Roles of DICER-LIKE and ARGONAUTE Proteins in *TAS*-Derived Small Interfering RNA-Triggered DNA Methylation[1[W]]

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Trans-acting small interfering RNAs (ta-siRNAs; TAS) emerge as a class of plant-specific small RNAs that are initiated from microRNA-mediated cleavage of *TAS* gene transcripts. It has been revealed that ta-siRNAs are generated by the sequential activities of SUPPRESSOR OF GENE SILENCING3 (SGS3), RNA-DEPENDENT RNA POLYMERASE6 (RDR6), and DICER-LIKE4 (DCL4), and loaded into ARGONAUTE1 (AGO1) proteins to posttranscriptionally regulate several target genes by messenger RNA cleavage in trans. Here, we showed a high cytosine DNA methylation status at ta-siRNA-generating loci in Arabidopsis (*Arabidopsis thaliana*), which is dependent on RDR6, SGS3, and DNA-DIRECTED RNA POLYMERASE V (PolV). More important, we found that DCL1 is the only DCL protein that is required for *TAS3* loci DNA methylation, and all four DCLs exert combinatory functions in the methylation of *TAS1* loci, suggesting a previously unknown role for DCL1 in directly processing *TAS* gene transcripts. Furthermore, we demonstrated that AGO4/6 complexes rather than AGO1 are responsible for *TAS* siRNA-guided DNA methylation. Based upon these findings, we propose a novel ta-siRNA pathway that acts at both the messenger RNA and chromatin level.

Small RNAs (sRNAs) are key players in eukaryotic RNA interference phenomena (Baulcombe, 2004). In general, sRNAs are preferentially sorted into one type of ARGONAUTE (AGO) protein to form RNA-induced silencing complexes (RISCs) and sequence-pairing transcriptionally or posttranscriptionally silences transcripts through epigenetic regulation, mRNA destabilization, and translation inhibition (Hutvagner and Simard, 2008; Ghildiyal and Zamore, 2009).

Plant microRNAs (miRNAs) are transcribed by RNA polymerase II and processed by DICER-LIKE1 (DCL1) protein in the nucleus from stem-loop structures, then associated with AGO1 according to their 5′-terminal uridine nucleotide to efficiently form RISCs in cytoplasm and perform target mRNA cleavage or translation repression (Voinnet, 2009).

Plant endogenous small interfering RNAs (siRNAs) have been reported so far as three subcategories: heterochromatic siRNAs (hc-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and trans-acting siRNAs (ta-siRNAs; TAS). The majority of hc-siRNAs are 24 nucleotides in length and are derived from transposons and repetitive elements. After being generated by the cooperative actions of plant-specific DNA-DIRECTED RNA POLYMERASE IV (PolIV), RNA-DEPENDENT RNA POLYMERASE2 (RDR2), and DCL3, hc-siRNAs are loaded into AGO4 and AGO6 complexes to trigger RNA-directed DNA methylation (RdDM; Qi et al., 2006; Zheng et al., 2007; Havecker et al., 2010; Law and Jacobsen, 2010). In the RdDM pathway, both PolV-dependent nascent scaffold transcripts and hc-siRNAs are essential to guide de novo cytosine DNA methylation, which maintains the silencing of repeats (Wierzbicki et al., 2008; Wierzbicki et al., 2009). Nat-siRNAs are products of processed natural antisense transcripts and have been shown to be involved in plant stress resistance and reproduction (Borsani et al., 2005; Katiyar-Agarwal et al., 2006; Ron et al., 2010). ta-siRNAs are initiated by DCL1-dependent miRNA-guided cleavage of noncoding transcripts, which are then stabilized by SUPPRESSOR OF GENE SILENCING3 (SGS3) and converted to double-stranded RNA (dsRNA) by the actions of RDR6 (Peragine et al., 2004; Vazquez et al., 2004; Xie et al., 2004; Allen et al., 2005; Yoshikawa et al., 2005; Axtell et al., 2006). Typically, the dsRNA products are processed by DCL4 into 21-nucleotide siRNA duplexes and one strand is then selectively recruited into AGO1 complexes to act on its targets as

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a slicer (Dunoyer et al., 2005; Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005; Adenot et al., 2006; Garcia et al., 2006; Henderson et al., 2006; Addo-Quaye et al., 2008). Consequently, it is generally believed that the biogenesis and actions of ta-siRNAs strictly require the sequential activities of DCL1, RDR6, SGS3, DCL4, and AGO1 (Ghildiyal and Zamore, 2009).

Four families of ta-siRNAs that are dispersed at eight genetic loci have been identified in Arabidopsis (*Arabidopsis thaliana*; Rajagopalan et al., 2006). *TAS1* and *TAS2* ta-siRNAs are initiated by miR173-AGO1 complexes and perform AGO1-mediated cleavage of complementary mRNA targets, such as members of pentatricopeptide repeats proteins (Montgomery et al., 2008b; Felippes and Weigel, 2009), whereas miR390 uniquely associates with AGO7 and functions in two different modes to process *TAS3* primary transcripts (Montgomery et al., 2008a). *TAS3* ta-siRNAs post-transcriptionally repress members of the *auxin response factor* gene family and regulate juvenile to adult transition during plant vegetative development (Fahlgren et al., 2006; Garcia et al., 2006; Marin et al., 2010). The formation of *TAS4* ta-siRNAs requires miR828, and their predicted targets encode myeloblastosis transcription factors, although their functions in plant development are still not clear (Rajagopalan et al., 2006).

