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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

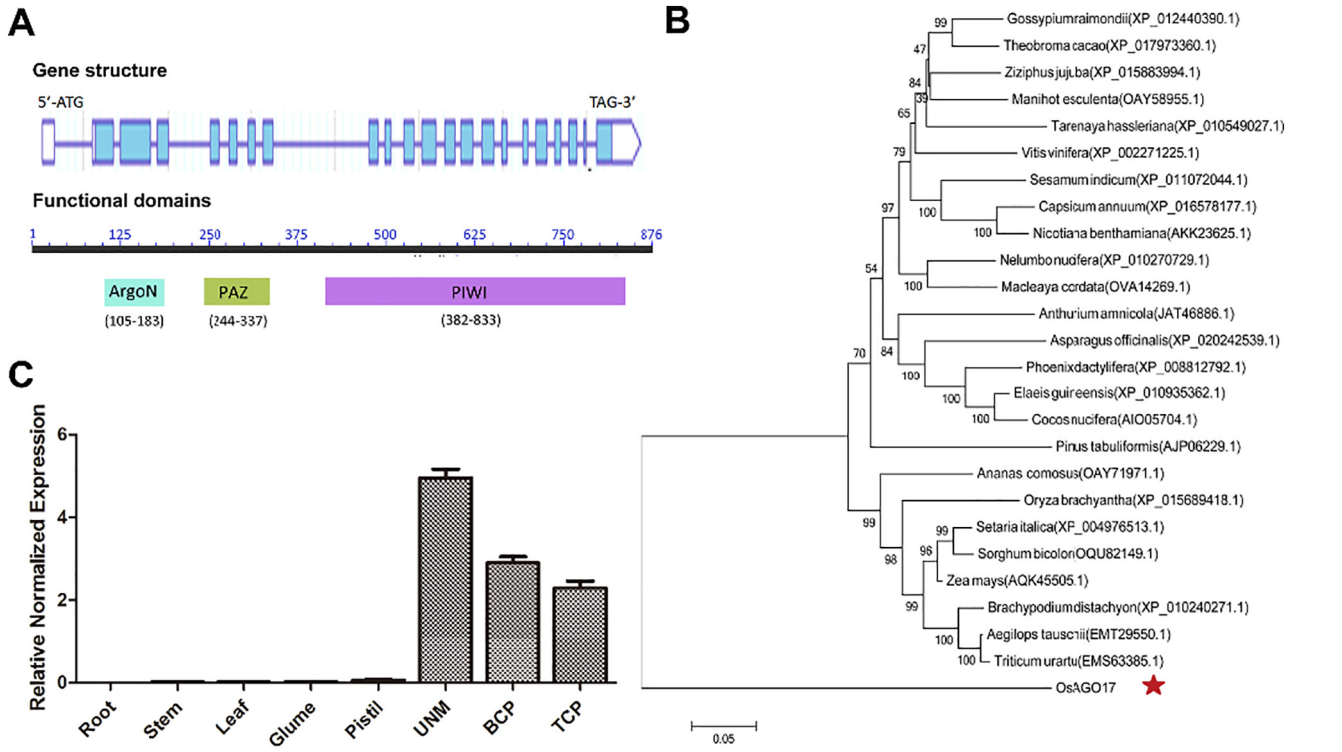

Fig. 1. Sequence and expression pattern analysis of *OsAGO17*. (A) Gene structure and functional domain analysis of *OsAGO17*. Blue boxes represent exons. (B) Phylogenetic analysis of *OsAGO17* and its homologs. *OsAGO17* is denoted by a red asterisk, and the scale bar indicates the branch length. (C) qRT-PCR analysis of the *OsAGO17* gene in different tissues of rice. *OsActin* was used as an internal control. The relative expression is represented as mean ± SE. UNM, uninucleate microspore; BCP, bicellular pollen; TCP, tricellular pollen.

other plant species were found in the NCBI database by conducting Protein BLAST. These genes are listed in Table S2. The phylogenetic tree was constructed using a full-length amino acid sequence of *OsAGO17* and homologous genes from other plant species (Fig. 1B). The results showed that only *OsAGO17* was grouped into one clade, thus indicating that *OsAGO17* was a rice-specific gene, which is consistent with the result given in previous study by Fang et al. [20].

