Arabidopsis C-Terminal Domain Phosphatase-Like 1 Functions in miRNA Accumulation and DNA Methylation

In Sil Jeong1,2, Emre Aksoy2, Akihito Fukudome2, Salina Akhter1, Akihiro Hiraguri3, Toshiyuki Fukuha3, Jeong Dong Bahk1*, Hisashi Koiwa2*

1 Division of Applied Life Science (BK21 Program), Graduate School of Gyeongsang National University, Jinju, Gyeongsangnam-do, Korea, 2 Department of Horticultural Sciences, Texas A&M University, College Station, Texas, United States of America, 3 Department of Applied Biological Sciences, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan

Abstract

Arabidopsis CTD-PHOSPHATASE-LIKE 1 (CPL1) is a protein phosphatase that can dephosphorylate RNA polymerase II C-terminal domain (CTD). Unlike typical CTD-phosphatases, CPL1 contains a double-stranded (ds) RNA-binding motif (dsRBM) and has been implicated for gene regulation mediated by dsRNA-dependent pathways. We investigated the role of CPL1 and its dsRBMs in various gene silencing pathways. Genetic interaction analyses revealed that cpl1 was able to partially suppress transcriptional gene silencing and DNA hypermethylation phenotype of ros1 suggesting CPL1 is involved in the RNA-directed DNA methylation pathway without reducing siRNA production. By contrast, cpl1 reduced some miRNA levels at the level of processing. Indeed, CPL1 protein interacted with proteins important for miRNA biogenesis, suggesting that CPL1 regulates miRNA processing. These results suggest that CPL1 regulates DNA methylation via a miRNA-dependent pathway.

Citation: Jeong IS, Aksoy E, Fukudome A, Akhter S, Hiraguri A, et al. (2013) Arabidopsis C-Terminal Domain Phosphatase-Like 1 Functions in miRNA Accumulation and DNA Methylation. PLoS ONE 8(9): e74739. doi:10.1371/journal.pone.0074739

Copyright: © 2013 Jeong et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from the BK21 program of the Ministry of Education and Science Technology of Korea, the National Science Foundation (MCB0950459), USDA-CSREES (2009-34402-19831) “Designing Food for Health”, and Grant-in-Aid for Scientific Research (number 24570044) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: koiwa@ineo.tamus.edu (HK); jdbahk@gnu.ac.kr (JDB)

Introduction

Plant gene expression is transcriptionally and post-transcriptionally regulated by a population of small RNAs. The small RNA biogenesis involves diverse factors that determine levels of specific type of small RNA [1–4]. Micro RNAs (miRNA) are 21-base long and their biogenesis starts from the transcription of MIR genes by RNA polymerase II (Pol II). The resulting pri-miRNAs are processed by a complex containing Dicer-like 1 (DCL1) [5], HYPONASTIC LEAVES1 (HYL1) [6,7] and SERRATE (SE) [8] leading to the production of a miRNA-miRNA* duplex [9,10]. siRNAs are generated from long dsRNAs, which are derived from transcription of inverted repeats, transposable elements, and conversion of single stranded RNAs by RNA-dependent RNA polymerases (RDRs) [11], and subsequent processing by various DCL proteins [12].

Among various roles of small RNAs, siRNA can promote DNA methylation via the canonical RNA-mediated DNA methylation (RdDM) pathway [13]. siRNAs bind to ARGONAUTE4, which in turn forms a complex with various factors, such as RNA polymerase V and KTF [14–17], required for defining RdDM targets loci, where DRM2 is recruited to catalyze DNA methylation [18,19]. Other factors such as RNA polymerase II (pol II) [20], mediator [21], splicing machineries [22], and DCL1 [23,24] also contribute to the establishment of the DNA methylation. Steady-state level of DNA methylation is determined by antagonizing activities of methylation and demethylation. In plants, some DNA demethylations are mediated by ROS1, which cleaves the DNA backbone to remove methyl-cytosine from the DNA double strand [25]. The ros1 plants exhibit DNA hypermethylation and enhanced transcriptional gene silencing (TGS) in various loci including promoter region of a transgene RD29A-LUC and its endogenous counterpart [25].

