Two novel NAC transcription factors regulate gene expression and flowering time by associating with the histone demethylase JMJ14

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ABSTRACT

The histone demethylase JMJ14 catalyzes histone demethylation at lysine 4 of histone 3 and is involved in transcriptional repression and flowering time control in Arabidopsis. Here, we report that JMJ14 is physically associated with two previously uncharacterized NAC transcription factors, NAC050 and NAC052. The NAC050/052-RNAi plants and the CRISPR-CAS9-mediated nac050/052 double mutant plants show an early flowering phenotype, which is similar to the phenotype of jmj14, suggesting a functional association between JMJ14 and NAC050/052. RNA-seq data indicated that hundreds of common target genes are co-regulated by JMJ14 and NAC050/052. Our ChIP analysis demonstrated that JMJ14 and NAC050 directly bind to co-regulated genes shared in jmj14 and NAC050/052-RNAi, thereby facilitating H3K4 demethylation and transcriptional repression. The NAC050/052 recognition DNA cis-element was identified by an electrophoretic mobility shift assay at the promoters of its target genes. Together, our study identifies two novel NAC transcription repressors and demonstrates that they are involved in transcriptional repression and flowering time control by associating with the histone demethylase JMJ14.

INTRODUCTION

Histone methylation affects transcription status on chromatin and is dynamically regulated through the addition and removal of methyl groups. The enzymes that contain an evolutionarily conserved SET domain (named after the three Drosophila histone methyltransferases Su(var)3–9, E(z) and Trithorax) catalyze histone lysine methylation (1,2). The JmjC domain-containing histone demethylases are responsible for the removal of methyl groups from methylated histone at lysine sites and are conserved in plants, animals and fungi (3,4).

In Arabidopsis, there are 21 JmjC domain-containing histone demethylases that have been named JMJ11–JMJ31 (5). Previous studies have demonstrated that the Arabidopsis JmjC domain-containing histone demethylases are involved in diverse biological processes (6). JMJ15/IBM1 (Increase in Bonsai Methylation 1), which is necessary for floral development, is responsible for H3K9 demethylation at actively transcribed genes and protects these genes from DNA methylation at CHG sites (7–9). JMJ30/JMJ5 positively affects circadian clock-regulated gene expression and is involved in controlling the circadian rhythm (10,11). The histone arginine demethylases JMJ20 and JMJ22 act as positive regulators of seed germination by mediating the removal of repressive histone arginine methylation and the transcriptional activation at GA3ox1 and GA3ox2 (12). ELF6/JMJ11 and REF6/JMJ12 are two close homologs that control flowering time and other developmental processes (11,13,14). REF6 acts as an H3K27me3 demethylase and contributes to the expression of genes involved in development and stress response (11). Although these JmjC histone demethylases were identified and characterized in Arabidopsis, further studies are required to clarify how conserved histone demethylases have different target genes and are involved in diverse biological processes.

The H3K4 demethylase JMJ14 is involved in repression of the floral integrator genes FT and SOC1 (15–17). Moreover, JMJ14 is required for maintenance of transcriptional silencing through an RNA-directed DNA methylation pathway (15,18). Interestingly, JMJ14 is involved in transcriptional repression of aberrant RNAs, which trigger post-transcriptional gene silencing (19). The close homologs of JMJ14, JMJ15 and JMJ18, regulate flowering time through reducing H3K4 trimethylation at the floral repressor gene FLC, resulting in the repression of FLC expression and the promotion of flowering time (20,21). The role
of JMJ15 and JMJ18 in flowering promotion is opposite to that of JMJ14 in flowering repression.

The mammalian histone demethylases usually interact with transcriptional regulators to control gene expression for specific target genes. The JmjC histone demethylase JHDM2A/JMJD1a interacts with the androgen receptor and cause the removal of H3K9 demethylation, resulting in the transcriptional activation of androgen receptor target genes (22). JARID1C/SMCX and the transcription repressor REST interact with each other and occupy the promoters of REST target genes (23). The depletion of JARID1C increases H3K4 trimethylation and simultaneously induces the expression of REST target genes that are implicated in X-linked mental retardation and epilepsy. JARID1a forms a complex with the CLOCK-BMAL1 transcription factors, influencing the circadian clock by activating the transcription of Per2 (24).

The plant-specific NAC (NAM, ATAF1 and CUC1/CUC2) proteins form one of the largest transcription factor families in plants (25–27). NAC transcription factors are involved in various biological processes, including development, hormone signaling, senescence and biotic and abiotic stress responses (26,27). NAC transcription factors contain a conserved N-terminal DNA-binding NAC domain and a diversified C-terminal transcription regulatory domain. Previous studies have demonstrated that many NAC proteins are transcriptional activators, whereas a few others are transcription repressors (26–29). NAC proteins are recruited to their target loci by directly binding the DNA-cis element in the promoter of their target genes (26,27).