The expression pattern of *OsAGO17* in rice was examined by qRT-PCR to understand the function of this gene in rice. The results revealed that *OsAGO17* was specifically expressed in rice pollen grains, with the highest expression in UNM. This gene was almost not expressed in root, stem, leaf, glume, and pistil (Fig. 1C), which confirmed that *OsAGO17* was specifically expressed in rice pollen grains.

3.2. Rice OsAGO17 downregulated by artificial microRNA

To determine the function of *OsAGO17* in rice, we selected the endogenous osa-miRNA319a precursor as the backbone for the construction and expression of amiRNAs to target the *OsAGO17* transcript. Using oligonucleotide-directed mutagenesis, we replaced the sequence of osa-miR319a with the designed amiRNA sequence (5'-TGGTGACGATCGTAGAGCCAG-3′), which targeted 21–41 nt of *OsAGO17* mRNA and mimicked the foldback structure of the endogenous osa-MIR159a with its fully complementary sequence as predicted by "RNAfold" (Vienna RNA package, http://www.tbi.univie.ac.at/RNA) for 23°C [37] (Fig. 2A). The strategy of downregulation of *OsAGO17* by the amiRNA approach is shown in Fig. 2B. The recombinant vector pH B-2 × 35S::pre-amiRNA was introduced into wild-type ZH11 by using the *A. tumefaciens*-mediated method. The homozygote transgenic rice lines were selected on the basis of hygromycin B resistance and PCR confirmation (data not shown) for further experimental analysis.

For detecting the expression of mature amiRNA in transgenic rice lines, the total RNA extracted from the rice lines at the TCP stage was used to perform small RNA blot. The results showed that different levels of mature amiRNA could be detected in transgenic rice lines (line 1–line 8) (Fig. 2C), which indicated that mature amiRNA was processed accurately from its precursor. Correspondingly, we detected the expression of *OsAGO17* in transgenic rice lines by qRT-PCR and found that the expression of *OsAGO17* in transgenic rice lines was much lower than that in wild-type ZH11 (Fig. 2D). A strong negative correlation was found between the expression levels of mature amiRNA and *OsAGO17* in transgenic rice lines, thus indicating that the expression of *OsAGO17* in the transgenic rice lines was specifically inhibited by the specific amiRNA. These results showed that we successfully conducted the downregulation of *OsAGO17* by amiRNA technology based on the endogenousosa-miRNA319a precursor.

3.3. Downregulation of OsAGO17 caused pollen abortion

To assess whether *OsAGO17* functions in pollen development, homozygous T3 generation transgenic rice lines (line 1–line 8) and wild-type ZH11 were planted in paddy fields. First, there were no significant differences between wild-type ZH11 and the transgenic rice plants at the vegetative growth stage (data not shown). It indicated that the downregulation of *OsAGO17* do not affect rice vegetative growth. However, when their pollen viability was detected by I2-KI staining, our results showed that mature pollen grains of wild-type ZH11 were round and black after I2-KI staining. Most pollen grains of the transgenic rice lines were shriveled and yellow, which indicated that the pollen fertility in transgenic rice lines was severely damaged as compared with that in the wild-type ZH11 (Fig. 3A).

Statistical results also showed that the percentages of strained pollen grains in transgenic rice lines were significantly lower than those of strained pollen grains in the wild-type ZH11 (Fig. 3B). Therefore, the results showed that the downregulation of *OsAGO17* significantly caused pollen abortion in transgenic rice lines. Moreover, the degree of pollen abortion was negatively correlated with the expression levels of *OsAGO17*. Taken together, our results suggested

