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An invisible switch for rice fertility: How phasiRNAs shape pollen development?

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Abstract

Small RNAs are essential for regulating gene expression, with specialized types playing key roles in reproduction. Shao et al. (2026) discovered that novel 5'-A 21-nt phasiRNA, *OsphasiR1_1*, acts as an *OsAGO2*-dependent switch for rice male fertility by cleaving *OsARF7* transcripts. Loss of this module causes *OsARF7* overexpression, disrupting pollen development. This discovery reveals specialized phasiRNA pathways beyond canonical *AGO5* functions, opening new avenues for climate-resilient hybrid breeding.

Keywords: PhasiRNA; OsAGO2; Male fertility; CRISPR; Rice reproduction; ARF

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Introduction

Plant small RNAs (sRNAs, typically 21-24 nucleotide in length) are critical for gene expression regulation, with distinct classes playing essential roles in reproductive development. In monocots such as maize and rice, a mysterious class of 21-nucleotide-long phased secondary small interfering RNAs (phasiRNAs) exists and is specifically expressed in the anthers [2]. The generation and accumulation of these reproductive phasiRNAs are closely related to male fertility in plants and expressed from hundreds and thousands of genomic loci known as PHAS loci. phasiRNAs originated from RNA Polymerase II derived precursors through a distinct biosynthesis pathway. In brief, phasiRNA biogenesis depends on a complex cascade involving transcription of PHAS loci, miRNA-guided cleavage, and subsequent processing by *Dicer-like* (*DCL*) proteins. The resulting mature phasiRNAs are selectively loaded onto distinct *ARGONAUTE* (*AGO*) proteins according to their length and the identity of their 5' terminal nucleotide to exert their functions [3]. Previous studies have established that disruption of key components of this pathway, such as *AGO1, DCL4,* and *AGO5*, leads to male sterility (Liu et al., 2020; Tamotsu et al., 2023). The existence of thousands of PHAS sites in plants has made it difficult to clarify whether a single phasiRNA possesses a specific biological function, and whether there are other atypical *AGO*- dependent regulatory modules that provided new insight into this unresolved question. Recently, Shao*, et al.* [1] shed the corner of *black box*

by identifying a 21-nt functional phasiRNA (referred as *OsphasiR1_1*) with a 5'-A (5'-adenosine monophosphate) terminal feature and investigated its positive regulatory role in rice male fertility by directing the *OsAGO2* mediated cleavage of *auxin-responsive factor 7 (OsARF7)*. This mechanism provides new insight into the link between phasiRNA biogenesis and function while highlighting their diverse *AGO*-dependent regulatory roles, thereby offering a framework for dissecting the molecular regulation of reproduction in monocotyledonous plants like rice and the creation of male-sterile lines.

Finding that crucial "5'-A" phasiRNA

To investigate the biological function of a specific 21-nt phasiRNA, Shao*, et al*. [1] unexpectedly discovered a 21-nt phasiRNA, named osphasiR1_1, that is functionally conserved across diverse rice varieties [Nipponbare (japonica), Zhongxian 3037 (3037, indica), and Huanghuazhan (HHZ, indica)] and possesses a 5'-A terminal feature, from degradome data of rice germ cells. To investigate the biological role of *osphasiaR1_1*, CRISPR/Cas9 knockout and short tandem target mimicry (STTM)-based knockdown approaches were used to disrupt or reduce expression of the *OsPHAS1* precursor locus. Phenotypic analysis showed that both the knockout mutant and the knockdown transgenic lines exhibited significant decreases in pollen fertility and seed setting rate, without compromising vegetative growth. These results indicate that *OsphasiR1_1* plays a specific positive regulatory role in maintaining male fertility in rice.

OsphasiR1_1 cleaves the target gene *OsARF7*

ARFs regulate plant growth and development with few involved in the reproductive development of rice. miRNAs precisely regulate ARF transcription factors (TF) to control auxin signaling essential for male fertility and anther development. In Arabidopsis, *miR160* targets *ARF17*, whose overexpression disrupts callose synthesis (via CalS5) and causes male sterility by impairing pollen wall formation; *miR160*-resistant *ARF17* similarly leads to sterility. *miR167* targets *ARF6/8*, inducing stamen defects and pollen germination failure when overexpressed [4]. Whether *OsphasiR1_1* can regulate male fertility in monocots by targeting ARFs remains unknown.

