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Identification of phasiRNAs in wild rice (*Oryza rufipogon*)

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Plant miRNAs can trigger the production of phased, secondary siRNAs from either non-coding or protein-coding genes. In this study, at least 864 and 3,961 loci generating 21-nt and 24-nt phased siRNAs (phasiRNAs), respectively, were identified in three tissues from wild rice. Of these phasiRNA-producing loci, or *PHAS* genes, biogenesis of phasiRNAs in at least 160 of 21-nt and 254 of 24-nt loci could be triggered by interaction with miRNA(s). Developing seeds had more *PHAS* genes than leaves and roots. Genetic constrain on miRNA-triggered *PHAS* genes suggests that phasiRNAs might be one of the driving forces contributed to rice domestication.

Keywords: *Oryza rufipogon,* phased siRNAs (phasiRNA), miRNA, small RNA, rice domestication

Abbreviations: miRNA, microRNA; siRNA, small-interfering RNA; phasiRNA, phased siRNA; tasiRNA, trans-acting siRNA; *TAS*, tasiRNA locus; *PHAS*, phasiRNA locus

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Endogenous small RNAs, which consist of miRNAs and siRNAs, are important regulators of organ development, stress responses and genomic stability in plants.[1,2] Plant miRNAs can trigger the production of phased, secondary siRNAs from either non-coding (such as *TAS*) or protein-coding genes. TasiRNAs are distinctive siRNAs, which are generated in 21-nucleotide (nt) phases in relative to the miRNA cleavage site. They act in trans to regulate gene expression at the post transcriptional level. Biogenesis of tasiRNAs is triggered by interaction of miRNA(s) with the precursor *TAS* transcripts at single or dual ends.[3-6] There are at least eight *TAS* loci that belong to four *TAS* families (*TAS1*–*TAS4*) in *Arabidopsis* and four *TAS3* loci have also been identified in rice.[7-9] Recent studies demonstrated that plant genomes are rich in phased siRNA (phasiRNA)-producing loci, or *PHAS* genes, and may harbor hundreds of these loci in protein-coding genes. For example, a bunch of 21-nt and 24-nt phasiRNAs triggered by miR2118 and miR2275, respectively, were identified in the developing inflorescence of rice.[10] miR482/2118-mediated cleavage of disease resistance *NBS-LRR* genes not only plays an important role in regulation of non-race specific disease resistance but triggers production of phasiRNAs that are able to regulate the expression of their targets in trans.[11] Recent study in rice illustrated that DCL4 is mainly responsible for processing of 21-nt tasiRNAs while the processing of 24-nt phasiRNAs requires OsDCL3b.[12]

We have previously investigated miRNAs in wild rice, *Oryza rufipogon,* using small RNAs isolated from leaves, roots and developing seeds.[13] Here we further investigated the 21-nt and 24-nt phasiRNA-producing loci in *O. rufipogon*. In the small RNA populations (except small RNAs mapped to house-keeping non-coding RNAs) from the three tissues investigated, only < 10% of small RNA reads were generated from miRNA loci, suggesting that > 90% of small RNAs are siRNAs. Using the draft genome of Dongxiang (*O. rufipogon*)[13] as the reference sequence and the pipelines (phase score > 1.4) described by Howell et al.,[14] we found that at least 864 and 3,961 loci were able to generate 21-nt and 24-nt phasiRNAs in the three tissues studied, respectively (**Table 1**). Of these phasiRNA-producing loci, at least 160 of 21-nt and 254 of 24-nt phsiRNA-producing loci had potential miRNA binding site(s) within the regions generating phased small RNA reads or their flanking 200-nt regions, suggesting a possible role of miRNAs in biogenesis of these phasiRNAs (two examples are shown in **Fig. 1A and B**). In these loci, the putative

Table 1. Number of loci generating 21-nt or 24-nt phasiRNAs in *O. rufipogon*

Category	Phase size (nt)	Leaf	Root	Developing seed	Total*	
PhasiRNA generating loci	21	265	75	649	864	(755)
(phase score > 1.4)	24	1,991	833	2,301	3,961	(3,448)
miRNA-triggered	21	28	8	124	160	(151)
	24	92	32	130	254	(250)
PhasiRNA generating loci	21	14	4	9	26	(17)
(phase score > 20)	24	6	4	79	84	(80)
miRNA-triggered	21	5	2	1	8	(7)
	24	1	1	16	18	(17)

