OsAG02 controls ROS production and the initiation of tapetal PCD by epigenetically regulating OsHXK1 expression in rice anthers

Shaoyan Zheng, Jing Liu, Lu Ma, Hai-long Wang, Hai Zhou, Erdong Ni, Da-gang Jian, Zhenlan Liu, and Chuxiong Zhuang

Proteins of the ARGONAUTE (AGO) family function in the epigenetic regulation of gene expression. Although the rice (Oryza sativa) genome encodes 19 predicted AGO proteins, few of their functions have thus far been characterized. Here, we show that the AGO protein OsAG02 regulates anther development in rice. OsAG02 was highly expressed in anthers. Knockdown of OsAG02 led to the overaccumulation of reactive oxygen species (ROS) and abnormal anther development, causing premature initiation of tapetal programmed cell death (PCD) and pollen abortion. The expression level of Hexokinase 1 (OsHXK1) increased significantly, and the methylation levels of its promoter decreased, in plants with knocked-down OsAG02 expression. Overexpression of OsHXK1 also resulted in the overaccumulation of ROS, premature initiation of PCD, and pollen abortion. Moreover, knockdown of OsHXK1 restored pollen fertility in OsAG02 knockdown plants. Chromatin immunoprecipitation assays demonstrated that OsAG02 binds directly to the OsHXK1 promoter region, suggesting that OsHXK1 is a target gene of OsAG02. These results indicate that OsHXK1 controls the appropriate production of ROS and the proper timing of tapetal PCD and is directly regulated by OsAG02 through epigenetic regulation.

AG02 | epigenetics | HXK1 | ROS | PCD

Rice (Oryza sativa L.) is the staple food for more than half the world’s population. It is estimated that rice production will need to increase by ~30% in 2030 to meet demands. Improving grain yield has become an important goal in rice breeding. Grain yield is affected by many genetic and environmental factors, such as pollen fertility and both abiotic and biotic stresses. Among these, pollen fertility is of particular importance. The anther is a portion of the stamen in which pollen grains develop. Rice anthers normally contain four compartments, known as lobes. Abnormal anther development will result in decreased pollen fertility, subsequently reducing yields (2–4). Therefore, improving our understanding of reproductive development (young microspore stage of pollen development) may help increase grain yields and facilitate rice breeding.

Rice anther development is a complex process (5–7) that can be divided into 14 stages according to morphological characteristics, which is consistent with that in Arabidopsis thaliana (5, 6, 8, 9). The tapetum, the innermost layer of the anther lobe, is derived from inner secondary parietal cells. The tapetum contributes to microspore release, nutrition, pollen wall synthesis, and pollen coat deposition (7, 10, 11); these functions are essential for pollen development (12). Tapetal cell fate can be divided into three phases: tapetum differentiation, tapetum formation, and apoptosis of tapetal cells. During the late stage of pollen development (stage 8 to stage 10), the tapetum undergoes programmed cell death (PCD). During PCD, tapetum degeneration occurs, along with the loosening of pollen from the surrounding tissue, the release of tapetal remnants, and the deposition of materials onto the pollen coating (13, 14). The excessive production of reactive oxygen species (ROS) in tapetal cells causes oxidative damage to proteins, lipids, and DNA, ultimately resulting in cell death. ROS can trigger PCD, and ROS levels are correlated with the regulation of cell death (15, 16). Timely PCD is critical for pollen development, and maintaining ROS homeostasis is crucial for cell growth and survival during various stages of anther development (6, 17).

The anther and pollen gene regulatory network is a complex system involving gene expression and interactions (8, 9). In recent years, significant progress has been made toward understanding the relationship between the expression of anther or pollen development-related genes and male fertility in Arabidopsis. These genes include DYSFUNCTIONAL TAPETUM (DYTT), ABORTED MICROSPOROGENESIS (AMS), MALE STERILITY1 (MS1), and MS18/MYB80 (18–24). There is a high degree of conservation with these in rice anthers, as demonstrated by the expression of OsAG02. We find that OsAG02 epigenetically regulates anther development by modulating DNA methylation modifications in the Hexokinase (OsHXK) promoter region. OsHXK1, in turn, affects anther development by regulating the production of reactive oxygen and the initiation of cell death in key anther structures. Identification of this epigenetic regulatory mechanism has implications for the production of hybrid crop varieties.

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Data deposition: Sequence data from this article can be found in the Rice Annotation Project (https://rapdb.dna.affrc.go.jp/viewer/gbrowse) and have been deposited in the GenBank database [Os04g0615700 (OsAG02), Os07g0446800 (OsHXK1), and Os10g0510000 (OsActin)]. The tapetal genes deposited in the GenBank database include accession nos. Os07g0496900 (OsDTT7), Os01g0293100 (OsTIP2), Os02g1205000 (OsDRY1), Os04g0559300 (OsLEY1), Os04g0706000 (OsMYB107), Os04g0849500 (OsTGA10), Os09g0449000 (OsPch1), Os10g0201700 (OsMADS3), and Os07g0543660 (OsDTC7). The OsRBOH genes deposited in the GenBank database include accession nos. Os07g0374200 (OsRBOH4/OsNODX), Os01g0360200 (OsRBOH4b), Os05g0528000 (OsRBOHc), Os08g0461800 (OsRBOHd), Os01g0835000 (OsRBOHf), Os08g0437000 (OsRBOHg), Os09g0438000 (OsRBOHh), Os12g0541300 (OsRBOHi), and Os11g0537400 (OsRBOHj).

