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MicroRNA mediates DNA methylation of target genes

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ABSTRACT

Small RNAs represented by microRNA (miRNA) plays important roles in plant development and responds to biotic and abiotic stresses. Previous studies have placed special emphasis on gene-repression mediated by miRNA. In this work, the DNA methylation pattern of microRNA genes (*MIRs*) was interrogated. Full-length cDNA and EST were used to confirm the entity of pri-miRNA. In parallel, miRNA in 24 nucleotides (nt) was pooled to detect chromatin modification effect by using bisulfite sequencing data. 97 *MIRs* were supported by full-length cDNA and 30 more were hit by EST. Notably, methylation levels of conserved *MIRs* were significantly lower than the non-conserved at all contexts (CG, CHG, and CHH). Additionally, a substantial part of 24-nt miRNA was able to induce target site methylation, providing a broader perspective for researchers.

1. Introduction

miRNA often serves as a gene expression regulator due to its participation in post-transcriptional gene silencing (PTGS) by either target-mRNA cleavage or translation repression [1,2], and it has been long-proposed to preferentially targets transcription factors (TFs) and enzymes that have critical functions in diverse aspects of plant biology [3,4]. Though great progress has been made in understanding the activities of miRNA and the identification of novel miRNA [5–7], the modulation associated with *MIRs* remains unclear.

DNA methylation, one of the epigenetic marks, plays a crucial role in the regulation of X-chromosome inactivation [8], transposon silencing [9], biotic stress response [10], gene expression [11], and genomic imprinting [12] in both plants and animals. Vrba et al. [13] found that DNA methylation participated in regulating cell type-specific miRNAs in human. In addition, a number of miRNAs in human are up-regulated after treatment with 5-aza-dC and/or 4-phenylbutyric acid [14–16], which highlights the conceivable connection of DNA methylation and miRNA expression. Aforementioned descriptions inspire us to look into the methylation pattern of *MIRs* in plants.

Besides, RNA-directed DNA Methylation (RdDM), firstly deliberated by Wassenegger et al. [17], has been paid more attention for its contribution in DNA methylation. To date, RdDM, R refers to siRNA, and whether miRNA bears the similar function are poorly understood, though a few miRNAs like osa-miR1863 do so [18,19]. In this study, we demonstrate that many more miRNAs also have a similar function in a genome-wide scale by using bisulfite sequencing data. The 24-nt miRNA was able to direct DNA methylation both in *cis* (loci they are originated) and in *trans* (target genes), which sheds fresh light on the diverse regulation modes of miRNA.

2. Materials and methods

2.1. Databases for sequences

The rice pre-miRNA and mature miRNA sequences were retrieved from miRBase (Release 19), and the whole genome sequences were downloaded from http://rice.plantbiology.msu.edu/ (Release 7) and http://www.phytozome.net/ (V8.0).

2.2. DNA methylation pattern of MIRs

1 or 2 kb upstream and downstream sequences of pre-miRNA were retrieved from rice genome. Promoters of *MIRs* were predicted by TSSP (http://linux1.softberry.com/berry.phtml). Then BLASTn with the cutoff *E* value of 10^{-10} was conducted to locate

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the full-length cDNA to *MIRs* followed by manual refinements. As for the EST data, to rule out the possibility of random matches, hits longer than 150 bp and >90% similarities were retained for further analysis. Subsequently SIM4 [36] was utilized to confirm the exon and intron splicing site in *MIRs*.

Bisulfite sequencing data of rice was downloaded from the GEO database under accession numbers: GSE22591, GSM497260, and GSM946552. DNA methylation was explored similar to [37]. In most cases, we restricted our analysis at comparable coverage level across tissues.

2.3. Target prediction of osa-miRNAs

Targets of osa-miRNAs were predicted by psRNATarget program (http://plantgrn.noble.org/psRNATarget/) with default parameters. Briefly, unique mature sequences of 21/24-nt osa-miRNAs were chosen to target Osativa_193_transcript.fa (JGI Genome). Gene ontology analysis was performed by WEGO [38].