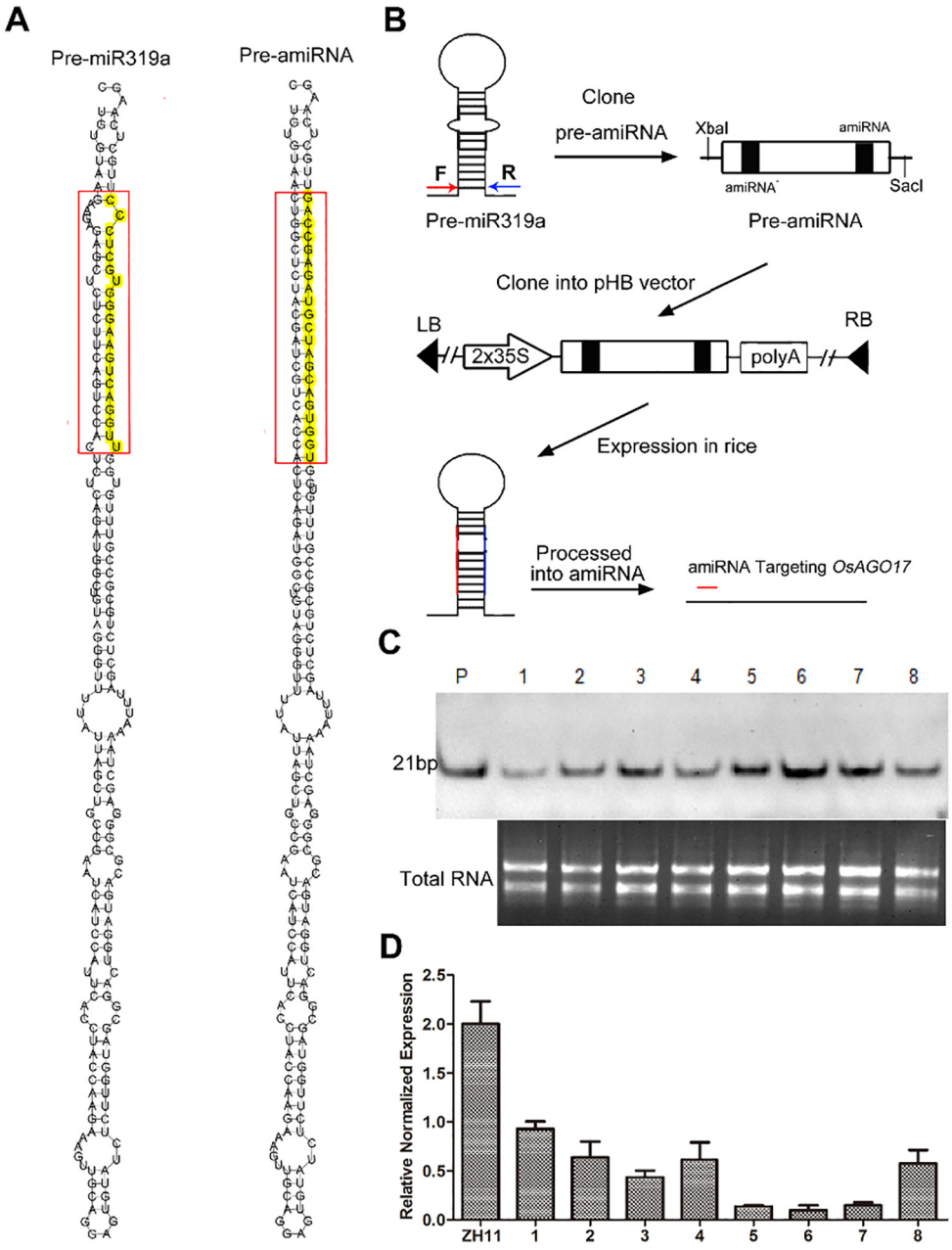

Fig. 2. Construction of amiRNA and analysis of transgenic rice lines. (A) Secondary structures of osa-miRNA319a precursor (pre-miR319a) and modified osa-miR319a precursor (pre-amiRNA) predicted by "RNA fold" (Vienna RNA package, http://www.tbi.univie.ac.at/RNA). The 21-nt sequence replaced in the transgene is boxed. The mature microRNA sequence is shown in yellow. (B) A schematic representation of amiRNA construction. The pre-amiRNA clone was transferred into the PHB vector under the control of the CAMV35S promoter between the *Xba*Iand *Sac*I restriction sites. The construct obtained was then transformed into rice to express pre-amiRNA and processed into mature amiRNA. (C) Detection of amiRNAs by the small RNA blotting method. Total RNA was extracted from eight transgenic rice lines (line 1–line 8). The 21-nt amiRNA fragment was artificially synthesized as the positive control (Note P). (D) Relative expression levels of *OsAGO17* in the eight transgenic rice lines. All expression levels of *OsAGO17* were normalized to the expression of *OsActin*. Each experiment was conducted thrice.

that *OsAGO17* may have functions in rice pollen development, and this may be essential for rice pollen grains.

