De novo DNA methylation induced by siRNA targeted to endogenous transcribed sequences is gene-specific and OsMet1-independent in rice

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Summary
Small interfering RNA (siRNA) is an essential factor for epigenetic modification of the genome. Recent studies have suggested that endogenous siRNAs induce DNA methylation, chromatin modification and chromatin inactivation at homologous sequences. We have shown that siRNAs targeted to promoter regions of endogenous rice genes induce strong DNA methylation of the targeted sequences, but transcriptional gene silencing is rarely observed. Here, an analysis of epigenetic modifications induced by RNAi targeted to transcribed regions of endogenous rice genes shows that the effects of siRNA are gene-specific, but that they tend to induce higher de novo DNA methylation of CpG dinucleotides than of other cytosines. However, loss of OsMet1 expression by RNAi did not significantly affect levels and patterns of de novo DNA methylation or post-transcriptional mRNA suppression. We also showed that sequence-specific de novo DNA methylation extended both 5′ and 3′ of the targeted sequences, but there was no significant extension of siRNA signals either 5′ or 3′. These results suggest that exogenous siRNAs are strong inducers of de novo DNA methylation in transcribed sequences of rice endogenous genes, but are insufficient to induce heterochromatin formation.

Keywords: RNAi, de novo DNA methylation, RdDM, rice, OsMet1.

Introduction
Small interfering RNAs (siRNA), which are processed from double-stranded RNA (dsRNA) by RNase III domain-containing enzymes such as Dicer, play a key role in gene silencing (Vaucheret, 2006). siRNA is incorporated into an RNA-induced silencing complex (RISC), which then recognizes and cleaves homologous target mRNA. In plants, strong methylation of suppressed gene sequences is associated with a similar phenomenon, known as post-transcriptional gene silencing (PTGS) (English et al., 1996; Ingelbrecht et al., 1994; Jones et al., 1998; Morel et al., 2000). Transposons are reactivated in mutants defective in RNA interference (RNAi) in Arabidopsis (Baulcombe, 2004). siRNA-mediated heterochromatin formation in Schizosaccharomyces pombe requires Dicer, Rdp1 and an RNA-induced initiator of transcriptional gene silencing complex that contains siRNA and ARGONAUTE 1 (AGO1) (Grewal and Jia, 2007). Thus, RNAi functions not only in post-transcriptional mRNA cleavage, but also in the epigenetic regulation of DNA sequences that are homologous with siRNAs (Wassenegger, 2005).

RNA-mediated epigenetic modifications are due to RNA-directed DNA methylation (RdDM) and RNAi-dependent heterochromatin formation. RdDM was first observed in transgenic tobacco plants that carried the potato spindle tuber viroid sequence as a transgene (Wassenegger et al., 1994). Infection and replication of potato spindle tuber viroid caused de novo DNA methylation of the corresponding transgene (Wassenegger et al., 1994). Sequence-specific de novo DNA methylation was also induced by PTGS and virus-induced gene silencing (VIGS) within the corresponding genomic region (Wassenegger, 2005). In plants, siRNAs show homology with endogenous heterochromatin repeats, transposable elements and non-repetitive sequences (Nozue et al., 2007; Qi et al., 2006; Xie et al., 2004). RdDM related endogenous 24 nt length siRNA is mainly produced by RNA polymerase IV (PolIV), RNA-dependent RNA polymerase 2
(RDR2), Dicer-like 3 (DCL3) and AGO4 in Arabidopsis (Matzke et al., 2007; Vaucheret, 2006). This 24 nt siRNA is associated with PTGS and VIGS, and acts as a de novo DNA methylation signal along with endogenous siRNA (Hamilton et al., 2002). Thus, exogenous siRNAs apparently mimic the function of endogenous siRNAs with respect to histone modifications within the corresponding euchromatin regions.