We have previously identified an Arabidopsis C-terminal domain (CTD)-phosphatase-like 1 (CPL1) by forward genetic screening using the RD29A-LUC reporter gene [26,27]. cpl1 causes hyperactivation of RD29A-LUC, opposite to ros1 in the same background [25]. CPL1 and its paralog CPL2 can dephosphorylate CTD of the pol II largest subunit specifically at the Ser5 of the heptad repeat sequence (Y1S2P3T4S5P6S7) suggesting their role in transcription elongation and mRNA maturation [28,29]. CPL1 regulates gene expression in various biological processes, including osmotic stress and iron deficiency [26,27,30]. However, little is known about how CPL1 regulates gene expression. Here we report roles of CPL1 in small RNA-mediated gene expression.

Materials and Methods

Primer sequences were listed in Table S1.

Plant Materials, Growth Condition, and Stress Treatments

Arabidopsis thaliana cpl1-2 (formerly fyu2-1), and ros1–1 mutants [25–27] in ecotype C24 carrying an RD29A-LUC reporter gene [31], and L1 line carrying post-transcriptionally silenced 35S-GUS
reporter (ecotype Columbia) [32] were used in this study. cpl1-2 transformed with gCPL1-FLAG transgene will be described elsewhere. Plants were grown on agar plates containing 1/2 Murashige and Skoog salts and 1% sucrose. Cold and ABA treatments were applied to 2-week-old plants as described [26]. Arabidopsis cell culture was induced and maintained as described [33]. Heat treatment was applied as described [34].

**Figure 1.** cpl1 partially suppresses ros1 gene silencing phenotype. RD29A-LUC luminescence image after cold treatment (0°C, 48 hr, A) and photograph showing kanamycin resistance (50 μg/ml, B) of WT, ros1, cpl1 ros1, and cpl1. (C) RT-qPCR analysis of the transcript levels of RD29A-LUC, endogenous RD29A and COR15A (left) and NPT II (right). (D) RT-qPCR analysis of the transcript levels of retrotransposons (left) and DNA transposons (right). Bars indicate standard errors of the mean (SEM) from three biological replicates. (E) RT-qPCR analysis of the transcript levels of ONSEN. Bars indicate standard errors of the mean (SEM) of two biological replicates. Different letters show significant differences (p<0.05, one-way ANOVA followed by Tukey’s HSD post hoc test).

doi:10.1371/journal.pone.0074739.g001

**Reporter Gene Assays**

In vivo luciferase activity was documented as described [26]. Cold stress (0°C, 48 hr) treated plants were sprayed with luciferin solution (0.01% TritonX-100, 1 mM Luciferin) and kept for 5 min in the dark. Image acquisition and processing were performed with Electron Multiplying Charge-Coupled Device camera (Cascade II, Photometrics) and the WinView software (Roper Scientific). β-
glucuronidase assay was performed as described [35]. Plants were transferred into staining solution that contains 0.5 mM K$_3$Fe(CN)$_6$, 0.5 mM K$_4$Fe(CN)$_6$, 0.3% v/v Triton X-100 and 2 mM X-gluc (Sigma-Aldrich), vacuum infiltrated for 5 min, and incubated at 37°C overnight. After staining, tissues were washed with 70% ethanol.

**RNA Analysis**

The total RNA and small RNA were extracted from 10-day-old seedlings using E.Z.N.A.® PF Micro RNA Kit and E.Z.N.A. miRNA Isolation Kit (Omega Biotek), respectively. For RT-qPCR analysis, small RNA samples were converted to cDNA using NGcode™ miRNA First-Strand cDNA Synthesis Kit (Life Technologies) and were subjected to qPCR analysis. qPCR were performed and analyzed as described [30] using the primers listed in Table S1.

Small RNA Northern blotting analysis was performed as described [1]. The small RNA samples (80 μg) were resolved on 17% polyacrylamide gel containing 7 M Urea in 1× TBE buffer and were transferred onto nylon filters. The filters were hybridized by 32P-labeled small RNA probes at 42°C in PerfectHyb™ Plus buffer (SIGMA). The filters were then washed three times with 2× SSC, 0.1% SDS at 42°C for 20 min and exposed to X-ray film. The sequence of oligonucleotide probes used for small RNA Northern blotting analysis are listed in Table S1.

