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 suppressing 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 RNaseasy 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 (N9). 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 oligoligation 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 (N9). 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.

<table>
<thead>
<tr>
<th>Target genes, chromosomes, and regions</th>
<th>Sequence (5′ to 3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-miR820</td>
<td>TGATGAAATCCTTACACATCTTG</td>
<td>sequencing</td>
</tr>
<tr>
<td></td>
<td>TGAAACGTGAACTACATCCTC</td>
<td>sequencing</td>
</tr>
<tr>
<td>C-kinase substrate</td>
<td>CGACTAAACCTCACTGATC</td>
<td>ChiP-qPCR</td>
</tr>
<tr>
<td></td>
<td>CAAAACATCTCTCTGTAA</td>
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</tr>
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<td>Centromere 8</td>
<td>CCGATATGCAAGAGGCAAGTC</td>
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<tr>
<td></td>
<td>CAAATCATCTCTCTAGTCC</td>
<td>ChiP-qPCR</td>
</tr>
<tr>
<td>CACTA (ORF1)</td>
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<td>ChiP-qPCR</td>
</tr>
<tr>
<td></td>
<td>GAAAATGAAGGCAAGTTGCC</td>
<td>ChiP-qPCR</td>
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<tr>
<td>pre-miR820</td>
<td>TGAAACGTGAGAGCTGAGATGTA</td>
<td>5′ RACE</td>
</tr>
<tr>
<td></td>
<td>TGAAACGTGAGAGCTGAGATGTA</td>
<td>5′ RACE</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>TCTTATGCTGAGAGGTCAC</td>
<td>ChiP-qPCR</td>
</tr>
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<tr>
<td></td>
<td>GAAATTTGCATCATTTACCA</td>
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<td>AGGAAACTAAACCGATGAA</td>
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<td>AGGAAACCTAAACCGATGAA</td>
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<td>GAAATTTGCATCATTTACCA</td>
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<td></td>
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<td>pre-miR820 (chr.10-1)</td>
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<td></td>
<td>TGTTAAGAATTTGTATCA</td>
<td>ChiP-qPCR</td>
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<td>pre-miR820 (chr.10-3 &amp; chr.12-3)</td>
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<tr>
<td></td>
<td>AGAATTTACCCGAGCAACAGCC</td>
<td>ChiP-qPCR</td>
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<tr>
<td>pre-miR820 (chr.12-1)</td>
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<td>ChiP-qPCR</td>
</tr>
<tr>
<td></td>
<td>GCTTGAGAACGAAGAACACAT</td>
<td>ChiP-qPCR</td>
</tr>
<tr>
<td>pre-miR820 (chr.12-2)</td>
<td>GAAATTTACCCGAGCAACAGCC</td>
<td>ChiP-qPCR</td>
</tr>
</tbody>
</table>

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 TEIs. 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, H3K4me3, H3K9ac) 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-
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, \( M\text{IR820b} \) 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

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**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 methylation...
loration 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. 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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