S.Z. and J.L. contributed equally to this work.
2To whom correspondence should be addressed. Email: zuangcn@scau.edu.cn.

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between the networks regulating pollen development in Arabidopsis and rice. In rice, diverse regulatory factors with key roles in tapetal PCD and anther development have been identified, including several transcription factors and protein kinases (6). For example, the altered expression of basic helix-loop-helix (bHLH) transcription factor genes has a strong effect on tapetal PCD. These bHLH factors include Undeveloped Tapetum 1 (UDT1/bHLH164), Tapetum Degeneration Retardation 1 (TDR1/bHLH5), TDR Interacting Protein 2 (TIP2), and Eternal Tapetum 1 (EAT1/TDT1/bHLH141) (10–29). The MYB transcription factor, MYB80/MYB103, is involved in tapetal PCD, thereby influencing anther or pollen development (30, 31). The rice MIKC-type MADS box transcription factor, MADS3, regulates ROS homeostasis during late anther development (15). Loss-of-function of the PHD finger protein gene, PERSISTENT TAPETAL CELL1 (PTC1), results in complete male sterility (11). In addition, two receptor-like kinases, DWARF AND RUNTISH SPIKELETS (DRUS1) and DRUS2, are involved in regulating PCD and reproductive growth (32).

Epigenetic factors, including DNA methylation, histone modification, and noncoding RNAs, are key regulators of plant development (33). Recent studies have suggested that an increase in global DNA methylation plays a role in tapetal PCD (34). Histone H2B monoubiquitination is another important epigenetic modification that modulates the transcriptional regulation of tapetal PCD in rice (35–36). Interestingly, noncoding RNAs are also associated with the regulation of tapetal PCD by functioning as posttranscriptional regulators (37). Although epigenetic modifications are essential for tapetal PCD, the underlying regulatory mechanisms remain largely unknown.

Proteins of the ARGONAUTE (AGO) family are major players in the epigenetic regulation of gene expression. Although many functions of AGO proteins have been reported in mammals, little is known about their functions in plants (38). In Arabidopsis, the AGO family comprises 10 members (39, 40). Well-studied AGOs—including AGO3, AGO4, AGO6, and AGO9—are responsible for regulating DNA methylation (40–44). Rice contains 19 predicted AGO proteins (45). Among these, AGO18 is required for plant resistance to evolutionarily diverse viruses (46). MEIOSIS ARRESTED AT LEPTOTENES1 (MEL1) epigenetically regulates the meiotic progression of meiocytes and is essential for meiosis (47). In addition, OsAGO4a, OsAGO4b, and OsAGO9 are involved in DNA methylation of target miRNAs, a process mediated by long miRNAs (48). Finally, the AGO1 homologs AGO1a, AGO1b, AGO1c, and AGO1d function in the miRNA pathway (49). Although AGO proteins might play roles in RNA-mediating gene silencing in rice, how they participate in PCD in anthers to affect male gametophyte development through DNA methylation is largely unknown.

Here, we performed a detailed functional characterization of OsAGO2 in rice. OsAGO2 was highly expressed in anthers before stage 12 during microspore development. Knockdown of OsAGO2 led to the overaccumulation of ROS, leading to the early initiation of PCD, abnormal anther development, and reduced pollen fertility. Interestingly, we found that the knockdown of OsAGO2 led to the up-regulation of OsHXK1 expression and that OsAGO2 directly regulates the expression of OsHXK1 via DNA methylation. Overexpression of OsHXK1 led to the early initiation of tapetal degeneration and reduced pollen fertility, which is similar to the phenotypes of plants with knocked-down OsAGO2 expression. These findings suggest that OsHXK1 positively regulates the appropriate production of ROS and proper timing of tapetal PCD through the epigenetic regulation of OsAGO2 expression.

**Results**

**Spatiotemporal Expression Pattern of OsAGO2.** OsAGO2 is a member of the AGO family, comprising 19 members in rice. OsAGO2 is located on chromosome 4 and contains three exons and two introns. The full-length 3,473-bp cDNA clone of OsAGO2 contains a 3,105-bp ORF encoding a 1,034 amino acid protein (NP_001053871) of 111 kDa. AGO family members can be divided into four groups, AGO1, AGO4, MEL1/AGO5, and ZIPPY/AGO7, according to their conserved domains (39, 43). OsAGO2 belongs to the ZIPPY/AGO7 clade and contains the PAZ and PIWI domains typical of AGO proteins. To explore the evolutionary relationships of AGO proteins in various species, we performed multiple sequence alignment of the amino acid sequences of the PAZ and PIWI domains of AGO2 in various species (SI Appendix, Fig. S1A) using Evolview (www.evolgenius.info/evolview). Based on this phylogenetic analysis, AGO2 protein sequences are relatively conserved among plants.