**DNA Methylation Analysis**

DNA methylation analysis EpiTect® Bisulfite Kit (QIAGEN) was performed as described [25]. Endogenous and transgene RD29A promoter fragments were amplified by PCR using the bisulfite-treated genomic DNA samples (2 μg) as template. The PCR products of two amplified promoters were cloned into pGEM-T easy vector (Promega) and 15 individual clones were sequenced for each sample. The primers used for bisulfite sequencing are listed in Table S1. PCR-based DNA methylation assays were performed as described [1,36]. Genomic DNA (500 ng) was digested with the methylation-sensitive restriction enzyme HaeIII overnight at 37°C or the methylated DNA-digesting enzyme MspBC for 1 hr at 37°C. The digested DNA was used to amplify the RdDM targets, including AtSN1, AtGP1, and AtMU1. The undigested genomic DNA was simultaneously amplified as control. PCR conditions were set at 94°C, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 40 s, and a final extension step at 72°C for 10 min. Three independent experiments were performed for AtMU1 and two independent experiments were performed for AtSN1 and AtGP1. PCR-based DNA methylation assays was performed using specific primers listed in Table S1.

**Co-immunoprecipitation Assay**

Arabidopsis calli expressing gCPL1-FLAG were homogenized in an extraction buffer containing 100 mM Tris–HCl pH 7.5, 2 mM EDTA, 25% glycerol, 2 mM DTT, 1 mM PMSF, 100 μg/l DNase, 50 μg/ml RNase, and 1× complete protease inhibitor cocktail. Protein extracts were centrifuged twice at 16,000 rpm at 4°C for 15 min, and protein concentration in the supernatant was determined by Bradford reagent assay. The cleared protein extract (110 μg protein) was incubated with the methylation-sensitive restriction enzyme HaeIII overnight at 37°C or the methylated DNA-digesting enzyme MspBC for 1 hr at 37°C. The digested DNA was used to amplify the RdDM targets, including AtSN1, AtGP1, and AtMU1. The undigested genomic DNA was simultaneously amplified as control. PCR conditions were set at 94°C, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 40 s, and a final extension step at 72°C for 10 min. Three independent experiments were performed for AtMU1 and two independent experiments were performed for AtSN1 and AtGP1. PCR-based DNA methylation assays was performed using specific primers listed in Table S1.

**Co-immunoprecipitation Assay**

Arabidopsis calli expressing gCPL1-FLAG were homogenized in an extraction buffer containing 100 mM Tris–HCl pH 7.5, 2 mM EDTA, 25% glycerol, 2 mM DTT, 1 mM PMSF, 100 μg/l DNase, 50 μg/ml RNase, and 1× complete protease inhibitor cocktail. Protein extracts were centrifuged twice at 16,000 rpm at 4°C for 15 min, and protein concentration in the supernatant was determined by Bradford reagent assay. The cleared protein extract (110 μg protein) was incubated with 5 μl anti-FLAG antibody (or anti-HYL1) for 5 min and, the immunocomplex was precipitated after incubation with 7 μl of protein A agarose resin overnight at 4°C with gently shaking and collected by centrifugation. After washing the immunoprecipitated proteins by TTBS were subjected to SDS-PAGE and specific proteins were detected by Western blotting using specific antibodies (anti-HYL1 and anti-FLAG).
Bimolecular Fluorescence Complementation (BiFC) Assay
cDNA fragments encoding a full-length CPL1 and a full-length HYL1 were cloned into BiFC vectors [37] to produce pCPL1-nYFP and pHYL1-cYFP. The transformation DNA mixtures contained the indicated combinations of 5 mg of each DNA preparation. Polyethylene glycol-mediated transformation of Arabidopsis protoplasts were performed as described [28].

Luciferase Complementation Imaging (LuCI) Assay
LuCI was performed as described [38]. CPL1, CPL11–714, CPL1699–967, HYL11–223 and SE fragments were cloned in pDONRzeo (Life Technologies) by Gateway BP reaction and then transferred into pDEST-NLUCGW or pDEST-CLUCGW [38] by Gateway LR reaction (Life Technologies). Resulting NLUC/CLUC constructs and a 35S-P19 construct (provided by Dr. Baulcomb) were introduced into Agrobacterium tumefaciens GV3101 cells [39].