RNA-mediated transcriptional gene silencing (TGS) is mediated by siRNAs derived from transgenes and targeted promoter regions, which induce RdDM and chromatin inactivation of homologous promoters in plants (Matzke et al., 2004; Mette et al., 2000). The activities of the methyltransferase genes Methyltransferase 1 (Met1), Chromomethylase 3 (Cmt3) and Domains rearranged methyltransferase (Drm1, Drm2) are also necessary for RNA-mediated TGS in Arabidopsis (Aufsatz et al., 2004; Cao et al., 2003). DRM2 is a major de novo DNA methyltransferase that has redundant functions in non-CpG maintenance methylation. MET1 is the homolog of the animal gene Dnmt1, and is required for maintenance and de novo DNA methylation of CpG in Arabidopsis (Aufsatz et al., 2004). PTGS is reactivated in Arabidopsis met1 mutants (Morel et al., 2000) and in OsMet1 knock-down rice cells (Teerawanichpan et al., 2004). These observations suggest that CpG DNA methylation is correlated with RNA silencing in plants.

In rice, exogenous siRNA strongly induced cytosine methylation in CpG, CpHpG and CpHpH contexts at each of the seven targeted promoters analyzed, but the 35S–GFP transgene only showed chromatin modifications and TGS, the seven targeted promoters analyzed, but the 35S–GFP transgene. These RNAi-mediated epigenetic modifications in rice. The results suggest that siRNA is a strong inducer of de novo DNA methylation of transcribed sequences in rice, and that the relative positions of DNA sequences in the genome may be important for the regulation of siRNA-mediated epigenetic modifications in rice.

**Results**

**De novo DNA methylation of transcribed sequences targeted by RNAi in rice**

Previously we reported that each of the seven highly homologous members of the OsRac gene family was specifically suppressed by its cognate RNAi (Figure 1a) (Miki et al., 2005), and that siRNAs induce DNA methylation at each of the endogenous target promoters, but rarely induce transcriptional suppression in rice (Okano et al., 2008). The question in the present study is whether RdDM occurs in transcribed sequences of endogenous genes that are suppressed. To address this question, DNA methylation analysis was performed using four independent RNAi transgenic lines of OsRac5 or OsRac7. OsRac5 and OsRac7 were specifically suppressed in these RNAi lines, and two size classes of siRNA were detected (Figure 1b,c). Southern hybridization was performed to identify de novo DNA methylation using cytosine methylation-sensitive restriction enzymes. Both symmetric (CpG and CpHpG; H is A, C or T) and asymmetric (CpHpH) cytosine residues were de novo methylated in both OsRac5 and OsRac7 RNAi transgenic rice lines (Figure 1d,e).

Interestingly, strong de novo DNA methylation was found at CpG sites in the target endogenous transcribed sequences. In contrast, cytosine methylation of the corresponding regions of the trigger transgenes was much higher than in endogenous target sequences for all three types of cytosine methylation (Figure 1f,g). In OsRac5 and OsRac7 RNAi transgenic rice, de novo DNA methylation was observed in corresponding genomic regions. These data suggest that de novo DNA methylation within transcribed sequences targeted by gene-specific RNAi is RNA-directed DNA methylation.

Most of the cytosine residues of the endogenous OsRac5 RNAi target region were methylated de novo in the OsRac5 RNAi lines (Figure 2a), with approximately 80% of CpG and 20% of CpHpG and CpHpH sites methylated (Figure 2b). However, the corresponding sequence of the trigger transgene was more heavily methylated at all CpG, CpHpG and CpHpH cytosine residues than the endogenous target region (Figure 2b).

Transgenic rice plants in which OsRac3, OsRac4, OsRac6, OsRac7 and OsGEN-L were silenced (Miki et al., 2005; Moritoh et al., 2005) were also analyzed (Figure 3). Plants in which OsRac3 and OsRac7 were silenced had higher CpG methylation of endogenous target regions than the two controls (Figure 3). In contrast, CpG, CpHpG and CpHpH cytosine methylation in the OsRac3 and OsRac7 trigger transgenes were all much higher than in the endogenous target regions. On the other hand, CpHpG and CpHpH were more highly methylated on the trigger transgene in OsRac6 silenced plants. In OsRac4 and OsGEN-L RNAi plants, small changes in DNA methylation were observed for all three types of cytosine methylation in the target endogenous sequence (Figure 3). These results showed that levels and patterns of DNA methylation are gene-specific, but that CpG dinucleotides were more readily methylated than the other cytosine residues in three of the six genes analyzed.
OsMet1 silenced transgenic rice

