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LETTER TO THE EDITOR

JMJ14 is an H3K4 demethylase regulating flowering time in *Arabidopsis*

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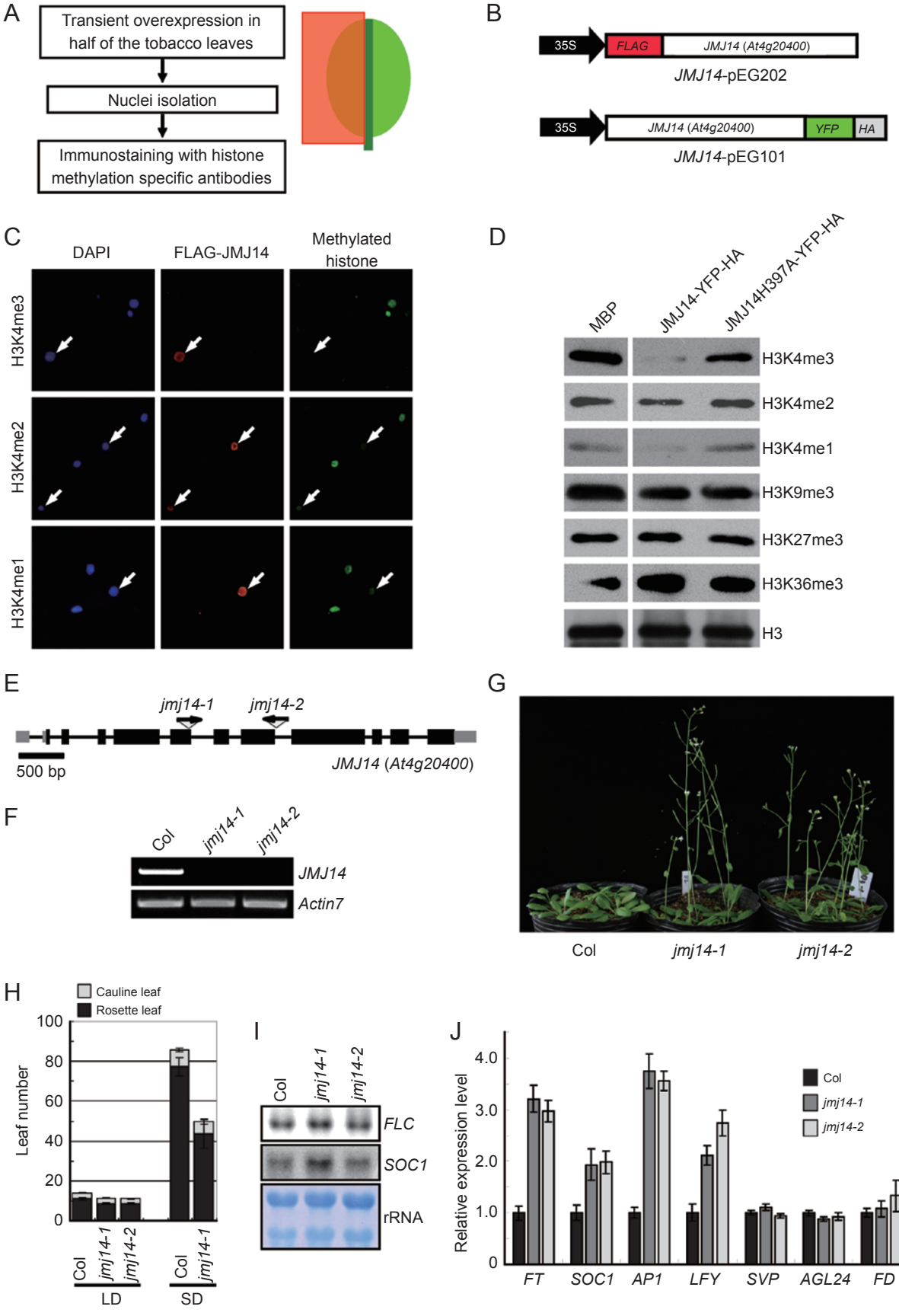
Dear Editor,

Histone lysine methylation plays an essential role in regulating chromatin functions such as transcription and heterochromatin formation. Histone H3 lysine 4 (H3K4) methylation is linked to active transcription [1, 2]. Recent findings in mammals have demonstrated that histone methylation is reversible by a family of Jumonji C (JmjC) domain-containing proteins. KDM5/JARID1 family proteins have been shown to be able to demethylate H3K4me1,2,3 in mammals [3]. Previously, we identified six proteins in Arabidopsis showing high sequence similarity to KDM5/JARID1 family proteins [4]. Here we demonstrate that one such protein, JMJ14, is an active histone H3K4 demethylase and is involved in flowering time regulation.