It is well known that miRNAs and ta-siRNAs associate with AGO1 and contribute to target gene silencing in an mRNA cleavage manner. Recently, however, we demonstrated that a class of 24-nucleotide-long miRNAs in rice (*Oryza sativa*) that are generated by DCL3 can be recruited into AGO4 complexes and perform transcriptional regulation of target gene expression by DNA methylation in addition to mRNA cleavage (Wu et al., 2010). It has been reported that ta-siRNA transcripts can generate 24-nucleotide small RNAs in plants (Allen et al., 2005; Khraiwesh et al., 2010), so whether they can trigger epigenetic modification as well raises an interesting question. It has been shown from integrated epigenome maps that *TAS* loci are potential DNA methylation sites (Lister et al., 2008). Our locus-specific bisulfite sequencing here confirmed this observation. Moreover, we found that RDR6, SGS3, and PolV are involved in this process. Importantly, we revealed that DCL1 and AGO4/6, but not DCL4 and AGO1, are required for *TAS*-derived siRNA-triggered DNA methylation, suggesting a previously unknown role for DCL1 in directly processing *TAS* gene transcripts. Based upon these present findings, we propose a novel model for the formation and association pathways of ta-siRNAs.

RESULTS

Status of DNA Methylation at *TAS* Gene Loci

First, we examined the siRNA distribution at *TAS* loci from published small RNA datasets and found that a subset of 24-nucleotide siRNAs were present as previously reported (Fig. 1, A and B; Supplemental Fig. S1A; Allen et al., 2005; Khraiwesh et al., 2010). To address whether *TAS*-derived siRNAs can trigger DNA methylation in cis, we performed bisulfite sequencing to examine the status of DNA methylation at several ta-siRNA-producing loci. Consistent with the observation by genome-wide DNA methylome sequencing (Lister et al., 2008), we detected high levels of cytosine methylation in all contexts (CG, CHG, CHH) at *TAS1a*, *TAS1c*, and *TAS3a* loci, and subtle methylation at the *TAS2* locus (Fig. 1, C and D; Supplemental Fig. S1B; Supplemental Table S1). The methylation sites were confined at regions close to the miRNA cleavage site at the 5′ end of the *TAS* genes, suggesting that the methylation was triggered by the siRNAs, which were generated at *TAS* loci (Fig. 1, C and D; Supplemental Fig. S1B). We did not find methylation at the *TAS4* locus, possibly because of the low abundant miR828 (5 reads per million) and the small amounts of siRNAs that are generated at this site (Supplemental Table S1).

Roles of RdDM Components in *TAS* Loci DNA Methylation

In Arabidopsis, silencing at endogenous transposons and repeats loci involves DNA methylation guided by homologous hc-siRNAs via the RdDM pathway. Central players in RdDM can be divided into two types: those for 24-nucleotide hc-siRNA biogenesis, including PolIV, RDR2, and DCL3, and those for triggering DNA methylation but not essential for hc-siRNA formation, containing PolV (Law and Jacobsen, 2010). To determine whether the pathway of *TAS*-derived siRNA-triggered DNA methylation is similar to RdDM, we examined ta-siRNA abundances and DNA methylation levels at *TAS3a* and *TAS1c* loci in mutants for RdDM components described above. In contrast with 24-nucleotide *AtSN1* and *SIMPLEHAT2* hc-siRNAs, no indicated mutants of RdDM components affected the 21-nucleotide miRNA or the *TAS3* ta-siRNA biogenesis (Fig. 2A). However, bisulfite sequencing revealed that the two types of RdDM components perform different actions when triggering *TAS* loci methylation. Compared with control plants, northern blots and small RNA datasets displayed that the *TAS3a* 24-nucleotide siRNAs were reduced in *dcl3-1* and *rdr2-2* mutants (Fig. 2, A and B), but the percentage of DNA methylation was not decreased in any of them (Fig. 2C; Supplemental Table S1). Meanwhile, we did not find that the methylation level of the *TAS3a* locus was compromised in the PolIV mutant (*nrpd1a-3*; Fig. 2C; Supplemental Table S1), in which the abundances of hc-siRNAs and 24-nucleotide *TAS3a* siRNAs were greatly reduced (Fig. 2, A and B), indicating that 24-nucleotide *TAS3a* siRNAs are not required for local DNA methylation and their actions could be functionally masked by other size classes of siRNAs. However, in the PolV mutant (*nrpd1b-11*), we found that the methylation of CHH contexts of the