Arabidopsis MET1, which shows not only maintenance but also de novo methylation activity for CpG sequences, is a well known methyltransferase (Aufsatz et al., 2004). We hypothesized that OsMet1 plays a role in RdDM induced by RNAi, because CpG is preferentially methylated within the target endogenous genes (Figures 2c and 3). The rice genome contains two Met1 homologs, OsMet1-1 and OsMet1-2 (Teerawanichpan et al., 2004). OsMET1-1 and -2 each have two bromo-adjacent homology (BAH) domains and one DNA methylase domain (catalytic domain), and these domains are highly conserved (Figure 4a and Figure S1a). OsMet1-1 and -2 were expressed in all tissues except small seedlings (Figure S2). Therefore, we generated OsMet1 RNAi transgenic rice plants (Mi) by targeting
544 bp of the highly conserved OsMet1-1 DNA methylase domain (Figure 4a and Figure S1b).

Previously, we have shown that RNAi triggers derived from highly conserved regions of homologous genes could suppress corresponding genes simultaneously, and that chimeric RNAi triggers could suppress two or more genes simultaneously (Miki et al., 2005). Because CpG is highly methylated in OsRac5 silenced plants, OsMet1-1 and OsRac5 chimeric RNAi transgenic rice plants (M5i) were generated to analyze de novo DNA methylation leading to RNAi in OsMet1 silenced plants (Figure 4b). Chimeric RNAi rice plants (G5i) targeting both GFP and OsRac5 were also generated as controls (Figure 4b). RT-PCR analysis was performed in three independent transgenic lines, and the results indicate that OsMet1-1 and -2 in Mi, OsMet1-1, -2 and OsRac5 in M5i, and OsRac5 in G5i were specifically suppressed (Figure 4c). Quantitative analysis of OsMet1-1, -2 and OsRac5 mRNA levels by real-time PCR in the Mi, M5i and G5i transgenic rice plants showed that the levels of targeted

![Figure 2](image1.png)

**Figure 2.** Bisulfite sequencing of endogenous OsRac5 targeted by RNAi. (a) Schematic representation of methylated cytosine residues of the OsRac5 RNAi targeted region. Four independent RNAi lines and wild-type (WT) were analyzed for the target gene. (b) Mean DNA methylation status. The methylation states of the cytosine residues within the OsRac5 RNAi target region were calculated in wild-type (WT), four independent RNAi lines, four independent control (OsRac7 RNAi) lines, and trigger transgenes of four independent RNAi lines.

![Figure 3](image2.png)

**Figure 3.** DNA methylation detected by bisulfite sequencing. Methylation states of cytosine residues were determined in the gene-specific RNA silencing transgenic plants. Endogenous target regions of wild-type (WT), four independent RNA silenced lines, four independent transgenic control lines, and trigger transgenes of four independent RNA silenced lines were analyzed.
mRNAs were <25% of those in the wild-type (Figure S3). Two classes of corresponding siRNAs were also detected in independent transgenic rice plants (Figure 4c). We then silenced the OsMet1 genes to determine whether they are essential for CpG methylation. Total DNA was digested with HpaII and hybridized with the CentO probe, which is a repeat sequence of the rice centromere. Reduction of CpG methylation was clearly detected at the CentO repeat in Mi and M5i, in which OsMet1 genes were suppressed, but not in wild-type or G5i plants (Figure 4d). CpG DNA methylation also decreased within the CRR2 retrotransponson (Figure S4). There were no alterations of DNA methylation at the CentO repeat when hybridizations were performed using a CpHpG methylation-sensitive restriction enzyme (Figure S4). These results indicate that OsMET1 enzymes play a key role in CpG methylation in rice. In the Arabidopsis met1-3 mutant, which has a complete loss-of-function allele of the AtMet1 gene, asymmetric DNA methylation is higher at the 5S rDNA repeat and gypsy-class LTR retro-elements (Mathieu et al., 2007). A slight increase in CpHpH methylation was observed when NlaIII-digested DNA was hybridized with the CentO probe in some OsMet1 silenced rice plants (Figure S4), suggesting that the OsMet1 genes are functional orthologs of AtMet1.

