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Brief Communication

Biogenesis of reproductive PhasiRNAs: exceptions to the rules

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Reproductive phased small interfering RNAs (phasiRNA) are a class of small RNAs specifically expressed in reproductive tissues, i.e., developing anthers (Liu *et al*., 2020). They are broadly present in both monocots and eudicots (Pokhrel *et al*., 2021a; Xia *et al*., 2019) and are important to male fertility (Araki *et al*., 2020; Teng *et al*., 2020; Zhang *et al*., 2020).

Reproductive phasiRNAs are classified as pre-meiotic and meiotic phasiRNAs according to their temporal accumulation in anthers (Zhai *et al*., 2015). In monocots, the pre-meiotic phasiRNAs are predominantly 21-nt in length and generated from a large number of noncoding precursor genes (*21-PHAS*) targeted by miR2118. These 21-nt pre-meiotic phasiRNAs likely function via the direction of mRNA cleavage, responsible for the transcriptome reprogramming in pre-meiotic anthers (Jiang *et al*., 2020; Zhang *et al*., 2020), and different pre-meiotic phasiRNAs are enriched in different cell types to perform distinct functions (Araki *et al*., 2020). Meiotic phasiRNAs are 24-nt long and produced from miR2275-targeted noncoding genes (*24-PHAS*). Although we know these 24-nt meiotic phasiRNAs are essential for male fertility, how they execute their function remains larger obscure. In eudicots, different miRNAs seem to be evolved to instigate the production of 21-nt pre-meiotic phasiRNA, i.e., miR11308 in wild strawberry (*Fragaria vesca*) and miR14051 in columbine (*Aquilegia coerulea*; Pokhrel *et al*., 2021a), and the production of 24-nt meiotic phasiRNAs can be triggered by miRNAs other than miR2275, including miR11308, miR482/2118 and miR14051 (Pokhrel *et al*., 2021b), indicating a greater diversity of miRNA triggers in eudicots and the high plasticity of reproductive phasiRNA production. Here, we reported, during our recent massive *PHAS* analyses of hundreds of plants, our discoveries on reproductive phasiRNAs of abnormal patterns, which broadens our horizon on the sophistication of reproductive phasiRNA production.

miR2275 targets a protein-coding gene to trigger the production of 21-nt phasiRNAs

In 2019, we found that the 24-nt reproductive phasiRNAs are broadly present in eudicots as in monocots (Xia *et al*., 2019). So far, miR2275 is considered as the master trigger of 24-nt reproductive phasiRNAs from noncoding *PHAS* loci. MiR2275 targets a large number of noncoding RNAs (*PHAS* precursors) preferentially expressed in meiotic anthers and instigates the production of 24-nt phasiRNAs (Figure 1a and Figure S1, Table S1). However, here we found an exceptional case in litchi (*Litchi chinensis*), in which miR2275 was found to target a protein-coding gene and trigger the production of profuse 21-nt phasiRNAs (Figure 1b, Table S2). This gene codes a Caffeoyl-shikimate esterase of 320 amino acids and is preferentially expressed in litchi flowers as well. Although there were considerable 24-nt sRNAs generated over the gene, most of these 24-nt sRNAs were not in-phase to the miR2275 cleavage site, indicating this gene is a canonical *21-PHAS* gene mainly generating 21-nt phasiRNAs. Therefore, the 22-nt long miR2275 can also serve as a typical miRNA trigger of 21-nt phasiRNA production, in addition to its established role in 24-nt reproductive phasiRNA production.

A conserved miRNA-*PHAS* module produces different types of phasiRNAs under different conditions

In plants, the well-conserved miRNA miR167 targets genes encoding *Auxin Responsive Factor 6/8* (*ARFs*) genes. In many cases, miR167 targets *ARF6* genes and triggers the production of 21-nt phasiRNAs simultaneously, probably due to some of the miR167 variants which can be 22-nt long and serve as phasiRNA triggers. As shown in Figure 1c (Table S3), rice miR167 targets *ARF6* gene (*LOC_Os02g06910*) and incites the production of abundant 21-nt phasiRNAs in non-reproductive tissues (i.e., leaves). Interestingly, the same gene can produce 24-nt phasiRNAs over the same region after miR167 cleavage site in rice anther (Figure 1c and Figure S2, Table S4). These results suggest that reproductive phasiRNAs can be generated from protein-coding genes, and the same *PHAS* gene/locus can be transitive on the production of either 21-nt or 24-nt phasiRNAs, independent of the miRNA trigger. This transition between 21-nt and 24-nt phasiRNAs is spatiotemporally specific.