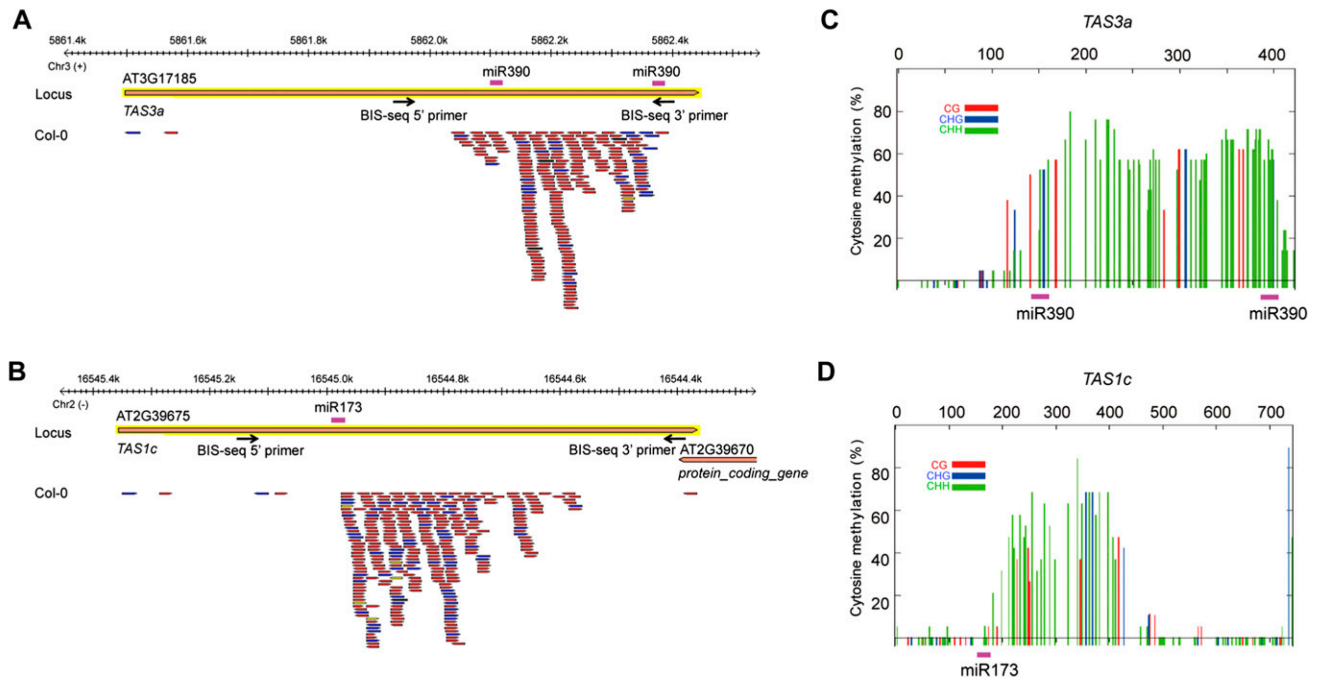
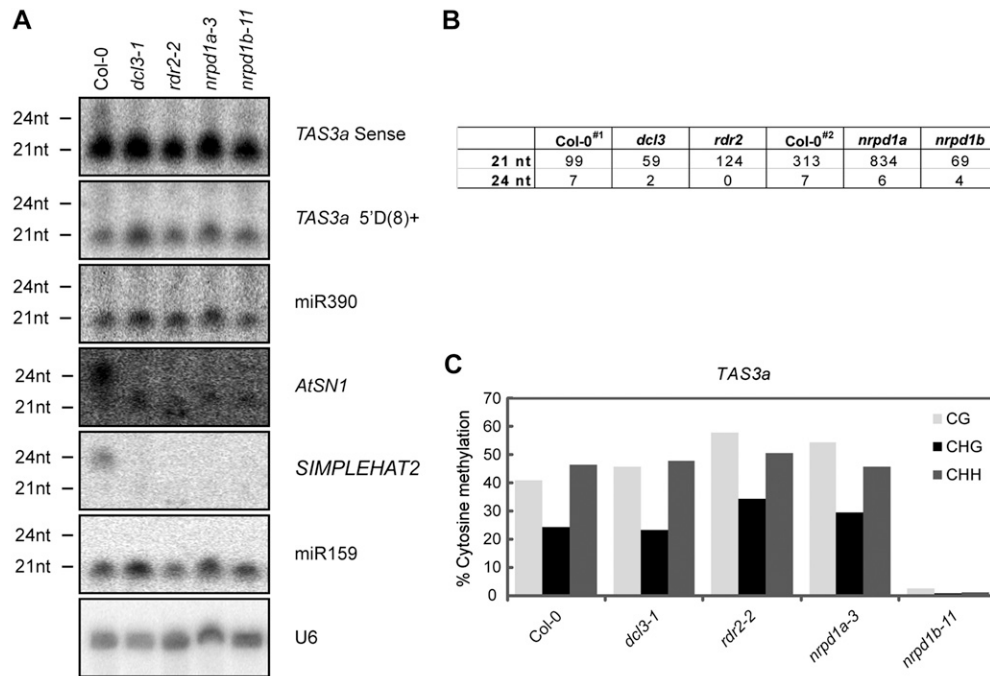


Figure 1. *TAS* sRNA distribution and DNA methylation at *TAS* gene loci. A, A diagram showing sRNAs that generated at the *TAS3a* locus in the Col-0 total sRNA dataset. B, *TAS1c* locus sRNAs. The yellow bar represents the *TAS* genomic region and the purple bar above it represents the miRNA target site. The bisulfite sequencing (BIS-seq) primer positions are indicated by black arrows. For sRNAs, red bars represent 21-nucleotide (nt) sRNAs, blue bars represent 24-nt sRNAs, black bars represent sRNAs that are longer than 24 nt, and yellow bars represent those that are over 100 reads per million. C, Analysis of DNA methylation at *TAS3a* locus by bisulfite sequencing in Col-0 plants. D, *TAS1c* locus DNA methylation. Sequencing data were analyzed using Kismeth software (Gruntman et al., 2008; http://katahdin.cshl.edu/homepage/kismeth/revpage.pl). The colored lines above the *x* axis show the percent methylation at individual cytosine sites. The colored lines below the *x* axis indicate the positions of cytosine sites. The positions corresponding to the miRNA complementary sites are designated by purple bars.

TAS3a locus was eliminated, and the levels of CG and CHG contexts were drastically reduced (Fig. 2C; Supplemental Table S1). As PolV appears to be involved in the synthesis of scaffold RNA to help recruit the RdDM effector complex to the corresponding chromatin (Wierzbicki et al., 2008; Wierzbicki et al., 2009), we consider that the association of PolV with the *TAS3a* scaffold transcripts is necessary for the *TAS3a* locus DNA methylation. But the accumulations of *TAS3a* 21-nucleotide siRNAs were not displayed reduced in *nrpd1b-11* from northern-blot analysis (Fig. 2A), suggesting that these scaffold transcripts may not be used for the production of 21-nucleotide siRNAs. Similar results were found at the *TAS1c* locus (Supplemental Fig. S2, A–C; Supplemental Table S1). These data therefore indicate that the effector counterparts, not 24-nucleotide siRNAs and their biogenesis processors in the RdDM pathway, are essential for *TAS* loci DNA methylation.

