Genes Genet. Syst. (2013) **88**, p. 105–112

Expression of the rice microRNA *miR820* is associated with epigenetic modifications at its own locus

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(Received 12 April 2013, accepted 8 May 2013)

Small RNAs, such as small interfering RNAs (siRNAs) or microRNAs (miRNAs), regulate gene expression at transcriptional and posttranscriptional levels in eukaryotes. miRNAs are processed from duplexes formed on single-stranded RNA. They regulate expression of their target gene either by cleaving mRNA or supressing translation. In general, the primary miRNA transcripts are synthesized by RNA polymerase II and processed similarly to mRNAs. *MIRNA* genes are usually located in transcriptionally active euchromatic regions. In contrast, siRNAs are processed from duplexes made of two RNA molecules. One of them is often derived from a transposable element (TE) or from repetitive sequences that reside in heterochromatic regions. The other strand is synthesized by the RNA-dependent RNA polymerase on the first strand as a template. siRNAs establish epigenetic marks in parasitic DNA such as TEs, thus they usually act in *cis*. The rice miRNA *miR820*, encoded by CACTA TEs (five copies, located on different chromosomes), reduces the expression of the *de novo* DNA methyltransferase gene *OsDRM2*. Because *miR820* is derived from silent TEs, in which the heterochromatic histone modifications are enriched, the mechanism of *MIR820* transcription could be expected to differ from typical miRNAs. Here we show that the primary transcript of *MIR820* is mainly derived from the CACTA TE copy on chromosome 7 (*MIR820b*). Histone modification and DNA methylation status around *MIR820b* differed from that of the other four loci. These unique epigenetic modifications in *MIR820b* were only found around the *miR820* coding region. We conclude that *MIR820b* transcription may depend on the unique epigenetic modifications, which in turn may be established by the action of *miR820* in *cis*. This suggests a dual function of *miR820* in *cis* and in *trans*.

Key words: histone modification, miRNA, posttranscriptional gene silencing, RNA-dependent DNA methylation, transcriptional gene silencing

INTRODUCTION

microRNAs (miRNAs) and small interfering RNAs (siRNAs) are small RNAs that regulate gene expression at both transcriptional and post-transcriptional levels in eukaryotes (Carthew and Sontheimer, 2009). The small RNAs are essential for plant development, environmental responses, and defense against genomic parasites such as transposable elements (TEs) and viruses (Plasterk, 2002; Almeida and Allshire, 2005; Aravin et al., 2007).

miRNAs are 21- or 22-nt-long RNA molecules processed from a duplex (formed on a single-stranded RNA) by Dicer-like protein 1 (DCL1) in plants (Kurihara and Watanabe, 2004; Kurihara et al., 2006). miRNAs selectively recognize their target mRNAs based on the base pairing, and cleave them or suppress translation (Voinnet, 2009; Sun, 2012). miRNA primary transcripts (pri-miRNAs) are synthesized by RNA polymerase II, and are processed into capped, spliced, and polyadenylated pri-miRNAs (which is similar to mRNA processing) (Lee et al., 2004). Actively transcribed *MIRNA* genes are usually located in euchromatic regions (similar to protein coding genes).

siRNAs (usually 20–30 nt long) originate from TEs, repetitive regions, other intergenic regions or transgenes. The initial siRNA transcripts are synthesized by RNA polymerase IV, a member of the plant-specific DNA-dependent RNA polymerases. These transcripts are

Edited by Yoshibumi Komeda
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converted into double-stranded RNA either by RNA-dependent RNA polymerases or by transcription from both strands, and are processed by DCL2–4 (Vazquez et al., 2008).

siRNAs are responsible for defense against genomic parasites such as TEs or viruses (Voinnet, 2008). siRNAs establish epigenetic marks (such as DNA cytosine methylation) in the parasitic DNA from which they originate, thus they usually act in *cis*. In plants, TE-derived siRNAs are loaded to Argonaute proteins. The complex is recruited to the locus from which the siRNA has originated by the guide RNAs transcribed by another plant-specific DNA-dependent RNA polymerase, RNA polymerase V (Cao et al., 2000; Cao and Jacobsen, 2002; Wierzbicki, 2012). Then the complex further recruits the downstream effectors, such as *de novo* DNA methyltransferase, DRM2, to establish and/or maintain epigenetic marks. Thus, siRNAs from TE loci induce RNA-directed DNA methylation, which results in epigenetic inactivation of TEs (Zilberman and Henikoff, 2004; Lisch, 2009; Matzke et al., 2009). This mechanism results in epigenetic silencing of most TEs (Feschotte et al., 2002).