3.4. Downregulation of OsAGO17 resulted in the reduction of grain yield in rice

Three transgenic rice lines (line 5, line 6, and line 7), which exhibited a low expression level of *OsAGO17* and a high pollen abortion rate, were used for further analysis to investigate the traits shown during reproductive stages. We compared the traits related to grain yield among the three transgenic rice lines and wild-type ZH11 during the ripening phases. All the three transgenic rice lines had a slightly lower plant height than the wild-type ZH11 (Fig. 4A, C), and their harvested panicles were slightly shorter than those of wild-type ZH11 (Fig. 4B, E). However, the significant difference is that the harvested panicles of the three transgenic rice lines had a large number of empty green seeds, thereby exhibiting a lower seed-setting rate than that of wild-type ZH11 (Fig. 4B, D). Further, no significant differences were found for the other agricultural traits including the number of florets per panicle, the number of primary branches per panicle and 100-grain weight among the three transgenic rice lines and wild-type ZH11 (Fig. 4F–H). Hence, the results showed that the downregulation

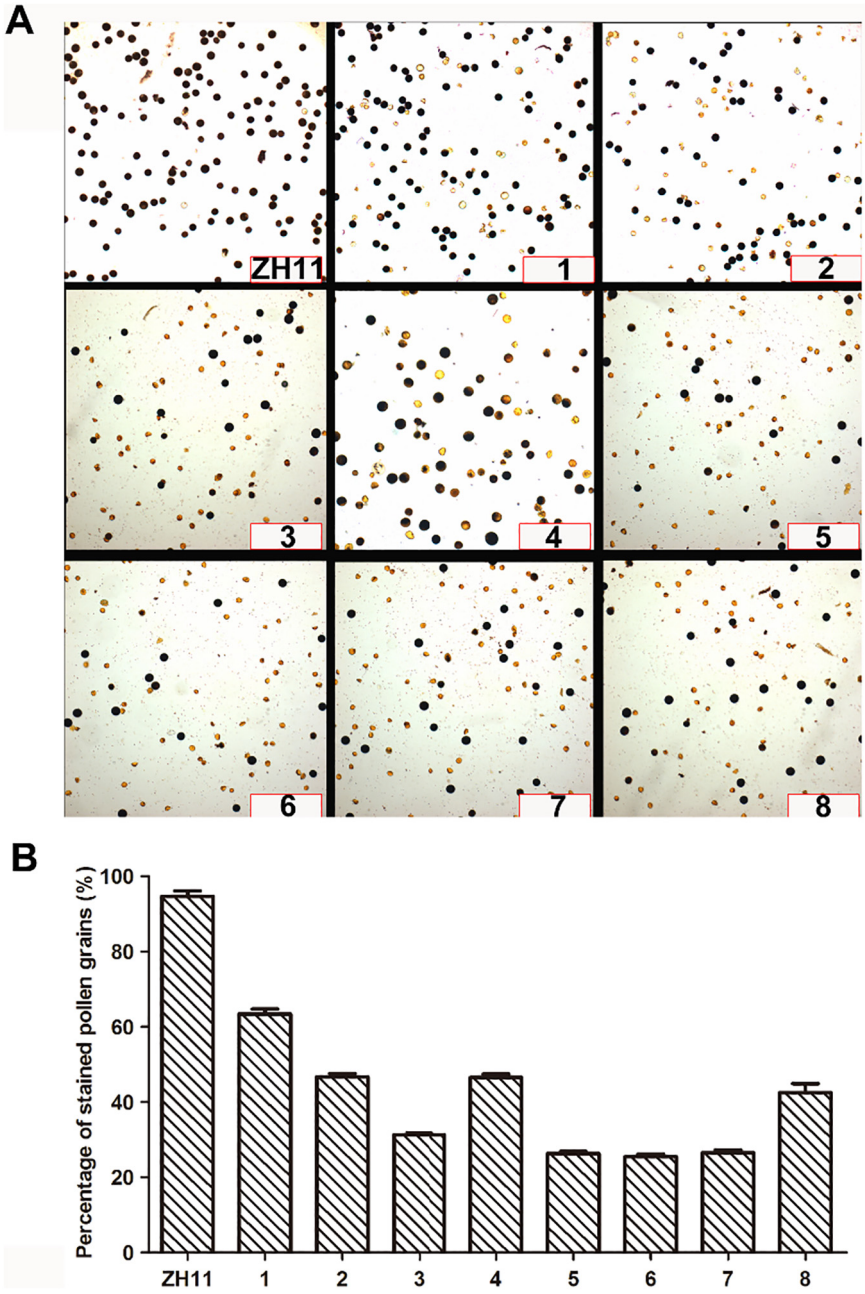

Fig. 3. Detection of pollen viability in transgenic rice lines and wild-type ZH11. (A) Detection of pollen viability by I2-KI staining. The pollen grains of eight transgenic rice lines and wild-type ZH11 at the mature pollen stage were stained with 1% I2-KI and immediately observed under a light microscope. (B) Percentage of stained pollen grains.