DCL4 Is Dispensable for ta-siRNA Generating Loci DNA Methylation

Because 24-nucleotide *TAS* siRNAs are not involved in *TAS* loci methylation, we wonder whether 21-nucleotide ta-siRNAs play a major role in this process. *TAS3* ta-siRNAs are produced from AGO7-dependent miRNA-directed cleavage of *TAS* transcripts and processed by an RDR6/SGS3/DCL4 biogenesis pathway (Yoshikawa et al., 2005; Montgomery et al., 2008a). We first examined *TAS3a* ta-siRNA accumulations in the mutants for these genes. Similar to previous reports (Yoshikawa et al., 2005), compared with control plants, *TAS3a* 21-nucleotide ta-siRNAs were significantly reduced in *dcl1-7*, *zip-1*, *rdr6-11*, *sgs3-11*, and *dcl4-2* mutants (Fig. 3A). We then examined the status of DNA methylation at the *TAS3a* locus in these mutants by bisulfite sequencing. As expected, all contexts of methylation were drastically reduced in *dcl1-7*, *zip-1*, *rdr6-11*, and *sgs3-11* mutants (Fig. 3B; Supplemental Table S1), indicating that *TAS* locus DNA methylation was indeed triggered by *TAS* sRNAs. However, the level of methylation at *TAS3a* locus was not reduced in the *dcl4-2* mutant (Fig. 3B; Supplemental Table S1), suggesting that DCL4 may be dispensable in the process of *TAS* loci methylation. To further test this case, we examined *TAS1c* ta-siRNA accumulation and DNA methylation in the *dcl1-7* and *dcl4-2* mutants. We found that although 21-nucleotide ta-siRNAs were reduced in both mutants



	Col-0#1	dcl3	rdr2	Col-0#2	nrpd1a	nrpd1b
21 nt	99	59	124	313	834	69
24 nt	7	2	0	7	6	4

Figure 2. 24-nucleotide hc-siRNA-independent *TAS3a* locus DNA methylation. A, Detection of indicated sRNAs in the wild type and various hc-siRNA-defective mutants. Specific oligonucleotide probes were used in small RNA northern-blot analysis, except for *TAS3a* sense, which was detected by a transcript probe. The RNA gel blots were stripped and reprobed several times. U6 small nuclear ribonucleoprotein was used as a loading control. The positions of RNA size markers, electrophoresed in parallel, are shown to the left of the blots. B, Relative abundance of the 21- and 24-nucleotide (nt)-class sRNAs at the *TAS3a* locus in control and indicated mutant sRNA datasets. Col-0$^{\#1}$ is the control for the *dcl3* and *rdr2* mutants and Col-0$^{\#2}$ for *nrpd1a* and *nrpd1b*. sRNAs of 20 to 22 nt are referred as the 21nt class, whereas 23- to 25-nt sRNAs are referred as the 24nt class. The abundance of sRNAs was calculated as reads per million. La-*er*, Landsberg *erecta*. C, DNA methylation status at the *TAS3a* locus in Col-0 and indicated mutants. Presented is the overall percentage of methylated cytosine sites in different sequence contexts.

(Supplemental Fig. S3A), the percentage of *TAS1c* locus methylation was increased from 16.4% to 23.9% in the *dcl4-2* mutant, whereas it significantly decreased to 1.2% in *dcl1-7* (Supplemental Fig. S3B; Supplemental Table S1), suggesting that DCL4 is not required for *TAS* loci DNA methylation, and it may compete for dsRNA substrates with other DCLs that are involved in the methylation of *TAS* loci. Thus, we hypothesize whether other DCL proteins can replace DCL4 to dice processed dsRNA of *TAS* transcripts into siRNAs, which in turn guide the DNA methylation.

Roles of DCLs in *TAS* Loci DNA Methylation

Because DCL2 proteins have surrogate roles when DCL4 activity is genetically compromised (Bouché et al., 2006; Deleris et al., 2006; Diaz-Pendon et al., 2007), the abundance of 21-nucleotide ta-siRNAs was much lower in the *dcl2/4* double mutant than in the *dcl4* single mutant (Fig. 3A; Supplemental Fig. S3A). However, in our bisulfite sequencing analysis, we found that the methylation levels at *TAS3a* and *TAS1c* loci were not decreased in the *dcl2/4* double mutant (Fig. 3C; Supplemental Fig. S3B; Supplemental Table S1). It has been reported that the *dcl3/4* double mutant also exhibits more severe developmental defects than the *dcl4* single mutant (Gasciolli et al., 2005), but we detected a slight increase in the percentage of DNA methylation in the *dcl3/4* double mutant compared with that in wild-type plants (Fig. 3C; Supplemental Fig. S3B; Supplemental Table S1). Consistent with earlier discoveries that DCL2, DCL3, and DCL4 function redundantly in ta-siRNA production (Henderson et al., 2006), our northern-blot assays confirmed the greatly decreased ta-siRNAs in *dcl2/3/4* triple mutants (Fig. 3A; Supplemental Fig. S3A). Despite this, the methylation at *TAS3a* locus remained at a comparable level in the *dcl2/3/4* mutant, suggesting that DNA methylation directed by *TAS3a*-derived siRNAs is not dependent on the redundant activities of DCL2, DCL3, and DCL4 (Fig. 3C; Supplemental Table S1). For the *TAS1c* locus, although no type of DNA methylation was compromised in *dcl2/4* and *dcl3/4* double mutants, we interestingly found that the CHG and CHH methylation level was reduced by 50% in the *dcl2/3/4* triple mutant, suggesting that DCL2, DCL3, and DCL4 may have redundant and cooperative actions on non-