Targeting the specific action site, degradome analysis and sequence complementarity analysis revealed that the target gene of *osphasiR1_1* is *OsARF7* (*OsARF7*, *LOC_Os02g35140*). 5' RACE assay precisely located *OsphasiaR1_1* cleavage site, and in loss-of-function mutant of osphasiR1-1, the expression level of *OsARF7* was significantly increased. To demonstrate that the decreased fertility was indeed due to osphasiaR1_1's inability to cleave *OsARF7,* Shao*, et al*. [1] generated *OsARF7* expression (*OsARF7-OE*) and *OsARF7* mutant (mutation in target site of *OsphasiaR1_1*) lines. The results showed that the OsARF7 mutant overexpression line had higher *OsARF7* expression, lower pollen fertility, and lower seed set rate, and 5' RACE confirmed that osphasiR1_1 can only cleave OsARF7-OE lines reduced/abolished in the mutated target site. RNAi knockdown of *OsARF7* in the *Osphas1-1* background significantly restored pollen fertility and seed set rate, while the *Osarf7-1* knockout mutant had normal fertility, indicating that osphasiR1_1 regulates male fertility by cleaving *OsARF7*. These analyses suggest that *Osphas1-1* safeguards male fertility in rice by directly mediating the cleavage of the *OsARF7* transcript.

OsphasiR1_1 relies on *OsAGO2* instead of *OsAGO5c*

Previous studies established that 21-nt reproductive phasiRNAs in monocots typically load into *AGO5* proteins (e.g., *MEL1/OsAGO5* in rice, *ZmAGO5* in maize) with a 5'-C feature to drive function [5, 6]. However, *OsAGO2* also plays a key role in rice anther

development: its deficiency elevates ROS levels and causes male sterility [7], its expression peaks with 24-nt meiotic phasiRNA accumulation [8]. Given that, Shao*, et al*. [1] presumed that 5'-A-containing osphasiR1_1 specifically associate with *AGO2*, this suggests this phasiRNA may function via a novel pathway independent of *AGO5*. CRISPR/Cas9 knockout mutants of *OsAGO2* and *OsAGO5c* were constructed using specifically associated with *AGO2*. Stem-loop RT-qPCR and Northern blot showed that *OsphasiaR1_1* expression was significantly decreased in the *Osago2* mutant, the expression level of its target gene *OsARF7* increased, and pollen fertility was severely impaired. On the other hand, no significant change was observed in the *osago5c* mutant, the level of *OsphasiaR1_1* and the cleavage of *OsARF7* were not affected. This strongly demonstrates that *OsphasiaR1_1* is loaded into the *OsAGO2* protein and relies on the latter to cleave the target gene. Likewise, RNAi knockdown of *OsARF7* in the *Osago2* mutant background significantly restored pollen fertility and seed set rate, further confirming that *OsphasiaR1_1* depends on *OsAGO2* cleavage of *OsARF7* to maintain male fertility.

From Molecular Regulation to Developmental Abnormalities

To investigate the cellular basis of *osphasiR1_1* regulation of male fertility, Shao*, et al*. [1] prepared the paraffin sections of anthers at different developmental stages. Histological sections revealed that in lines overexpressing osphasiR1_1 loss-of-function mutants, germ cells exhibited vacuolization during meiosis at stage S7, shrinkage and disintegration in subsequent S8/S9 stages, followed by anther atrophy and pollen abortion in stage S12. Further DAPI staining revealed abnormal meiosis in mutant pollen mother cells, with chromosome adhesion and incomplete bivalent formation during diakinesis. Chromosomal bridges appeared in anaphase I of meiosis, resulting in abnormal tetrads containing micronuclei and chromosome fragments after division. This indicates that osphasiR1_1 deletion or *OsARF7 target-site mutant lines* severely interferes with meiosis, leading to abnormal male germ cell development.

Premature "Awakening" Disrupts Normal Development

Analysis of the spatiotemporal expression dynamics of *OsphasiaR1_1* and *OsARF7* at developmental stages of anthers revealed that the wild type, osphasiaR1_1 peaked in early meiosis (M2 stage) and then rapidly declined. Moreover, OsARF7 expression was suppressed during M2 stage period and then gradually increased. In mutants lacking *OsphasiaR1_1*, *OsARF7* was abnormally highly expressed in early anthers. This "premature awakening" likely disrupted the subsequent developmental program, ultimately leading to male sterility [1].

OsARF7 is a transcriptional activator that overactivated fertility-related genes

Shao*, et al*. [1] showed *OsARF7* acts as a transcriptional activator of key fertility genes like *Ubl404* (*LOC_Os09g31031*) and *cHSP70-4* (*LOC_Os03g16920*). It directly binds their promoters to drive expression. The *OsphasiaR1_1–OsAGO2* module limits *OsARF7* mRNA via cleavage, preventing overactivation of these genes and ensuring normal fertility.

Conclusion

The study by Shao*, et al*. [1] reveals for the first time a novel regulatory module for male fertility in rice, composed of *OsphasiaR1_1-OsAGO2-OsARF7* (Figure 1). It expands the previous understanding that 21-nt phasiRNA functions solely through *AGO5*, demonstrating that 5'-A phasiRNA can also perform important biological functions by loading *AGO2*, filling a gap in the phasiRNA pathway. Simultaneously, this

research deepens our understanding of how the auxin signaling pathway finely regulates reproductive development. Future in-depth research on this module, and even artificial manipulation, may provide entirely new molecular tools for the creation and improvement of sterile lines in hybrid breeding.

