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Research article

Downregulation of *OsAGO17* by artificial microRNA causes pollen abortion resulting in the reduction of grain yield in rice

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ABSTRACT

Background: Pollen development is an important reproductive process that directly affects pollen fertility and grain yield in rice. Argonaute (AGO) proteins, the core effectors of RNA-mediated silencing, play important roles in regulating plant growth and development. However, few AGO proteins in rice were reported to be involved in pollen development. In this study, artificial microRNA technology was used to assess the function of *OsAGO17* in pollen development.

Results: In this study, *OsAGO17*, a rice-specific gene, was specifically expressed in rice pollen grains, with the highest expression in uninucleate microspores. Downregulation of *OsAGO17* by artificial microRNA technology based on the endogenous osa-miRNA319a precursor was successfully achieved. It is found that downregulation of *OsAGO17* could significantly affect pollen fertility and cause pollen abortion, thus suggesting that *OsAGO17* functions in rice pollen development. In addition, the downregulation of *OsAGO17* mainly caused a low seed-setting rate, thereby resulting in the reduction of grain yield, whereas the downregulation of *OsAGO17* did not significantly affect rice vegetative growth and other agricultural traits including number of florets per panicle, number of primary branch per panicle, and 100-grain weight. Furthermore, the result of subcellular localization analysis indicated that the OsAGO17 protein was localized to both the nucleus and the cytoplasm.

Conclusion: These results represent the first report of the biological function for *OsAGO17* in rice and indicate that *OsAGO17* may possibly play crucial regulatory roles in rice pollen development. It helps us to better understand the mechanism of pollen development in rice.

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1. Introduction

Rice is an important staple food crop and a model plant for conducting a developmental study in monocots [1]. The development and fertility of rice pollen, which are important for double fertilization, directly affect grain yield in rice [2, 3]. Pollen development is a complex biological process regulated by the elaborate coordination of many genes and requires the coordinated participation of various cell and tissue types [4, 5, 6, 7]. Moreover, studies have shown that small RNAs including microRNAs, siRNAs, and phasiRNA abound in rice pollen and play a broad regulatory role during pollen development [8, 9, 10, 11]. However, small RNAs are loaded to Argonaute (AGO) family proteins, and these RNAs exert their functions after forming RNA-induced silencing complexes and guiding the RNA or DNA targets through base pairing [12, 13, 14, 15]. Hence, AGO proteins are the key components in small RNA-mediated gene silencing, which is one of the several important mechanisms that regulate plant growth and development [16, 17].

In general, AGO proteins consist of a variable N-terminal domain and conserved C-terminal PAZ, MID, and PIWI domains [18, 19]. The PIWI domain adopts an RNaseH fold and harbors a conserved catalytic triad, Asp-Asp-His/Asp-Asp-Asp, which exhibits endonuclease activity. However, not all AGO proteins catalyze small RNA-directed

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endonucleolytic cleavage of target RNAs [20]. Rice contains 19 AGO proteins, and most of them have been functionally characterized. These proteins include OsAGO1s, MEL1, and OsAGO4s, which can be phylogenetically divided into four subgroups: AGO1, MEL1, AGO7, and AGO4 [20, 21, 22, 23, 24, 25]. However, OsAGO18 and OsAGO17 do not cluster into the four subgroups, and OsAGO17 has been found only in rice. Moreover, our previous study showed that *OsAGO17* is specific for male gametophyte in rice as indicated by comparison microarray analysis [9]. However, the functions of the *OsAGO17* gene remain unknown.

To investigate the functions of the *OsAGO17* gene, we conducted the downregulation of the *OsAGO17* gene by the artificial microRNA (amiRNA) approach, which is considered as an efficient alternative tool for downregulating endogenous genes with high specificity and stable inheritance [26, 27, 28] and has been performed successfully in model plants including *Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa,* and *Chlamydomonas reinhardtii* [29, 30, 31, 32]. Furthermore, we found that the downregulation of *OsAGO17* could affect pollen fertility and cause pollen abortion, thus resulting in the reduction of grain yield in rice. Moreover, we confirmed that the OsAGO17 protein was specifically expressed in rice pollen grains according to the results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) and that this protein was localized to both the nucleus and the cytoplasm according to the results of subcellular localization analysis. This work may aid in understanding the functions of the OsAGO17 protein and be greatly beneficial for improving plant growth and crop grain yield.