Thus, there are many differences between miRNAs and siRNAs (Ambros et al., 2003; Meyers et al., 2008): (1) miRNAs are made from single transcripts, whereas siRNAs are produced from two RNA molecules. (2) The length of most miRNAs is 21 nt, whereas that of siRNAs is 24 nt in plants. (3) miRNAs act on *trans* targets, whereas siRNAs act in *cis* (although there are some exceptions such as ta-siRNAs). (4) miRNAs regulate gene expression post-transcriptionally, whereas siRNAs induced transcriptional silencing. (5) Because siRNAs arise from various genomic locations, their molecular diversity is much higher than that of miRNAs. (6) The expression levels of miRNAs, especially those conserved among various plant species, are usually higher than those of most siRNAs. (7) Small RNA profiling in many plant species revealed that the content of siRNA is much higher than that of miRNA.

Several small RNAs cannot be easily classified as miRNAs or siRNAs. One such example is rice *miR820* (Chellappan et al., 2010; Wu et al., 2010; Nosaka et al., 2012). It is classified as a miRNA because it is produced from a single transcript with potential fold back structure and because it cleaves its *trans* target mRNA encoding DRM2. In addition, *miR820* is highly expressed as with other conserved miRNAs (miRBase; http://www.mirbase.org/). However, it also has similar character to siRNAs. For example, it is originated from transposons, its size is 22 or 24 nt, and these two forms are processed by DCL1 and DCL3, respectively (Cao et al., 2000; Cao and Jacobsen, 2002; Sharma et al., 2009; Henderson et al., 2010). In a previous study, we have shown that the role of *miR820* is to enable TEs to suppress *OsDRM2*, the major effector of host defense (Nosaka et al., 2012). However, it is still unknown how *MIR820* is transcribed.

In this study, we show that most of CACTA TEs carrying *MIR820* are transcriptionally inactive and harbor chromatin relatively enriched in silent histone marks. High expression of *miR820* solely depends on transcription from one CACTA TE on chromosome 7, *MIR820b*. Epigenetic marks, such as DNA methylation and histone modification in the *MIR820b* region, show a unique pattern different from four other copies. We conclude that *MIR820b* transcription may depend on epigenetic modifications, which may be in turn established by the action of *miR820* in *cis*. Thus, *miR820* appears to act both on *cis* and *trans* targets, and its own transcription is under epigenetic control.

MATERIALS AND METHODS

Plant materials and growth conditions Rice cultivars Nipponbare and Yukihikari were used. Plants were grown in soil or in tissue culture boxes at 29°C under continuous light.

RNA purification, PCR and sequencing Total RNA was purified from seedlings with the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA (50 ng) was reverse-transcribed by using Omniscript Reverse Transcriptase (QIAGEN) with random primers (N_9). PCR was performed by using *Ex Taq* DNA polymerase (TaKaRa). Primers used for RT-PCR are listed in Table 1. Amplified bands were gel-purified, cloned, and sequenced.

5′ RACE Total RNA (3 μg) was subjected to RNA oligo ligation with the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. The oligo-ligated RNA was reverse-transcribed by using Omniscript Reverse Transcriptase (QIAGEN) with random primers (N_9). PCR and nested PCR were performed by using *Ex Taq* DNA polymerase (TaKaRa). Primers used for 5′ RACE PCR are listed in Table 1. Amplified bands were gel-purified, cloned, and sequenced.

ChIP-qPCR Chromatin immunoprecipitation was performed as described previously (Miura et al., 2009) with the anti-H3K4me2 (ab1012), anti-H3K4me2me3 (ab6000), anti-H3K9ace (ab12179), and anti-H3K9me2 (ab1220) antibodies (Abcam). The immunoprecipitates were analyzed by quantitative PCR (qPCR) on a StepOnePlus Real-Time PCR system (Applied Biosystems). The qPCR reactions contained 5 μl 2× SYBR Premix *Ex Taq* II (Tli RNase H Plus) (TaKaRa), 0.5 μl DMSO, 0.2 μl 50× ROX reference dye, 1 μl immunoprecipitated DNA, and 400 nM of each primer. Each reaction was run in triplicate using a three-step cycling program (95°C for 15 s, 55°C for 30 s, 72°C for 30 s; 50 cycles). qPCR specificity was checked

Table 1. Primers and probes used in this study. Regions 1–3 (see Fig. 3A) are indicated after the chromosome numbers