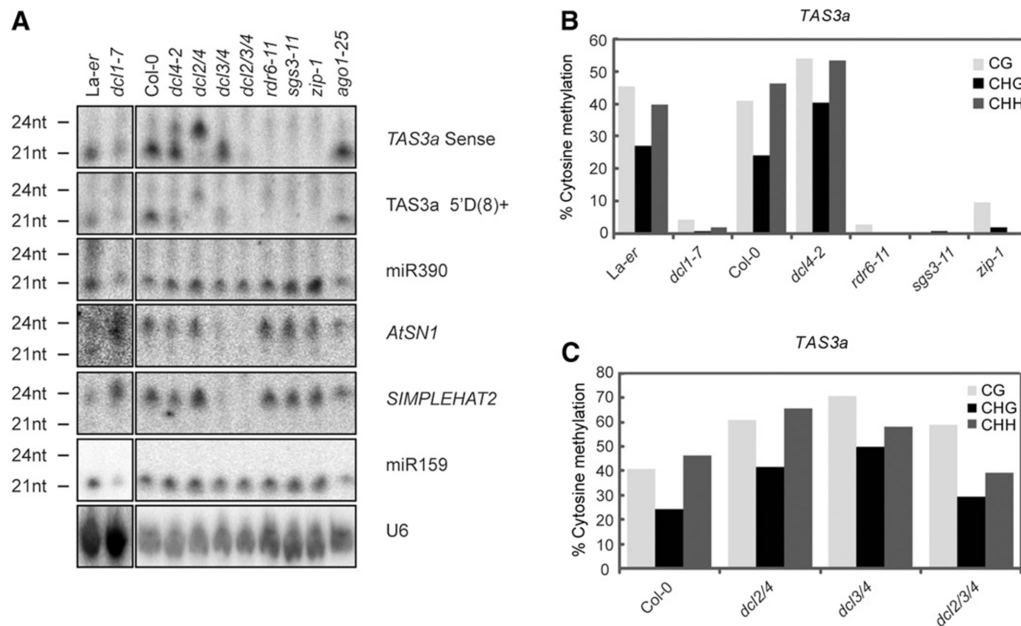


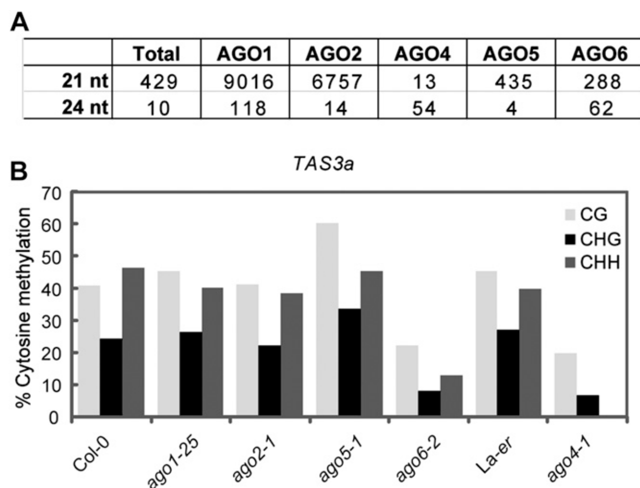
Figure 3. DCL1-dependent and *TAS* siRNA-directed *TAS3a* locus DNA methylation. A, Detection of indicated sRNAs in the wild type and various ta-siRNA defective mutants. Method and labeling are the same as in Fig. 2A. La-*er*, Landsberg *erecta*. B, DNA methylation status at the *TAS3a* locus in the control and the mutants for *DCL1*, *DCL4*, *RDR6*, *SGS3*, and *AGO7*. Presented is the overall percentage of methylated cytosine sites in different sequence contexts. La-*er*, Landsberg *erecta*. C, DNA methylation status at the *TAS3a* locus in the control and indicated *DCL* double or triple mutants.

CG methylation at the *TAS1*c locus (Supplemental Fig. S3C; Supplemental Table S1).

Taken together, our results support the idea that DCL proteins play different roles in *TAS* loci DNA methylation; DCL1 is probably the major dicer required for *TAS3a*, and all four DCLs play redundant roles in *TAS1c* methylation.

TAS-Derived siRNAs Are Associated with AGO4 Proteins to Direct DNA Methylation

To exert their functions, sRNAs must associate with one type of AGO protein to form RISCs. AGO1 preferentially associates with small RNAs with a 5′-terminal uridine and is the most important AGO required for sRNAs-mediated posttranscriptional gene regulation (Mi et al., 2008; Vaucheret, 2008). Previous genetic and biochemical studies showed that ta-siRNAs are loaded into AGO1 complexes to slice their target mRNAs (Baumberger and Baulcombe, 2005; Qi et al., 2005). Therefore, we first tested the methylation status at the *TAS3a* locus in the *ago1-25* mutant, which is defective in posttranscriptional gene silencing and has pleiotropic developmental and morphological defects (Morel et al., 2002). However, we did not detect any reduction in DNA methylation when compared with that in wild-type plants (Fig. 4B), suggesting that the methylation was not achieved through AGO1 complexes. We then analyzed the *TAS3a* siRNA distribution from published AGO-associated small RNA datasets. We found that siRNAs from *TAS* loci were differentially associated with different AGOs. As shown in Figure 4A, most 21-nucleotide siRNAs from the *TAS3a* locus were associated with AGO1 and AGO2 (Fig. 4A). Because *TAS3a* locus methylation was not reduced in the *ago1-25* mutant, we examined the methylation level in a loss-of-function AGO2 mutant, *ago2-1*, (Lobbes et al., 2006), and found that it still remained unaffected (Fig. 4B; Supplemental Table S1), suggesting that AGO1 and AGO2 proteins are not the effectors for ta-siRNA generating loci DNA methylation. Although a subset of *TAS3a* sRNAs are enriched in AGO5 dataset (Fig. 4A), the DNA methylation level of the *TAS3a* locus was not reduced in the *ago5-1* mutant (Katiyar-Agarwal et al., 2007), when compared with wild-type control plants (Fig. 4B; Supplemental Table S1). AGO4 and AGO6 belong to the same clade proteins and play redundant roles in hc-siRNA-controlled epigenetic modifications. In AGO4 and AGO6 sRNA datasets, we found that a small number of 21-nucleotide *TAS* siRNAs were present (Fig. 4A). *ago4-1*, harboring a frameshift mutation in the AGO4 P-element induced wimpy testis domain, blocks hc-siRNA-directed DNA methylation and causes severe loss of AGO4 function (Zilberman et al., 2003). A loss-of-function mutant of AGO6, *ago6-2*, has been demonstrated that was compromised in asymmetric DNA methylation at multiple endogenous transposon and repeat loci (Zheng et al., 2007). Thus, we determined the *TAS3a* methylation status in the *ago4-1* and *ago6-2* mutants. Strikingly, a



	Total	AGO1	AGO2	AGO4	AGO5	AGO6
21 nt	429	9016	6757	13	435	288
24 nt	10	118	14	54	4	62

