**RESEARCH COMMUNICATION**

**JMJ14, a JmjC domain protein, is required for RNA silencing and cell-to-cell movement of an RNA silencing signal in Arabidopsis**

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**Abstract**

JMJ14 is a histone H3 Lys4 (H3K4) trimethyl demethylase that affects mobile RNA silencing in an Arabidopsis transgene system. It also influences CHH DNA methylation, abundance of endogenous transposon transcripts, and flowering time. JMJ14 acts at a point in RNA silencing pathways that is downstream from RNA-dependent RNA polymerase 2 (RDR2) and Argonaute 4 (AGO4). Our results illustrate a link between RNA silencing and demethylation of histone H3 trimethylsine. We propose that JMJ14 acts downstream from the Argonaute effector complex to demethylate histone H3K4 at the target of RNA silencing.

Supplemental material is available at http://www.genesdev.org.

Received February 14, 2010; revised version accepted March 26, 2010.

In plants, RNA silencing of a non-cell-autonomous nature is evident in transgenic and virus-infected plants. Transgene silencing may initiate spontaneously or be induced in localized regions and subsequently spread throughout the plant [Palauqui et al. 1997; Voinnet and Baulcombe 1997; Voinnet et al. 1998]. Similarly, virus-induced silencing has effects beyond the infected tissue, and it is thought that the silencing signal is an RNA species. This RNA would move short distances through the plasmodesmata and systemically through the phloem [Voinnet et al. 1998].

Movement of the silencing signal is also associated with epigenetic modifications such as DNA cytosine methylation at targeted DNA loci. This RNA-directed DNA methylation (RdDM) is characterized by methylation of cytosines in all sequence contexts: CG, CHG, and CHH, where H is A, T, or C. In contrast, RNA-independent methylation affects those residues that are present in a CG or CHG context. Various DNA cytosine methyltransferases, histone-modifying enzymes, and nuclear-localized RNA silencing proteins are required for RdDM [Cao and Jacobsen 2002b; Jackson et al. 2002; Li et al. 2006; Pontes et al. 2006]. In addition, several plant-specific proteins are required, most notably subunits of novel RNA polymerases termed Pol IV and Pol V [Herr 2005; Kanno et al. 2005b]. Pol IV and Pol V share the same second-largest subunit, NRFP2/NRPE2, but are distinguished by their unique largest subunits, NRFP1 and NRPE1, respectively. Many of the other subunits are shared with Pol II [Huang et al. 2009; Lahmy et al. 2009]. Pol IV produces and/or amplifies the small RNA trigger, whereas Pol V acts downstream from this step to facilitate de novo methylation at the small RNA targeted site [Kanno et al. 2005a].

RdDM is also associated with histone modification, as well as DNA methylation at Arabidopsis loci, where CHG methylation by CHROMOMETHYLASE 3 (CMT3) is dependent on an SRA-SET domain protein, KRYPTONITE (KYP/SUVH4) [Cao and Jacobsen 2002a; Jackson et al. 2002]. KYP catalyzes methylation of histone H3 Lys9 (H3K9), providing a binding site for the chromodomain of CMT3 [Lindroth et al. 2004]. Two other SRA-SET proteins [SUH5 and SUH6] that methylate H3K9 also contribute [Ebb et al. 2005; Ebb and Bender 2006]. The relationship between histone and DNA modification also operates in the reverse direction. Thus, KYP and SUH6 bind directly to DNA methylated at CHG sites through their SRA domains [Johnson et al. 2007].

