphatidylinositol (GPI)-anchor signal at its C terminus, and a plastocyanin-like domain (Fig. 4 A). To investigate the subcellular localization of the OsUCL23 protein, GFP was fused to the N-terminal, C-terminal, or central region of OsUCL23 as shown in the diagram (Fig. 4 A). These fusion molecules were expressed under the control of the Cauliflower mosaic virus 35S promoter and were transformed into rice protoplasts. As the GPI anchor is often responsible for membrane localization (22), we coexpressed the endoplasmic reticulum (ER)-yellow fluorescent protein (YFP) marker CD3-958, the Golgi-YFP marker CD3-966, the nucleus-mCherry marker HY5, and the cytoplasm marker pUC-mCherry with the N-GFP-UCL23-C fusion protein in protoplasts to enable subcellular localization studies of the GFP- OsUCL23 constructs (SI Appendix, Fig. S6).

After incubating the transformed cells for 12 h, we monitored the localization of the fusion proteins using a confocal microscope. The fluorescent signals exhibited a spotty distribution, partially overlapping with the ER marker but not the Golgi marker (SI Appendix, Fig. S6). The distribution of OsUCL23 was similar to that of the organelles in the endomembrane system. We then determined whether OsUCL23 colocalized with the prevacuolar compartment and multivesicular body (PVC and MVB, respectively, which respond to brefeldin A) marker VSR2 or the trans-Golgi network (which responds to mannosylated marker SYP61) (23–25). The fluorescence signals of GFP-OsUCL23 overlapped with the VSR2 marker but not with the SYP61 marker, indicating that OsUCL23 localizes to PVCs and MVBs (SI Appendix, Fig. S6 and Fig. 4B). We further analyzed the effect of different domains of OsUCL23 on its subcellular localization.

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When the GFP protein was fused to different parts of OsUCL23, we found that both parts of the OsUCL23 protein give the fusion protein the ability to localize to PVC/MVBs (Fig. 4A and B). PVCs and MVBs have been reported to function in the degradation, trafficking, and mobilization of various cellular components (23); thus, OsUCL23 might affect pollen intine development by regulating the function of these organelles.

To understand the role of OsUCL23 during microspore development, we then performed qRT-PCR using gene-specific primers to analyze its temporal expression pattern. WT anthers at the late microspore stage, early binucleate pollen stage, and late binucleate pollen stage were collected and used for an analysis of OsUCL23 and OsmiR528 expression. The results showed that OsUCL23 was expressed at low levels in the late binucleate pollen stage, during which the intine begins to accumulate (SI Appendix, Fig. S7A). OsmiR528 showed the opposite expression pattern to OsUCL23 (SI Appendix, Fig. S7A). β-Glucuronidase (GUS) activity analysis of OsmiR528 and OsUCL23 also showed that the promoter activity of OsmiR528 increased from the vacuolated stage to the mature stage, whereas OsUCL23 exhibited the opposite pattern and decreased beginning in the binucleate pollen stage (Fig. 4C and SI Appendix, Fig. S7 B and C), indicating that OsmiR528 might function during sporogenesis. We further performed in situ hybridization of OsmiR528 and OsUCL23 to analyze their spatial expression patterns. Similar to the GUS signals, OsmiR528 gradually accumulated to a high level from the binucleate pollen stage to the mature stage, whereas the abundance of OsUCL23 mRNAs decreased during OsmiR528 accumulation (Fig. 4D). In addition, OsUCL23 mRNAs existed in both the pollen grains and tapetum, but OsmiR528 was expressed mainly in the pollen grains (Fig. 4D). These results indicated that OsmiR528-mediated OsUCL23 degradation plays a role in intine development during the late binucleate pollen stage.