*Number in parentheses, number of tissue-specific loci.

miRNA cleavage sites (between positions 10 and 11 in the predicted miRNA target sites) were always found at a position corresponding to a phase register. When an extremely strict phase score (> 20) was applied, 21-nt and 24-nt phasiRNAs were still identified in 26 and 84 *PHAS* genes, respectively (**Table 1**). In comparison, miRNA-binding sites were more likely to be found in the 21-nt *PHAS* genes than in the 24-nt *PHAS* genes. Previously, 21-nt and 22-nt miRNAs have been reported to be the triggers for tasiRNA biogenesis. In this study, we found that miRNAs with a length of 23-nt or 24-nt were also potential trigger for biogenesis of 21-nt and 24-nt phasiRNAs. Moreover, at least 10 *PHAS* genes seem to be triggered by *O. rufipogon*-specific miRNAs, such as oru-miRX42. For the phasiRNA-producing loci without miRNA trigger identified for the time being, we speculate that at least some of them could be actually triggered by miRNA(s) that are yet to be identified. Meanwhile, whether or not certain types of siRNAs are able to initiate the biogenesis of phasiRNAs remains an open question.

The numbers of *PHAS* genes seem to be different in the three tissues analyzed and the highest number of *PHAS* genes was observed in developing seeds (**Table 1**). The percentage of phasiRNAs in the small RNA populations varied widely among three tissues, ranging from 10% in leaves to nearly 50% in developing seeds, suggesting that *PHAS* genes and consequently phasiRNAs could play a more important role in seed development than in leave and root development in wild rice. We also noticed that a significant proportion of *PHAS* genes seemed to be tissue-specific. For example, 176 of the 265 21-nt *PHAS* genes identified in leaves were unique to the leaf tissue. In addition to non-coding transcript, such as *TAS3*, protein-coding genes were also able to generate miRNA-triggered phasiRNAs in wild rice. According to gene ontology (GO) analysis, no obvious functional classification bias was observed for these protein-coding genes, a phenomenon different from that in *Picea abies*, in which 21-nt phasiRNAs were mainly identified in disease resistance genes.[15] To know the potential functions of phasiRNAs, potential targets were predicted for the top expressed phasiRNAs in each *PHAS* genes. We found that these potential target genes cover a wide range of functional categories (data not shown), suggesting that if these phasiRNAs were functional, they would be involved in a diverse biological events.

Several miRNA-like long hairpin loci which also generate phasiRNAs have been characterized in developing seeds of rice.[16,17] In wild rice, we found orthologs of the two miRNA-like long hairpin genes (*MIR436* and Os06 g21900) previously identified in the cultivated rice Nipponbare.[15] Similarly, small RNAs generated from these two loci showed a phased distribution pattern (phase score = 35.0 and 59.3 for *MIR436* and Os06 g21900, respectively).

The genetic diversity of *PHAS* genes between the wild rice (*O. rufipogon*, Dongxiang) and the cultivated rice (*O. sativa,* Nipponbare) was further investigated using 58 *PHAS* gene pairs (phase score > 20) based on their mutation rates (SNPs/kb). As a control, the mutation rates were also estimated for 580 intergenic regions randomly selected from the wild and cultivated rice genomes. A significantly lower genomic variation (t-test) was observed in the *PHAS* genes than in the intergenic regions (**Fig. 1C**), indicating genetic constrain in the *PHAS* genes, probably due to artificial selection.