JMJ14 is involved in transcriptional gene silencing and flowering time regulation (15–18), but we do not know how JMJ14 specifically functions at a subset of genes but not others. Given that several histone demethylases exist in prokaryotes (19–21), we asked whether JMJ14 associates with any other proteins in flowering regulation. Given that several histone demethylases exist in prokaryotes (19–21), we asked whether JMJ14 associates with any other proteins in flowering regulation. The plant-specific NAC (NAM, ATAF1 and CUC1/CUC2) proteins form one of the largest transcription factor families in plants (25–27). NAC transcription factors are involved in various biological processes, including development, hormone signaling, senescence and biotic and abiotic stress responses (26,27). NAC transcription factors contain a conserved N-terminal DNA-binding NAC domain and a diversified C-terminal transcription regulatory domain. Previous studies have demonstrated that many NAC proteins are transcriptional activators, whereas a few others are transcription repressors (26–29). NAC proteins are recruited to their target loci by directly binding the DNA-cis element in the promoter of their target genes (26,27).

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**MATERIALS AND METHODS**

**Plant materials, constructs and growth conditions**

The Arabidopsis materials included the wild-type (WT) Col-0, jmj14 (Salk_135712C) and NAC050/52-RNAi plants. Two inverted copies of the NAC050 cDNA fragment (+489—+1077) were separately inserted into the RNAi vector pFGC5941 and transformed into the WT and jmj14 plants for the knockdown of NAC050 and NAC052 in Arabidopsis. This fragment of the NAC050 cDNA sequence is highly similar to NAC052, but is different from any other genes. Thus, both NAC050 and NAC052 were knocked down in the NAC050/52-RNAi plants. We generated the native promoter-driven JMJ14–3xFlag, NAC050–3xMyc and NAC052–3xMyc constructs in the modified pCAMBIA1305 backbone. The length of the promoters for JMJ14, NAC050 and NAC052 is 1538, 1438 and 1772 bp, respectively. The sequences of the primers used in amplification of JMJ14, NAC050 and NAC052 are shown in Supplementary Table S1.

**Affinity purification and mass spectrometric analysis**

We collected flowers at stages 1–15 of floral development from 1-month-old plants for isolating protein extract. Protein extraction was performed according to the method previously described (31) and subjected to the affinity purification of JMJ14–3xFlag using an anti-Flag antibody. For the affinity purification, Anti-Flag M2 Affinity Gel (Sigma, A2220) was added to the protein extract and incubated in lysis buffer at 4°C for 2–4 h. The agarse-bound proteins were precipitated by centrifugation and washed four times, followed by an elution with the 3xFlag peptide (Sigma, F4799). The eluted proteins were run on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and visualized by silver staining (Sigma, PROT-SIL1). The proteins were extracted from the gel and analyzed by tandem mass spectrometry as previously described (31). A search was conducted to see if the obtained peptide sequence data could be found in the International Protein Index database of Arabidopsis on the Mascot server (Matrix Science Ltd., London, UK).

**Co-IP and gel filtration**

The NAC050–3xMyc and NAC052–3xMyc constructs were separately transformed into JMJ14–3xFlag transgenic plants. The offspring seedlings harboring both the JMJ14–3xFlag and NAC050–3xMyc or NAC052–3xMyc transgenes were used to determine whether JMJ14 interacts with NAC050 or NAC052 by co-IP. Anti-Flag M2 Affinity Gel (Sigma, A2220) was added to the protein extract and incubated at 4°C for 2–4 h. The agarse-bound proteins were...
precipitated by centrifugation and washed four times. The precipitate was suspended, boiled in 1× SDS sample buffer and run on an SDS-PAGE gel for western blotting. The protein extracts from JMJ14-3xFlag, NAC050-3xMyc and NAC052-3xMyc transgenic plants were used for gel filtration. The protein extracts were loaded onto a Superose 6 10/300 GL column (GE Healthcare, 17–5172–01) and harvested once per 500 μl. The indicated fractions were run on a 10–12% SDS-PAGE gel and subjected to western blotting. Anti-Flag (Abmart, M20008L) and anti-Myc antibodies (Abmart, M20002L) were used in the western blotting for both the co-IP and gel filtration assays.

Yeast two-hybrid assay

The full-length cDNA sequences of JMJ14, NAC050 and NAC052 were separately cloned into the yeast vectors pGADT7 and pGBK7T to examine the interactions between JMJ14, NAC050 and NAC052. The full-length cDNA of JMJ14 (2865 bp) was amplified and cloned into the pGADT7 vector between the XmaI and XhoI sites and into the pGBK7T vector between the XmaI and PstI sites. The full-length cDNAs of NAC050 (1341 bp) and NAC052 (1353 bp) were separately cloned into both the pGADT7 and pGBK7T vectors between the EcoRI and BamHI sites. Truncated JMJ14 and NAC050 sequences were cloned into pGADT7 or pGBK7T to determine the domains in JMJ14 and NAC050 that are required for the interaction.