2.4. RdDM induced by 24-nt osa-miRNA

Algorithm psRNATarget was applied to seek the coordinates in the target genes. Then local methylation status was interrogated confined to sites with at least five and not more than 50 read coverage. RdDM was defined for the region that is proximal to MBS (including MBS) with at least five differentially methylated cytidines at CHH sites.

3. Results and discussion

3.1. Identification of MIRs and their promoters in rice

There are 591 rice pre-miRNAs deposited in miRBase (http://www.mirbase.org, Release 19). To explore the promoter distribution of *MIRs*, 2 kb upstream and 2 kb downstream flanking sequences of pre-miRNA were pooled from the MSU rice genome (http://rice.plantbiology.msu.edu/). The adjacent pre-miRNAs (span within 4 kb) were merged as one by reason that some pri-miRNA may contain several pre-miRNAs. Eventually, 548 *MIR* sequences were retained that covered all 591 pre-miRNAs. The promoters of *MIRs* were predicted by TSSP (a plant promoter recognition program). And results showed that the majority of *MIRs* possessed the core promoter motif TATA-box (376/548), which was much higher than protein coding genes [20], suggesting that the primary transcripts of *MIRs* were transcribed by polymerase II (Pol-II). Furthermore, more than half promoters presented within 500 bp upstream of pre-miRNA (Fig. 1A), which was consistent with a previous study [21].

To investigate the characteristics of rice pri-miRNA, full-length cDNA [22] was mapped against these *MIRs*. We observed that only 17.7% (97/548) *MIRs* were supported by the full-length cDNA. A rational explanation was that the remaining *MIRs* might be tissue-specific, developmental-stage specific, or stress induced. For this reason, rice EST data collected from NCBI was mapped to *MIRs*, and another 30 *MIRs* were supported by EST. Then SIM4 was utilized to confirm the splicing site of exon and intron. Intriguingly, some *MIRs* harbored the canonical GT-AG splicing structure that would distinguish exon from intron (Fig. 1B).

The length of rice pri-miRNA varied widely from 200 to 3000 nt, with the average of 1000 nt, which was a little longer than that of maize [23] based on the 97 full-length cDNAs and 30 EST supported *MIRs*. Besides, 5′ region (from the transcriptional start site to pre-miRNA) of pri-miRNA was generally shorter than the 3′ region (from pre-miRNA to transcriptional stop site), with a mean length of 321 and 671 nt, respectively.

3.2. Hypomethylation in conserved MIRs

Aforementioned pri-miRNA characteristics inspired us to explore the DNA methylation profile of the *MIR* promoter (1 kb upstream of pre-miRNA) and gene body (pre-miRNA and 1 kb downstream). DNA methylation status at CG, CHG, and CHH contexts were interrogated, and cytosines with read depth $\geqslant 5$ and $\leqslant 50$ were retained for further analysis. Aggregate DNA methylation of promoters and gene bodies were compared between conserved miRNAs (based on the conservation between rice and *Arabidopsis*) and non-conserved miRNAs. The methylation level of *MIR* promoters was the highest at CG (12.46%, 42.53%), then at CHG (3.46%, 14.55%), and the lowest at CHH (1.43%, 3.34%) both in conserved and non-conserved *MIRs* (Fig. 2A). Notably, the methylation level of non-conserved *MIRs* was corresponding to the genomic level [24,25]. Furthermore, the methylation level at CG, CHG, and CHH sites of non-conserved *MIRs* was three, four, and twofold higher than the conserved, respectively ($P < 0.05$, Mann Whitney test). Surprisingly, the methlylation level within gene body was very similar to that of the promoter, that is, conserved *MIRs* were significantly lower than the non-conserved ($P < 0.05$, Mann Whitney test) (Fig. 2B). This trend was also held true in *Arabidopsis* (data not shown).

The bulk of conserved *MIRs* were hypomethylated suggested that they were more resistant to methylation, whereas the non-conserved were prone to suffer from methylation. It is widely accepted that hypomethylation in the promoter region tends to correlate with transcription. The hypomethylation of conserved *MIRs* can thus facilitate their escape from the regulation of DNA methylation since ancient conserved miRNAs often constitutively expressed and/or expressed at high levels [26]. Conversely, species-specific *MIRs* were hypermethylated both in promoters and gene bodies. Previous studies demonstrate that a substantial part of novel evolved miRNAs probably have not been incorporated into the extant regulatory network for their deleterious to plants, and express at a low level to tolerate their existence [4,26]. Thus, DNA methylation may contribute to the repression of newly evolved miRNA.