JMJ14 encoded by At4g20400 shows the highest sequence similarity to human KDM5/JARID1 family histone demethylases, suggesting that JMJ14 might be an active histone H3K4 demethylase. To test this prediction, we developed an *in vivo* assay to analyze its histone demethylase activity (Figure 1A). In brief, we first overexpressed JMJ14 in half of a 5-week-old tobacco leaf by injecting Agrobacteria carrying a FLAG-tagged *JMJ14* driven by the 35S promoter (Figure 1B). The other half of the leaf was not infiltrated and served as a control. Then nuclei were isolated from the leaf 2 days after infiltration and stained for specific histone modifications (Figure 1C). 4,6-Diamidino-2-phenylindole (DAPI) staining indicates the location of nuclei in each field. Anti-FLAG antibody was used to visualize the expression of the putative histone demethylase, thus showing which cells were successfully transfected. Histone demethylation activity was then monitored by comparing immunostaining between transfected and non-transfected cells. Using this method, we analyzed the demethylase activity of JMJ14. In FLAG-stained cells where JMJ14 was overexpressed, H3K4me3 and H3K4me2 modifications were hardly detectable, and H3K4me1 was much lower compared to cells without FLAG labeling (Figure 1C). In addition, when one conserved iron-binding residue of JMJ14, His397, was mutated to Ala (H397A), the H3K4 demethylation activity was abolished (Supplementary information, Figure S1). In contrast, no differences of mono-, di- or tri-methylation levels at H3K9, H3K27 and H3K36 were observed between cells with or without FLAG labeling (Supplementary information, Figure S2). This demonstrates that JMJ14 is an H3K4-specific demethylase and the conserved iron-binding residue is absolutely required for its enzymatic activity.

The demethylation activity was also shown using an *in vitro* assay. JMJ14-YFP-HA and JMJ14H397A-YFP-HA proteins were transiently overexpressed in tobacco leaves and immunoaffinity purified using anti-HA antibody. Then, an *in vitro* demethylation assay was carried out using calf thymus histone as a substrate. The results were consistent with our *in vivo* assays that JMJ14 but not JMJ14H397A demethylated H3K4me3,2,1, while H3K9me3, H3K27me3 and H3K36me3 were not affected (Figure 1D). Taken together, these results suggest that JMJ14 has the enzymatic activity and specificity to reverse H3K4me3, H3K4me2 and H3K4me1. Moreover, JMJ14 was localized to cell nuclei in both tobacco leaves and transgenic Arabidopsis expressing the JMJ14-YFP-HA fusion protein (Supplementary information, Figure S3), further supporting the conclusion that JMJ14 is a histone demethylase *in vivo*.

To investigate the roles of JMJ14 in plant development, we obtained two T-DNA insertion mutants, *jmj14-1* and *jmj14-2*, from the SALK T-DNA collection. The *JMJ14* gene (At4g20400) contains 12 exons and encodes a 954-amino-acid polypeptide with distinct domains, including the JmjN domain, JmjC domain, C5HC2 zinc finger (zf-C5HC2), F/Y-rich N-terminus (FYRN) and F/Y-rich C-terminal (FYRC) domains [4]. The T-DNAs inserted into the sixth exon (*jmj14-1*) and the eighth intron (*jmj14-2*) of *JMJ14*, respectively (Figure 1E). *JMJ14* full-length cDNA was not detectable in either of these two mutants by RT-PCR (Figure 1F). Compared to the wild-type plants, both of the mutants showed an early flowering phenotype under long day (LD, 16-h light and 8-h dark) conditions (Figure 1G and 1H).