Target genes, chromosomes, and regions	Sequence (5′ to 3′)	Purpose
pre-miR820	TGATGAATATCCTTACCAATCTTG	sequencing
	TGATAACGTAMGAACTACACCTCC	sequencing
C-kinase substrate	CGACTAAACCACTCCAATCATC	ChIP-qPCR
	CCAATCAAAACTTCTCCTGTAA	ChIP-qPCR
Centromere 8	CCGATATGCCAAAGAGCGAGTC	ChIP-qPCR
	CAAATCATCTATCCTCAAGTCC	ChIP-qPCR
CACTA (ORF1)	GTATGAATATGGCAAGCCGTT	ChIP-qPCR
	GAAACTGAAGGCGAAGTTTGC	ChIP-qPCR
pre-miR820	GGACACTGACATGGACTGAAGGAGTA	5′ RACE
	TGATAACGTAMGAACTACACCTCC	5′ RACE
pre-miR820 (chr.1-1)	AATGGGTCAGAGACACAACAA	ChIP-qPCR
	TCTTATCGTCGGAAGGGTCAC	ChIP-qPCR
pre-miR820 (chr.1-2)	GTTTGATAACAGAACAACTAC	ChIP-qPCR
	GAAATTATTCATCATTTACCA	ChIP-qPCR
pre-miR820 (chr.1-3)	TAGTTCGTACGTTATCATGCC	ChIP-qPCR
	AGGAACTAAACCGACTAGTAA	ChIP-qPCR
pre-miR820 (chr.7-1)	AATGGGTCAGAGACACAACAT	ChIP-qPCR
	TCTTATCATCGGAAGGGTCAT	ChIP-qPCR
pre-miR820 (chr.7-2)	GCTTGAGAACAGAACAACTAA	ChIP-qPCR
	GCAATTATTCATCATGAACCC	ChIP-qPCR
pre-miR820 (chr.7-3)	TAGTTCGTACGTTATCATGCT	ChIP-qPCR
	AGCAACTAAACCGACTAGTAG	ChIP-qPCR
pre-miR820 (chr.8-1)	AATGGGTCAGAGACACAACAG	ChIP-qPCR
	TCTTATCGTCGAAAGGGTCAA	ChIP-qPCR
pre-miR820 (chr.8-2)	GCTTGAGAACAGAACAGCTAC	ChIP-qPCR
	GAAATTATTCATCATTTACCA	ChIP-qPCR
pre-miR820 (chr.8-3)	TAGTTCTTACGTTATCATGCC	ChIP-qPCR
	AGGAACTAAACCGACCAAGCC	ChIP-qPCR
pre-miR820 (chr.10-1)	AATGGATCAGAGAACAGAACAG	ChIP-qPCR
	TCTTATCGTCGGAAGGGTCAC	ChIP-qPCR
pre-miR820 (chr.10-2)	GCTTGAGAACAGACAACTAC	ChIP-qPCR
	TGGTAAGGATATTCATCACC	ChIP-qPCR
pre-miR820 (chr.10-3 & chr.12-3)	TAGTTCGTACGTTATCATGCC	ChIP-qPCR
	AGGAACTAAACCGACCAAGCC	ChIP-qPCR
pre-miR820 (chr.12-1)	AATGGGTTAGAGACACAACAG	ChIP-qPCR
	TCTTATCGTCAGAAGGGTCAC	ChIP-qPCR
pre-miR820 (chr.12-2)	GCTTGAGAACAGAACAACTAC	ChIP-qPCR
	GAAATTATTCATCATTTACCA	ChIP-qPCR

for each run with a dissociation curve at 95°C–60°C. The data were analyzed by using the standard curve method. The enrichment relative to input DNA was used to normalize the qPCR output. Primers used for qPCR are listed in Table 1. The *C-kinase substrate* and *Centromere 8-30* (*Cen 8-30*) genes were used as controls for euchromatic and heterochromatic genes, respectively (Nagaki et al., 2004); PCR primers were as designed by these authors.

Bisulfite sequencing Total RNA (10 μg) from Yukihikari seedlings was subjected to bisulfite treatment with the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. Primers used for bisulfite sequencing are listed in Table 1.

qPCR Relative expression levels were quantified by qPCR performed by using the One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) on a StepOnePlus Real-Time PCR system (Applied Biosystems). The reactions contained 5 μl 2× One Step SYBR RT-PCR Buffer 4, 0.5 μl DMSO, 0.4 μl PrimeScript One step Enzyme Mix 2, 0.2 μl 50× ROX reference dye, the equivalent of 50 ng total RNA, and 400 nM of each primer. Each reaction was run in triplicate. The mixtures were reverse-transcribed at 42°C for 5 min, and amplified by using a two-step cycling program (95°C for 5 s, 60°C for 20 s; 40 cycles). qRT-PCR specificity was checked for each run with a dissociation curve at 60°C–95°C. The data were analyzed by the standard curve method. The housekeeping gene *OsGAPDH* was used to normalize the qRT-PCR output. Primers used for qPCR are listed in Table 1.