To shed more light on the DNA methylation pattern of *MIRs*, in parallel, we explored their methylation status in rice five tissues (mature leaves, shoot, root, endosperm, and embryo). We found that aggregate DNA methylation of promoters were indistinguishable among four tissues (mature leaves, shoot, root, and embryo) at CG and CHG contexts for non-conserved *MIRs* ($P > 0.05$, t test), which was quite different from *Populus trichocarpa* [27]. CHH methylation, however, was enriched in embryo and mature leaves for *MIRs*, which was in line with MITE [25], suggesting that they both suffered enhanced regulation from RdDM. Moreover, we also noticed that hypomethylation occurred more frequently at all contexts in endosperm ($P < 0.05$, t test; Fig. 3A–C), which was in full agreement with genome-wide scale analysis [25]. A similar trend was observed for the conserved *MIRs* promoters except for the CG sites ($P > 0.05$, t test). As for the gene bodies, aggregate DNA methylation was somewhat similar with promoters for non-conserved *MIRs* (Fig. 3D–F). In the case of conserved *MIRs*, however, DNA methylation was indistinguishable at all contexts (CG, CHG, and CHH) among all the five tissues (Fig. 3D–F).

3.3. 24-nt miRNA directed DNA methylation of target genes

Previous study only identifies five genes that display DNA methylation around the 24-nt miRNA target sites [18], which prompts us to explore the DNA methylation profile of other 24-nt miRNA targets by using bisulfite sequencing data. A total of 325 targets were obtained for the 24-nt miRNAs (miRBase, Release 19), including 39 transposable elements. Then DNA methylation

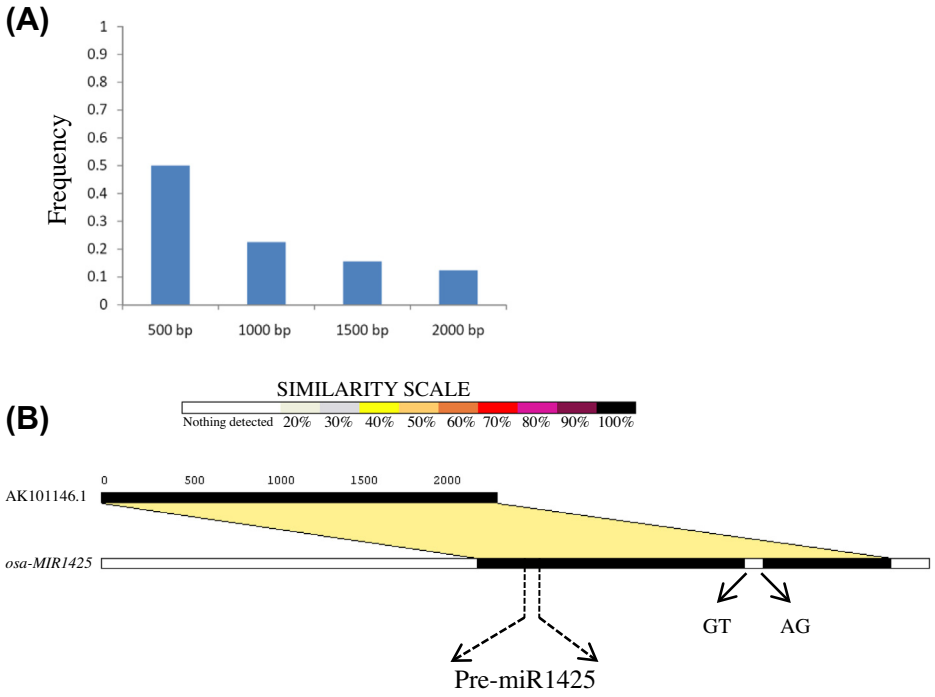

Fig. 1. Promoter distribution and gene structure of *MIRs* in rice. (A) Distance distribution of promoters relative to pre-miRNAs. Figures below column diagram corresponding to the span of promoters to pre-miRNAs. (B) Intergenic *MIRs*, same as protein coding genes, also possess GT-AG in their splicing sites.