A link between histone modification and DNA methylation is implied not only by the phenotype of mutations in SRA-SET proteins, but also by the function of Jumonji C (JmJC) proteins found in diverse eukaryotes, including humans, yeast, and plants that are H3 lysine demethylases. Arabidopsis encodes 21 JmJC domain proteins, including some that may affect DNA methylation [Lu et al. 2008]. One of them [IBM1/JMJ25] prevents the spread of DNA methylation at certain genomic loci [Saze et al. 2008]. KDM1/LSD1—another protein in this group—demethylates methylated H3K4 [H3K4me1] and dimethylated H3K4 [H3K4me2] residues [Shi et al. 2004]. Other JmJC group proteins [ELF6/JMJ11 and REF6/JMJ12] either delay or accelerate flowering time, and they demethylate dimethylated/trimethylated H3K9 (H3K9me2/3) and H3K36me2/3, respectively [Noh et al. 2004]. MEE27/JMJ25 is required for embryo development, and belongs to the KDM5/JARID1 subgroup of JmJC proteins [Pagnussat et al. 2005]. KDM5/JARID1 proteins are histone demethylases using H3K4me1, H3K4me2, and H3K4me3 substrates [Christensen et al. 2007; Iwase et al. 2007; Lee et al. 2007; Seward et al. 2007].

In this study, we describe genetic evidence to implicate a KDM5/JARID1 protein, JMJ14, in RNA silencing. It is associated with a mobile signal of silencing in a pathway that is associated with methylation of the DNA at the target locus of RNA silencing. JMJ14 is also implicated in silencing of endogenous transposable elements in a pathway that is dependent on RNA-dependent RNA polymerase 2 (RDR2) and Argonaute 4 (AGO4) and control of flowering time. Based on an epistasis analysis in which the subcellular location of RDR2 and AGO4 location was assayed in wild-type and jmj14 plants, we propose that JMJ14 acts downstream from the Argonaute effector proteins.
complex to demethylate histone H3K4me3 residues at the target of RNA silencing.

Results and Discussion

A JmjC domain-containing protein, JAM39/JMJ14, is required for RNA silencing

To identify RNA silencing components involved in cell-to-cell movement of an RNA silencing signal in Arabidopsis, we used plants in which a transgenic photobleaching phenotype was due to spread of an RNA silencing signal into the mesophyll from the phloem (Smith et al. 2007). The target of silencing in this system was the endogenous phytoene desaturase (PDS), and we refer to the silencer transgene as JAP [Smith et al. 2007]. We demonstrated previously that chromatin silencing components NRPD1 (nuclear Pol IV), RDR2, and CLSY1 (an SNF2 domain-containing protein) are required for this mobile silencing effect based on the characterization of nrpd1, rdr2, and clsy1 mutants. Here, we characterize an additional mutant, jam39, with a similar phenotype of reduced photobleaching and lower levels of PDS siRNAs (Fig. 1). We mapped jam39 to At4g20400, which encodes the predicted JMJ14 protein with Jumonji C (JmIC), JmIN, C5HC2 Zinc finger, F/Y-rich C terminus, and F/Y-rich N terminus domains (Fig. 1). At4g20400 was first named as JM14 [Lu et al. 2008], and subsequently as JM14 [Jeong et al. 2009] and PKDM7B [Yang et al. 2010]. We acknowledge precedence and follow the Lu et al. (2008) nomenclature.

To confirm jam39 was due to mutation of At4g20400/JMJ14, we complemented the mutant phenotype of jam39 by transformation with a fragment of At4g20400 consisting of the promoter, coding region, and 3′ untranslated region (UTR) [Supplemental Fig. 1]. We also demonstrated that two T-DNA insertion alleles, jmj14-1 [salk_135712] and jmj14-2 [salk_136058], had a loss of photobleaching phenotype in the presence of the JAP transgene (Fig. 1). The mutant from our screen is jmj14-3.

Sequence alignment of JMJ14 and four other closely related proteins—AtJM15/MEE27/At2g34880, AtJM16/At1g08620, AtJM18/At1g30810, and AtJM19/At2g38950—revealed a high degree of similarity to the KDM5/JARID1 subgroup of JmjC proteins [Supplemental Fig. 2; Lu et al. 2008] in the JmIC domain and in a 61-amino-acid C5HC2 Zinc finger domain [Supplemental Fig. 3]. The C5HC2 domain has eight potential zinc ligand-binding residues, and may bind DNA or RNA. However, unlike the KDM5/JARID1 group, JMJ14 lacks a PHD domain that preferentially binds H3K9me3 [Iwase et al. 2007], and it has FYRN and FYRC domains at the N and C termini [Supplemental Fig. 3]. Interestingly FYRN and FYRC domains are normally found in trithorax and its homologs, a group of histone H3K4 methyltransferases [Finn et al. 2006].