For cloning the truncated JMJ14 sequences into pGADT7, all the truncated JMJ14 sequences were amplified from the above pGADT7-JMJ14 plasmid. The JMJ14-a/b/c fragments were amplified using the primers JMJ14ADB-Xma I and JMJ14AD-a/b/c-R, whereas the JMJ14-e/d/f fragments were amplified using the primers JMJ14AD-d/e/f-F and JMJ14AD-Xho I. These fragments were digested with corresponding restriction enzymes and cloned into the pGADT7 vector. For cloning the truncated NAC050 sequences into pGBK7T, all the truncated NAC050 sequences were amplified from the pGBK7T-NAC050 plasmid. NAC050-a/c were amplified using the primers NAC050ADB-EcoR I and NAC050BD-a/c-R, whereas NAC050-DB-b/d were amplified using the primers NAC050BD-b/d-F and NAC050ADB-BamH I. The primers that were used for cloning the full-length and truncated sequences are listed in Supplementary Table S1. Different combinations of the pGADT7 and pGBK7T constructs were transformed into the yeast strain Y1348. The positive strains were selected from a synthetic dropout medium minus Trp and Leu (SD-TLH) and then used for a growth assay on SD-TLH (the synthetic dropout medium minus Trp, Leu and His) supplemented with 20 mM 3-AT. These strains were simultaneously grown on SD-TL as controls.

Analyses of RNA transcripts by reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from 2-week-old seedlings that were grown on MS medium plates as previously described (31). The RNA was treated with DNase to remove DNA contamination and subjected to quantitative RT-PCR using a one-step RT-PCR kit (Takara, RR018A). Oligo-dT was used as the primer for reverse transcription. The expression of ACT2 was examined as an internal control. The expression of each gene that was tested in this study was normalized to the expression of ACT2. ‘No RT’ indicates that the RNA samples were directly used as templates to amplify the control gene. The primers that were used for the PCR are listed in Supplementary Table S1.

Electrophoretic mobility shift assay (EMSA)

The full-length NAC050/052 cDNA sequences were cloned in frame with the 5′-terminal GST sequence in the pGEX-4T-1 vector. Meanwhile, the NAC050 cDNA sequences of the N-terminal NAC domain (1–678 bp) and the C-terminal regulatory domain (679–1341 bp) were cloned in the pGEX-4T-1 vector and the corresponding truncated NAC050 proteins were defined as NAC050-N and NAC050-C, respectively.

The GST-fusion proteins were expressed in the E. coli strain BL21 (Invitrogen), purified with Glutathione Sepharose 4B (GE Healthcare,17075601) and used for EMSA. One microgram of purified protein was incubated with 0.08 nM of each double-stranded DNA oligo in a binding buffer at 25°C for 30 min. The binding buffer contained 25 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM ethylene-diaminetetraacetic acid (pH 8.0), 12.5 mM MgCl2, 1 mM DTT, 0.5% (w/v) bovine serum albumin and 5% (w/v) glycerol. The binding reaction mixture was loaded onto a 7% non-denaturing polyacrylamide gel at 80 V for 2 h, and the bound DNAs were visualized by ethidium bromide staining.

ChIP assay

The association of JMJ14, NAC050 and H3K4me3 with chromatin was determined using a ChIP assay. Two-week-old seedlings were subjected to cross-linking in 0.5% formaldehyde under vacuum conditions. Thereafter, the nuclei were extracted from the seedlings and sonicated for chromatin extraction. Anti-Flag M2 Affinity Gel (Sigma,
A2220) and Anti-Myc Affinity Gel (Sigma, A7470) were used for the IP of JMJ14–3xFlag and NAC050–3xMyc, respectively. For H3K4me3 ChIP, anti-H3K4me3 antibody (Millipore, 05–745) were conjugated with Protein A agarose (Millipore, 16–157) followed by IP. The precipitate was analyzed by quantitative PCR. The ChIP-PCR experiments were biologically repeated and the same results were obtained. Showing is the results of three technical replicates from a representative experiment. The association of these proteins with the actin gene ACT2 was examined and used as an internal control. The primers that were used for the PCR are listed in Supplementary Table S1. H3K4me3 ChIP-seq analysis was performed as previously described (33,34).