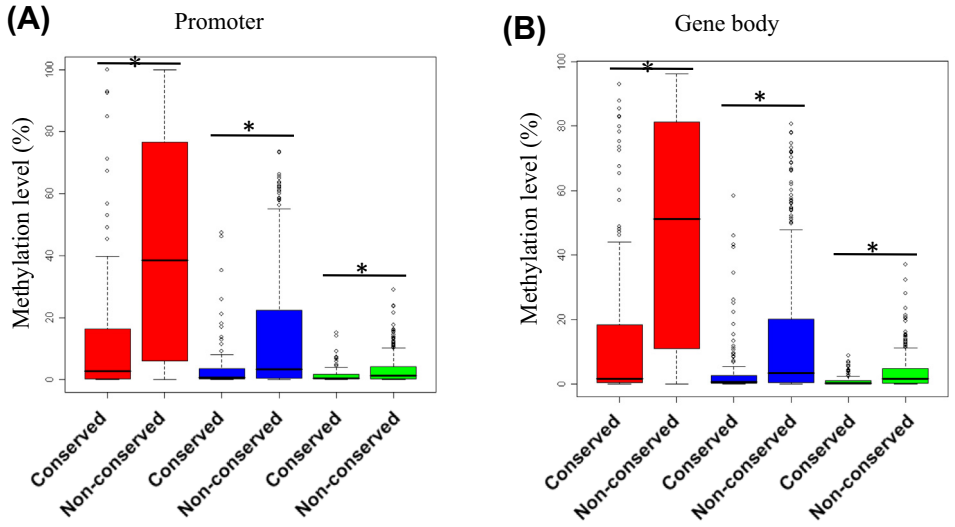

Fig. 2. Methylation variation between conserved *MIRs* and the non-conserved in promoter (A) and gene body (B). CG, CHG, and CHH methylation were shaped in red, blue, and green, respectively. Horizontal lines in the box indicated the median, and the top and bottom of the boxes correspond to the second and third quartiles. * Indicated $P < 0.05$ via Mann Whitney test. Bisulfite sequencing data were downloaded from GEO under accession number GSM946552. To improve reliability, we only considered the cytidines at all contexts with read depth $\geqslant 5$ and read depth $\leqslant 50$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

profile of all target genes was investigated and intriguingly, 65 genes showed enhanced CHH methylation around miRNA binding site (MBS) (Fig. 4, Table S1, and Supplemental file 1). In particular, over half members of the osa-miR812 family were capable to induce MBS methylation despite of the huge sequence differences (Fig. S1). We also found that osa-miR1862c, osa-miR1863b, osa-miR1867, osa-miR2121b, osa-miR5150, and osa-miR5831 were strong initiators to induce MBS methylation (Table S1). It is noteworthy that our results were probably underestimated because of our strict target prediction parameters, thus Os02g05890 and Os07g41090 (putatively targeted by miR1876) showing DNA methylation around MBS [18] were omitted in our result. Furthermore, unlike RdDM of host gene mediated by primary siRNA and secondary siRNA spread in a unidirectional manner (3′-direction) [28,29], DNA methylation directed by miRNA exhibited a greater propensity to extend MBS bidirectionally (commonly limited within 200 bp).

These two classes of small RNAs share the same size, in particular, their roles both depend mainly on DICER-LIKE (DCL), Argonaute (AGO), and Pol [18,30]. Sometimes it may obscure the boundary between miRNA and siRNA. Nevertheless, regulatory module differs considerably. Unlike miRNA transcribed by Pol II