RESULTS

CACTAs carrying *MIR820* are epigenetically silenced *MIR820* is a TE-encoded gene that targets *OsDRM2*, a gene required for the host defense against parasitic DNA such as TEs (Nosaka et al., 2012). No transpositionally active TEs have been reported in the Nipponbare genome under standard cultivation conditions. Histones associated with inactive TEs bear modifications typical for heterochromatin, and transcription in these regions is low. However, *miR820* is highly expressed from CACTA TEs. In order to clarify the mechanism of *MIR820* transcription from silent TEs, we determined the chromatin state in the transposase regions of CACTAs carrying *MIR820* by using ChIP-qPCR with primers that amplify all five copies simultaneously (Fig. 1). The recovery of chromatin by the antibodies recognizing the euchromatic marks (H3K4me2, H3K4me2me3, H3K9ace) was low. In contrast, high recovery was observed for the heterochromatic mark (H3K9me2) (Fig. 1). Thus, all five copies of CACTAs carrying *MIR820* seem to be in the silenced chromatin context at least in this region.

***MIR820* is mostly transcribed from one of the five CACTA copies** Although the alignment of the sequences of CACTAs carrying *MIR820* showed that they are very similar, we found several single-nucleotide poly-

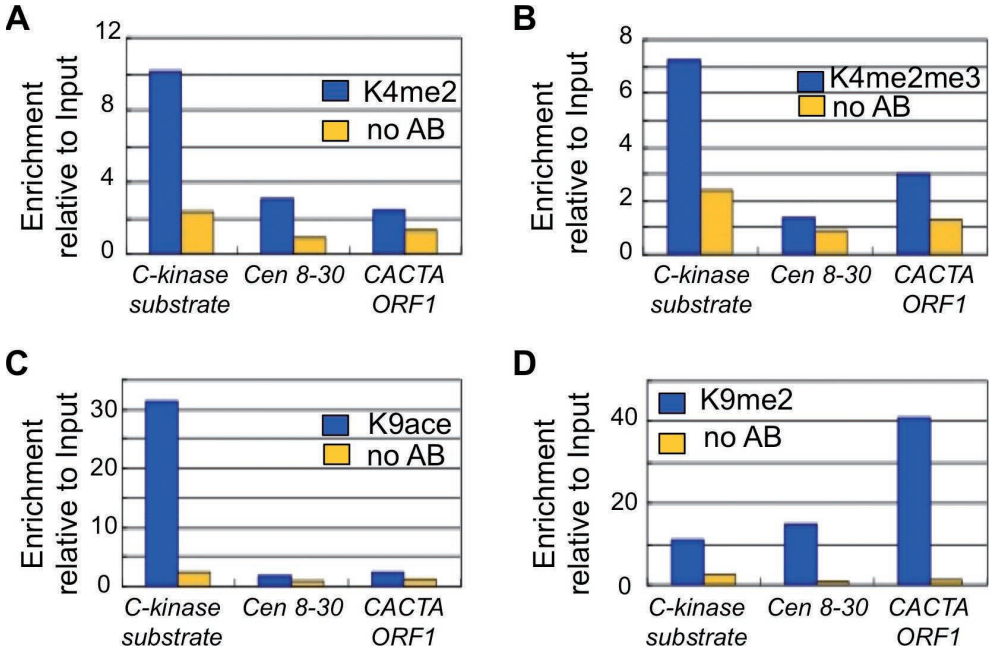

Fig. 1. *miR820*-CACTA is located in a heterochromatic region. Chromatin immunoprecipitation was performed with antibodies that detect active euchromatic marks (H3K4me2 (A), H3K4me2 and H3K4me3 (B), and H3K9ace (C)) or a constitutive heterochromatic mark (H3K9me2 (D)). As a negative control, immunoprecipitation was performed without an antibody (yellow boxes). *C-kinase substrate* and *Centromere 8-30* were used as controls for euchromatic and heterochromatic regions, respectively. Values are means of three technical replicates.