To characterize the expression of OsAGO2, we measured its transcript levels in various tissues by qRT-PCR and β-glucuronidase (GUS) activity analysis. The cytological descriptions of different stages of rice anther development used in this study are based on those described by Zhang et al. (9). Strong expression of OsAGO2 was detected in anthers before stage 12 during microspore development (SI Appendix, Fig. S24). Analysis of GUS activity directed by the OsAGO2 promoter revealed strong GUS staining in anthers from stages 4–11 (SI Appendix, Fig. S2B), confirming the expression patterns obtained by qRT-PCR. To further explore the spatial and temporal expression patterns of OsAGO2 during anther development, we performed RNA in situ hybridization using WT plants. Strong in situ signals were observed in tapetal cells from stages 6–9 (SI Appendix, Fig. S2C). Signals were also detected in the epidermis (from stages 5–6), endothecium, meiocytes, and microspores from stages 5–9. As a negative control, hybridization with the OsAGO2 sense probe did not produce any detectable signal. These results suggest that OsAGO2 might be involved in anther development in rice.

To investigate the subcellular localization of OsAGO2, we fused the yellow fluorescent protein (YFP) sequence to the C terminus of OsAGO2 and expressed the fusion protein (OsAGO2-YFP) under the control of the CaMV35S promoter. We transformed this construct into shoot protoplasts of Zhonghua11 (O. sativa L. ssp. japonica, cv. Zhonghua11, ZH11) and detected the localization of the YFP fusion protein in cytoplasm and nuclei based on the colocalization of YFP fluorescence and DAPI staining (SI Appendix, Fig. S1B). The presence of OsAGO2-YFP in the cytoplasm and nuclei was also verified by immunoblotting (SI Appendix, Fig. S1C). These results demonstrate that OsAGO2 is highly expressed in tapetal cells and its encoded protein is mainly localized to the cytoplasm and nuclei.

**Phenotypic Analysis of OsAGO2 Knockdown Plants.** To explore the function of OsAGO2 in tapetal cells, we constructed an OsAGO2 antisense vector containing the gene-specific OsAGO2 fragment driven by the native promoter and a CRISPR/Cas9 vector targeting the OsAGO2 gene. We then transformed Zh11 calli with these two constructs (50, 51). Nine independent transgenic lines were obtained, including four antisense and five CRISPR/Cas9 lines (50, 51). Three independent transgenic lines (OsAGO2-GFP) were obtained by microinjection using embryonated chicken eggs (SI Appendix, Figs. S2A and S3). For each knockdown line, the expression levels of OsAGO2 were verified by qRT-PCR and western blotting (SI Appendix, Figs. S1A and S2B). Two transgenic lines (OsAGO2-GFP) were selected for further study, and were named ago2-1, ago2-2, Casago2-1, and Casago2-2 (Fig. 1D). There are two rice proteins (OsAGO2, OsAGO3) in the ZIPPY/AGO7 subfamily and they share high sequence similarity with each other. Because the PAZ and PIWI domains of OsAGO2 share high sequence similarity with those of OsAGO3 (SI Appendix, Fig. S1A), we also examined the expression levels of OsAGO3 in ago2-1, ago2-2, Casago2-1, and Casago2-2 plants by qRT-PCR. OsAGO3 expression was not altered in the antisense or CRISPR/Cas9 mutants (SI Appendix, Fig. S4A). Therefore, our antisense and CRISPR/Cas9 systems caused considerable suppression of OsAGO2 without having obvious effects on its highly similar homolog, OsAGO3.

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These four antisense and CRISPR/Cas9 lines exhibited delayed growth (SI Appendix, Fig. S4C) and reduced fertility compared with WT, with shrunken, shortened stamens containing distorted pollen grains (Fig. 1A). The seed-settling rates of ago-2-1, ago-2-2, Casago2-1, and Casago2-2 were 5.6%, 23.6%, 8.2%, and 12.8%, respectively, which were greatly reduced compared with the rate of 95% observed in WT plants (Fig. 1E and SI Appendix, Fig. S4D). We also examined pollen viability by iodine/potassium iodide (I₂/KI) (Fig. 1B) and fluorescein diacetate (FDA) staining (Fig. 1C). The pollen grains of ago-2-1, ago-2-2, Casago2-1, and Casago2-2 appeared lighter than WT when stained with I₂/KI (Fig. 1B), and very few were viable, in contrast to the almost full viability of WT pollen grains (Fig. 1C). Taken together, these results indicate that the knockdown of OsAGO2 results in abnormal anther appearance, compromised pollen viability, and reduced seed development in rice.

**Loss-of-Function of OsAGO2 Causes Defective Anther Development.** To further examine the defects in anther development of OsAGO2-knockdown plants, we examined semithin sections of anthers by light microscopy. At stage 5 of anther development, no obvious differences were observed between OsAGO2-knockdown and WT plants (SI Appendix, Fig. S5). Compared with normal WT anther development (Fig. 2A–G), in OsAGO2-knockdown plants, cytoplasm loosening and vacuolation were observed in microsporocytes and tapetum from stages 6–8 (Fig. 2A1–A4, B1–B4, and C1–C4). At stage 9, the tapetum swelled severely, and irregular microspores were observed (Fig. 2D1–D4). At stage 10, as the swollen, inward-growing tapetum enlarged, the microspores were squeezed into an irregular shape (Fig. 2E1–E4). At stage 11, the anthers of knockdown plants showed diffuse vacuolation, and the microspores were distorted (Fig. 2F1–F4). Until stage 12, the number of round microspores decreased, and some microspores developed abnormally, perhaps due to the reduced accumulation of starch and other materials (Fig. 2G1–G4). These results indicate that ago-2-1, ago-2-2, Casago2-1, and Casago2-2 anthers have defective microspores and tapetal cells, thus affecting pollen development.