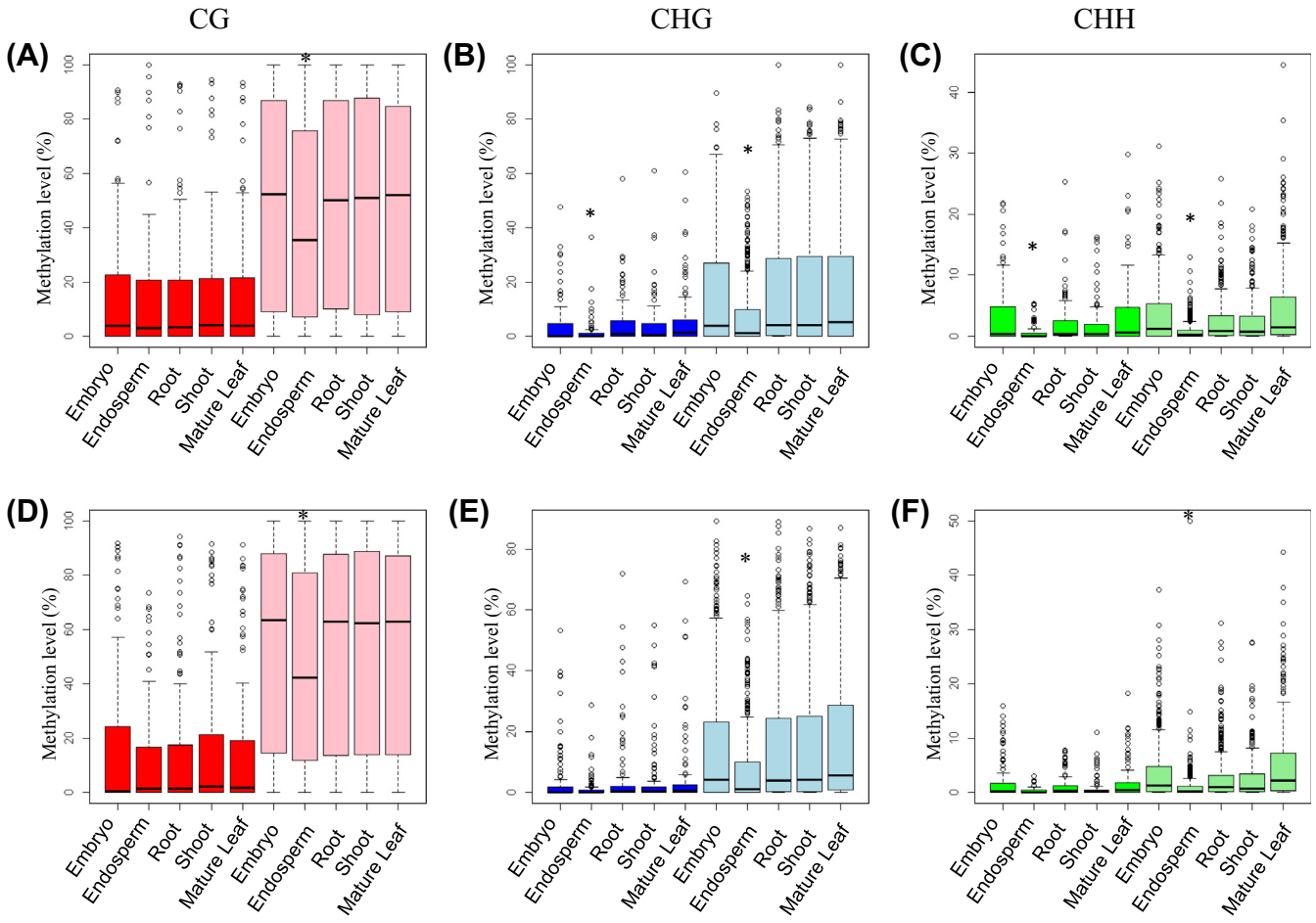

Fig. 3. Comparison of *MIR* methylation levels among rice five tissues. For A–C, promoter methylation level between conserved and non-conserved in the contexts of CG, CHG, and CHH in rice five tissues. As for D–F, gene body methylation level between conserved and non-conserved in the contexts of CG, CHG, and CHH. Red: conserved CG methylation; Pink: non-conserved CG methylation; Blue: conserved CHG methylation; Light blue: non-conserved CHG methylation; Green: conserved CHH methylation; Light green: non-conserved CHH methylation. Bisulfite sequencing data were downloaded from GEO under accession number GSE22591 and GSM497260. We restricted our analysis within cytidine read coverage $\geqslant 2$ and in comparable level among those tissues. Each column represents one tissue. * indicated $P < 0.05$ via a two-tailed *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(a few by Pol III), generation of siRNA relies on RNA-dependent RNA polymerase 2 (RDR2), Pol IV, and Pol V [30,31]. miRNA, on the one hand, in line with siRNA, was able to methylate its host gene in a locus-specific manner (so-called regulation in *cis*). On the other hand, miRNA can also mediate DNA methylation of its targets (so-called regulation in *trans*); namely 24-nt miRNA like osa-miR5831 can direct DNA methylation both in *cis* and in *trans* (Fig. S2). Xie et al. [32] have proposed that *rdr2* mutant correlates with declining of accumulation of heterochromatic siRNA, while 24-nt miRNA almost remains unchanged, suggesting that miRNA employs an independent regulatory cascade other than siRNA. These findings, in combination with a previous study [18], implying that 24-nt miRNA is indeed implicated in RdDM. Similarly, piwi interacting RNA (piRNA) has been shown to be involved in DNA methylation in animals [33,34]. It is thus tempting to believe that these small non-coding RNAs act as genome buffers and fine-tune their targets to keep the plasticity and flexibility of the organism.