To test interactions, GV3101 cells carrying the various NLUC/CLUC constructs were prepared as follows. Cells grown on solid LB medium supplemented with 50 μg/ml kanamycin were inoculated in 10 ml of liquid LB kanamycin medium. After 20 h incubation, cells were harvested by centrifugation at 4000 rpm for 10 min and re-suspended in fresh activation medium containing 10 mM MES/KOH (pH 5.6), 10 mM MgCl2 and 150 μM acetosyringone. Cell suspensions were mixed to achieve a final OD600 of 0.4 for NLUC/CLUC constructs and 0.15 for the P19 helper strain. The 100 μl of NLUC, CLUC and P19 cell suspension mixtures were infiltrated into leaves of 4- to 7-week-old Nicotiana benthamiana plants. Luminescence images were taken 3d after infiltration. Leaves were infiltrated with luciferin solution (10 mM MES/KOH, pH 5.6, 10 mM MgCl2 and 100 μM luciferin) and images were acquired using an EMCCD camera and processed by WinView software.

Yeast two-hybrid Assays
For the yeast two-hybrid analysis, CPL1, HYL1 and SE fragments were amplified by PCR and cloned into pDONRzeo by the Gateway BP reaction. Gateway compatible two-hybrid vectors, pBUTEGW and pGADGW, were prepared by inserting Gateway cassette A (Life Technologies) into the Smal site of pBute [40] or pGAD.c1 [41], and were used to clone CPL1, HYL1 and SE fragments by Gateway LR reactions. Lithium acetate-mediated transformation of yeast strain PJ69-4A was performed as described [42]. After transformation, yeast were plated on synthetic dropout media (SD) composed of nitrogen base, 2% glucose and a dropout supplement without uracil and leucine (-UL) and incubated at 28°C for 48 hr. 2×10^6 cells of colonies growing on SD/-UL and their diluted cells (2×10^4 cells) were transferred onto SD composed of nitrogen base, 2% glucose, a dropout supplement without uracil, leucine, histidine and adenine (-ULHA) and incubated at 28°C for 48 h.

Results and Discussion
The cpl1 Suppresses Transcriptional Gene Silencing by ros1
Overexpression of the cold-stress-inducible RD29A-LUC reporter is a hallmark phenotype of cpl1 mutants. The expression level of RD29A-LUC is controlled by many factors including transcriptional gene silencing via RdDM. Since CPL1 has dsRBMs at its C-terminus, we tested if CPL1 is involved in dsRNA mediated gene regulations, i.e., transcriptional and post-transcriptional gene silencing (TGS and PTGS). The cpl1-2 line (hereafter referred as cpl1) was crossed with RD29A-LUC ros1–1 plants (hereafter referred as ros1) and with 35S-GUS L1 plants, representative systems to test TGS and PTGS in plants, respectively. As shown in Figures 1A to C, the expression levels of RD29A-LUC and 35S-NPTII were substantially decreased in

Figure 3. Differential accumulation of small RNAs in cpl1 under cold stress. (A) Northern blotting analyses of small RNAs in WT and cpl1 under stress conditions. Two-week-old plants were treated with cold (0°C) for 48 h or with 100 μM ABA for 3 h. U6 was used as loading controls. The different letters on the right side indicate independently prepared membrane blots. (B and C) Time course analyses of cold response of select pri-miRNA (B) and mature miRNA (C) levels by RT-qPCR. Bars indicate standard errors of the mean (SEM) of two biological replicates. *p<0.05, Student’s t-test between mean values of cpl1 and Col-0 for the same conditions.

doi:10.1371/journal.pone.0074739.g003
ros1 plants, resulting in lack of cold-induced luminescence and kanamycin sensitivity. Interestingly, in cpl1 ros1 double mutants, expression of RD29A-LUC but not 35S-NPTII and kanamycin resistance phenotype was partially restored. These results indicate that cpl1 suppresses gene silencing caused by ros1, but in a target specific manner.

To test the specificity of antagonistic interaction between ros1 and cpl1, expression levels of various transposable elements that are targets of gene silencing in ros1 were analyzed. Consistent with the previous report [43], ros1 downregulates expression of both transposons (Figure 1D, right) and retrotransposons (Figure 1D, left) tested. Individual transposable elements produced a unique expression profile in the different genetic backgrounds tested. In general, retrotransposons were down and up-regulated in ros1 and cpl1, respectively, and show intermediate levels in cpl1 ros1. This is indicative of an antagonistic effect of CPL1 and ROS1. Interestingly, expression of ONSEN, a heat inducible copia-like retrotransposon [34], was enhanced in cpl1 but was not affected in ros1 (Figure 1E). By contrast, both increases and decreases in cpl1 were observed for transcripts encoded by DNA transposons.
of the pri-miRNA levels (Figure 3C). Interestingly, some miRNA of several miRNA were decreased upon cold treatment regardless (Figure 3B). Consistent with the Northern blotting analysis, levels 171 (Figure 3A) but not other small RNAs tested (miR157, 159, as siRNA1003 and REP2, and miRNA160, 161, 164, 168, and in cold-treated wild type but not in cpl1. Unexpectedly, the siRNA level was slightly decreased treatment (hormonal inducer of RD29A) by Northern blotting RD29A promoter siRNA level during cold stress and ABA production of RD29A promoter siRNA, we tested if cpl1 affects DNA methylation of some but not all RdDM targets.