Figure 4. siRNAs are associated with AGO4 family proteins to direct *TAS* loci DNA methylation. A, Relative abundance of 21- and 24-nucleotide (nt) classes of sRNAs at the *TAS3a* locus in total, AGO1, AGO2, AGO4, AGO5, and AGO6 sRNA datasets. The abundance of siRNAs was calculated as reads per million. B, DNA methylation status at *TAS3a* locus in the control and the mutants for *AGO1*, *AGO2*, *AGO4*, *AGO5*, and *AGO6*. Presented is the overall percentage of methylated cytosine sites in different sequence contexts. La-*er*, Landsberg *erecta*.

great reduction of *TAS3a* methylation was found in these two mutants (Fig. 4B; Supplemental Table S1), suggesting that AGO4 family proteins are the effectors for triggering *TAS* loci DNA methylation. To rule out the possibility that AGO4 may mediate DNA methylation in a locus-specific manner, we examined the methylation level at the *TAS1c* locus in *ago4-1* and found that AGO4 was also responsible for *TAS1c* locus methylation (Supplemental Fig. S4, A and B; Supplemental Table S1). Taken together, these results demonstrate that *TAS*-derived siRNAs are associated with AGO4 family proteins to direct DNA methylation in cis.

DNA Methylation Does Not Impact the Expression of *TAS* Gene Transcripts

As described above, PolV and AGO4 are the essential components in *TAS*-derived siRNA-directed DNA methylation pathways. To explore whether DNA methylation can affect the expression of *TAS* transcripts, we examined the transcription level of *TAS* in the mutants of *PolV*, *AGO4*, and their corresponding wild-type plants. We found the expression of retroelement *AtSN1* was dramatically increased in *nrpd1b-11*and *ago4-1*; however, the transcripts of *TAS3a* displayed comparably in these mutants (Fig. 5, A and B). Similar results were found for the *TAS1c* locus (Supplemental Fig. S5, A and B). These data suggest that high DNA methylation status that occurs at *TAS* loci has no obvious effect on the regulation of *TAS* gene transcription.

DISCUSSION

After being converted by RDR6 and SGS3, miRNA-mediated cleavage products of *TAS* precursor transcripts can form stable dsRNA substrates. Previous studies revealed that DCL4 is essential for processing these dsRNAs to generate ta-siRNAs, which can be loaded into AGO1 complexes to guide target mRNA cleavage. In this paper, we presented that high DNA methylation can occur in *TAS* loci through a pathway involving AGO4/6 and PolV, suggesting that a complicated ta-siRNA function pathway may exist in plants. Intriguingly, although most ta-siRNAs were diminished in *rdr6*, *sgs3*, *dcl1*, and *dcl4* mutants, the status of the DNA methylation level at *TAS* loci was quite different; it was totally compromised in *rdr6*, *sgs3*, and *dcl1* mutants, but still persisted in the *dcl4* mutant. The complete lack of *TAS* methylation

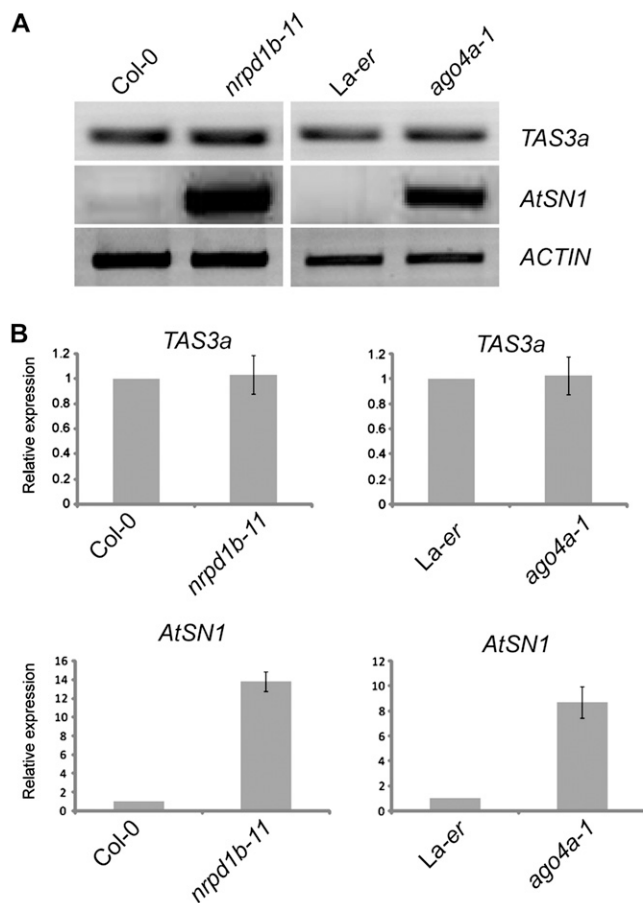


Figure 5. DNA methylation does not have effect on the expression of *TAS* transcripts. A, Reverse transcription PCR analysis of *TAS3a* transcripts in the indicated mutant and the corresponding wild-type plants. *AtSN1* was also used as a control for verification of respective mutant background. *ACTIN* was detected in parallel and used as a loading control. B, Relative expression levels of *TAS3a* and *AtSN1* transcripts. The expression levels were normalized using the signal from the *ACTIN* gene. The average (± SD) values from three biological duplicates of quantitative reverse transcription PCR are shown. La-*er*, Landsberg *erecta*.

observed, as in the *dcl1* mutant, is probably due to the facts that (1) *TAS* loci cannot be targeted by miRNAs anymore and causes *TAS* siRNA production to be abolished, which is reminiscent of *TAS3a* DNA methylation status in *zip-1* (Fig. 3B), and (2) DCL1 may compete for the dsRNAs produced by RDR6 to generate siRNAs for directing DNA methylation. However, it is difficult to explain why DCL4 is not required for the methylation of *TAS* loci, as DCL4 is primarily responsible for *TAS* transcript processing.