**ChIP-seq data analysis**

For H3K4me3 ChIP-seq data analysis, reads were mapped to the Arabidopsis genome (TAIR10, [http://www.arabidopsis.org](http://www.arabidopsis.org)) with Bowtie (v0.12.7) with at most two mismatches (35). The package MACS (v1.4.2) was used to identify regions of H3K4me3 enrichment in jmj14 and NAC050/052-RNAi as compared to the WT (36). Only peaks with P-value <0.001 and fold-change >2 were identified as differentially enriched regions. To determine the relationship between H3K4me3 enrichment and differentially expressed genes, we used R to plot the H3K4me3 pattern for differentially expressed genes. Each gene was divided into 50 intervals (2% for each interval), and the 1-kb regions upstream and downstream of each gene were divided into 20-bp intervals. Normalized reads in each interval was graphed to indicate the distribution of H3K4me3 across genes.

**CRISPR-CAS9-mediated mutation**

The DNA sequences of NAC050 and NAC052 are highly similar to each other. To produce a gRNA that directs their common target sequence GAAGGTTAAT-GATCTCCAGA, we synthesized two DNA oligos: oligo1 and oligo2 (Supplementary Table S1), which were annealed to form a double-stranded DNA and then inserted into the cloning vector harboring the psgRNA-CAS9-At fragment (37). Thereafter, the whole psgRNA-CAS9-At fragment was digested by EcoRI and HindIII and inserted into the corresponding sites of pCAMBIA1300 for agrobacterium-mediated transformation in Arabidopsis. The T1 transgenic plants were grown on MS medium supplemented with 30 mg/l hygromycin, and the resistant positive seedlings were selected for sequencing the target sequences of NAC050 and NAC052. The homozygous mutants were acquired from T2 transgenic plants.

**Transient luciferase reporter assay**

A luciferase gene was driven by the promoter sequences of AT2G18720 (~562 ~ 52 bp), AT2G21640 (~884 ~ 470 bp), AT5G16020 (~488 ~ 1 bp) and AT1G02580 (~500 ~ 22 bp) at the KpnI-BamHI site of the modified pCAMBIA1300 vector. Moreover, we generated a construct, in which the luciferase reporter gene was driven by a minimal 35S promoter with an upstream GAL4-binding site. The reporter can be activated by the GAL4-DDBD-VP16 fusion protein. The 5xNAC050-binding motif (CTTGGTCGCCAGGAA) as well as its mutant variant (CTTGGTCCCGCCGAA) were separately ligated with 3’-end of the 35S minimal promoter in the regulator construct to determine the function of the NAC050-binding motif. The luciferase reporter assay was performed in Arabidopsis mesophyll protoplasts as described by Sheen’s group ([http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html](http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html)) with minor modifications. The luciferase activity was measured with Dual-Luciferase® Reporter Assay System (Promega, E1910). Three technical replicates from a representative experiment were shown.

**RESULTS**

**JMJ14 is physically associated with the NAC transcription factors NAC050 and NAC052**

To study how JMJ14 functions in vivo, we generated a construct harboring a native promoter-driven JMJ14 genomic sequence with its C-terminus in frame with the 3xFlag epitope tag and transformed the cassette into Arabidopsis. The expression of the JMJ14–3xFlag transgene in transgenic plants was determined by a western blotting assay (Supplementary Figures S1 and S2). We demonstrated that the JMJ14–3xFlag transgene complements the early flowering phenotype of the jmj14 mutant (Supplementary Figure S1), suggesting that the JMJ14–3xFlag transgene functions as well as the corresponding endogenous gene. Based on previous transcriptome data (Supplementary Table S2), NAC050/052 and JMJ14 are highly expressed in floral organs and moderately expressed in other organs. Thus, flowers were collected from 1-month-old plants and used for affinity purification of JMJ14–3xFlag by anti-Flag antibody-conjugated agarose. Purified proteins were subjected to a mass spectrometric assay. Two previously uncharacterized NAC transcription factors, NAC050 (AT3G10480) and NAC052 (AT3G10490), were identified in co-purified proteins (Figure 1A). The two NAC genes form tandem repeats on the genome and may originate from a recent duplication. The N-terminal NAC domains of NAC050 and NAC052 are highly conserved in the NAC family proteins, while their C-terminal regions are similar to each other but are distinct compared to those of other NAC family proteins (Supplementary Figure S3).

To confirm the association between JMJ14 and the two NAC proteins, we introduced the native promoter-driven NAC050–3xMyc and NAC052–3xMyc transgenes into the JMJ14–3xFlag transgenic plants. The offspring plants harboring both JMJ14–3xFlag and NAC050–3xMyc or NAC052–3xMyc were used for co-IP. The protein extracts were precipitated by anti-Flag antibody followed by western blotting assay. The results indicate that JMJ14 can interact with both NAC050 and NAC052 (Figure 1B). To clarify whether the possible DNA binding ability of JMJ14 or NAC050/052 is required for the interaction between JMJ14 and NAC050/052, we performed co-IP after the protein extracts were treated with DNase (Supplementary Figure S2) and found that the addition of DNase has no effect...
on the interaction between JMJ14 and NAC050/052, indicating that the interaction is independent of the binding of JMJ14 and NAC050/052 to chromatin. By gel filtration, we found that NAC050 and NAC052 were co-eluted at the fractions between 17 and 21 (Figure 1C), suggesting that the two NAC proteins may form a tight complex in vivo. The result are consistent the yeast two-hybrid data indicating two NAC proteins may form a tight complex.