DNA Methylation Level at RdDM Target Loci is Reduced in cpl1
In ros1, RD29A-LUC is silenced by DNA methylation. To test if cpl1 restores RD29A-LUC expression by decreasing DNA methylation levels, we performed bisulfide sequencing analysis of the endogenous and the transgene RD29A promoters. As shown in Figure 2A, extensive cytosine methylation of both RD29A promoters in all sequence contexts (CG, CHG, and CHH; H represents A, T, or C) were observed in ros1. In cpl1 ros1, the CG methylation level was reduced 25% in transgene RD29A promoter and 16% in endogenous RD29A promoter. The partial reversion of DNA methylation is consistent with the partial release of gene silencing at these loci.

DNA methylation of RD29A promoter occurs via RdDM. We therefore tested DNA methylation levels of other RdDM targets, i.e., AAtMU1, AAtGPI and AAsN1 using PCR-based assays (Figure 2B). As reported previously, a DNA transposon AAtMU1 is heavily methylated in wild type and ros1. However, consistent with the higher expression levels, cpl1 and cpl1 ros1 mutants showed decrease in AAtMU1 methylation level. By contrast, no alteration was detected in methylation levels of AAtGPI and AAsN1 in cpl1. Together, these results indicate that cpl1 mutation affect DNA methylation of some but not all RdDM targets.

cpl1 Affects Small RNA Levels
Since silencing of RD29A/RD29A-LUC in ros1 is dependent on production of RD29A promoter siRNA, we tested if cpl1 decreases RD29A promoter siRNA level during cold stress and ABA treatment (hormonal inducer of RD29A) by Northern blotting (Figure 3A). Unexpectedly, the siRNA level was slightly decreased in cold-treated wild type but not in cpl1. Similar decreases were observed only in wild type for several additional small RNAs, such as siRNA1003 and REP2, and miRNA160, 161, 164, 168, and 171 (Figure 3A) but not other small RNAs tested (miR157, 159, 167, 173, 390, and TAS1, 2, and 3, (Figure S2)).

Time course analysis of select miRNA and pri-miRNA levels using RT-qPCR revealed that cold treatment increased the level of pri-miRNA171a expression but not other pri-miRNAs tested (Figure 3B). Consistent with the Northern blotting analysis, levels of several miRNA were decreased upon cold treatment regardless of the pri-miRNA levels (Figure 3C). Interestingly, some miRNA levels in cpl1 were lower than those in wild type even before cold treatment, and remained low during the cold treatment.

The inconsistency in miRNA levels in cpl1 detected by Northern blotting (maintain untreated WT levels) and RT-qPCR (always low) was recently explained by Manavella et al [45]; RT-qPCR reflects the levels of correctly processed miRNA, whereas Northern blotting detects both correct and incorrect forms of miRNA in cpl1. Based on this model, in wild type, cold stress decreases overall miRNA levels, although most of them are correctly processed. Interestingly, miR171a level decreases even its precursor pri-miR171A level increased. This is indicative that processing of pri-miRNA to mature miRNA rather than transcriptional induction of pri-miRNA is a regulatory step for defining miRNA levels during cold stress. This regulation appears impaired in cpl1, because correct processing of miRNA is constitutively lower in cpl1 and total miRNA level remained largely unchanged regardless of the cold stress.