OsUCL23 Interacts with a POT Family Protein to Regulate Flavonoid Metabolism. To further demonstrate the molecular mechanisms by which OsUCL23 regulates the development of the pollen intine, we next identified OsUCL23-interacting flavonoid genes using a yeast 2-hybrid system and a rice young panicle cDNA library. Fragments from 6 proteins (LOC_Os03g14610, LOC_Os01g14670, LOC_Os03g06840, LOC_Os10g33170, LOC_Os06g01210, and LOC_Os03g17570) showed positive results. We further verified the interaction between the full-length proteins of the 6 genes and OsUCL23 protein, respectively, and only the POT protein (LOC_Os10g33170) was proved to have authentically interacted with OsUCL23 (Fig. 5A). POT proteins are an evolutionarily conserved family of proton-coupled transport proteins spanning cellular membranes. A remarkable feature of the POT family is the diversity and extent of the natural substrates that they recognize. In mammals, fungi, and bacteria, POT proteins are predominantly peptide transporters, but in plants, the family has diverged to recognize nitrites, plant defense compounds, and hormones (26, 27).

We hypothesized that OsUCL23 might interact with POT proteins on the membranes of PVCs and MVBs to mediate the transport of metabolites during pollen grain development. To address this hypothesis, we first analyzed the subcellular localization of POT proteins in protoplasts. Similar to OsUCL23, POT showed perfect colocalization with the PVC/MVB marker VSR2 (Fig. 4B and SI Appendix, Fig. S6). To validate the in vivo interaction between the OsUCL23 and POT proteins, we then performed bimolecular fluorescence complementation (BiFC) in rice protoplasts. The results indicated that POT and OsUCL23 interact with each other in PVC/MVBs (Fig. 5A). We also analyzed the expression pattern of POT during anther development by in situ hybridization. The abundance of POT mRNAs was higher than that of OsmiR528 and OsUCL23 mRNAs, and they exhibited a similar expression pattern to OsUCL23 mRNAs (Fig. 4D). These mRNAs were highly accumulated at the vacuolated stage and decreased during pollen development in the pollen grains and tapetum (Fig. 4D), indicating similar roles for OsUCL23 and POT. The results showed that POT and OsUCL23 interact with each other in PVC/MVBs during early sporogenesis but decrease beginning in the binucleate pollen stage.

Second, to identify which metabolite may be affected by OsUCL23 and OsmiR528 in anthers, we performed 3 repetitions of high-throughput quantification of the metabolites present in WT, OXmiR528, mir528, uc123, and OXUCL23 anthers at MPS, respectively, using a liquid chromatography-electrospray ionization-tandem mass spectrometry system and screened the metabolites that were the following: 1) complementarily changed between transgenic plants with higher OsUCL23 abundance (OXUCL23 and mir528) and those with lower OsUCL23 abundance (uc123 and OXmiR528); 2) consistently changed between transgenic plants with a similar OsUCL23 expression pattern (OXUCL23 and mir528, uc123, and OXmiR528); 3) among the 1,000 metabolic compounds detected in rice anthers, fewer than 200 were annotated. These metabolites included flavonoids, amino acids and their derivatives, fatty acids, nucleic acids and their derivatives, and other compounds. We analyzed only the changes in the levels of annotated metabolites. Following this standard, we screened 53 metabolites belonging to 14 classes, and 22 of them belonged to the flavonoid class, indicating that OsmiR528 and OsUCL23 mainly affected flavonoids. Among these 22 flavonoids, 17 were up-regulated in OXUCL23 anthers; 16 varied as expected in at least 3 samples; and 8 were up-regulated in both OXUCL23 and mir528 samples and down-regulated in both OXmiR528 and uc123 samples, indicating that they might be directly regulated by OsmiR528 and OsUCL23 (SI Appendix, Fig. S7D). We further performed staining with the flavonol-specific dye diphenylboric acid-2-aminoethyl ester (DPBA) to monitor the accumulation pattern of flavonol in mutants and WT plants during anther development. DPBA staining showed that OXmiR528 and uc123...
pollen grains accumulated more flavonol at the binucleate pollen stage, but the flavonol content decreased to a lower level than in WT plants at the mature pollen stage, which was consistent with the metabolomics results, whereas OXPOT and POT-overexpressing (OXPOT) plants (Fig. 5B). Flavonoids are required for pollen germination and tube growth and are constituents of the pollen intine. Flavonol contents in OXPOT and POT-overexpressing plants were similar to those of the WT plants, which exhibit fewer aborted pollen grains and higher seed-setting rates than the WT plants (Fig. 5C–H). Taken together, our results indicate that OsUCL23 interacts with a POT protein in PVC/PMVs, which together regulate intine deposition, pollen development, and seed-setting rate in rice.