of *OsAGO17* mainly caused a low seed-setting rate, which resulted in grain yield reduction in the three transgenic rice lines, and the phenotype was stable for later generations (data not shown).

3.5. OsAGO17 localized in the nucleus and the cytoplasm

The results of signal peptide prediction showed that OsAGO17 has no signal peptide. To further investigate the subcellular localization of OsAGO17, we constructed a fusion protein of the full-length *OsAGO17* coding region and the cDNA-encoding GFP. The OsAGO17-GFP fusion construct gene and the GFP gene alone were both driven under the control of the CaMV35S promoter (Fig. 5A) and were introduced into *Arabidopsis* protoplast cells isolated from *Arabidopsis* mesophyll cells. Three biological replicates were performed in this experiment. The results showed that OsAGO17 was found in both the nucleus and the cytoplasm (Fig. 5B). Thus, we speculate that OsAGO17 may have functions in both the nucleus and the cytoplasm or only in the nucleus and just synthesis in the cytoplasm.

4. Discussion

AGO proteins are the effectors of RNA interference, which is the most important mechanism in regulating gene expression at the transcriptional or post-transcriptional level in eukaryotes. Rice genome contains 19 AGO family members, and among these members, biological functions have been investigated for AGO1s, AGO4s, MEL1, and AGO18 [22, 23, 24, 25, 38, 39]. OsAGO17 is only found in rice and was predicted to be specifically expressed in pollen grains [9, 21]. However, the biological function of *OsAGO17* remains unknown.