In summary, thousands of *PHAS* genes, hundreds of them containing putative miRNA binding site(s), were identified in wild rice, and like *MIRNA* genes, *PHAS* genes might also be one of the driving forces that contributed to rice domestication.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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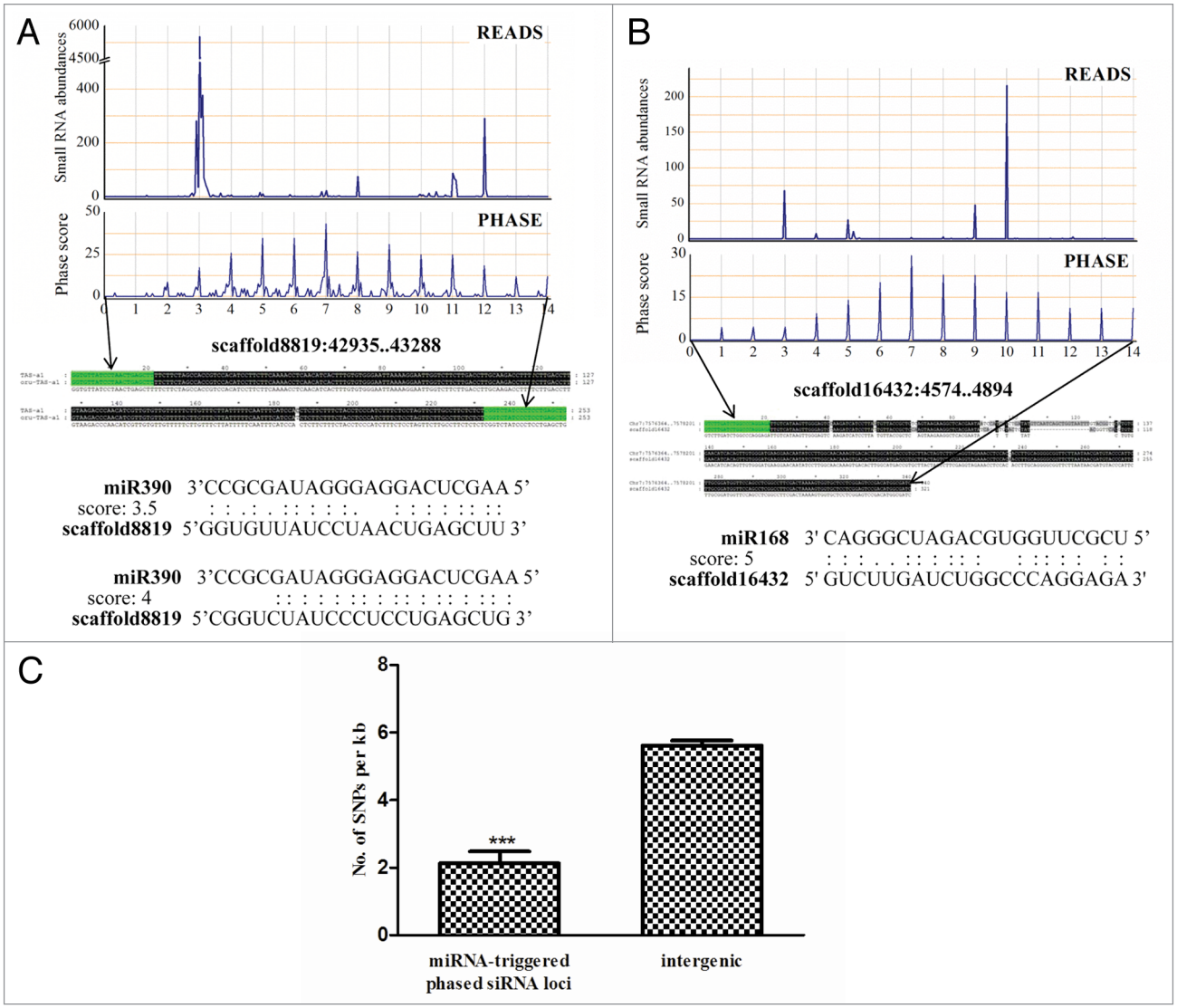

Figure 1. miRNA-triggered phasiRNA production in *O. rufipogon*. Two putative *TAS* genes targeted by miR390 (**A**) and miR168 (**B**) are shown. The upper panel shows the small RNA abundances and phasing score distributions of the wild rice *TAS* genes. The lower panel shows the alignments between miRNAs and their targets; in the alignment, two dots indicate matches, missing dots indicate mismatches and G:U wobble pairs are indicated with a dot. (**C**) Comparison of genomic variations of the wild and cultivated rice between *PHAS* genes and intergenic regions. ***p < 0.0001.

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