OsMet1 does not affect RNAi-associated DNA methylation

There was no difference in the DNA methylation levels of endogenous OsRac5 sequences between wild-type and Mi as determined by hybridization (Figure 4e). De novo DNA methylation was also observed in M5i and G5i, but there was no significant difference in methylation at the three cytosine patterns between M5i and G5i (Figure 4e). Detailed analysis of methylation by bisulfite sequencing demonstrated that endogenous OsRac5 was strongly methylated in M5i and G5i, but not in wild-type or Mi. In M5i, CpG was heavily
methylated, as were OsRac5 RNAi transgenic rice and G5i, even though its OsMet1 genes were silenced (Figure 5a). There were no significant differences in mean DNA methylation levels between M5i and G5i with respect to cytosine patterns (data not shown). Thus, all cytosine residues were compared between M5i and G5i. These results showed that methylation of CpHpG and CpHpH, but not CpG, were clearly reduced in M5i (Figure 5b). OsMet1 may thus slightly affect non-CpG methylation, but is not involved in CpG methylation induced by RNAi in rice.

**Spreading DNA methylation**

The Aci site that is located upstream of the RNAi-targeted region was specifically methylated in OsRac7 RNAi transgenic rice plants (Figure 1e; asterisk). We confirmed that the methylation at this Aci site was specifically induced by OsRac7-targeted RNAi using another probe (data not shown). These results suggest that RNAi-induced de novo DNA methylation could spread beyond the targeted sequence. Thus, we analyzed approximately 350 bp on each side of the OsRac5 RNAi targeted region by the bisulfite sequencing method, which showed that de novo DNA methylation extended both 5¢ and 3¢ from the RNAi target.

![Figure 5. DNA methylation analysis of endogenous OsRac5 targeted by RNAi in OsMet1 silenced lines. (a) Schematic representation of methylated cytosine residues of the OsRac5 RNAi-targeted region. The same sequence as in Figure 2(a) was analyzed after bisulfite treatment. (b) Comprehensive representation of methylation status in the OsRac5 RNAi-targeted region of M5i and G5i. The relative values were calculated by subtracting the value of G5i from the value of M5i for each cytosine residue.](image)

![Figure 6. Bisulfite sequencing around the endogenous OsRac5 RNAi-targeted region. (a, b) Distribution of DNA methylation beyond the RNAi targeted sequence. DNA methylation of the 348 bp upstream (a) or 331 bp downstream (b) of the endogenous target OsRac5 was analyzed by bisulfite sequencing. Four independent RNA silenced lines and one wild-type (WT) plant were analyzed for the corresponding regions.](image)
region (Figure 6a,b). OsRac6 showed extended de novo methylation, but only in the 3’ direction (data not shown). We were not able to amplify the corresponding fragments by PCR after bisulfite treatment in other RNAi lines. Bisulfite sequencing of OsRac5 (Figure 6a,b) and OsRac6, and Southern hybridization analysis of OsRac7 (Figure 1g and data not shown), suggest that some de novo DNA methylation extension occurs in rice.

The maintenance function of OsMet1 genes may be involved in de novo DNA methylation extension, because there is no evidence for transitive RNAi of endogenous genes in rice (Miki et al., 2005). Bisulfite sequencing was used to determine whether OsMet1 is involved in methylation extension, but there was no methylation in either the 5’ or 3’ regions of wild-type or Mi plants. Extension was detected in both 5’ and 3’ directions in Mi5 and G5i, but there were no significant differences between the two lines (Figure S5). These results suggest that OsMet1 genes are unlikely to be involved in the RNAi-mediated extension of DNA methylation.