**Down-Regulation of OsAGO2 Leads to ROS Overaccumulation and the Early Initiation of Tapetal PCD.** PCD is an important feature of anther development in rice. In OsAGO2-knockdown anthers, abnormal degeneration of tapetal cell was observed (Figs. 2 and
3), likely due to PCD. To determine whether the altered OsAGO2 expression in knockdown plants affects tapetal PCD, we examined DNA fragmentation in the plants using a TUNEL assay. In WT tapetal cells, TUNEL+ signals were not detected at stages 6 or 7 (Fig. 4 A and B), began to appear at stage 8, were strongest at stage 9 (Fig. 4 C and D), and gradually weakened from stages 10–12 (Fig. 4 E–G). In contrast, in ago2-1, ago2-2, Casago2-1, and Casago2-2 tapetal cells, TUNEL+ signals were observed as early as stage 6 (Fig. 4 A1–A4), suggesting that tapetal PCD begins prematurely in OsAGO2-knockdown plants. The signals became increasingly stronger from stages 7–9 and peaked at stage 8 (Fig. 4 B1–B4, C1–C4, and D1–D4). Signals were still present at stages 10 and 11 and became weaker at stage 12 (Fig. 4 E1–E4, F1–F4, and G1–G4). TUNEL+ signals were also detected in both the endothecium layer and microspores of these lines from stages 6–9 (Fig. 4 A1–A4, B1–B4, C1–C4, and D1–D4). These results indicate that PCD is initiated earlier in OsAGO2-knockdown anthers compared with WT.

ROS can induce PCD in plants (52, 53). We therefore measured ROS production in plants during anther development based on superoxide anion and hydrogen peroxide (H2O2) levels by nitro blue tetrazolium (NBT) and 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) staining. Consistent with the results of the TUNEL assay, ROS levels in WT anthers were high at stages 8 and 9 and decreased at stage 10 (Fig. 5A). However, in ago2-1 and Casago2-1 anthers, high ROS levels were detected from stages 6–12 (Fig. 5B and C). Histological analysis of anther sections revealed that superoxide anion was mainly localized to the tapetum and microspores (Fig. 5E). Measurement of H2O2 levels also revealed higher ROS level in ago2-1 and Casago2-1 vs. WT anthers from stages 8–12 (Fig. 5F). These results imply that downregulating or altering the expression of OsAGO2 leads to the overaccumulation of ROS in anthers.

Characterization of OsHXK1 as a Candidate Target Gene of OsAGO2.

To identify target genes of OsAGO2, we performed microarray analysis of WT and ago2-1 spikelets at stage 5. Because AGO proteins negatively regulate gene expression at the transcriptional or posttranscriptional levels (54), we focused on up-regulated genes in ago2-1 plants. Fifty genes were found to be up-regulated (>twofold and \( q < 0.05 \) with a false discovery rate of 0.05%) in two replicate screenings. Among these 50 genes, OsHXK1 (Os07g0446800) was up-regulated 2.96-fold in ago2-1 anthers. Hexokinase (HXK) is an essential enzyme that phosphorylates glucose and fructose. In Arabidopsis, HXK1 is thought to contribute to the generation of ROS (55, 56). We reasoned that OsHXK1 might be a target of OsAGO2.
To analyze the expression patterns of OsHXK1, we performed qRT-PCR using WT, ago2-1, and Casago2-1 anthers. OsHXK1 was expressed at extremely low levels during anther development in WT plants. In contrast, OsHXK1 expression was significantly higher in ago2-1 and Casago2-1 plants, especially at stages 5–12 (SI Appendix, Fig. S7A). We also performed RNA in situ hybridization of WT, ago2-1, and Casago2-1 anther sections. Consistent with the qRT-PCR results, in situ hybridization revealed that OsHXK1 was expressed at significantly higher levels in ago2-1 and Casago2-1 tapetal cells than in WT at stages 8 and 10 (SI Appendix, Fig. S7B). OsHXK1 was mainly localized to the cytoplasm (SI Appendix, Fig. S7C). These results suggest that OsHXK1, which is up-regulated in ago2-1 and Casago2-1 anthers, is a target gene of OsAGO2.

**Overexpression of OsHXK1 Causes Defective Anther Development and Early Initiation of PCD.** To determine whether increasing the expression of OsHXK1 influences tapetum and pollen development, we constructed an OsHXK1 overexpression vector and transferred it into ZH11 rice plants. Two independent transgenic lines were selected, which we named OEHXK1-1 and OEHXK1-2 (SI Appendix, Fig. S7D and Table S1). We examined the pollen viability in the plants by I2/KI staining (SI Appendix, Fig. S7E). As shown in SI Appendix, Fig. S7E, ~75% and 74% of the pollen grains from OEHXK1-1 and OEHXK1-2 plants were stained lighter than those of the WT, respectively, indicating that pollen viability was reduced in OEHXK1-1 and OEHXK1-2 plants. The seed-setting rates of OEHXK1-1 and OEHXK1-2 plants were 14.1% and 27%, respectively, which were greatly reduced compared with the rate of 94.7% in WT plants (SI Appendix, Fig. S7F). These results strongly indicate that increasing OsHXK1 expression affects pollen fertility.