We performed a yeast two-hybrid assay to examine the interaction between JMJ14 and the two NAC proteins, demonstrating that JMJ14 not only interacts with NAC050 but also with NAC052 (Figure 2A). We also determined whether JMJ15 and JMJ18, two close homologs of JMJ14 (Supplementary Figure S4), interact with NAC050 and NAC052 by yeast two-hybrid. The results indicate that JMJ15 and JMJ18 fail to interact with the two NAC proteins (Supplementary Figure S5), suggesting the functional specificity of JMJ14 in the association with NAC050 and NAC052.

Our gel filtration assay indicated that NAC050 and NAC052 form ~440-KDa complexes, which are much greater than the sizes of their monomers (Figure 1C). Thus, we performed a yeast two-hybrid assay to test whether the two NAC transcription factors interact with each other. The results indicate that NAC050 interacts not only with NAC050 but also with NAC052, whereas NAC052 cannot interact with NAC052 (Figure 2A). Based on the approximate size of the NAC050/052 complex, we propose that NAC050 and NAC052 may form tetramers or even higher molecular weight oligomers in Arabidopsis.

To determine the domain of JMJ14 that is required for association with the two NAC proteins, we cloned a series of truncated JMJ14 sequences for the yeast two-hybrid assay (Figure 2B). The FYRC domain of JMJ14 by itself (JMJ14-f) is sufficient for the interaction of JMJ14 with NAC052 but not with NAC050 (Figure 2B and C). For the interaction of JMJ14 with NAC050, not only the FYRC domain but also the two other domains JmjC and C5HC2 are required (Figure 2B and C). The truncated JMJ14 sequences JMJ14-a and JMJ14-b without the FYRC domain cannot interact with NAC050 and NAC052 (Figure 2B and C). Therefore, the FYRC domain is necessary for the interaction of JMJ14 with both NAC050 and NAC052 as determined by the yeast two-hybrid assay. We further investigated whether the FYRC domain is required for the interaction of JMJ14 with NAC050 and NAC052 in Arabidopsis. A truncated JMJ14 genomic sequence was constructed in frame with 3xFlag and transformed into NAC050–3xMyc and NAC052–3xMyc transgenic plants (Figure 2D). The truncated JMJ14 genomic sequence encodes a JMJ14 fragment that is equivalent to JMJ14-a used in the yeast two-hybrid assay (Figure 2B and C). The plants that express both JMJ14-a-3xFlag and NAC050–3xMyc or NAC052–3xMyc were used to determine the interaction of JMJ14-a with NAC050 and NAC052 by co-IP. The results indicate that NAC050–3xMyc and NAC052–3xMyc co-precipitate with JMJ14–3xFlag but not with JMJ14-a-3xFlag (Figure 2D), suggesting that the FYRC domain is required for the interaction of JMJ14 with NAC050 and NAC052 in Arabidopsis. To determine which domain in NAC050 is necessary for the interaction of JMJ14 with NAC050, we cloned a series of truncated NAC050 sequences in the pGAL4-BD vector and used for the yeast two-hybrid assay (Supplementary Figure S6A). None of the truncated NAC050 sequences could interact with JMJ14 (Supplementary Figure S6B), suggesting that the full-length NAC050 is required for the interaction with JMJ14.

![Figure 1](https://example.com/figure1.png)  
Figure 1. JMJ14 associates with NAC050 and NAC052 in vivo. (A) The NAC transcription factors NAC050 and NAC052 were identified in proteins that were co-purified with JMJ14 in a mass spectrometric assay. The protein extract was isolated from flowers of 1-month-old plants. (B) The interaction of JMJ14 with NAC050 and NAC052 was determined by co-IP. The JMJ14–3xFlag transgenic plants were crossed with NAC050–3xMyc and NAC052–3xMyc transgenic plants. The offspring harboring both of the epitope tags was used in the co-IP analysis. The parent JMJ14–3xFlag, NAC050–3xMyc and NAC052–3xMyc transgenic plants were used as controls. Two-week-old seedlings were used for protein extract. (C) Gel filtration assay of the protein extracts from JMJ14–3xFlag, NAC050–3xMyc and NAC052–3xMyc transgenic plants. The protein extract was isolated from flowers of 1-month-old plants.
Figure 2. The FYRC domain is required for the interaction of JMJ14 with NAC050 and NAC052. (A) The interaction of JMJ14 with NAC050 and NAC052 was determined by the yeast two-hybrid assay. The full-length cDNA sequences of JMJ14, NAC050 and NAC052 were cloned into the yeast vectors pGADT7 and pGBK7. The constructs were transformed into the yeast strain Y1348 as indicated and then subjected to a growth assay on SD-TLH (synthetic dropout medium minus Trp, Leu and His) supplemented with 20 mM 3-AT as well as on SD-TL. (B) Diagram of the full-length and truncated versions of JMJ14. The conserved domains JmjN, JmjC, C5HC2 and FYRC are indicated. (C) Different JMJ14 cDNA fragments were cloned into pGADT7 or pGBK7 vectors. The constructs harboring each of the JMJ14 sequences, as well as pGBK7-NAC050 or pGBK7-NAC052, were co-transformed into the yeast strain Y1348 for growth assays. (D) Either the full-length JMJ14–3xFlag or the truncated JMJ14-a–3xFlag transgene was introduced into NAC050–3xMyc or NAC052–3xMyc transgenic plants. The transgenic plants were used to determine the interaction of the full-length and truncated JMJ14 with NAC050 or NAC052 by co-IP. The protein extract for co-IP was isolated from 2-week-old seedlings.