Moreover, MBS methylation of some genes such as Os03g50470 can be induced simultaneously by several miRNAs (Fig. 4D). Interestingly, when we dissected the methylation status among these MBSs, their intensities were found to vary considerably across different target genes and intragentic regions (Fig. 4 and Supplemental file 1). Some showed only sporadic CHH methylation while others exhibited biased methylation blocks (>30% methylation level at CHH sites) in the vicinity of MBS, contrasted sharply with the methylation desert outside MBS region. This led us to hypothesize that differentially methylated regions in the vicinity of MBS were non-random distribution of methylcytidines, but it was of RdDM consequence. We further partitioned target sequences into three types, that is, 200 bp centered at MBS (type I), 200 bp flanking sequences (type II, 100 bp downstream and upstream of type I), and the whole target gene (type III). As expected, we observed that the CHH methylation level of the type I was significantly higher than that of type II and type III ($P < 0.001$, Mann Whitney test), which underscored the interaction between 24-nt miRNAs and targets.

In plants, it is clear that almost conserved miRNAs are 21-nt, while a lot of, if not all, 24-nt miRNAs identified in rice are lineage-specific. Our results showed that the DNA methylation level of non-conserved *MIRs* was significantly higher than that of conserved ones and a large number of 24-nt miRNA targets showed enhanced CHH methylation around MBS (Figs. 2 and 4). More in-depth analysis was therefore performed to account for their targets functional variance. Gene Ontology (GO) enrichment analysis showed that 866/1361 (64%, 21-nt miRNA) and 210/325 (64%, 24-nt miRNA) targets were covered by GO assignments (Fig. S4). Remarkably, the aggregate GO architecture differed sparingly between targets of 21-nt and 24-nt miRNA under "Biological Process"