Crystal structure of a KDM4A/JHDM3A catalytic core sequence shows that Fe(II) is chelated by three residues [His188, Glu190, and His276] within the JmIC domain [Supplemental Fig. 3]. Two additional residues [Thr185 and Lys206] are required for αKG binding [Chen et al. 2006; Klose et al. 2006]. Substitutions of the Glu that binds to Fe(II) to Asp, the Thr that binds to αKG to Tyr or Phe, and the Lys that binds to αKG to Arg are compatible with histone demethylation activity [Klose et al. 2006; Agger et al. 2007; De Santa et al. 2007; Hong et al. 2007; Lan et al. 2007]. Based on these combined observations, we predicted that JMJ14 has H3K4 histone demethylation activity that is dependent on these conserved Fe(II)- and αKG-binding amino acids [Supplemental Fig. 3]. This prediction has been confirmed recently by in vivo and in vitro analysis of JMJ14 [Jeong et al. 2009; Lu et al. 2010].

**JMJ14 acts at a far downstream stage of RNA silencing pathway mutants**

Previously, we identified non-CG DNA methylation at the endogenous PDS locus in a region complementary to the JAP transgene. NRPD1 is required for non-CG (CHG and CHH) methylation and JAP-induced photobleaching [Smith et al. 2007]. In jm14 mutants, based on bisulphite sequence analysis, we found that the CHH methylation at this endogenous PDS locus was reduced by as much as in nrpd1 and rdr2 [Fig. 1]. In contrast, CHG methylation was not affected in jm14 mutants, and was similar to the wild-type control with the JAP transgene [Fig. 1]. As expected, nrpd1 and rdr2 mutants had reduced amounts of CHG methylation at the endogenous PDS [Fig. 1]. CG sites were highly methylated in wild type and all mutants, jm14, nrpd1, and rdr2 [Fig. 1]. From this result, we infer that JM14 may act at the effector stage of RNA silencing rather than siRNA biogenesis and, based on the
similarity of this protein to a histone demethylase, we propose that it may influence epigenetic modification of targeted DNA.

Based on epistatic interactions affecting the subcellular localization of proteins, the components of the Pol IV silencing pathway act in the sequence [NRPD1/CLSY1]–RDR2–DCL3–AGO4–NRPE1 (Pontes et al. 2006). Therefore, to place JMJ14 in the silencing pathway, we analyzed the subcellular localization of RDR2 and AGO4 in nuclei of wild type and jmj14 mutants. Upstream silencing component RDR2 forms a crescent along the inner perimeter of the nucleolus and at foci throughout the nucleus (Pontes et al. 2006) in wild-type plants that was unaffected in jmj14 mutants. AGO4 localizes to nucleolus-adjacent Cajal bodies and nuclear foci, called AB bodies (Li et al. 2006; Pontes et al. 2006), and this pattern was also unaffected in most of the jmj14 and the JAP nuclei examined [Fig. 2]. These results are therefore consistent with the hypothesis that JMJC14 acts far downstream from siRNA biogenesis in the silencing pathway.

To confirm this conclusion, we used Western blotting to assess the accumulation of AGO4 in the jmj14 mutants. AGO4 is less abundant in rdr2 and rdp2 mutants, indicating that there is a link between the accumulation of the AGO4 effector of silencing and upstream siRNA biogenesis [Li et al. 2006; Pontes et al. 2006]. In contrast, in jmj14 mutants, the AGO4 protein levels were similar in jmj14 and wild type [Fig. 2], consistent with the action of this protein in the downstream part of the JAP transgene silencing pathway.

