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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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. MJM14 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 trimethyllysine. We propose that MJM14 acts downstream from the Argonaute effector complex to demethylate histone H3K4 at the target of RNA silencing.

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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 meth-

yltransferases, 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 two 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, NRDP2/NRPE2, but are distinguished by their unique largest subunits, NRDP1 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 (SUVH5 and SUVH6) that methylate H3K9 also contribute (Ebbs et al. 2005; Ebbs and Bender 2006). The relationship between histone and DNA modification also operates in the reverse direction. Thus, KYP and SUVH6 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, MJM14, 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. MJM14 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 MJM14 acts downstream from the Argonaute effector

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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 MJ14 protein with Jumonji C (JmjC), JmjN, C5HC2 Zinc finger, F/Y-rich C terminus, and F/Y-rich N

terminus domains (Fig. 1). At4g20400 was first named as MJ14 (Lu et al. 2008), and subsequently as MJ4 (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 MJ14 and four other closely related proteins—AtMJ15/MEE27/At2g34880, AtMJ16/At1g08620, AtMJ18/At1g30810, and AtMJ19/At2g38950—revealed a high degree of similarity to the KDM5/JARID1 subgroup of JmjC proteins (Supplemental Fig. 2; Lu et al. 2008) in the JmjC 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, MJ14 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 JmjC domain (Supplemental Fig. 3). Two additional residues (Thr185 and Lys206) are required for α KG 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 α KG to Tyr or Phe, and the Lys that binds to α KG 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 MJ14 has H3K4 histone demethylation activity that is dependent on these conserved Fe(II)- and α KG-binding amino acids (Supplemental Fig. 3). This prediction has been confirmed recently by *in vivo* and *in vitro* analysis of MJ14 (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 *jmj14* 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 *jmj14* 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, *jmj14*, *nrpd1*, and *rdr2* (Fig. 1). From this result, we infer that MJ14 may act at the effector stage of RNA silencing rather than siRNA biogenesis and, based on the

Figure 1. MJM14/JAM39 is required for SUC2-PDS (*JAP*)-induced silencing of *PDS* and DNA methylation. (A) Leaf phenotypes in *JAP* background. (B) Detection of *PDS* siRNAs in *jam39/jmj14-3* and controls *nrpd1* and *rdr2*. (C) Schematic representation of MJM14 locus and protein. Thick lines indicate exons and thin lines indicate introns. Protein domains shown are JmjN (white hexagon), JmjC (black rectangle), C5HC2 Zinc finger (white oval), FYRN (white diamond), and FYRC (white pentagon). (D) Cytosine methylation at the endogenous *PDS* locus assayed by bisulphite sequencing. At least 18 clones were analyzed for each line.

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 JM14 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

Figure 2. Nuclear localization of effector components AGO4 and RDR2 are not perturbed in *jmj14*. (A) Immunolocalization of AGO4 was performed on nuclei derived from JAP3 and mutants *jmj14-3* and *ago4*. DAPI was used as a DNA counterstain. (B) Immunolocalization of RDR2 was performed on JAP3, and mutants *jmj14-3* and *rdr2*. *n* = number of nuclei analyzed; (%) percentage of nuclei with representative immunolocalization pattern. Bar, 5 μ m. (C) Detection of AGO4 protein abundance by Western analysis. AGO1 abundance was used as a loading control.

consistent with the hypothesis that JM14 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 *nrpd1* and *rdr2* 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 JM14 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 JM14 acts in the downstream part of the JAP silencing pathway.

The effect of JM14 on endogenous RNA, DNA methylation, and flowering

To find out whether JM14 also affects silencing at endogenous loci, we used high-throughput sequencing of siRNA from seedling and floral tissues in wild-type and *jmj14* plants. However, there were no loci at which JM14 had a significant effect. This lack of an effect was confirmed by small RNA Northern analysis of selected microRNAs (miRNAs), *trans*-acting siRNAs (tasiRNAs), and siRNAs (Fig. 4). From these data, it seemed likely that JM14 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 JM14 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 JM14 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

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.

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 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, 5S, 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. *MspI* digestion is sensitive to cytosine methylation. Quantification of triplicate samples was performed, of which one representative sample is shown.

Figure 5. Flowering time of *jmj14* mutants and controls Columbia and JAP. (A) Average leaf number of plants grown under long-day photoperiod 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 CCTGAAGAAGACAGCCTGACTTG and GCTCCCAGAGGCA GTAAGTAG revealed two products, 200 and 264 bp, in Columbia, and a single 464 product in *jmj14-3*. Mutant alleles used were *ago4* (*ago4-3*), *nrdp1* (*nrdp1a-5*), *nrdp1e* (*drd3-1*), and *rdr2-5*.

Plasmid constructs

A complementing 6.2-kb genomic clone of JM14/At4g20400 was amplified using oligonucleotide primers *attB1* (AGCAACATGTGTGTGACC) and *attB2* (GATCTAGACGAGGATGATCTCAC), cloned into pGGW2-35S and transformed into *jmj14-3* using *Agrobacterium tumefaciens* GV3101-mediated transformation. Vector pGGW2-35S was constructed after XbaI digestion of pGGW2 to remove the 35SCaMV promoter and self-ligation of the remaining vector. Gateway recombination sequence *attB1* is ACAAGTTTGTACAAAAAGCAGGCT and *attB2* is ACCCAG CTTTCTGTACAAAGTGGT.

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 GGGCTTGAATGTTTGATTCTC and

CTTCAACAGTCCACGCAGAAGC. RT-PCR detection of retrotransposon AtSN1 and DNA transposon AtMu1 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).

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