HXK1 possesses hexokinase catalytic activity in Arabidopsis, in which Gly\(^{109}\) and Ser\(^{182}\) are the key catalytic sites (55). To determine the relationship between OsHXK1 hexokinase catalytic activity and the altered phenotypes of plants, we generated three catalytically inactive overexpression lines (OEHXK1D1, OEHXK1D3, and OEHXK1D8). Based on the key catalytic sites in Arabidopsis, OsHXK1 was mutated at two active sites, Gly\(^{109}\) → Asp\(^{109}\) (G109D) and Ser\(^{182}\) → Ala\(^{182}\) (S182A) in these overexpression plants. These overexpression lines all displayed increased OsHXK1 expression and normal fertility, like WT plants (SI Appendix, Fig. S8). These results suggest that the catalytic activity of OsHXK1 is necessary for the altered phenotypes in the overexpression lines.

To examine the characteristics of pollen abortion in OEHXK1-1 and OEHXK1-2 anthers, we examined transverse semithin anther sections by light microscopy (Fig. 5A–C). OEHXK1-1 and OEHXK1-2 anthers were considerably different from WT anthers. The following abnormalities were observed: cytoplasm vacuolization, expanded tapetum, and irregular tetrads appeared (from stages 6–8); the tapetum swelled severely and began to form hill-like shapes on the inner surface (stage 9); and microspores were squeezed into irregular shapes and became distorted (stages 10–12). To determine the effect of overexpression of OsHXK1 on tapetal PCD, we examined DNA fragmentation using the TUNEL assay (Fig. 5D–F). Enhanced positive signals were observed in OEHXK1-1 and OEHXK1-2 tapetum from stages 6–12, when obvious signals were also present in microspores and other anther wall tissues (Fig. 6 E and F). These results suggest that overexpressing OsHXK1 leads to early initiation of tapetal degeneration and subsequent pollen abortion. A ROS production assay also revealed that ROS levels increased in OEHXK1-1 anthers from stages 6–12 (Fig. 5D). Similarly, ROS levels were higher in OEHXK1-1 anthers than in WT anthers (Fig. 5 E and F). Microscopy of transverse semithin anther sections, TUNEL assays, and NBT and H2DCF-DA assays showed that the characteristics of anther development in OEHXK1-1 plants were similar to those in OsAGO2-knockdown plants. These results imply that overexpressing OsHXK1 leads to the overaccumulation of ROS, thereby damaging the anthers in the OEHXK1 overexpression lines. Therefore, it appears that OsHXK1 positively controls proper ROS production, the timing of tapetal PCD, and microspore development, with the phenotypes of ago2-1, ago2-2, Casago2-1, and Casago2-2 plants similar to those of OEHXK1 overexpression plants.

Changes in the Expression of Genes Involved in Tapetal PCD and ROS Production in ago2-1 Anthers. Based on the early initiation of PCD in ago2-1, ago2-2, Casago2-1, and Casago2-2 anthers, we reasoned that some genes critical for anther development might be affected in these plants. Therefore, we used qRT-PCR to examine the expression of nine genes involved in anther development, including OsUDT1 (25), OsZIP2 (26, 27), OsTDR1 (10), OsEAT1/DTD (28, 29), OsMYB80/OsMYB103 (20–22), OsTGA10 (57, 58), OsPTC1 (11), OsMADS3 (15), and OsDTC1 (59) (SI Appendix, Fig. S9).
OsAGO2 Directly Regulates OsHXK1 Expression via DNA Methylation.

Because the phenotypes of OsHXK1-1 plants were similar to those of ago2-1 and Casago2-1 plants, we reasoned that OsHXK1 is directly regulated by OsAGO2. To investigate this hypothesis, we knocked down the expression of OsHXK1 using CRISPR/Cas9 and RNA interference (RNAi) in plants in the ago2-1 background. For each knockdown method, two lines were selected for further analysis (A2CHI-1 and A2CHI-2 for CRISPR/Cas9; A2Hi-1 and A2Hi-2 for RNAi) (SI Appendix, Table S1). The progeny plants showed normal growth and development, with decreased OsHXK1 and OsAGO2 expression compared with WT (SI Appendix, Fig. S11 A and B). Bisulfite sequencing revealed a similar reduction in methylation levels to those in ago2-1 plants (SI Appendix, Fig. S11C). I2/KI staining showed that pollen fertility was restored in the progeny plants (SI Appendix, Fig. S11 D and E). These results suggest that OsAGO2 and OsHXK1 interact and that OsHXK1 is regulated by OsAGO2.

Because OsHXK1 is not targeted by any known rice miRNAs, it appears that OsAGO2 does not regulate OsHXK1 at the posttranscriptional level. Through genomic sequence analysis, we found that OsHXK1 contains a GC-rich promoter. Therefore, we hypothesized that epigenetic regulation, particularly DNA methylation, may be responsible for the relationship between OsAGO2 and OsHXK1. To explore the interaction of OsAGO2 with OsHXK1, we analyzed the methylation level in the promoter region of OsHXK1 in ago2-1 and Casago2-1 plants. In plants, cytosine methylation primarily occurs in three sequence contexts: CG, CHG, and CHH (H = A, T, or C). To analyze the methylation status of OsHXK1 in ago2-1 and Casago2-1 DNA, we performed a bisulfite conversion reaction using a sequence starting 2,000-bp upstream of the transcriptional start site of OsHXK1. In the OsHXK1 promoter region, the levels of CG, CHG, and CHH methylation in spikelets were lower in ago2-1 and Casago2-1 plants than in WT plants (Fig. 7 A and B). We performed quantitative pyrosequencing to further assess the methylation pattern of the OsHXK1 promoter in cytosine sites from −856 to −382 bp. Eight CG positions (from position 1 to position 8) in ago2-1 and Casago2-1 plants displayed lower CG levels from stages 5–12 in ago2-1 and Casago2-1 anthers compared with WT anthers (SI Appendix, Fig. S9 A–F). These results suggest that OsAGO2 might mediate a pathway in the regulatory network for anther development.