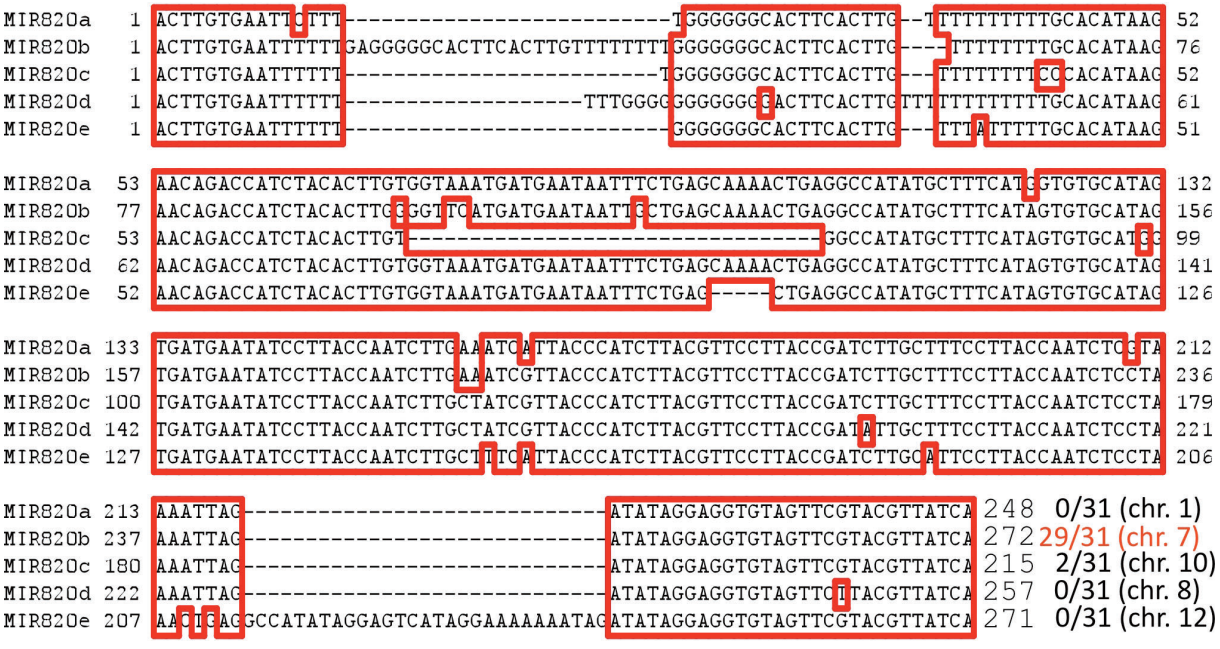

Fig. 2. *pre-miR820* is mainly transcribed from *MIR820b* on chromosome 7. The nucleotide sequence alignment of *pre-miR820* transcribed from different chromosomes is shown. The red boxes indicate the conserved residues. The numbers next to the alignment denote the number of clones with the nucleotide sequence of each *pre-miR820* out of 31 clones sequenced.

morphisms (SNPs) and insertions/deletions among the five copies (Fig. 2). We used these polymorphisms to distinguish the primary transcripts of *MIR820* of different origin. We amplified the corresponding fragments by RT-PCR with primers that recognize all five copies, followed by cloning and sequencing. Among 31 clones obtained, 29 and 2 clones had sequences identical to the *MIR820b* and *MIR820c* regions on chromosomes 7 and 10,

respectively (Fig. 2). Thus, *MIR820b* is mainly transcribed.

Histone modifications in the *MIR820* loci Because *miR820* is actively produced from CACTA on chromosome 7, it is possible that the local histone modifications in the