siRNA-induced de novo DNA methylation causes chromatin modifications leading to de novo CpG methylation (Vaillant and Paszkowski, 2007); thus sequences adjacent to the cognate siRNA sequence may be methylated due to localized chromatin modifications. To determine whether siRNA induces histone modifications on the endogenous OsRac5 RNAi target region, chromatin immunoprecipitation (ChIP) assays (Nagaki et al., 2004; Okano et al., 2008) were performed on the endogenous OsRac5 RNAi target region using two independent OsRac5 RNAi transgenic rice plants, with wild-type and OsRac7 RNAi lines as controls. Histone modifications of the cognate region in the OsRac5 RNAi lines were not significantly different from the two controls with respect to acetylation of lysine 9 of histone H3 or dimethylation of lysines 4 and 9 of histone H3 (Figure S6), although DNA methylation was slightly elevated. Furthermore, plants silenced for OsRac6 or OsRac7 by RNAi did not have chromatin modifications within the RNAi target region that were any different from those of the controls (data not shown). These results suggest that de novo DNA methylation is established on the cognate endogenous transcribed region, but no chromatin modifications are induced.

Discussion

DNA is highly methylated in regions of DNA associated with PTGS or VIGS in plants (English et al., 1996; Ingelbrecht et al., 1994; Jones et al., 1998; Morel et al., 2000), but some studies have shown that PTGS and VIGS does not induce RdDM (Jones et al., 1999; Sonoda and Nishiguchi, 2000). In addition, siRNAs derived from transgenes and promoter regions can induce RdDM and TGS at homologous promoters (Matzke et al., 2004; Mette et al., 2000; Okano et al., 2008). In Arabidopsis, RNA-mediated TGS requires not only de novo DNA methylation, but also histone H3 deacetylation, dimethylation of lysine 9 of histone H3 and monomethylation of lysine 27 of histone H3 (Auffsatz et al., 2002; Cao et al., 2003; Huettel et al., 2006). In rice, siRNAs targeted to promoter regions induce de novo DNA methylation, deacetylation of histones H3 and H4, demethylation of lysine 4 of histone H3, dimethylation of lysine 9 of histone H3 and TGS (Okano et al., 2008). As mentioned above, siRNAs are considered to be signal molecules that induce epigenetic modifications such as DNA methylation and chromatin modifications. Our results indicate that siRNA directly induces de novo DNA methylation, and patterns and degrees of DNA methylation are gene-specific. In three of the six genes examined, CpG dinucleotides were specifically methylated, but this CpG methylation does not depend on the OsMet1 genes. We also show that both 5’ and 3’ regions outside the siRNA corresponding sequences were de novo methylated.

CpG methylation in transcribed sequences

We show here that de novo DNA methylation occurs within endogenous genes targeted by RNAi. In particular, CpG dinucleotides are highly methylated compared with CphPg and asymmetric cytosine residues in some genes. In contrast, there was strong cytosine methylation in the CpG, CphPg and CphH contexts on the trigger transgene regions, even though their sequences were identical. One possible reason for these differences in DNA methylation patterns may be that the RdDM machinery recognizes structural differences between the endogenous and transgene DNA. For example, transitive RNAi of a GFP transgene was clearly demonstrated, but has not been observed in endogenous rice genes (Miki et al., 2005). Therefore, it is possible that endogenous genes are protected from hypermethylation, possibly by a demethylation enzyme such as ROS1 (Gong et al., 2002). Another possibility is that histone modifications occur only on trigger transgenes. In the Arabidopsis ago4 mutant, RNAi of endogenous genes was observed at a higher efficiency than in wild-type (Zilberman et al., 2004). These findings suggest that the RdDM machinery is involved in establishing DNA methylation on RNAi trigger transgenes in cis, as well as siRNAs derived from the RNAi trigger transgene by RNA polymerase II. However, target endogenous genes are only methylated by siRNA in trans.