Furthermore, we observed that DNA methylation at *TAS3a* loci was not changed in *dcl2*, *dcl3*, or *dcl4* double or triple mutants. There are three ways in which DNA methylation at *TAS3a* loci was possibly comparable in the wild type and *dcl2/3/4* triple mutants. (1) DCL1 may directly process a portion of *TAS3a* transcripts to generate siRNAs for guiding DNA methylation. (2) siRNAs generated at other loci can trigger *TAS3a* DNA methylation in trans. (3) miR390 can replace *TAS3a* siRNAs to trigger DNA methylation in *dcl2/3/4* triple mutants. To test the second possibility, we examined the DNA methylation status in a *tas3a-1* mutant, which has only 40% of wild-type *TAS3a* transcripts (Adenot et al., 2006; Marin et al., 2010). As expected, the CHG and CHH methylation level was drastically reduced in *tas3a-1*, suggesting that *TAS3a* DNA methylation is indeed triggered by local siRNAs in cis, rather than by other siRNAs in trans (Supplemental Fig. S6; Supplemental Table S1). Although miR390 is initiated with a 5′ end A and can be partially loaded into the AGO4 complex (Qi et al., 2006; Mi et al., 2008), we do not believe this is the case because the DNA methylation region in *dcl2/3/4* was not confined in miR390 target sites but was extended to the whole *TAS* siRNA-generating region as in wild-type plants (Supplemental Fig. S7, A and B). Despite the fact that it is difficult to test whether DCL1 can directly process a portion of *TAS* transcripts, several pieces of evidence support this possibility. (1) DCL4 is not required for *TAS* loci methylation. (2) The methylation level of the *TAS3a* locus does not change in *dcl2/3/4* triple mutants. (3) There are still some siRNAs generated at *TAS3a* and *TAS1c* loci in *dcl2/3/4* mutants that may guide DNA methylation (Supplemental Fig. S8, A and B). Together, we hypothesize that DCL1 and DCL4 may cooperatively process *TAS* transcripts and produce siRNAs that are required for DNA methylation and targeting mRNA cleavage, respectively. Given that DCL1 and DCL3 function respectively as 21-nucleotide and 24-nucleotide sRNA producers (Xie et al., 2004; Qi et al., 2005), it is reasonable to consider that *TAS* loci DNA methylation is triggered by 21-nucleotide but not 24-nucleotide sRNAs. The role of DCL1 in guiding transposon DNA methylation also has been reported (Laubinger et al., 2010), suggesting that DCL1-dependent siRNA-mediated DNA methylation is not occasional.

Although *TAS3a* locus DNA methylation was not changed in *dcl2/3/4* mutants (Fig. 3C), the methylation level in*TAS1c* locus displayed 50% reduction in *dcl2/3/4* mutants (Supplemental Fig. S3C). These results indicated that distinct DCLs were required in the process of different *TAS* loci methylation. For the *TAS3a* locus, DCL1-generated siRNAs are sufficient to trigger DNA methylation, but at the *TAS1c* locus DCL1 and DCL2/3/4 may exert combinatory functions to process siRNAs, which are involved in DNA methylation in cis. Whether the different roles of DCLs in *TAS3a* and *TAS1c* loci methylation are due to being targeted by different miRNAs (miR390 and miR173) and loaded in different effector complexes (AGO7 and AGO1) needs to be further elucidated.

Most *TAS*-derived siRNAs are loaded into AGO1 and AGO2 complexes (Fig. 4A; Supplemental Fig. S4A), whereas ta-siRNAs must be sorted into AGO1 complexes for posttranscriptional target regulation. However, we observed that the level of *TAS* loci DNA methylation persisted in *ago1-25* and *ago2-1* mutants (Fig. 4B; Supplemental Fig. S4B), suggesting that the effector complexes for directing *TAS* loci DNA methylation are different from those for ta-siRNA-mediated posttranscriptional target regulation. Our further study

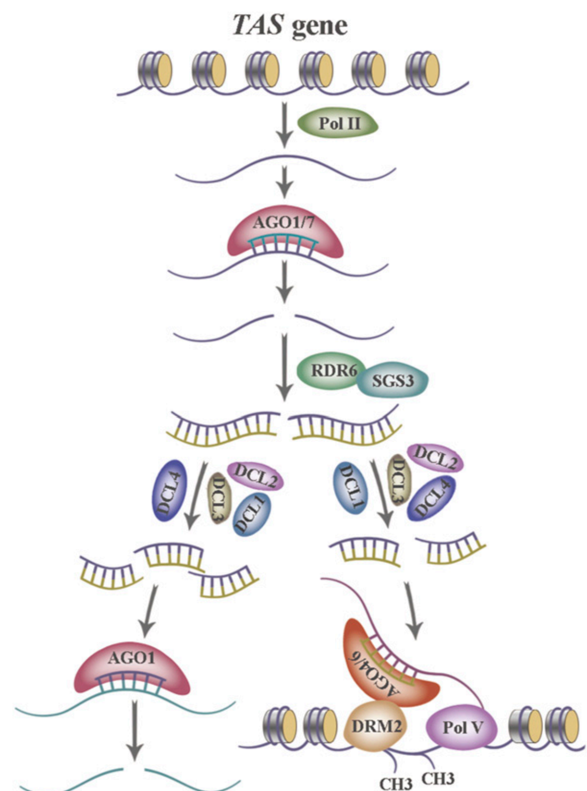


Figure 6. A schematic model for ta-siRNA biogenesis and action pathway. *TAS* genes are transcribed by PolII, and the transcripts are cleaved by miRNA-AGO1/7 interacting complexes. The products are then stabilized and converted into dsRNA by SGS3 and RDR6. Some dsRNAs are processed by DCL4 with the redundant activities of DCL1/2/3 to form ta-siRNA-AGO1 cleavage complexes for posttranscriptional target regulation, whereas others are processed by DCL1 with DCL2/3/4 and loaded into AGO4/6 complexes, recruiting other RdDM effectors to trigger DNA methylation.

demonstrated that AGO4/6 is responsible for this process. Despite the fact that only a small number of *TAS* siRNAs are recruited into AGO4/6 complexes (Fig. 4A and Supplemental Fig. S4A), especially the 21-nucleotide ones, they are able to trigger DNA methylation. The capability of a small number of siRNAs in triggering target site DNA methylation through AGO4 complexes also has been observed for those generated from nonconserved miRNA sites (Chellappan et al., 2010). Because AGO4 possesses cleavage activity in Arabidopsis (Qi et al., 2006), we envision that 24-nucleotide siRNAs generating at *TAS* loci may take part in the process of target mRNA cleavage or DNA methylation in trans, although we cannot absolutely exclude the possibility that they may also direct DNA methylation in cis.