JMJ14 is functionally associated with NAC050 and NAC052 in flowering time regulation

Previous studies indicated that the jmj14 mutant shows an early flowering phenotype, suggesting that JMJ14 is involved in the repression of flowering (13,15,16). We examined whether JMJ14 is functionally associated with NAC050 and NAC052 in flowering time regulation. No mutant of NAC050 and NAC052 is available in the Arabidopsis Biological Resource Center. To determine the biological function of the two NAC proteins, we generated an RNAi construct targeting NAC050. Because of the high sequence similarity between NAC050 and NAC052, both of the NAC genes were reduced by the RNAi construct (Figure 3A). In the two individual NAC050/052-RNAi lines 8 and 11, the expression of NAC050 and NAC052 was effectively knocked down (Figure 3A). The NAC050 cDNA fragment used for the RNAi construct was selected from the diversified region rather than from the conserved region of the NAC family genes to avoid affecting gene expression of other NACs. In the jmj14 mutant, the expression of NAC050 and NAC052 is increased (Figure 3A). This feedback effect of jmj14 indicates that NAC050 and NAC052 are functionally associated with JMJ14. The early flowering phenotype was not only found in jmj14 but also in the two NAC050/052-RNAi lines. The numbers of rosette leaves upon flowering are comparable between jmj14 and the two NAC050/052-RNAi lines but are significantly less than those in the WT (Figure 3B). We transformed the 35S-NAC050 construct into WT plants and determined the flowering time of the transgenic plants. RT-PCR indicated that NAC050 was markedly overexpressed in two independent 35S-NAC050 transgenic plants (Figure 3C). As expected, the flowering time of the two NAC050 overexpression lines was significantly delayed compared to that of the WT (Figure 3D). These results suggest that NAC050 and NAC052 are functionally associated with JMJ14 and contribute to repression of flowering.

The early flowering phenotype of jmj14 was previously reported to be associated with increased expression of floral integrator genes (13,16). Quantitative RT-PCR indicated that the transcript levels of the floral integrator genes FT, LFY and PI were weakly increased by jmj14 as well as by the two individual NAC050/052-RNAi lines (Figure 3E), suggesting that the early flowering phenotype caused by jmj14 and NAC050/052-RNAi is correlated with the increased expression of these floral integrator genes. We further determined whether the overexpression of NAC050 in the 35S-NAC050 transgenic plants represses the expression of FT, LFY and PI. The results show that the expression of FT and PI is reduced in the NAC050 overexpression lines relative to that in the WT (Figure 3E), suggesting that NAC050 represses the expression of FT and PI. The delayed flowering time in the NAC050 overexpression lines is correlated with the reduced expression of FT, PI and possibly other uncharacterized floral integrator genes. Different from FT and PI, the expression level of LFY is comparable between the WT and the NAC050 overexpression lines (Figure 3E), suggesting that the WT expression level of NAC050 is sufficient for properly repressing the expression of LFY.

Involvement of JMJ14 in flowering time control was previously reported by several independent groups, but the un-
The transcript levels of JMJ14 are shown. (B) Shown are the WT, jmj14, NAC-RNAi-8 and NAC-RNAi-11 plants that were grown on soil under long-day conditions. The flowering time was assessed by counting rosette leaves. At least 20 plants of each genotype were used to count the rosette leaves. The average and standard deviation are indicated in the chart. (C) The transcript level of NAC050 was determined by RT-PCR in the WT and two individual 35S-NAC050 transgenic lines #10 and #12. The actin gene was used as a control. ‘No RT’ stands for amplification of the actin gene without reverse transcription. (D) The effect of jmj14 and NAC050/052-RNAi on the expression of floral integrator genes. The expression of the floral integrator genes FT, LFY and PI in the WT, jmj14 and two individual NAC050/052-RNAi lines was determined by quantitative RT-PCR. The experiment was biologically repeated and the results of three technical replicates from a representative experiment are shown. (F) The effect of jmj14 and NAC050/052-RNAi on the expression of FT in different tissues of 1-month-old plants. The results of three replicates are shown. (G) The CRISPR-mediated nac050/052 double mutant shows an early flowering phenotype. Soil-grown plants were photographed 3 weeks after planting.