The RNA silencing machinery induces heterochromatin formation in eukaryotes (Grewal and Jia, 2007; Wassenegger, 2008). There is genome-wide reduction of DNA methylation and alteration of chromatin modifications in dcl3, rdr2, ago4 and polIV mutants of Arabidopsis (Matzke et al., 2007; Wassenegger, 2005). siRNAs targeted to promoter regions induce TGS in Arabidopsis, tobacco, petunia, maize
and rice. RdDM has been observed in siRNA-mediated TGS in all plant species examined, but chromatin modifications of targeted promoters have only been reported in Arabidopsis and rice (Cigan et al., 2005; Jones et al., 1999; Melquist and Bender, 2003; Mette et al., 2000; Okano et al., 2008; Sijen et al., 2001). Based on these results, it was possible that RNAi targeted to endogenous transcribed sequences also induced chromatin modifications on the corresponding endogenous target sequence. However, no chromatin modifications such as acetylation of lysine 9 of histone H3 or dimethylation of lysines 4 and 9 of histone H3 were induced on the targeted sequences, although de novo DNA methylation was clearly detected (Figure S6). In the current study, none of the RNAi targeted regions showed high levels of dimethylation of lysine 27 of histone H3 (data not shown).

In transgenic rice plants in which siRNAs were targeted to promoter sequences, not only CpG but also the other cytosines were also strongly methylated within homologous endogenous and transgene promoter sequences (Okano et al., 2008). Steady-state DNA methylation levels are low in the promoter regions and 3' UTR of Arabidopsis (Zhang et al., 2006). However, basal levels of histone modifications are clearly different between promoters and sequences transcribed by RNA polymerase II (Shilatifard, 2006). These findings suggest that positional information is important for the epigenetic regulation of genomic sequences (Fischer et al., 2008; Johnson et al., 2007). It is possible that these contrasting observations are due to differences between the promoter and transcribed regions, and that cytosines in CpHpG and CpHpH are weakly methylated or demethyltransferases such as ROS1 removes target sequence methylation in some genes (Gong et al., 2002). Transcribed regions may also be protected from chromatin modifications by some unknown mechanism. However, the possibility that other types of chromatin modifications that were not examined in this study are induced by RNAi remains to be tested.

Function of OsMet1 genes in DNA methylation in rice

Two OsMet1 genes were identified in the rice genome that have previously been shown to be required for PTGS of the GFP transgene (Teerawanichpan et al., 2004). However, no biological function of OsMet1 genes has been reported. We generated transgenic rice in which both OsMet1 genes are silenced (Figure 4c), and showed that CpG methylation is clearly reduced on the centromere repeats and CRR2 retrotransposons in OsMet1 silenced transgenic rice (Figure 4 and Figure S4), and that asymmetric DNA methylation at the CentO repeats is slightly elevated in these plants (Figure S4). These results suggest that the OsMet1 genes are the functional orthologs of Arabidopsis Met1 (Vaillant and Paszkowski, 2007). However, further analysis is required to understand the detailed biological functions of OsMET1.

OsMet1 is not required for de novo CpG DNA methylation induced by RNAi

PTGS is gradually released in Arabidopsis met1 mutants (Morel et al., 2000). In rice, PTGS of the GFP transgene is suppressed in rice callus in which an OsMet1 RNAi construct was introduced by particle bombardment (Teerawanichpan et al., 2004). We thus wished to determine whether the OsMet1 genes are required for de novo DNA methylation in endogenous gene-targeted RNAi of rice. We show here that suppression of OsRac5 measured by real-time PCR was not altered in the OsMet1 silenced lines (Figure 4c and Figure S3). It has previously been shown that PTGS and DNA methylation on the GFP gene do not change under VIGS of endogenous Met1 in transgenic tobacco (Jones et al., 2001). Furthermore, initiation of GFP-targeted VIGS and de novo DNA methylation in the corresponding cognate region were observed when 35S–GFP transgenic tobacco plants were simultaneously infected with RVX-P and TRV–Met1 (Jones et al., 2001). Our results in rice support these findings, suggesting that these phenomena occur in monocot plants as well as dicots. PTGS and VIGS cause the reduction of target mRNA accumulation, but these plants did not show a null mutant phenotype (Wesley et al., 2001). Therefore, it is possible that a residual MET1 activity in the Met1 suppressed plants is sufficient to establish and maintain DNA methylation caused by RNAi on the targeted sequences.