Both types of reproductive phasiRNAs are produced from the same *PHAS* loci simultaneously

In wild strawberry, miR11308 was found to trigger the production of 21-nt and 24-nt phasiRNAs from distinct *PHAS* genes/loci (Pokhrel *et al*., 2021a,b). Here we found that 21-nt and 24-nt phasiRNAs can be biosynthesized from the same tissue

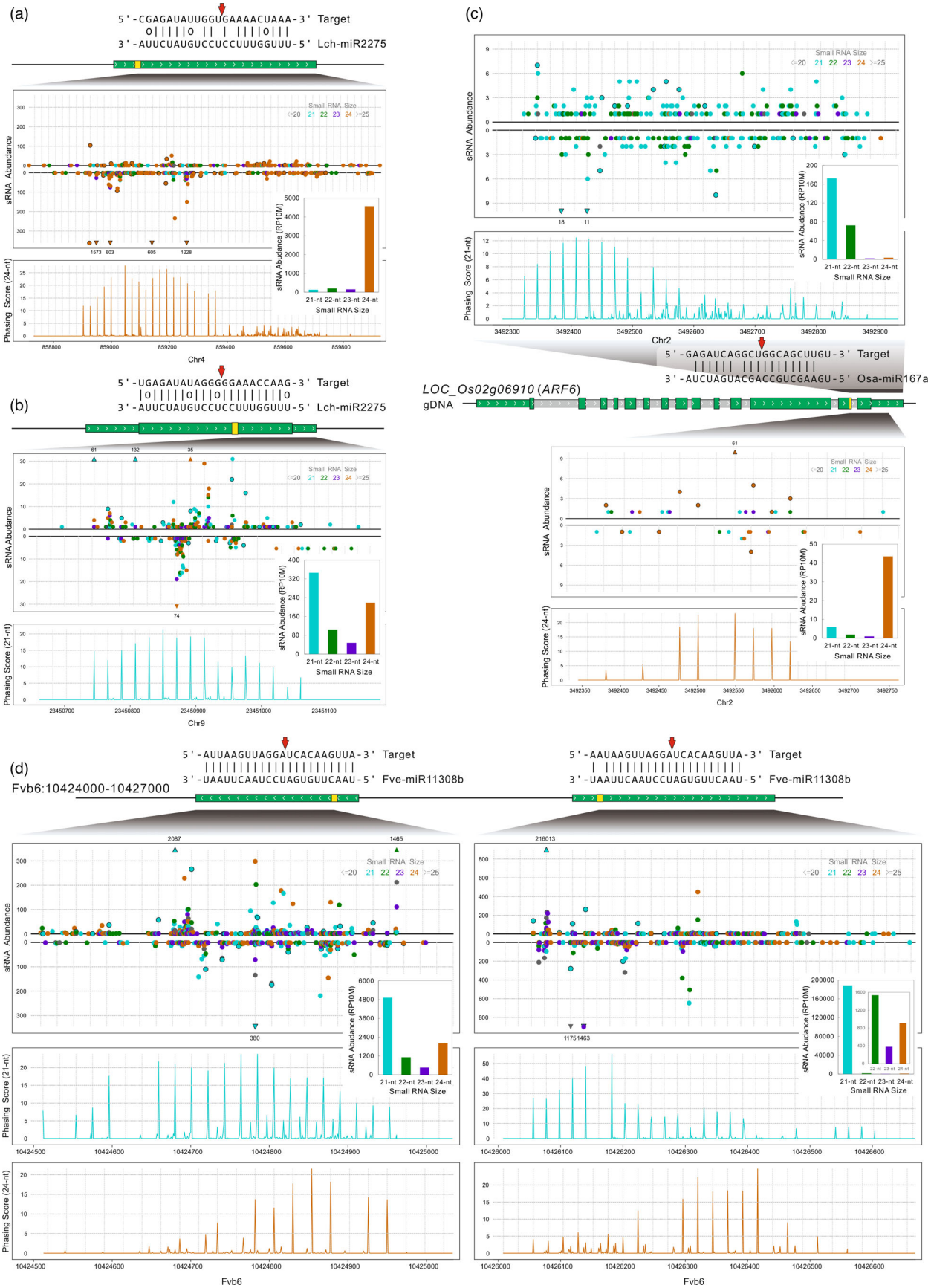
(a)
5'-CGAGAUAUUGGUGAAAACUAAA-3' Target
3'-AUUCUAUGUCCUCCUUUGGUUU-5' Lch-miR2275
sRNA Abundance
Small RNA Size
sRNA Abundance (RP10M)
21-nt 22-nt 23-nt 24-nt
Small RNA Size
Phasing Score (24-nt)
Chr4
(b)
5'-UGAGAUAUAGGGGGAAACCAAG-3' Target
3'-AUUCUAUGUCCUCCUUUGGUUU-5' Lch-miR2275
Phasing Score (21-nt)
Chr9
(c)
LOC_Os02g06910 (ARF6)
gDNA
5'-GAGAUCAGGCUGGCAGCUUGU-3' Target
3'-AUCUAGUACGACCGUCGAAGU-5' Osa-miR167a
Chr2
(d)
Fvb6:10424000-10427000
5'-AUUAAGUUAGGAUCACAAGUUA-3' Target
3'-UAAUUCAAUCCUAGUGUUCAAU-5' Fve-miR11308b
5'-AAUAAGUUAGGAUCACAAGUUA-3' Target
3'-UAAUUCAAUCCUAGUGUUCAAU-5' Fve-miR11308b
Fvb6