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underlying mechanism is controversial (13,15–17). Jeong et al. reported that JMJ14 directly targets the floral integrator gene FT and acts in histone H3K4 demethylation and transcriptional repression at the locus. However, another study indicated that the effect of jmj14 on FT expression is unrelated to H3K4me3 (16), which argues against the direct role of JMJ14 in the repression of FT. We found that although the expression of FT, LFY and PI is increased in jmj14, H3K4me3 of the three genes is not significantly altered in jmj14 (Supplementary Figure S7), suggesting that FT, LFY and PI are not the direct targets of JMJ14. Based on the online transcriptome data (38), we found that JMJ14 and NAC050/052 are co-expressed in different tissues during various developmental stages (Supplementary Tables S2 and Figure S8), supporting the notion that JMJ14 and NAC050/052 act together. We performed quantitative RT-PCR to determine whether jmj14 and NAC050/052-RNAi affect the expression of FT in different tissues including shoot apex, rosette leaf, stem and flower. We found that the expression of FT is upregulated not only by jmj14 but also by NAC050/052-RNAi in all the tissues tested in 1-month-old plants (Figure 3F), suggesting that JMJ14 and NAC050/052 affect the expression of FT in different tissues during various development stages. Furthermore, we obtained jmj14 ft and jmj14 fve double mutants by crossing jmj14 with two late-flowering mutants ft and fve, respectively. The flowering time is markedly promoted in jmj14 fve compared to fve, confirming that JMJ14 is involved in flowering time regulation (Supplementary Figure S9). Surprisingly, the jmj14 mutation promotes flowering time even in the ft background (Supplementary Figure S9). The result suggests that the involvement of JMJ14 in flowering time control is at least partially through a FT-independent pathway.

To confirm the function of NAC050/052 in flowering time regulation, we mutated NAC050 and NAC052 by the
CRISPR-CAS9 system and obtained a nac050/052 double mutant (Figure 3G; Supplementary Figure S10A–D) (37), in which a cytosine nucleotide is separately inserted into both NAC050 and NAC052 and leads to frame shifts of NAC050 and NAC052 (Supplementary Figure S10C and D). As expected, the nac050/052 double mutant shows an early flowering phenotype relative to the WT (Figure 3G). The effect of nac050/052 on flowering time is comparable with that of jmj14, suggesting that NAC050/052 and JMJ14 are functionally associated in flowering time regulation.

JMJ14 and NAC050/052 regulate a large number of common target genes

To understand the mechanism underlying the collaboration between JMJ14 and NAC050/052, we isolated total RNA from 2-week-old seedlings and performed RNA-seq to identify differentially expressed genes in NAC050 and NAC052 (Supplementary Figure S12A–E). H3K4me3 plot indicates that H3K4me3 is enriched at 5′-ends of transcribed regions (Supplementary Figure S11). In general, NAC050/052-RNAi either does not affect their expression in all the tested tissues or affects in some specific tissues to various extents, suggesting that the involvement of JMJ14 in repression of these genes may occur in both NAC050/052-dependent and NAC050/052-independent manners. The expression of JMJ14 target genes are prone to be ubiquitously regulated by JMJ14, whereas the function of NAC050/052 is likely dependent on specific target loci, developmental stages and tissues.