cpl1 Interacts with HYL1 and SERRATE
The above results indicated that CPL1 is required for proper biogenesis of some miRNAs. Since miRNA are produced from hairpin-shaped dsRNA precursors and CPL1 contains dsRBMs in its C-termini, CPL1 may interact with dsRNA or other dsRBM proteins involved in production of small RNA. However, we did not detect reproducible dsRNA-binding activity in CPL1 dsRBM (data not shown). To test if CPL1 C-terminus functions in protein-protein interaction with dsRBM proteins, we performed co-immunoprecipitation assays using epitope-tagged CPL1 expressed at endogenous level (cpl1-FLAG) and antibodies against dsRBM proteins, namely, HYL1, DRB2, DRB3, DRB4, DRB5, DCL1, DCL3, and DCL4. Bi-directional co-immunoprecipitation successfully detected CPL1 interacting with HYL1, but not with other dsRBM proteins (Figures 4A, B, and data not shown). This interaction was further confirmed by BiFC and LaCl analyses, establishing that the CPL1-HYL1 complex was localized in nuclei (Figures 4C, D), and CPL1999-1009 containing C-terminal dsRBMs but not CPL1C1-714 containing the catalytic domain was sufficient. Interestingly, no CPL1-HYL1 interaction was detected using yeast two-hybrid analysis (Figure 4E). By contrast, SE, an interactor of HYL1 [46], could bind to CPL1 with the same specificity to HYL1 in planta, and did so in yeast as well (Figures 4D, E). Together, CPL1 forms a complex with HYL1 and SE via the C-terminal dsRBM-containing region, therefore is a part of miRNA producing complex. Interestingly, cpl1 mutants do not exhibit typical miRNA-deficient phenotype like hyl1 or se mutants, and the expression levels of miRNA target genes were similar to wild type (data not shown). The mild phenotype of cpl1 plants may be due to overlapping function of CPL1 and its paralog CPL2. The cpl1 cpl2 double mutant is lethal, perhaps partially due to the lack of essential miRNA/siRNA similar to severe dcl1 allele [47].

According to the classification of DNA methylation levels in various Arabidopsis RdDM mutants [48], the cpl1 phenotype likely belongs to “weakly reduced” or “affect only small proportion” category. It is rather surprising that cpl1 affects DNA methylation and expression levels of RdDM targets since RdDM is generally mediated by siRNA. However, Laubinger et al [24] reported that dcl1, which predominantly affects miRNA biogenesis, also affects DNA methylation. Since HYL1 and SE affect DCL1 function [49], it seems likely that weakly reduced DNA methylation in cpl1 ros1 plants is due to a compromised DCL1-dependent DNA methylation mechanism. Further analyses of target specificity of CPL1-dependent DNA methylation may reveal different branch of DNA methylation pathways.

Supporting Information

Figure S1 Post-transcriptional silencing of 35S-GUS transgene was intact in cpl1-2. GUS activity of 24 -day-old L1 and L1 cpl1-2 plants were visualized by X-gluc. (PDF)

Figure S2 Northern blotting analyses of small RNAs in WT and cpl1 under stress conditions. Two-week-old plants were treated with cold (0°C) for 48 h or with 100 µM ABA for 3 h. U6 was used as loading controls. The different letters on the right side indicate independently prepared membrane blots. (PDF)
Table S1  Oligonucleotide primers used in this study.

Author Contributions
Conceived and designed the experiments: ISJ JDB HK. Performed the experiments: ISJ EA AF SA. Analyzed the data: ISJ JDB HK. Contributed reagents/materials/analysis tools: AH TF. Wrote the paper: ISJ JDB HK.

References
1. Zheng X, Zhu J, Kapoor A, Zhu JK (2007) Role of Arabidopsis AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. EMBO J 26: 1691–1701.
2. Zheng X, Pontes O, Zhu J, Miki D, Zhang F, et al. (2008) ROASi is an RNA-binding protein required for DNA demethylation in Arabidopsis. Nature 455: 1259–1262.
3. He XJ, Hsu YF, Zhu S, Wierzbicki AT, Pontes O, et al. (2009) An Effector of RNA-Directed DNA Methylation in Arabidopsis Is an ARGONAUTE 4- and RNA-Binding Protein. Cell 137: 498–508.
4. Bies-EEhve N, Pontier D, Lahmy S, Picart G, Vega D, et al. (2009) RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family. EMBO Rep 10: 649–654.

48. Stroud H, Greenberg MV, Feng S, Bernatavichute YV, Jacobsen SE (2013) A novel role of the Arabidopsis SERRATE2 protein in active DNA demethylation. Proc Natl Acad Sci U S A 110: 9970–9975.
49. Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear RNA-Binding Protein. Cell 137: 498–508.