Discussion

Unlike most miRNAs, miR528 has evolved various functions in developmental regulation and stress responses by targeting distinct protein-coding genes (12–16). However, the functions of miR528 in cleaving monocopper targets and fertility are unknown (31). In this study, we revealed that OsmiR528 is essential for plant reproductive growth by targeting a monocopper target, OsUCL23. The miR528 knockout mutant showed aborted pollen grains at the late binucleate pollen stage, which decreased its seed-setting rate. We showed that OsmiR528 affects pollen development by directly targeting OsUCL23 and regulating the deposition of the pollen intine. Taken together, our results add regulation of gametogenesis to the diverse known roles of miR528.

OsUCL23 is a type-I arabinosylation (AGP) uclacyanin protein, which belongs to the phytocyanin family. Compared with other phytocyanins, uclacyanins are relatively small blue copper proteins that bind a single copper atom (32). Some members of the family are specifically localized in the cell plate and function in signal recognition and cell-wall biosynthesis. Treating Nicotiana

Fig. 5. Interaction of OsUCL23 with a POT protein in PVC/PMVs and phenotype analysis of POT gain-of-function and loss-of-function transgenic plants. (A) BiFC and yeast 2-hybrid analysis between OsUCL23 and POT. For the yeast 2-hybrid analysis, the POT and OsUCL23 open reading frames were fused in frame to the pGBKT7 and pGADT7 vectors, respectively, to construct POT-BD and AD-UCL23. Both constructs were used to transform to the Y2HGold Yeast Strain (Clontech). Hybridizations between POT and pGBK7 vector (POT-BD), the pGADT7 vector and OsUCL23 (AD-UCL23), and the pGBK7 vector and pGBK7 vector (AD-BD) were used as negative controls. (Scale bars, 2 μm.) (B) DPBA staining showed that pollen grains in OXPOT anthers were nonviable and intine defective, and the seed-setting rate of OXPOT was significantly lower than that of WT plants (Fig. 5 C–H). Thus, pot plants are similar to ucl23 plants, which exhibit fewer aborted pollen grains and higher seed-setting rates than the WT plants (Fig. 5C–H). Taken together, our results indicate that OsUCL23 interacts with a POT protein in PVC/PMVs, which together regulate intine deposition, pollen development, and seed-setting rate in rice.
tube growth and pollen tube are involved in plant reproductive development such as pollen forming a transporter complex to regulate metabolite transport. Animals and bacteria, POT proteins are involved in the uptake of one of the most promiscuous nutrient transport systems. In an grains. PVC and MVB transportation, which is required for viable pollen OsUCL23 might regulate the deposition of the intine by affecting and MVBs were also reported to function in the formation of the primary cell wall and cell plates (36, 37). We inferred that OsUCL23 might regulate the deposition of the intine by affecting PVC and MVB transportation, which is required for viable pollen grains.

We further identified a POT protein that interacted with OsUCL23 in PVC/MVBs. POT family transporters represent one of the most promising nutrient transport systems. In animals and bacteria, POT proteins are involved in the uptake of peptides for both metabolism and growth. Here, we showed that this protein colocalized with OsUCL23 in PVC/MVBs, potentially forming a transporter complex to regulate metabolite transport. OsMrS28 and OsUCL23 could regulate the levels of flavonoids and amino acids in the anthers. Flavonols are plant-specific compounds required for pollen germination and tube growth and are constituents of the pollen wall (28, 29). Previous studies have suggested that disordered intine formation would cause pollen tube burst (19). We propose that OsUCL23 and the POT protein might form a transporter complex on the PVCs and MVBs and regulate pollen grain metabolism, particularly flavonoid transport, during pollen intine development. The study revealed the functions for OsMrS28 and uclacyanin in pollen grain development.

Materials and Methods

Detailed description is provided in SI Appendix for plant growth conditions, generation of transgenic rice plants, semithin sections, transmission electron microscopy, examination of gene expression by qRT-PCR analysis, Northern blot analysis, histochemical GUS staining, cytological analysis, metabolic profiling and transient expression assay, and BiFC. Data Availability. The data that support the findings of this study are included in the main text and SI Appendix.

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