A
Transient overexpression in half of the tobacco leaves
Nuclei isolation
Immunostaining with histone methylation specific antibodies
B
35S
FLAG
JMJ14 (At4g20400)
JMJ14-pEG202
35S
JMJ14 (At4g20400)
YFP
HA
JMJ14-pEG101
C
DAPI
FLAG-JMJ14
Methylated histone
H3K4me3
H3K4me2
H3K4me1
D
MBP
JMJ14-YFP-HA
JMJ14H397A-YFP-HA
H3K4me3
H3K4me2
H3K4me1
H3K9me3
H3K27me3
H3K36me3
H3
E
jmj14-1
jmj14-2
500 bp
JMJ14 (At4g20400)
F
Col
jmj14-1
jmj14-2
JMJ14
Actin7
G
Col
jmj14-1
jmj14-2
H
Cauline leaf
Rosette leaf
Leaf number
100
80
60
40
20
0
Col
jmj14-1
jmj14-2
LD
Col
jmj14-1
SD
I
Col
jmj14-1
jmj14-2
FLC
SOC1
rRNA
J
Relative expression level
4.0
3.0
2.0
1.0
0
Col
jmj14-1
jmj14-2
FT
SOC1
AP1
LFY
SVP
AGL24
FD

Floral transition is one of the most important developmental processes of higher plants and must be executed properly for maximum reproductive success. In Arabidopsis, flowering time is controlled by internal and environmental cues and can be affected by four major pathways, namely photoperiod, vernalization, gibberellic acid signaling, and autonomous pathways [5]. *FLC* is a major repressor of flowering. The Short Vegetative Phase (SVP) protein was shown to physically interact with FLC to repress flowering [6]. Floral signals are integrated by floral integrators *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION 1 OF CONSTANS* (*SOC1*), *AGAMOUS-LIKE 24* (*AGL24*), *APETALA1* (*AP1*) and *LEAFY* (*LFY*). FD, a bZIP protein, interacts with FT in the shoot apex to promote *SOC1* expression [7]. To explore the pathway in which JMJ14 acts, we further assayed the *jmj14-1* phenotype in short day (SD) conditions. The *jmj14-1* mutant was early flowering under SD conditions; however, it is much later than that in LD conditions (Figure 1H), suggesting that *jmj14* mutants respond normally to photoperiod. The response of *jmj14-1* to different photoperiods is similar to that of *FLC*-dependent pathway mutants. Therefore, we investigated the *FLC* transcript level in *jmj14* mutants. The *FLC* expression level in *jmj14-1*was similar to that in wild-type plants, indicating that *JMJ14* regulation of flowering time acts independently from *FLC* expression and it probably regulates downstream floral integrators (Figure 1I). To test this hypothesis, we examined the expression levels of floral integrators that act cooperatively with *FLC* or downstream of *FLC*, including *SOC1*, *LFY*, *FT*, *AP1*, *SVP, AGL24*, and *FD*. RNA gel blot analysis showed a slight elevation of *SOC1* transcripts (Figure 1I). Quantitative RT-PCR analysis showed that transcripts of *FT*, *SOC1*, *LFY* and *AP1* were 2- to 4-fold increased from wild-type levels in *jmj14* mutants, whereas the expression of *SVP*, *AGL24* and *FD* remained unchanged (Figure 1J). Thus, the early flowering phenotype of *jmj14* mutants was associated with increased expression of floral integrators.

As H3K4me3 is a hallmark of active transcribing regions and JMJ14 is a histone H3K4 demethylase, we wondered whether overexpression of *FT*, *AP1* and *SOC1* was caused by increased H3K4 methylation due to loss of JMJ14. A chromatin immunoprecipitation (ChIP) assay was used to examine the H3K4 methylation status of *FT*, *AP1* and *SOC1* chromatin loci. Chromatin regions used in ChIP assays were as previously reported, except for the *FT* promoter region (*FT* P) and *SOC1* D (Supplementary information, Figure S4). ChIP results showed that H3K4me2 and H3K4me3 methylation levels were not significantly different between *jmj14* mutants and wild type at the *AP1* locus, the *FT* locus or the *SOC1* locus (Supplementary information, Figure S4). Therefore, JMJ14 repression of flowering time might not act through directly demethylating *FT*, *AP1* or *SOC1* loci.