In a further test, we recombined jmj14 with ago4 in the JAP background. Single ago4 mutants exhibited enhanced photobleaching that is not affected by loss of function in either RDR2 or CLSY1 (Smith et al. 2007). Our interpretation of this result is that AGO4 mediates self-silencing of the JAP transgene. The enhanced phenotype in the single ago4/mutants is because this self-silencing pathway is perturbed, and it persists in the double mutants because the requirement for upstream proteins RDR2 and CLSY1 is bypassed. If JMJ14 acts in the upstream part of the pathway, like RDR2 or CLSY1, then the jmj14 ago4 double mutant would exhibit the enhanced silencing phenotype. However, it did not: The enhanced silencing was greatly reduced [Fig. 3]. This double-mutant analysis is therefore a further confirmation that JMJ14 acts in the downstream part of the JAP silencing pathway.

**The effect of JMJ14 on endogenous RNA, DNA methylation, and flowering**

To find out whether JMJ14 also affects silencing at endogenous loci, we used high-throughput sequencing of siRNA from seedling and floral tissues in wild-type and jmj14 mutants. However, there were no loci at which JMJ14 had a significant effect. This lack of an effect was confirmed by small RNA Northern analysis of selected microRNAs [miRNAs], trans-acting siRNAs [tasiiRNAs], and siRNAs [Fig. 4]. From these data, it seemed likely that JMJ14 acts similarly to RNA-binding regulators of flowering FCA and FPA (Baurle et al. 2007): These proteins are required for JAP-induced photobleaching and transgene-directed methylation of the endogenous PDS DNA, but they did not affect the abundance of endogenous siRNA.

The similar action of JMJ14 and these flowering time regulators was reinforced by the analysis of long RNA transcripts of retrotransposons AtSN1 and Solo LTR [long terminal repeat] by quantitative RT–PCR. These long RNAs are up-regulated in fpa and fca plants [Baurle et al. 2007], and they are similarly increased in jmj14 mutants. AtSN1 RNA was increased 75-fold more than wild type in jmj14 and nrpd1 mutants. A solo LTR was several-hundred-fold more abundant than wild type in jmj14 and nrpd1 mutants [Fig. 4].

Intergenic region 1 [IG1] RNA is also a target of the RNA silencing pathway that affects AtSN1 and Solo LTR. The IG1 RNA is up-regulated in nrpd1 and jmj14 mutants, although the abundance of the associated small RNAs [Huettel et al. 2006] in sequence data sets was not affected by JMJ14 function. However, it was striking that the long IG1 RNA increased to a greater extent in jmj14 mutants [Fig. 4] than in nrpd1.

The activation of AtSN1 and Solo LTR in jmj14 is also associated with loss of symmetric and asymmetric DNA methylation. Additionally, consistent with the hypothesis that JMJ14 acts far downstream from siRNA biogenesis in the silencing pathway.
methylation at the corresponding genomic loci [Hamilton et al. 2002], as found previously in nrpd1 [Herr et al. 2005]. Using an assay that combines digestion of genomic DNA using DNA methylation-sensitive restriction enzymes and semiquantitative PCR (Fig. 3), we found a loss of DNA methylation at these loci in jmj14 similar to nrpd1. We also assayed DNA methylation at solo LTRs using the PCR assay and found a loss of DNA methylation in jmj14 similar to nrpd1 (Fig. 4).

A link between JMJ14 and FPA/FCA was made through our observations that all three genes affect flowering. However, under long- and short-day conditions, the jmj14 mutants flowered earlier than the controls, approximately six rosette leaves, respectively (Fig. 5), whereas fca and fpa mutants were later flowering [Quesada et al. 2003]. This difference can be explained by the distinct activity of FPA/FCA and JMJ14 on the flowering time pathways. FPA/FCA suppress the FLC master regulator of flowering [Quesada et al. 2003], whereas JMJ14 does not affect FLC. Instead, it promotes expression of a second regulator, FT [Jeong et al. 2009, Lu et al. 2010].