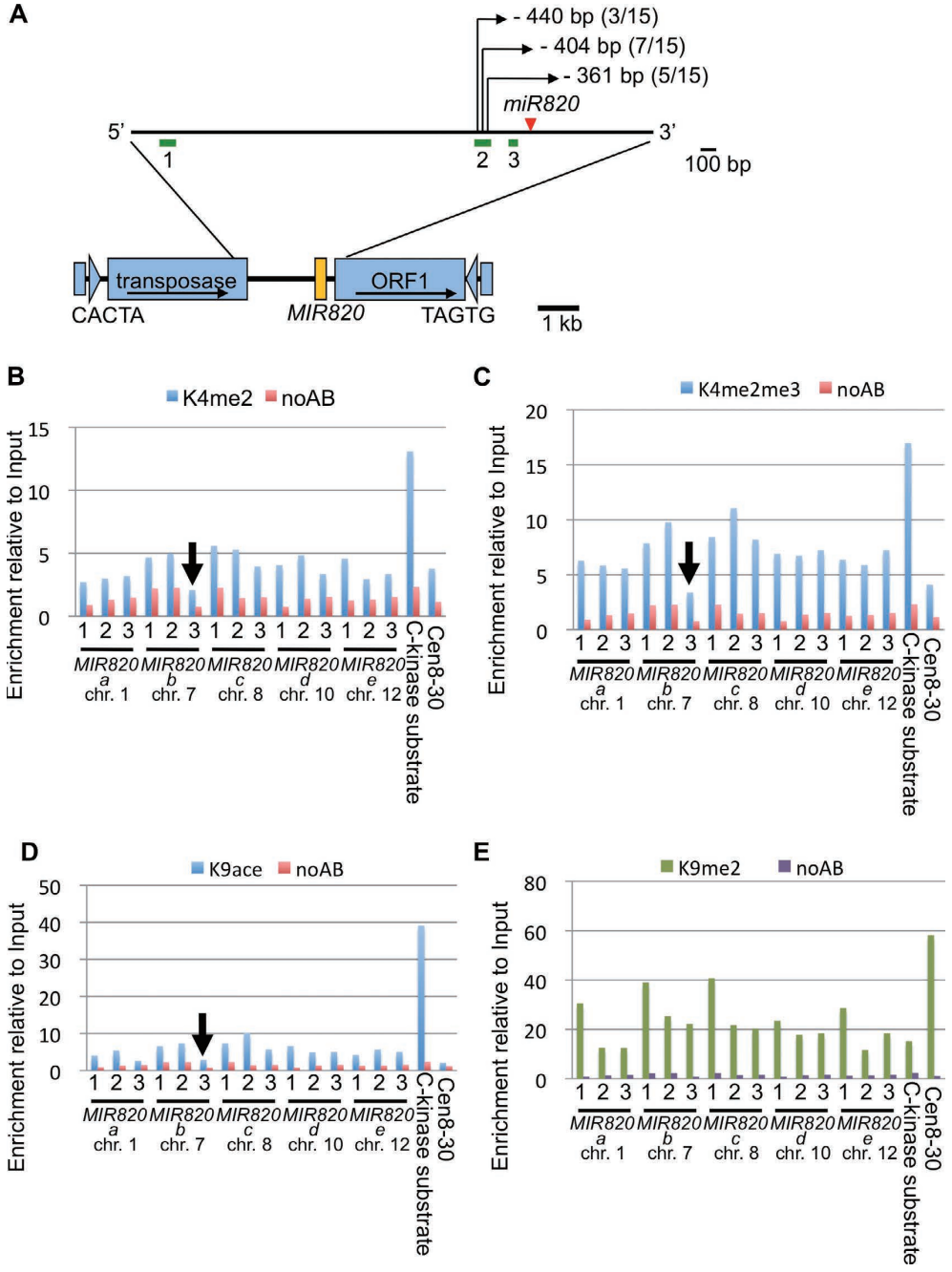

Fig. 3. Characterization of *pre-miR820* transcripts. (A) Location of *miR820* within the *CACTA* transposon (lower panel) and the position of the 5′ ends of *pre-miR820* transcripts (upper panel). The black line indicates the *CACTA* transposon genomic region. The red triangle indicates the position of *miR820*. The right-angled arrows show the position of the 5′ ends of *pre-miR820* transcripts. The numbers following the arrows show the position of the 5′ end of each transcript relative to the 5′ end of *miR820*. The numbers of clones sharing the same 5′ ends are shown in parentheses (out of 15 clones analyzed). ChIP-qPCR was performed in the regions shown by the green lines; the numbers (1–3) correspond to the numbers under the *x* axes in (B–E). ChIP-qPCR was performed with antibodies against H3K4me2 (B), H3K4me2 and H3K4me3 (C), and H3K9ace (D), which detect active euchromatic marks, and H3K9me2 (E), which detects a constitutive heterochromatic mark. Equal amounts of input DNA and the immunoprecipitates with or without antibodies (no AB) were analyzed and normalized against input DNA. Values are means of three technical replicates. *C-kinase substrate* and *Centromere 8-30* are controls for euchromatic and heterochromatic regions, respectively.

promoter region and/or in the vicinity of the *MIR820b* transcriptional unit could be different from the other four copies. Therefore, we tried to identify the transcription start site of *MIR820b* by 5′ RACE analysis. We could not detect any PCR product amplified from 5′-capped or triphosphate RNAs (data not shown), but we successfully amplified the 5′ monophosphate RNA. Three positions of 5′ ends of *MIR820b* transcripts were detected at approximately 400 bp upstream of the *miR820* coding region (Fig. 3A). Because the 5′ monophosphate end is produced by nuclease digestion, it is possible that these amplified fragments are not primary transcripts but are processed. However, these regions amplified by modified 5′ RACE are at least in the transcriptional units.

Next, we examined the histone modification state around the transcriptional unit of each *MIR820a-e* on chromosomes 1, 7, 8, 10, and 12, respectively, by ChIP-qPCR (Fig. 3, B–E). We designed three pairs of PCR primers that amplify the 3′ end of the transposase ORF, the region recovered as the 5′ end of *MIR820*, and the region just upstream of the *miR820* coding region (Fig. 3A; regions 1–3, respectively). The recovery of chromatin by the antibodies recognizing the euchromatic marks (H3K4me2, H3K4me2me3, H3K9ace) was low in these regions for all five *MIR820* copies compared to a region in the *C-kinase substrate* gene (an actively transcribed gene), and mostly higher than that of *Cen8-30* (an authentic heterochromatic sequence in rice) (Fig. 3, B–D). The recovery of chromatin in these three regions by the H3K9me2 antibody was higher than (in particular, in region 1 from all five copies) or similar to that for *C-kinase substrate* (Fig. 3E). Only region 3 of *MIR820b* on chromosome 7 showed low active marks compared to all other regions in all CACTAs carrying *MIR820*. There was no obvious change in the silent marks of histone modification in this region among five copies. Thus, the histones in region 3 on chromosome 7 have a unique modification pattern.