2. Materials and methods

2.1. Plant materials and growth conditions

The Japonica rice variety Zhonghua 11 was used as the wild type, and all rice materials were grown in paddy fields during natural growing seasons in Chengdu, China. Pollen at uninucleate microspore (UNM), bicellular pollen (BCP), and tricellular pollen (TCP) stages was isolated and purified from wild rice spikelets according to the methods given by Peng et al. [9]. Pollen precipitation was collected and stored in liquid nitrogen after identification under an inverted fluorescence microscope (Olympus IX71).

2.2. Sequence alignment and phylogenetic analysis

The BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to obtain the homologs of *OsAGO17*. Phylogenetic analysis was conducted using MEGA software version 5.0. For phylogenetic tree construction, 10,000 bootstrap replicates were performed. The secondary structure of the OsAGO17 protein was analyzed in the Conserved Domain Database of NCBI [33].

2.3. RNA preparation and qRT-PCR

Total RNAs were isolated using the TRIzol reagent (Invitrogen), and the cDNA was synthesized with the oligo(dT) primer according to the manufacturer's instruction given in PrimeScript™ RT reagent kit (Takara). qRT-PCR was conducted with SsoFast™ EvaGreen® Supermix on the Bio-Rad CFX96 PCR system. Each sample was analyzed in three biological replicates. The reactions using gene-specific primers were performed as follows: 95°C for 2 min, 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. *OsActin* was used as the internal control gene to normalize all data, and the expression levels of *OsAGO17* were calculated by the relative quantification method (ΔΔCt of the CFX Manger 3.0). All primers are listed in Table S1.

2.4. Artificial microRNA construction and rice transformation

The amiRNA targeted to *OsAGO17* was designed using Web MicroRNA Designer (WMD3) based on the TIGR v5 rice genome annotation. amiRNA was constructed based on the endogenous osa-miRNA319a precursor, which was cloned using pre-miR319a-F and pre-miR319a-R primers. Pre-amiRNA was obtained by overlapping PCR amplified with the primers pre-amiR-*Xba*I-F and pre-amiR-*Sac*I-R and then by cloning them into the PHB vector under the CAMV35S promoter by digestion with the restriction enzymes *Xba*I and *Sac*I. The construct obtained was introduced into the EHA105 *Agrobacterium tumefaciens* strain and then transformed into wild-type ZH11 according to the method described by Hieiet al. [34]. Transgenic rice lines were selected on the basis of hygromycin B resistance and confirmed by PCR with the primers Hygromycin-F and Hygromycin-R. All primers are listed in Table S1.

2.5. Detection of mature amiRNA

Eight transgenic rice lines were used to detect the expression of mature amiRNA. For the detection of mature amiRNA, total RNAs were extracted from the TCP stage of the transgenic rice lines and wild-type ZH11 by using TRIzol Reagent (Invitrogen). The experimental workflows followed the method described by Li et al. [35]. The hybridization probes complementary to amiRNA were synthesized and labeled at 5′ terminals with biotin. The sequence of the designed probe is listed in Table S1.

2.6. Phenotypic analysis of transgenic rice lines

The phenotype of the whole plant and reproductive organs was photographed with a Nikon D80 digital camera. The spikelets of rice were randomly collected at the heading stage. Each phenotypic analysis was performed at least thrice. The anthers before anthesis were dissected in a drop of 1% I2/KI solution to examine the pollen viability. The number of pollen grains was counted under an inverted microscope (Olympus IX71), and the stained pollen grains were counted as mature pollen grains.

2.7. Subcellular localization of the OsAGO17 protein

SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) was conducted for signal peptide prediction of *OsAGO17*. The coding sequence of *OsAGO17* was fused to the C-terminal sequence of green fluorescent protein (GFP) by cloning into the pBI221-GFP vector. Under the control of the CaMV35S promoter, the OsAGO17-GFP fusion construct and the GFP alone (as the control) were introduced into mesophyll protoplasts, which were freshly isolated from the well-expanded leaves of wild-type *Arabidopsis* according to a previously described method [36]. After DAPI staining, the protoplasts were observed under a confocal laser scanning microscope (Leica TCS SP2). Primers used for cloning are listed in Table S1.

3. Results

3.1. Sequence and expression pattern analysis of OsAGO17

Genomic DNA sequence obtained from Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/index.shtml) showed that *OsAGO17*, the locus identifier LOC_Os02g07310 (Chr2:3755492-3748363), consists of 22 exons and 21 introns. Protein sequence analysis revealed that OsAGO17 contained a variable N-terminal domain and conserved C-terminal PAZ and PIWI domains (Fig. 1A), which are the characteristic domains of AGO family proteins. This finding indicated that OsAGO17 is a member of AGO family proteins. On the basis of protein sequence homology, 25 genes from