The precise role of *TAS* loci DNA methylation remains obscure, as we did not find differences in *TAS* gene expression between the wild-type plants and the *ago4-1* and *nrpd1b-11* mutants, which are defective in *TAS* loci DNA methylation (Fig. 5; Supplemental Fig. S5). Although the methylation may not exert transcriptional influence in cis, it is possible that this methylation plays a role in facilitating DCLs to generate moderate siRNAs in *TAS* loci. We also propose that this DNA methylation may recruit chromatin-remodeling factors to change local histone modification status. ta-siRNAs may also function as transcriptional regulators to modulate their targets through DNA methylation in trans, which may affect target gene expression. Further investigations are warranted to explore the exact roles of *TAS* siRNA-guided DNA methylation.

Based on our genetic results on *TAS* loci DNA methylation, we propose an alternative ta-siRNA biogenesis and action pathway (Fig. 6). In Arabidopsis, ta-siRNAs are a hallmark of the RDR6-dependent 21-nucleotide secondary siRNAs that are commonly considered to be generated by DCL4 and to participate in posttranscriptional silencing of exogenous targets through AGO1 (Cuperus et al., 2010). Our discovery of the pathway of *TAS* siRNA-triggered DNA methylation may extend to all secondary siRNA formation and function mechanisms. The cross functions of different secondary siRNA pathways in plants may increase the efficiency in target silencing. Therefore, it is imperative to investigate the equilibrium between transcriptional and posttranscriptional gene regulations contributed by these noncoding RNAs.

MATERIALS AND METHODS

Plant Materials

The mutant alleles of *dcl3-1*, *dcl4-2*, *dcl2/4*, *dcl3/4*, *dcl2/3/4*, *nrpd1a-3*, *nrpd1b-11*, *rdr6-11*, *sgs3-11*, *zip-1*, *ago1-25*, *ago6-2*, and *tas3a-1* used in this study were of a Columbia-0 (Col-0) genetic background, whereas *dcl1-7* and *ago4-1* were of a Landsberg *erecta* background. All of them have been described previously. Plants were grown in soil in chambers, and experimental materials are 40-d-old whole plants.

DNA Extraction and Bisulfite Sequencing

Total genomic DNA isolation and bisulfite sequencing were performed as described (Wu et al., 2010). Bisulfite conversion rate for the sample was over 99%. Primers used for bisulfite sequencing are described in Supplemental Table S2, and the sequences of bisulfite-sequenced regions are provided in Supplemental Figure S9.

RNA Extraction and Small RNA Northern Blot

Total RNA was isolated from plants using Trizol reagents (TaKaRa). After being enriched by the polyethylene glycol precipitation method, small RNAs were separated by 15% denaturing PAGE and transferred onto Hybond N^+ membrane. [^{32}P]ATP-labeled oligonucleotides complementary to small RNA sequences were used as probes. [^{32}P]UTP-labeled T7 polymerase transcript probes corresponding to *TAS* sense transcripts were generated by Riboprobe in vitro Transcription Systems (Promega). A synthetic [^{32}P] end-labeled RNA oligo was used as a size marker. The hybridization was performed as described (Wu et al., 2009). Probe sequences and primers for in vitro transcription are described in Supplemental Table S2.

Quantitative Reverse Transcription PCR

Total RNA was extracted with the Trizol reagent (Invitrogen) from 4-week-old soil-grown plants. Total RNA was treated with RNase-free DNase I (Promega) to remove DNA and reverse-transcribed by M-MLV reverse transcriptase (Promega) using gene-specific primer. Quantitative PCR was performed with SYBR Premix EX Taq (TaKaRa). *ACTIN* mRNA was detected in parallel and used for data normalization. The primers used for quantitative PCR are listed in Supplemental Table S2.

Computational Analysis

Annotation of ta-siRNAs was performed using Arabidopsis Small RNA Project Database (http://asrp.cgrb.oregonstate.edu) and The Arabidopsis Information Resource 8 (http://www.arabidopsis.org/). Small RNA datasets used in this study are as described previously (Rajagopalan et al., 2006; Mi et al., 2008; Mosher et al., 2008; Havecker et al., 2010).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. High DNA methylation status at *TAS1a* loci.

Supplemental Figure S2. *TAS1c* locus DNA methylation is not triggered by 24-nucleotide hc-siRNAs.

Supplemental Figure S3. DCL4 is not required for *TAS* siRNA-directed *TAS1c* locus DNA methylation.

Supplemental Figure S4. siRNAs are associated with AGO4 family proteins to direct *TAS1c* locus DNA methylation.

Supplemental Figure S5. DNA methylation of the *TAS1c* locus does not affect *TAS1c* transcript expression.

Supplemental Figure S6. *TAS3a* locus DNA methylation is decreased in the *tas3a-1* mutant.

Supplemental Figure S7. *TAS3a* locus DNA methylation in the *dcl2/3/4* mutant is not triggered by miR390.

Supplemental Figure S8. Relative abundance of 21- and 24-nucleotide class of sRNAs at *TAS3a* and *TAS1c* loci in wild-type and *dcl2/3/4* mutant small RNA datasets.

Supplemental Figure S9. Genomic sequences of the loci that are examined in this study by bisulfite sequencing.

Supplemental Table S1. DNA methylation status at *TAS* gene loci in the wild type and indicated mutants.

Supplemental Table S2. Primers and probes used in this study.

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