***MIR820b* on chromosome 7 has a high level of asymmetric cytosine methylation around the *miR820* coding region** Because the histone modification state around the *miR820* coding region of *MIR820b* on chromosome 7 was different from those on other chromosomes, we suspected that the DNA methylation in this region could also be different. We analyzed the DNA methylation of *MIR820a-d* (including *miR820* and *miR820** coding regions) by bisulfite sequencing (Fig. 4). In all copies tested, the ratio of cytosine methylation at most of CG and CHG sites around *miR820* and *miR820** coding regions was 80–100%. On *MIR820a*, *MIR820c* and *MIR820d*, the average DNA methylation at CHH sites within this region was 4.0, 2.6 and 3.8%, respectively, and was much lower (or even undetectable) than at the adjacent CG or CHG sites, whereas on *MIR820b*, the average DNA methylation at CHH sites within this region was 14.4%.

Thus, both the histone modifications and DNA methy-

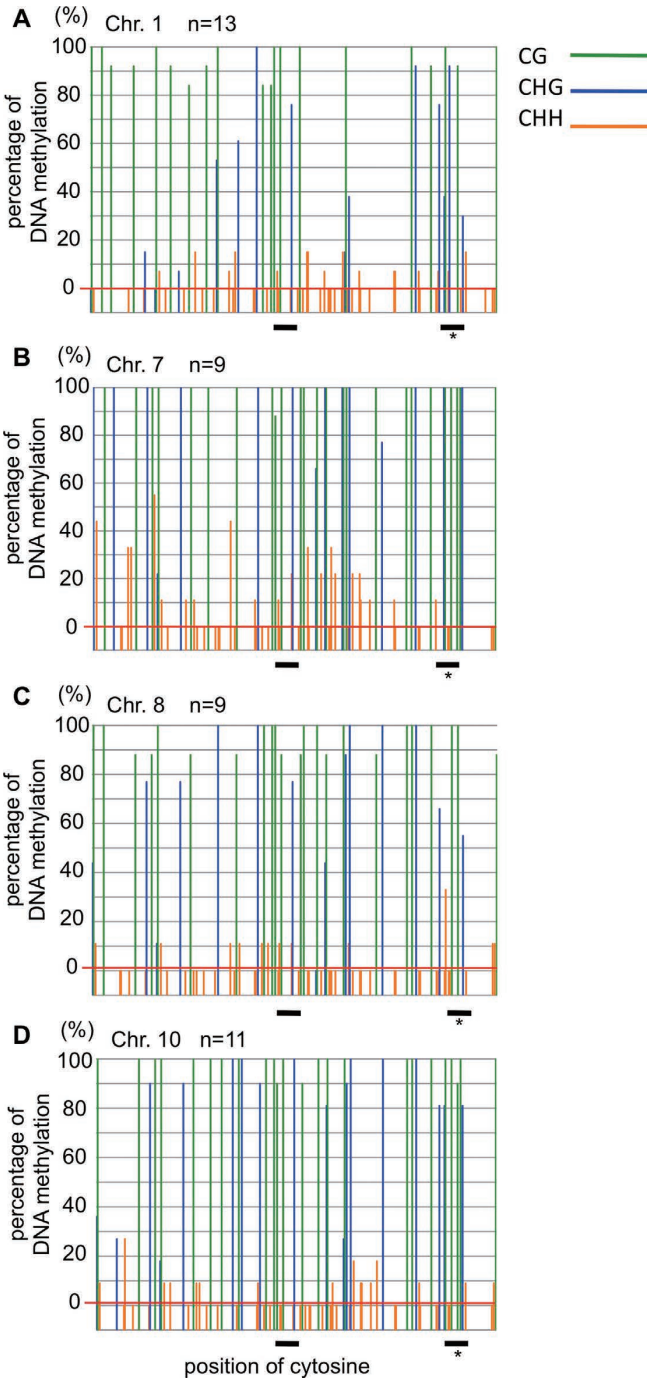

Fig. 4. DNA methylation in the *pre-miR820* region. Bisulfite sequencing analysis of *pre-miR820a* (A), *pre-miR820b* (B), *pre-miR820c* (C), and *pre-miR820d* (D) regions on their respective chromosomes. The colored lines show the positions of individual cytosines; those extending above the horizontal line indicate methylated sites. The height of the colored lines indicates the percentage of methylation. *miR820* and *miR820** are indicated by black bars without and with an asterisk, respectively. The n value represents the number of clones analyzed.

lation around the *miR820* coding region on *MIR820b* on chromosome 7 are different from other copies.