The *FT* locus has been previously shown to possess

Figure 1 JMJ14 is a histone H3K4 demethylase that represses flowering. **(A)** Scheme of the *in vivo* histone demethylation assay is shown on the left and a diagram of an infiltrated leaf is shown on the right. The red shadow indicates the part of the leaf infiltrated. **(B)** Diagrams of constructs used in FLAG-JMJ14 and JMJ14-YFP-HA transient expression. **(C)** Overexpression of FLAG-JMJ14 reduced H3K4me3, H3K4me2 and H3K4me1. Histone methylation was visualized by immunostaining with rabbit polyclonal modification specific antibodies followed by Alexa Fluor 488 conjugated goat anti-rabbit (green), which is shown in the right panels. Nuclei transfected with FLAG-JMJ14 were detected by immunostaining with FLAG M2 antibody followed by Alexa Fluor 555 conjugated goat anti-mouse (red) and are shown in the middle panels. Nuclei were revealed by DAPI staining (blue) and are shown in the left panels. More than 50 pairs of transfected nuclei versus non-transfected nuclei were observed with the same results. Arrows indicate nuclei transfected with FLAG-JMJ14. **(D)** JMJ14-YFP-HA but not JMJ14H397A-YFP-HA, which contains histidine 397 to alanine mutation in the Fe(II) binding site, can demethylate H3K4 methylation on calf thymus histone *in vitro*. However, JMJ14-YFP-HA has no effect on H3K9me3, H3K27me3 and H3K36me3. MBP (maltose binding protein) was used as a no enzyme control. The methylation status was detected by immunoblots with methylation-specific antibodies. Immunoblot with histone H3 showed equal loading. **(E)** Gene structure of the *JMJ14* transcribed unit and T-DNA insertion site of *jmj14* mutants. Black bars, gray bars and black lines indicate coding exons, UTRs and introns, respectively. T-DNA insertions are indicated by triangles, directions of which are indicated by arrows. Bar equals 500 bp. **(F)** RT-PCR of full-length *JMJ14* revealed that transcription of *JMJ14* was eliminated in *jmj14* mutants. *Actin7* was used as an internal control. **(G)** *jmj14* mutants show early flowering under LD (long day, 16-h light, 8-h dark) conditions. **(H)** *jmj14* mutants were early flowering under both LD and SD (short day, 8-h light, 16-h dark) conditions. Black cylinders represent rosette leaf number and gray cylinders represent cauline leaf number. Error bars represent 2× standard error. **(I)** RNA gel blot analysis of *FLC* (upper) and *SOC1* (middle) in 10 DAG seedlings grown under LD. Membrane post transfer stained with methylene blue revealing rRNA was used as a loading control (bottom). **(J)** Quantitative RT-PCR of *FT*, *SOC1*, *AP1*, *LFY*, *SVP*, *AGL24*, and *FD* using 10 DAG seedlings grown under LD. Transcript levels were first normalized to *Actin7* expression levels in each sample, and then normalized to the expression of the Col sample. Black bars represent Col, dark gray bars represent *jmj14-1* and light gray bars represent *jmj14-2*. Error bars represent 2× standard error.

H3K4me3, which could be repressed by H3K27me3 catalyzed by CURLY LEAF (CLF) [8]. In *clf* mutants, H3K4me3 was increased 2- to 4-fold at the *FT* locus. However, we note that *clf* mutants had about a 200-fold increase in *FT* transcription [8]. In this work, we showed that *jmj14* mutants had about a 3-fold increase in *FT* transcription, which is unlikely to be caused by a significant change in H3K4 tri-methylation. Recently, one related study reported that H3K4me3 was increased at the *FT* transcription initiation region and that JMJ14 (called JMJ4 in their study) associated with the *FT* promoter directly [9]. One possibility of this difference is that materials of different developmental stage were used in the experiments. Jeong *et al*. used 57-day-old SD grown plants, whereas we used 10-day-old LD grown plants. In addition, they showed that a 200-bp I region of *FT* promoter was enriched four times by JMJ14-FLAG; in contrast, an overlapping 200-bp G region showed no enrichment in our assays.

Although the JmjC domain is essential for histone demethylase activity, such activity may not always be associated with the function of cognate proteins. For example, the function of Lid2, a yeast homolog of KDM5/JARID1, in euchromatin does not require its demethylation activity but requires an intact JmjC domain [10]. As for JMJ14's function in repressing flowering, the requirement of its demethylation activity remains to be tested in further study. If repressing *FT*, *SOC1*, *AP1* and *LFY* expression by JMJ14 requires its demethylation activity, we would also expect that some histone H3K4 methyltransferases should be involved in promoting their expression.

In conclusion, our study has revealed that JMJ14 is a histone H3K4 demethylase that is important for preventing early flowering through repression of the floral integrators *FT*, *AP1*, *SOC1* and *LFY* during the vegetative growth phase.

(Experimental materials and methods are depicted in the Supplementary information, Data S1)

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)