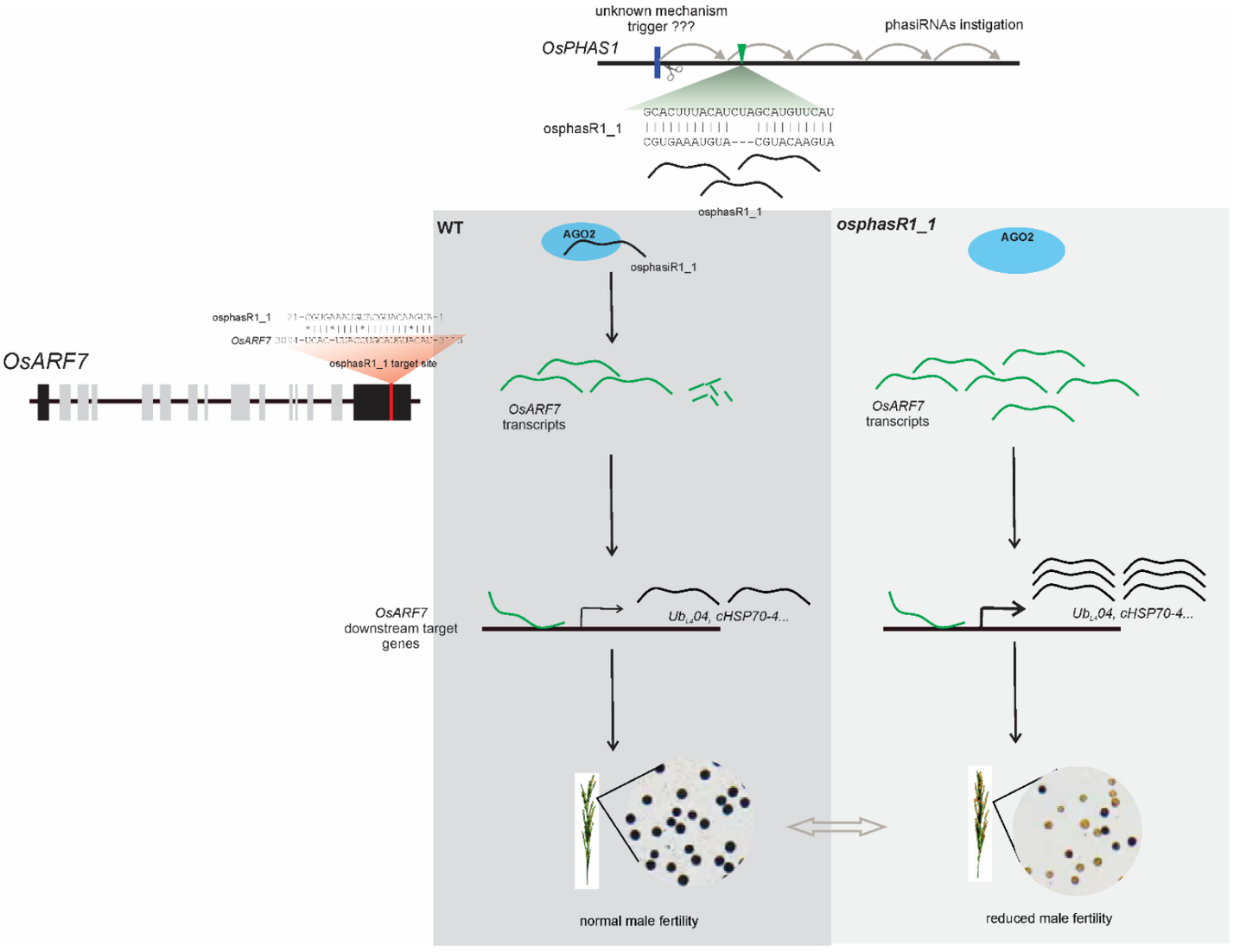

Figure 1. In wild-type rice, OsphasiaR1_1 loads into OsAGO2 to cleave OsARF7 transcripts, keeping its levels in check to prevent overactivation of fertility genes like UbL404 and cHSP70-4. Without OsphasiaR1_1 or functional OsAGO2, OsARF7 accumulates, hyperactivates those targets, and causes male germ cell defects plus poor fertility.

Data availability

Not applicable

Acknowledgments

Not applicable

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contribution

The author(s) confirm contribution to the paper as follows: SM: writing-original draft, conceptualization

supervision, writing, review, and editing. LJK and SJ: review and editing, conceptualization and reviewing. All authors discussed the content, reviewed the results and approved the final version of the manuscript.

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