OsHXK1 function in a similar regulatory pathway during anther development.

Appendix, Fig. S9). These genes encode bHLH, MYB, PHD-finger, and MADS box transcription factors. Six of these genes (OsUDT1, OsTIP2, OsTDR1, OsEAT1/DDT, OsMYB80/OsMYB103, and OsTGA10) were significantly down-regulated in ago2-1 and Casago2-1 anthers compared with WT anthers (SI Appendix, Fig. S9 A–F). These results suggest that OsAGO2 might mediate a pathway in the regulatory network for anther development.

Optimal ROS levels are required for tapetal PCD. Plant NADPH oxidase genes known as respiratory burst oxidase homolog (RBOH) genes play a role in ROS generation. The proper timing of tapetal PCD is ensured by a transcriptional network, including proper ROS patterns produced by RBOHs (53). The rice genome contains nine RBOH family members, including OsRBOHA/OsNOX, OsRBOHB, OsRBOHC, OsRBOHD, OsRBOHF, OsRBOHG, OsRBOHH, and OsRBOHJ. To determine the reason for the increase in ROS levels in the mutants, we carried out qRT-PCR analysis to examine the expression levels of the OsRBOH genes. As shown in SI Appendix, Fig. S10 A, B, D, and E, the OsRBOH genes were expressed at higher levels from stages 5–12 in ago2-1 and Casago2-1 anthers compared with WT. These results suggest that the changes in the expression patterns of OsRBOH genes lead to increased ROS accumulation in ago2-1 and Casago2-1 anthers, which might serve as signaling molecules in tapetal PCD.

To investigate the relationship between OsAGO2 and OsHXK1, we analyzed the expression levels of the nine genes involved in anther development mentioned above in OsHXK1-1 plants. All nine genes were also down-regulated in OsHXK1-1 plants vs. WT (SI Appendix, Fig. S9). Furthermore, the expression levels of the OsRBOHs in OsHXK1-1 plants were also higher from stages 5–10 compared with WT anthers (SI Appendix, Fig. S10). Therefore, several anther development–related and OsRBOH family genes exhibited changes in expression in ago2-1 and Casago2-1 anthers similar to those in OsHXK1-1 anthers. These results suggest that OsAGO2 and OsHXK1 function in a similar regulatory pathway during anther development.
methylation levels than those of WT plants (SI Appendix, Fig. S11F). These results suggest that OsAGO2 regulates the methylation status of the OsHXK1 promoter.

To validate the binding of OsAGO2 to the OsHXK1 promoter, we performed chromatin immunoprecipitation (ChiP) assays and analyzed the resulting purified ChiP DNA samples by qRT-PCR of specific regions at the OsHXK1 promoter. Four DNA sequences (S2, S5, S6, and S7) were enriched by OsAGO2 when using an anti-FLAG antibody. However, sequences P1 to P3 lacking OsAGO2-binding sequences did not associate with OsAGO2 (Fig. 7 C and D). These results suggest that OsAGO2 binds to the OsHXK1 promoter and directly regulates its expression via DNA methylation, thereby ensuring proper PCD and pollen development.

We propose a model to describe the function of OsAGO2 in rice pollen development, as shown in Fig. 7E. According to this model, OsHXK1 is an important regulator of ROS production and PCD in tapetal cells. OsAGO2 controls OsHXK1 expression through methylation of its promoter, thereby ensuring the proper timing of tapetal PCD and normal pollen fertility. Our results reveal a regulatory pathway for pollen development in rice.

**Discussion**

**OsAGO2 Is a Critical Regulator of Tapetum Development.** Male reproductive development is a key step in determining grain yield and production in rice. The male gametophyte developmental process is broadly conserved across angiosperms. Anther development is a highly precise, complex process involving diverse gene regulatory pathways. It is important to uncover the gene regulatory networks that function during tapetum differentiation and PCD. Several genes have been shown to be associated with tapetal function and pollen development in rice (5, 7, 9), including OsUDT1/bHLH164, OsTDRI/bHLH5, OsTIP2/bHLH142, OsEAT1/DTD1/bHLH141, OsDTC1, OsMYB80/OsMYB103, OsPTC1, and OsMADS3 (10, 11, 15, 25–28, 59). Among these, OsTDRI controls tapetum development and degeneration (10). OsUDT1 plays a crucial role in the differentiation of secondary parietal cells to mature tapetal cells (25). OsTIP2 regulates tapetal PCD and pollen wall development (26, 27). OsDTC1 also acts as a key regulator of tapetal PCD through the inhibition of a ROS scavenger (59). OsMYB80/OsMYB103 is involved in ROS accumulation in the entire tapetum, thereby influencing the late stage of anther development (20, 22). OsPTC1 regulates tapetal PCD and pollen development (11). The absence of these genes can lead to abnormal tapetal PCD and male sterility.