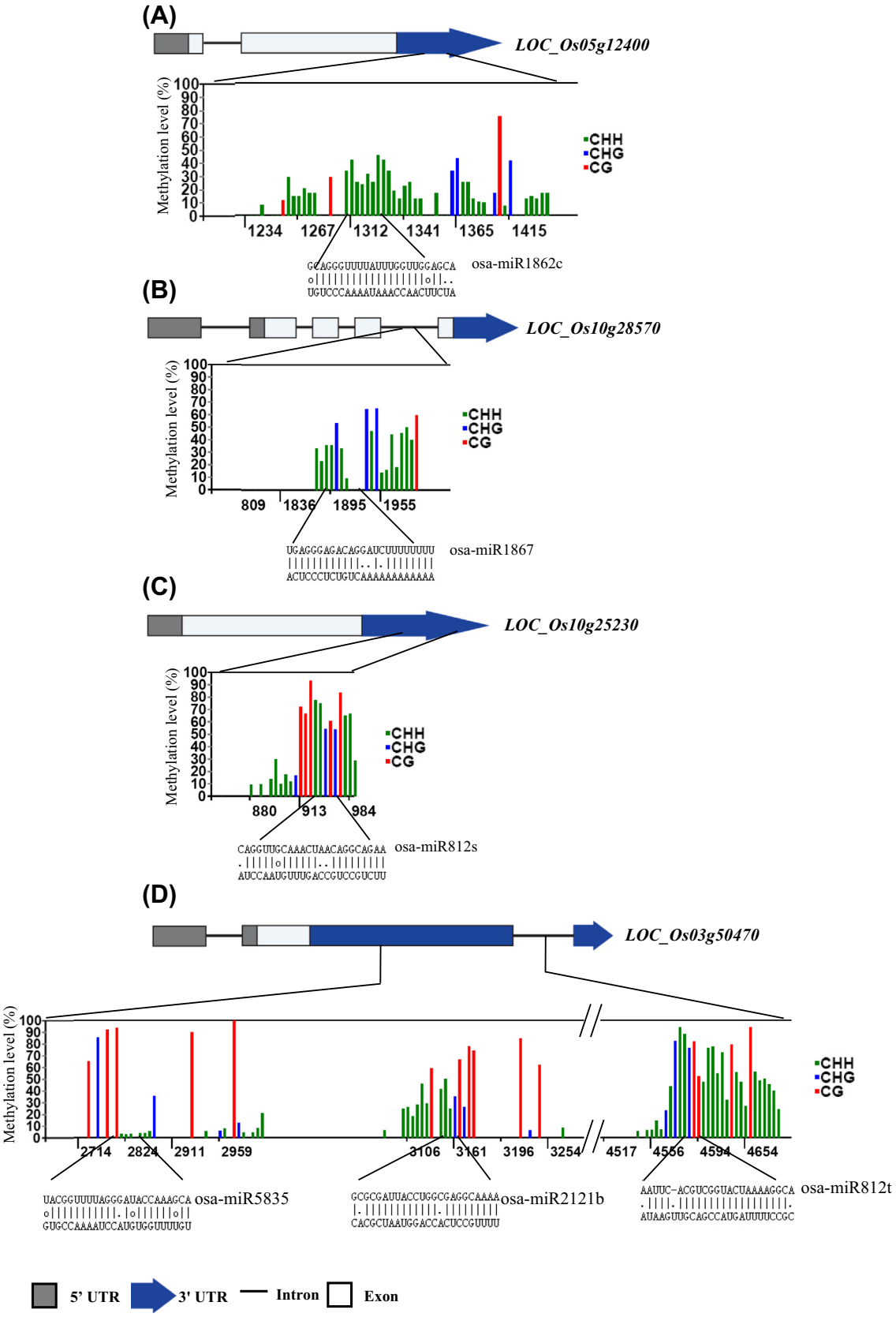

Fig. 4. RdDM induced by 24-nt miRNA. Bisulfite sequencing data were downloaded from GEO under accession number GSM946552. Cytidines with read depth $\geqslant 5$ and read depth $\leqslant 50$ were retained for further analysis. Evidently enhanced DNA methylation was observed proximal to (including) miRNA binding sites (MBS). In addition, it was evident that RdDM intensity varied considerably either in different intragentic regions or different targets.

term. By contrast, enzyme regulator under "Molecular Function" term as well as endomembrane system, envelope, and organelle envelope under "Cellular Component" term were absent in 24-nt targets (Fig. S4). Although some 24-nt miRNAs indeed lack targets, the remaining ones, akin to 21-nt, rarity of difference was observed in target functional category (Fig. S4). These observations, in combination with previous findings that selection is weak or neutral for the lineage-specific miRNA, support the notion that young miRNA is not always associated with target degradation, but in other unknown function due to its restricted expression [26,35].

In this study, we dissected the methylation landscape of *MIRs* in plants. Besides, 24-nt miRNA, to some extent, akin to heterochromatic siRNA, has emerged as a novel methylation mediator. Even so, the underlying molecular mechanism remains elusive. Indeed, siRNA-like miRNA, associated with DNA methylation, provides a unique perspective to the further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.171.

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