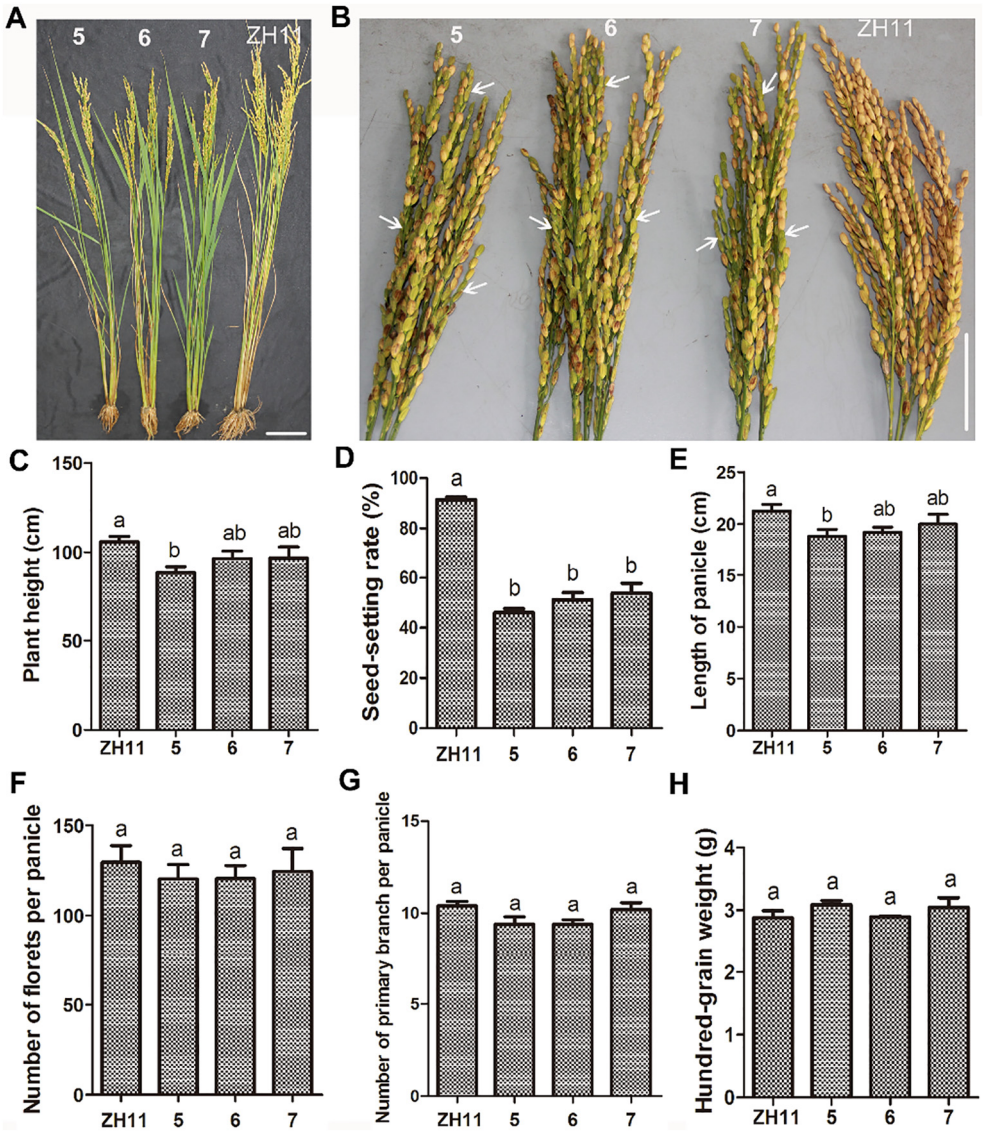

Fig. 4. Agricultural traits of transgenic rice lines and wild-type ZH11. (A) Comparison of transgenic rice lines and wild-type ZH11 grown in paddy fields at the grain-filling stage. Bar = 10 cm. (B) The harvested panicles of transgenic rice lines and wild-type ZH11. White arrows indicate empty seeds, Bar = 5 cm. (C) Plant height, (D) Seed-setting rate, (E) Length of panicle, (F) Number of florets per panicle, (G) Number of primary branches per panicle, and (H) 100-grain weight of three transgenic rice lines and wild-type ZH11. Bars represent mean ± SE based on at least five replicates. Values with the same letter within columns are not significantly different according to Duncan's multiple range tests at $P < 0.05$.

In this study, the bioinformatic analysis revealed that OsAGO17 had the characteristic domains of AGO family proteins and was confirmed to be uniquely found in rice. qRT-PCR confirmed that OsAGO17 is specifically expressed in rice pollen grains, where a number of pollen-specific microRNAs played a broad regulatory role during male gametophyte development [9, 10]. In addition, the result of subcellular localization analysis showed that OsAGO17 was localized in the nucleus and the cytoplasm. Hence, we reasonably hypothesize that OsAGO17 may be associated with microRNAs and functions in the cytoplasm and/or the nucleus during rice pollen development.

To confirm this hypothesis, we successfully used the endogenous osa-miRNA319a precursor to design the specific amiRNA-targeting *OsAGO17* and consequently to downregulate *OsAGO17*. amiRNA technology is a powerful tool to silence endogenous genes based on microRNA precursors. Several endogenous microRNA precursors have been successfully used to express amiRNAs that will target specific genes including MIR159a [40], MIR171a [41], MIR172a, and MIR319a [30]. However, most endogenous microRNA precursors are obtained from *A. thaliana,* and only the endogenous microRNA precursors of rice OsMIR528, OsMIR395, and OsMIR390 were used as the backbone to design amiRNA [32, 42, 43]. As the precursor of *A. thaliana* miRNA319a, a conserved microRNA, was successfully designed for amiRNA expression and the design principles for amiRNAs were generalized by Schwab et al. [30], we confirmed that the osa-miRNA319a precursor can also be used as the backbone for amiRNA expression in rice.