Figure 1 Production of 21-nt and 24-nt reproductive phasiRNAs is transitive in plants. (a) The noncoding *PHAS-24* locus targeted by miR2275 generates 24-nt phasiRNAs in litchi. In the upper is a schematic diagram of the noncoding gene, the green squares represent the gene structure with the white arrow representing the direction of transcription, while the yellow box marks the target site of miR2275. Base pairing of miRNA:target is shown above with the red arrow indicating the cleavage site. Size distribution of sRNAs is presented in the middle with each dot representing a small RNA. sRNAs of different sizes are colour-coded (21-nt: light-blue, 22-nt: green, 23-nt: purple, 24-nt: orange, other sizes: grey). Phasing scores of 24-nt phasiRNAs are shown in the bottom. Grey lines indicate the phased positions with 21-nt or 24-nt spacers. The bar chart in the insert shows the cumulative sRNA abundance according to sRNA sizes. (b) A protein-coding gene targeted by miR2275 generates 21-nt phasiRNAs in litchi. The thinner green squares of the gene represent the 5′-UTR and 3′-UTR, respectively, while the larger green square represents the coding region. Phasing scores of 21-nt phasiRNAs are shown in the bottom. (c) The gene (*ARF6*) targeted by miR167a can generate both 21- and 24-nt phasiRNAs from the same trigger site in rice. (d) *PHAS* targets of miR11308b trigger produce both 21-nt and 24-nt phasiRNAs in wild strawberry.

simultaneously (Figure 1d and Figure S3). For example, over the two *PHAS* loci targeted by Fvb-miR11308, both types of phasiRNAs can be detected from the library, i.e., anthers at stage 8 (an-8), with both 21-nt and 24-nt sRNAs are in good phase to the corresponding miRNA cleavage site (Figure 1d, Table S5). These results indicate that *PHAS* transcripts cleaved by miRNA can be directed into both 21-nt and 24-nt phasiRNA biosynthesis pathways simultaneously.

Biogenesis of most phasiRNA in plants is processed via the 'one-hit' model, in which mRNA of *PHAS* genes/loci is firstly cleaved by a 22-nt miRNA trigger. Usually, a certain trigger miRNA or *PHAS* gene/locus is associated with only one type of phasiRNAs, either 21-tn or 24-nt. However, in this study, we observed abnormalities regarding the general rules of reproductive phasiRNA production: miR2275 can target both protein-coding and noncoding genes/loci to trigger the production of 21-nt or 24-nt phasiRNA; neither the miRNA trigger, nor the *PHAS* gene/locus is the determinant of which type of phasiRNAs (21-nt or 24-nt) is biosynthesized downstream, indicating the process of reproductive phasiRNA production in plants is of a much greater flexibility than we have already known. Perhaps some other unknown cofactors exist to steer the double-strand precursor RNAs to the action of different Dicer-like proteins, and those cofactors are likely under strict spatiotemporal regulation. These findings deepen our understanding of phasiRNA biogenesis, which will be helpful for engineering the phasiRNA pathway as an effective tool to introduce artificial gene silencing in plants.

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Conflict of interest

The authors declare no competing interest.

Author contributions

R.X. designed the research; J.Z., C.C., G.L., P.C. and Y.L. performed the experiments; J.Z. and R.X. wrote the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Materials and methods.
Figure S1 Lc-miR2275 targets both coding and noncoding *PHAS* genes in litchi.
Figure S2 miR167a-*ARF6* phasiRNA pathway in rice.
Figure S3 Fvb-miR11308b-*PHAS* pathway generate both 21-nt and 24-nt phasiRNA simultaneously in wild strawberry.
Figure S4 Northern blot analyses of 24-nt phasiRNAs in flower buds of litchi.

Table S1 Mapping sRNA-data of the *24-PHAS* locus (Figure 1a) in litchi.
Table S2 Mapping sRNA-data of the *21-PHAS* locus (Figure 1b) in litchi.
Table S3 Mapping sRNA-data of 21-nt phasiRNAs in the *PHAS ARF6* gene (Figure 1c) in rice.
Table S4 Mapping sRNA-data of 24-nt phasiRNAs in the *PHAS ARF6* gene (Figure 1c) in rice.
Table S5 Mapping sRNA-data of the *PHAS* locus (Figure 1d) in strawberry.