**JMJ14 in chromatin silencing**

A straightforward interpretation of the results presented here invokes JMJ14 in the downstream part of silencing pathways that affect various endogenous loci, including PDS, AtSN1, Solo LTR, and IG1 (Fig. 3). Presumably, as JMJ14 is a histone H3K4 demethylase [Supplemental Fig. 2; Jeong et al. 2009, Lu et al. 2010], its role would lead to the loss of activating epigenetic marks associated with H3K4 methylation at the chromatin of target loci. This interpretation therefore implies that RNA silencing at AtSN1, Solo LTR, IG1, and PDS is either transcriptional or cotranscriptional, and is influenced by structural changes at the chromatin level.

Loss of a positive epigenetic mark is normally associated with gain of a negative mark at either the chromatin or DNA level and, consistent with this idea, there is increased methylation of DNA at AtSN1, Solo LTR, and PDS target loci (Figs. 1, 4). At the PDS transgene locus, this change could be indirect and influenced by reduced siRNA production in the jmj14 mutants [Fig. 1]. However, at the endogenous loci, where the level of siRNA is unaffected by the JMJ14 mutations (Fig. 4), there may be a more direct linkage. Inactivating epigenetic marks associated with modification of H3K9 or H3K27 might also be involved, and so that loss of the corresponding histone methyltransferase functions would give loss of the PDS silencing phenotype in the JAP lines. However, mutation of histone methyltransferase KYP had no effect on the PDS silencing phenotype (data not shown), and one of the other Arabidopsis SRA/SET domain proteins might be involved.

It is striking that mutation of JMJ14 has no effect on siRNA production from endogenous loci, but it does influence the level of siRNA from the PDS loci (Figs. 1, 3). To account for this observation, there must be a difference between the response of the endogenous and transgene loci to JMJ14-mediated epigenetic modification. Presumably, the endogenous AtSN1 and Solo LTR loci produce siRNA irrespective of whether JMJ14 is active, whereas the JAP transgene must be a better source of siRNA in the presence rather than the absence of active JMJ14. According to this idea, the JAP transgene would differ from the endogenous AtSN1 and Solo LTR loci in that there would be a positive feedback system affecting siRNA production. Silencing at all of these loci would be affected by JMJ14 acting at the downstream effector step. However, the silencing of the PDS locus would be reinforced in the presence of JMJ14 if the loss of the H3K4-activating epigenetic mark promoted further rounds of siRNA production.

**Figure 3.** JMJ14 and not NRPD1A is required for JAP-induced enhanced photobleaching. [A] Single mutants nrpd1, clsy1, or jmj14-3 in a JAP background. Controls plants Col and JAP are also shown. [B] Double mutants of nrpd1, clsy1, or jmj14 in an ago4 JAP genetic background. Control plant JAP ago4 is also shown. All plants were grown under long-day conditions.

**Figure 4.** Characterization of endogenous siRNAs and detection of increased AtSN1, solo LTR, and IG1 abundance in jmj14. (A) Detection of siRNAs derived from AtSN1, LTR, SS, tasiRNA 1511, and miRNAs 159 and 319 in JAP3, jmj14, and nrpd1 mutants. (B) Detection of AtSN1 transcripts in JAP, jmj14, and nrpd1 mutants by quantitative PCR. (C,D) Detection of solo LTR abundance (C) and IG1 abundance (D) by quantitative PCR. (E) Analysis of DNA methylation at AtSN1 by PCR. AluI digestion is sensitive to cytosine methylation. Reduction of methylation results in reduced levels of the PCR product. (F) Analysis of DNA methylation at solo LTR. Mspl digestion is sensitive to cytosine methylation. Quantification of triplicate samples was performed, of which one representative sample is shown.
a single 464 product in GTAAGTAG revealed two products, 200 and 264 bp, in Columbia, and EcoRI digestion of the PCR product amplified using oligonucleotide Lys, and was detected by PCR and restriction enzyme digestion assay. was a G-to-A transition changing conserved amino acid 387 from Glu to is SALK_136058, and (nrpd1 previously (Smith et al. 2007). RT–PCR detection of JMJ14 mRNA was with oligo-dT. RT–PCR detection of the JAP transgene was described here as JAP, was described previously (Smith et al. 2007). JMJ14/jmj14-3 and mutants and controls Columbia and JAP. [A] Average leaf number of plants grown under long-day photoperiodic conditions. White bars indicate rosette, and the black bar indicates cauline leaves. [B] Representative Columbia and jmj14-3 plants grown under long-day conditions. Roots were removed for photographing. [C] Average leaf of plants grown under short-day conditions. Average flowering time was calculated from at least 16 plants, and error bars show standard deviations.