In the present study, we found that OsAGO2 is highly expressed in rice tapetal cells from stages 5–11. Down-regulation of Osago2 led to excessive ROS accumulation, which triggered PCD of tapetal cells. The PCD patterns were also found to be altered in these lines, with early initiation of PCD at stage 6 accompanied by DNA fragmentation in other cell layers.

The altered ROS accumulation and PCD patterns in ago2-1, ago2-2, Casago2-1, and Casago2-2 anthers suggested that some genes critical for anther development might be affected in these lines. We therefore analyzed the transcript levels of six anther development-related genes, including OsUDT1, OsTIP2, OsTDRI, OsEAT1, OsMYB80/OsMYB103, and OsTGA10. Most of these genes are factors in the anther development regulatory networks in rice. The transcript levels of these six genes were markedly lower in ago2-1 and Casago2-2 anthers than in WT. In
WT plants, the expression of these tapetum-specific genes terminated at stage 12. However, their expression was completely suppressed in ago2-1 and Casago2-1 anthers. In previous studies, the mutations of these genes led to delayed tapetal PCD (10, 20, 22, 25–29, 57, 58). However, we demonstrated that knocked down OsAGO2 expression led to the early initiation of tapetal PCD, suggesting that the process of OsAGO2-mediated tapetal PCD might be different from that of the genes mentioned above.

Notably, although tapetal degradation began earlier in the knockdown plants, the degradation process was similar to that of WT plants and continued until stage 12. A similar phenomenon was also observed in studies of photoperiod-sensitive male sterility in rice (37, 60–62). Perhaps different mechanisms control PCD initiation and tapetal degradation. Normal tapetal degradation is regulated and coordinated by many other genes. Although altered OsAGO2 expression led to the early initiation of PCD, the degradation process was not affected in tapetal cells of the OsAGO2 mutants due to the down-regulation of various genes, such as OsUDT1, OsTIP2, OsTDR1, OsEAT1, OsMYB80/ OsMYB103, and OsTGA10.

OsHXK1 is a Critical Regulator of ROS Production and PCD in the Tapetum. Tapetal cells undergo PCD during the late stages of anther development. The correct timing and appropriate regulation of tapetal PCD are essential for pollen development and plant reproduction. Premature or delayed tapetal PCD and cellular degeneration can lead to male sterility (10, 11). In the present study, PCD was initiated early in OEHXK1-1 and OEHXK1-2 anthers. Enhanced TUNEL+ signals were observed in the tapetum of these lines from stages 6–10. In addition to tapetal cells, strong TUNEL+ signals were also detected in microspores and anther wall layers, causing the abnormal timing and tissue-specificity of PCD. These results suggest that OsHXK1 regulates tapetal PCD. Interestingly, HXK1 is also involved in regulating senescence and PCD in Arabidopsis (55, 56). These findings indicate that the regulatory patterns of tapetal PCD by OsHXK1 are conserved in plants.

During anther development, ROS production plays a role in regulating tapetal PCD, but how ROS regulate tapetal development remains unclear. Plant NADPH oxidase, also known as RBOH, is an important generator of ROS (63–67). In Arabidopsis, RBOH is critical for tapetal PCD (63). Several studies have shown that ROS generation during tapetum degeneration is associated with its DNA methylation state, we performed bisulfite sequencing analysis. The ChIP assay showed that OsAGO2 binds to the promoter of OsHXK1. We propose that OsHXK1 expression is directly regulated epigenetically by DNA methylation-mediated OsAGO2-induced silencing, along with altered ROS production, which is modulated via an OsHXK1-dependent pathway.

ROS Are Required for PCD Initiation and Inhibited Tapetal Degradation. We observed abnormal tapetal degradation in plants with down-regulated OsAGO2 expression and plants with up-regulated OsHXK1 expression. We compared the results of TUNEL analysis and cytological observation and found that the initiation of tapetal PCD was significantly advanced in these lines, while tapetal cell degradation was significantly delayed. In fact, similar phenomena were observed in other studies. In an Arabidopsis MYB80 mutant, TUNEL signals in anthers appeared early (stages 9–13), whereas they were detected from stages 10–12 in WT plants (22). In OsDEX1 rice mutants, TUNEL signals appeared early (stage 7) and continued to stage 8a, with persistent tapetum observed at stage 11. In contrast, in WT plants, TUNEL signals appeared at stage 8b and were detected until stage 9, and tapetal degradation was completed at stage 11 (74). Based on these results, tapetal degradation can be divided into two stages: PCD initiation and execution. Different signals and pathways are involved in the initiation and execution of PCD, causing the execution phase to appear after the initiation phase. The precise timing of these two phases is crucial for pollen development. The appearance of TUNEL signals indicates the initiation of tapetum degradation. However, the earlier initiation process observed in the mutant and transgenic lines examined in the present study does not imply that the execution process is accelerated in these plants. ROS production is a key component of PCD initiation and execution. In Arabidopsis, loss-of-function of RBOH resulted in the delayed appearance of TUNEL signals, while the overexpression of RBOHE resulted in increased ROS accumulation and both the earlier appearance and delayed disappearance of TUNEL signals (63). In WT rice plants, ROS began to accumulate at stage 8, reached a peak at stage 9, and were maintained at low levels following a sharp decline. At the same time, TUNEL signals emerged at stage 8 and disappeared at stage 10, while tapetal cells were completely degraded at stage 12. However, in dtc1 mutant plants, ROS accumulation decreased without any apparent peaks.
There were no TUNEL signals at any stage, and tapetum degradation was delayed (59). Furthermore, there were no obvious differences in ROS accumulation or tapetum degradation between MADS3 mutants and WT plants before stage 9. Tapetum cells were completely degraded at stage 11 in WT plants, while they were still observed at stage 12 in mutant plants (15). In the present study, ROS were maintained at high levels in anthers at different stages of pollen development in plants with down-regulated OsAGO2 expression, as well as plants overexpressing OsHXK1, causing TUNEL signals to appear in advance and delaying tapetum degradation. These results indicate that high ROS levels during early anther development are required for tapetum degradation. The rapid reduction of ROS levels after the initiation of PCD is also essential for normal tapetum degradation.