DISCUSSION

Because TEs are potentially harmful for their host, most of them are silenced by the host defense machinery, and they are located in heterochromatic regions. This defense machinery is activated by siRNAs derived from genomic parasites such as TEs. These siRNAs guide the silencing machinery to the genomic region where they originated, or to the homologous sequence(s) in the genome to epigenetically silence them. Our previous report showed that *miR820* acts against the host silencing machinery by attenuating *OsDRM2* (Nosaka et al., 2012). Thus, it seemed plausible that CACTA TEs carrying *MIR820* could be active; however, so far there has been no evidence of active DNA transposons in Nipponbare under standard cultivation conditions. Here we found that *MIR820b* is actively transcribed from a CACTA TE copy on chromosome 7. This finding raised the question of why only this copy is capable of transcription, and how it differs from the four other copies.

The ChIP-qPCR analysis and bisulfite sequencing around *MIR820* regions gave us some hints on this issue. Histones in region 3 of the *MIR820b* locus on chromosome 7 have low levels of active marks (H3K4 di/tri methylation and H3K9 acetylation). Despite this, *MIR820b* is the major transcribed copy. We found that the low level of active marks in this copy correlates with the high level of asymmetric cytosine methylation (CHH) in the same region. Although we are unable to depict the molecular framework of how this pattern of epigenetic modifications in region 3 allows transcription of *MIR820b*, it is plausible that these unique modifications allow transcription from epigenetically silenced TE.

A unique feature of *miR820* is that it exists as both 22- and 24-nt species. We and another group have shown that *miR820* cleaves *OsDRM2* mRNA, and also induces DNA methylation at the *miR820* recognition site in the *OsDRM2* locus. In the present study, the high level of CHH methylation was observed mostly within and around the *miR820* coding region of *MIR820b* on chromosome 7. This pattern of DNA methylation may also be induced by *miR820*. The high level of CHH methylation is only observed in *miR820* encoded on chromosome 7, whereas the corresponding regions on chromosomes 1, 8, and 10 should be also recognized by *miR820*. One possible explanation is that *miR820*-directed CHH methylation is coupled with transcription, and this is why *miR820* acts in *cis* but has no effect on *miR820* coding regions on other chromosomes.

We propose a model for the mode of action of *miR820* (Fig. 5). *Pri-miR820* transcripts derived from *MIR820b* on chromosome 7 are processed into mature *miR820*.

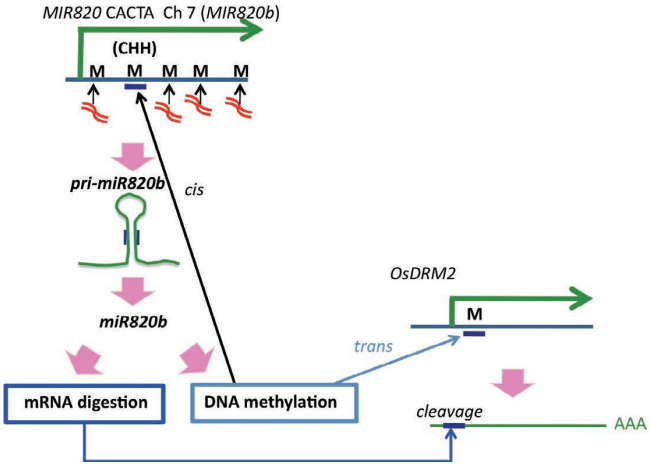

Fig. 5. A model for the action of *miR820*. *pri-miR820* transcripts are mainly transcribed from the *MIR820b* locus on chromosome 7. *miR820* processed from *pri-miR820b* induce CHH DNA methylation (M) in the *MIR820b* genomic region (in *cis*). This could allow *miR820* transcription from this locus. *miR820* also induces *OsDRM2* mRNA digestion and DNA methylation at the *miR820* target site in the *OsDRM2* genomic region (in *trans*). Thus, *miR820* has two functions, regulating its own transcription in *cis* and *OsDRM2* expression in *trans*.

This induces both *OsDRM2* mRNA cleavage and DNA methylation at the *miR820* target site in the *OsDRM2* genomic region (in *trans*). *miR820* also induces CHH methylation in the *miR820* genomic region of *MIR820b* on chromosome 7 (in *cis*) and epigenetically regulates its own transcription. Thus, *miR820* is a unique miRNA that acts both in *cis* and in *trans*, and its own transcription is under epigenetic control. Typically, TE-derived small RNAs are siRNAs that act in *cis*, whereas miRNAs act in *trans*. Our data confirms that *miR820* has features resembling both miRNA and siRNA, possibly because it is encoded by a parasitic gene that uses the host machinery to counteract silencing.

This work was supported in part by a Grant-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant #21027017), and PRESTO program from JST to YS.

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