In transgenic plants simultaneously silenced for OsMet1 and OsRac5, DNA methylation of CpHpG and CpHpH was slightly reduced, but CpG methylation was not altered (Figure 5b). One possible reason for this is the lower level of OsRac5 siRNA detected in M5i compared with G5i transgenic rice (Figure 4c). However, a similar relationship between siRNA and DNA methylation levels was observed in OsRac7 RNAi transgenic rice lines (Figure 1c,e). Thus, we conclude that the slight reduction of DNA methylation on the CpHpG and asymmetric cytosine residues is due to suppressed OsMet1. RNAi may initially induce de novo DNA methylation on CpG, and DNA methylation of the other cytosines might be established later (Mathieu et al., 2005). It was previously reported that methylation of CpHpG as well as CpG was decreased in Arabidopsis met1 mutants (Chan et al., 2005; Mathieu et al., 2007; Saże et al., 2003). Therefore, in the present study, reduction of CpG methylation maintenance activity in OsMet1 silenced lines may be the cause of reduced methylation of CpHpG and CpHpH. Our results indicate that OsMet1 genes may not play a key role in RNAi-mediated de novo DNA methylation in rice. De novo DNA methylation associated with RNAi may be established at the cognate sequence by DRM2 and CMT3 orthologs in rice as shown in Arabidopsis (Cao et al., 2003).
De novo DNA methylation extension

In this study, we observed that de novo DNA methylation associated with RNAi extended approximately 300 bp in both the 5’ and 3’ directions from the endogenous target. Similar results were found in transgenic plants that showed PTGS (English et al., 1996; Ingelbrecht et al., 1994; Zilberman et al., 2004). In tobacco, DNA methylation extends beyond the target sequence when the GFP transgene is targeted by VIGS. This spreading of DNA methylation was only observed for the GFP transgene. Thus, RdDM may occur outside target sequences associated with transitive RNA silencing (Jones et al., 1999). There is, however, no evidence for transitive RNAi of endogenous genes in rice (Miki et al., 2005). To test this observation, an RNase protection assay, which is more sensitive than Northern hybridization, was performed, but no detectable spreading of siRNA signals in either the 5’ or 3’ direction was detected (Miki et al., 2005). RDR is unlikely to synthesize new dsRNA on the cognate sequences because extension of de novo DNA methylation occurred in the 7th intron of OsRac5 and downstream of its 3’ UTR. Therefore, the de novo DNA methylation extension observed in this experiment is unlikely to be associated with transitive RNAi. It is possible that extension may depend on chromatin modifications of the region induced by siRNA signals. However, we did not detect any significant alterations of chromatin modifications in these regions (Figure S6); however, it is possible that other types of modifications were induced. Another possibility is that the heterochromatin establishment pathway works on not only trigger transgenes but also target endogenous genes with ‘long’ siRNAs. It has been reported that regions upstream of a target sequence are methylated de novo in association with 35S promoter-targeted VIGS in tobacco (Jones et al., 1999). A similar mechanism may be at work in these cases.

DNA methylation has been observed on transposons as well as surrounding sequences (Khodosevich et al., 2004). The activity of transposable elements was shown to affect expression of neighboring genes (Kashkush et al., 2003). It is likely that de novo DNA methylation associated with RNAi is established not only on the cognate siRNA regions, but also on the surrounding sequences through siRNA-mediated DNA methylation.

Experimental procedures

Constructs and plant materials

All of the RNAi-triggering constructs were produced using inverted repeat regions that were amplified using specific primers (Table S1) subcloned into pENTR/D-TOPO (Invitrogen, http://www.invitrogen.com) to yield entry vectors (OsRac5, 246 bp; OsRac7, 221 bp; OsMrt1, 544 bp; GFP, 544 bp). RNAi constructs carrying fragments of endogenous genes were produced using pANDA (Miki and Shimamoto, 2004). The final RNAi vectors were produced by an LR clonase reaction between an entry vector and the pANDA vector. Transgenic rice plants were generated by Agrobacterium-mediated transformation of rice calli (cv. Kinmaze), performed as described previously (Hiei et al., 1994). Plants were selected on the basis of hygromycin resistance and regenerated from transformed calli. Regenerated transgenic rice plants were grown in a greenhouse at 28°C.