**Methods**

**Plant Materials and Growth Conditions.** All transgenic and WT rice plants (O. sativa ssp. japonica cv. Zhonghua11, ZH11) used in this study were grown in the paddy field, and named ago2-1, ago2-2, Casago-1, Casago-2, OEHXK1-1, and OEHXK1-2.

**Characterization of Transgenic Plant Phenotypes.** Anthers from different developmental stages were collected and confirmed by examining septum thin sections (10). TEM was performed as described in Li et al. (10). Anthers from different developmental stages, as defined by Zhang et al. (9), were collected. Pollen viability was analyzed by 1% trypan blue staining. FDA staining of pollen was performed according to a previous study (74).

**Vector Construction and Plant Transformation.** To construct the OsAGO2 anti-sense vector, an ~300-bp reverse fragment of the OsAGO2 cDNA sequence driven by its native promoter (~2.3 kb) was inserted into the modified pCAMBIA1300 vector (50). To construct the OsHXK1 overexpression vector, a 1,497-bp fragment of the OsHXK1 cDNA sequence driven by the Ubiquitin promoter was inserted into the p′Lox vector (75). To construct the RNAi vector for OsHXK1, an ~300-bp fragment of the cDNA sequence was inserted into the pYLRNAI vector (76). To generate the inactive OEHXK1 mutants, the 109th glycine (Gly109) and 182nd serine (Ser182) were mutated to asparagine (Asp109) and alanine (Ala182), respectively. The WT cDNA sequence was used as a template for PCR amplification. These oligonucleotides create nucleotide substitutions to induce amino acid changes. The mutagenized cDNAs were cloned into the p′Lox vector as previously described (75).

To construct the ProAGO2::GUS vector, the OsAGO2 native promoter was inserted into the pCAMBIA1300G vector (50). To generate the CRISPR/Cas9 vector, the OsAGO2 and OsHXK1 target sequences were constructed using pYLRNA-OsU6 and pYLgRNA-OsU6, as previously described (77). To generate the 355:YFP-OsAGO2 and 355:YFP-OsHXK1 vectors, full-length OsAGO2 and OsHXK1 cDNAs were amplified and fused with the N terminus of YFP in the pUC18 vector. To construct the CRISPR/Cas9 vector, the full-length OsHXK1 cDNA was amplified and fused with the N terminus of FLAG in the p′Lox vector driven by the Ubiquitin promoter (76).

All constructs were confirmed by sequencing, introduced into Agrobacterium tumefaciens EHA105 cells and transformed into ZH11 by Agrobacterium-mediated transformation (50, 51). All primers used for vector construction are listed in SI Appendix, Table 53.

**qR-PCR Assay and Expression Analysis.** RT-PCR and quantitative PCR were performed as previously described (74). Primers used for qRT-PCR assay are listed in SI Appendix, Table 53.

1. Zheng XH, Peng SB, Wang F, Huang NR (2004) Using heterosis and hybrid rice to increase yield potential in China. Rice: life: Scientific perspectives for the 21st century. Proceedings of the World Rice Research Conference (International Rice Research Institute, Los Baños, Philippines).
2. Airshad MS, et al. (2017) Thermal stress impacts reproductive development and grain yield in rice. Plant Physiol Biochem 115:57–72.
3. De Sterne N, Geelen D (2014) The impact of environmental stress on male reproductive development in plants: Biological processes and molecular mechanisms. Plant Cell Environ 37:1–18.
4. Kang H, et al. (2016) Overexpression of wheat ubiquitin gene, Ta-Ub2, improves grain yield. J Exp Bot 67:1497–1505.
5. Zhang DB, Wilson ZA (2009) Stamen specification and anther development in rice. Chin Sci Bull 54:2342–2353.
6. Wilson ZA, Zhang DB (2009) From Arabidopsis to rice: Pathways in pollen development. J Exp Bot 60:1479–1492.
7. Zheng et al. PNAS Latest Articles | 9 of 10
8. Sanders PM, et al. (1999) Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sex Plant Reprod 11:297–322.
9. Zhang D, Luo X, Zhu L (2011) Cytological analysis and genetic control of rice anther development. J Genet Genomics 38:279–286.
10. Li N, et al. (2006) The rice tapetum degeneration retardation gene is required for tapetum degradation and anther development. Plant Cell 18:2999–3014.
11. Li H, et al. (2011) PERSISTENT TAPETAL CELL1 encodes a PHD-finger protein that is required for tapetal cell death and pollen development in rice. Plant Physiol 156:613–624.
12. Parish RW, Li SF (2010) Death of a tapetum: A programme of developmental altruism. Plant Sci 178:73–89.