Materials and methods

Plant material

Transgenic Arabidopsis thaliana (Columbia ecotype SUC2-PDS line #3, described here as JAP, was described previously [Smith et al. 2007]. JM14/At4g20400 alleles described are as follows: jmj14-1 is Salk_135712, jmj14-2 is SALK_136058, and jmj14-3 is an EMS-induced mutation. Allele jmj14-3 was a G-to-A transition changing conserved amino acid 387 from Glu to Lys, and was detected by a PCR and restriction enzyme digestion assay. EcoRI digestion of the PCR product amplified using oligonucleotide primers CCTTGAAGAAGCCCTGACTTG and GCTCCCAAGGCGA GTAAATGC revealed two products, 200 and 264 bp, in Columbia, and a single 464 product in jmj14-3. Mutant alleles used were ago1 [ago1-3], nrpd1 [nrpd1-1a-5], nrpd1le [nrpd1le-1d], and xrd-1-5.

Plasmid constructs

A complementing 6.2-kb genomic clone of JM14/At4g20400 was amplified using oligonucleotide primers attB1 (AGCAACATGTGTGTGACC) and attB2 (GATCTAGACGAGGATGATCTCAC), cloned into pGWI2-35S and transformed into jm14-3 using Agrobacterium tumefaciens GV3101-mediated transformation. Vector pGWI2-35S was constructed by self-ligation of the remaining vector. Gateway recombination sequence attB1 is ACAAGTTTGTACAAAAAGCTGAC and attB2 is ACCACG CTTCTGTGACAAAGTGC.

RNA and protein analysis

Solexa small RNA cloning was performed using Illumina Solexa kit as described by the manufacturer. Total RNA was isolated from 10-d-old seedlings, and ~ 10 µg of mirVana |Ambion]-purified RNA was used for ligation to adapters and amplification of small RNA fragments. Illumina sequencing was performed at Cambridge Research Institute UK, Cambridge. RT–PCR was performed using an Invitrogen SuperScript III kit as per the manufacturer’s recommendations using 5 µg of total RNA and with oligo-dT. RT–PCR detection of the JAP transgene was described previously (Smith et al. 2007). RT–PCR detection of JM14 mRNA was performed using primers GGGCCTTGAATGTTTGGATTCTC and CTCTCAACAGTCCTACCCGGAAGTC. RT–PCR detection of retrotransposons 10831 and DNA transposon AtMosl by RT–PCR was described previously (Herr et al. 2005; Baurle et al. 2007). Nuclear immunolocalization of proteins AGO4 and RDR2 was performed as described (Pontes et al. 2006).

Acknowledgments

We thank the Genomics and Bioinformatics core facilities (CRUK Cambridge Research Institute) for Illumina sequencing. This work was supported by the Gatsby Charitable Foundation and the EU Sixth Framework Programme Integrated Project SIROCCO (LSHG-CT-2006-037900). O.P. was supported by the Edward Mallinckrodt Jr. Foundation. I.S. received a Marie Curie Incoming International post-doctoral fellowship and a Wellcome Trust VIP Award. D.C.B. is supported as a Royal Society Research Professor. C.W.M is funded by Commonwealth and NSERC scholarships. Experiments were designed by I.S. and D.C.B., and were performed by I.S., O.P., L.M.S., and C.W.M. The manuscript was prepared by I.S., C.W.M., and D.C.B.

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*Genes Dev.* 2010, 24: Access the most recent version at doi:10.1101/gad.579910

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