RNA extraction and RT-PCR

For RNA or DNA extraction and ChIP assays, leaf tissues or suspension cultures were ground in liquid nitrogen using a Shake-Master Auto version 2.0 (BioMedicalScience Inc., http://www.bmsci.com). Total RNA was extracted by using Sepasol-RNA I Super (an equivalent of Trizol) (Nacalai Tesque Inc., http://www.nacalai.co.jp). For the synthesis of first-strand cDNA, 1 μg total RNA was reverse-transcribed using oligo(T) poly primers and Super-Script II (Invitrogen) in 25 μl total volume. Aliquots (1 μl) of synthesized first-strand cDNA were used for PCR analysis with various sets of gene-specific primers (Table S1).

DNA extraction and hybridization

Genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method, and 10 μg of genomic DNA were used for Southern blot analysis. DNA digested with restriction enzymes was loaded onto 1.5% Tris-Acetate-EDTA (TAE) agarose gels, electrophoresed at 50 V for 3 h and blotted on Hybrid-N+ membranes (Amersham Biosciences, http://www5.amershambiosciences.com/). The probes used for the detection of DNA methylation were DNA fragments amplified using specific primers (Table S1) and labeled with 32P-dCTP. The probe used for the detection of CentO was a 32P-dCTP-labeled mixture of CentO sequence DNA oligomers (Table S1). Hybridization signals were detected using a BAS-2500 bioimaging analyzer (Fuji, http://www.fujifilm.jp/index.html).

Bisulfite sequencing

BamHI-digested total DNA was analyzed by sodium bisulfite genomic sequencing using a BisulFast DNA modification kit for methylated DNA detection (TOYOBO, http://www.toyobo.co.jp/index.htm) according to the manufacturer’s protocol. A 1 μl aliquot of bisulfite-treated DNA was used for each PCR reaction. PCR was performed in 50 μl total volume using Blend Taq polymerase (TOYOBO). Sequenced fragments were amplified with primers specific for each region (Table S1). Amplified PCR products were subcloned into a pCRII-TOPO vector (Invitrogen) according to the manufacturer’s instructions. For each region, 16 independent top-strand clones were sequenced from each sample. Sequencing was performed using a Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, http://www.appliedbiosystems.com/) and an ABI 3100 sequencer following the manufacturer’s protocol.

Northern blotting for siRNA

siRNA detection was performed as described previously (Hamilton and Baulcombe, 1999; Miki and Shimamoto, 2004). Low-molecular-weight RNA was enriched from an aliquot of 50 μg total RNA by polyethylene glycol precipitation. Probes used for the detection of siRNA were DNA fragments amplified using specific primers (Table S1) and labeled with 32P-dCTP. DNA oligomers were used as
molecular size markers for siRNAs. The probe used for detection of SS rRNA was a 32P-dCTP-labeled mixture of SS rRNA sequence DNA oligomers (Table S1). Hybridization signals were detected using a BAS-2500 bioimaging analyzer (Fuji).

Accession numbers

The accession numbers for the sequences described in this study are as follows: OsRac3, BAA84494; OsRac4, AK061102; OsRac5, AK067504; OsRac6, AK100842; OsRac7, AK058414; Ubq, D12629; OsMet1-1, AF462029; OsMet1-2, BK001405; OsGEN-L, AK063534; SS rRNA, D26370.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Amino acid and nucleotide sequences of the OsMET1 genes.

Figure S2. Expression analysis of OsMet1-1 and OsMet1-2 in rice.

Figure S3. Quantitative mRNA expression analysis in Mi, M5i and G5i.

Figure S4. Southern hybridization analysis in Mi, M5i and G5i.

Figure S5. Distribution of DNA methylation around the target sequence.

Figure S6. ChIP analysis at the OsRac5 RNAi targeted region.

Table S1. Primers, annealing temperatures and number of cycles for PCR used in this study.

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References


DNA methylation by RNAi in rice