As a result of the specifically expressed amiRNA, *OsAGO17* transcripts in the transgenic rice lines were downregulated. In addition, most of their pollen grains underwent abortion, which resulted in a low seed-setting rate and reduced rice grain yield. This finding indicated that *OsAGO17* had an important function in rice pollen development. A number of genes involved in the regulation of rice pollen development have been identified and characterized during the past decade [3, 44, 45]. Using high-throughput deep sequencing, a complex microRNA-targeting interaction network has been implicated in the regulation of rice pollen development [9, 10]. OsAGO17 functions as an effector protein of microRNA and was bound in rice pollen grains; thus, its downregulation can affect pollen fertility

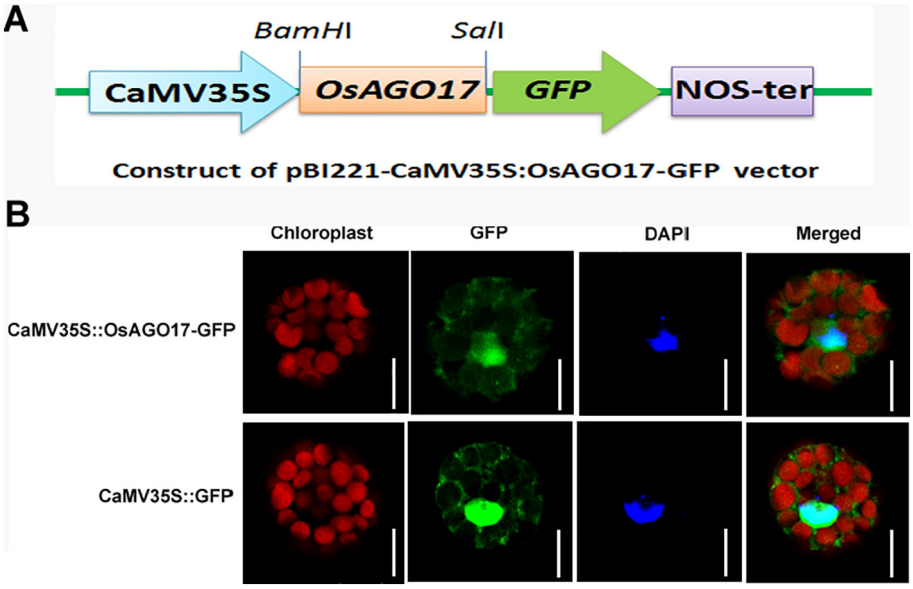

Fig. 5. Subcellular localization analysis of *OsAGO17*. (A) Schematic diagram of the OsAGO17-GFP fusion protein under the control of the CAMV35S promoter. (B) OsAGO17-GFP fusion protein transiently expressed in *Arabidopsis* protoplasts. Chloroplast, chlorophyll autofluorescence; GFP, GFP fluorescence; DAPI, nucleus stained with DAPI; Merged, combined fluorescence from chloroplast, GFP, and DAPI. Bar = 10 μm.

and cause pollen abortion, as shown in this study. However, the mechanism of OsAGO17 functioning in rice pollen grains needs to be further studied.

In summary, we first successfully conducted the downregulation of the rice-specific gene *OsAGO17* by using the amiRNA-mediated approach based on the osa-miRNA319a precursor, which caused pollen abortion and rice grain yield reduction. We also found that OsAGO17 was specifically expressed in rice pollen grains and localized in both the nucleus and the cytoplasm. Taken together, our results indicated that OsAGO17 plays a vital role in rice pollen development. Our findings may help us to understand the functions of *OsAGO17* in rice pollen development and lay the foundation for further studies on *OsAGO17*.

Conflict of interest

The authors have declared no conflict of interest.

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Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.07.001

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