The heat map of the differentially expressed genes indicates that the effects of jmj14 and NAC050/052-RNAi on gene expression are highly similar to each other (Figure 4C). A large number of genes are co-upregulated or co-downregulated in the jmj14 and NAC050/052-RNAi plants. We identified 938 and 1470 genes that are significantly upregulated in the jmj14 and NAC050/052-RNAi plants, respectively (P < 0.01 and log2 (fold change of reads) > 0.5). More than half (494/938, 52.7%) of the 938 upregulated genes in the jmj14 mutant overlap with the 1470 upregulated genes in the NAC050/052-RNAi plants (Figure 4D; Supplementary Tables S4 and S5). Of the 389 downregulated genes in jmj14, nearly half (192/389, 49.4%) overlap with the 1559 downregulated genes in NAC050/052-RNAi (Figure 4D; Supplementary Tables S4 and S5). In jmj14 and NAC050/052-RNAi, the expected numbers for overlapping upregulated and downregulated genes by chance are 40 and 18, respectively. The numbers are significantly less than those of observed overlapping genes, suggesting a functional association between JM14 and NAC050/052. To confirm the effect of jmj14 and NAC050/052-RNAi on co-upregulated genes as determined by RNA-seq, we selected 10 of them for validation by quantitative RT-PCR (Figure 4E). We found that all the 10 genes are also co-upregulated in our quantitative RT-PCR results (Figure 4F), confirming the RNA-seq data. Moreover, we determined whether co-upregulated genes identified in 2-week-old seedlings as determined by RNA-seq are also upregulated in tissues of adult plants. We found that three co-upregulated genes identified in 2-week-old seedlings are markedly upregulated by jmj14 in all the tested tissues from 1-month-old plants, and the effect of NAC050/052-RNAi on the expression of these genes is variable in different tissues (Supplementary Figure S11). In general, NAC050/052-RNAi either does not affect their expression in all the tested tissues or affects in some specific tissues to various extents, suggesting that the involvement of JMJ14 in repression of these genes may occur in both NAC050/052-dependent and NAC050/052-independent manners. The expression of JMJ14 target genes are prone to be ubiquitously regulated by JMJ14, whereas the function of NAC050/052 is likely dependent on specific target loci, developmental stages and tissues.

JMJ14 and NAC050/052 are required for the H3K4 demethylation of their common target genes

JMJ14 is a histone H3K4 demethylase that is required for removing H3K4 trimethylation, a histone modification that is related to transcriptional activation (16). We performed H3K4me3 ChIP-seq to determine whether the effect of jmj14 and NAC050/052-RNAi on gene expression is related to H3K4me3. The results show that H3K4me3 is preferentially present in euchromatic regions of each chromosome (Supplementary Figure S12A–E). H3K4me3 plot indicates that H3K4me3 is enriched at 5′-ends of transcribed regions following the transcription start site (Supplementary Figure S12F), which is consistent with previous studies (33,34).

Generally, jmj14 and NAC050/052-RNAi have no significant effect on H3K4me3 at the whole genome level (Supplementary Figure S12A–F). However, we identified in total 555 genes that showed a significantly increased H3K4me3 in jmj14, whereas only 62 genes show decreased H3K4me3 (Supplementary Tables S6 and S7), which is consistent with the role of JMJ14 in H3K4 demethylation (13,16,17). At least some of the identified 555 genes may be direct targets of JMJ14, whereas the decrease of H3K4me3 in the 62 genes is likely due to an indirect effect of jmj14 on gene repression. In the 555 H3K4me3 hypermethylated genes, 130 genes overlap with the 938 genes that are upregulated in jmj14 (Figure 5A). The overlap is significantly (P < 0.01) higher than expected by chance. Simultaneously, only 5 of the H3K4me3 hypermethylation genes overlap with the 389 downregulated genes (Figure 5B). Several previous reports suggest that JMJ14 is a JmjC-type histone H3K4me3 demethylase (13,16,17). It is likely that the change of H3K4me3 levels in jmj14 as well as in the RNAi lines of its related NAC proteins is directly caused by the reduced action of JMJ14. However, we cannot rule out the possibility that the change in H3K4me3 reflects an indirect effect of gene expression.

For 494 co-upregulated genes in jmj14 and NAC050/052-RNAi plants, the effect of jmj14 and NAC050/052-RNAi
on gene expression is comparable (Figure 5C), but the H3K4me3 level is increased in jmj14 and to a lesser extent in NAC050/052-RNAi plants (Figure 5D). The results suggest that NAC050 and NAC052 are involved in transcriptional repression through both histone demethylation-dependent and demethylation-independent pathways. To confirm the H3K4me3 ChIP-seq data, we selected some H3K4me3 hypermethylated genes for validation by ChIP-PCR (Figure 5E). Only those genes with a long intergenic region (>1 kb) were selected to exclude the influence of upstream genes. Consistent with the H3K4me3 ChIP-seq data (Figure 5D), our quantitative ChIP-PCR results indicate that jmj14 and NAC050/052-RNAi derepress H3K4me3 at 5′-ends of transcribed regions but not at intergenic regions (Figure 5E; Supplementary Table S1).

In our H3K4me3 ChIP-seq results, a number of upregulated genes in jmj14 and NAC050/052-RNAi were not identified as being H3K4me3 hypermethylated (Figure 5A). We randomly selected six of these genes and determined their H3K4me3 levels by H3K4me3 ChIP-PCR. The results indicate that the H3K4me3 levels of the six genes are more or less increased in jmj14 and to a lesser ex-

Figure 4. Effect of jmj14 and NAC050/052-RNAi on gene expression as determined by RNA-seq. (A) The plots show the RNA reads that matched NAC050 and NAC052. The RNA reads were plotted in the WT and NAC050/052-RNAi plants. (B) The JMJ14 RNA reads were plotted in the WT and jmj14 plants. RNA reads of a flanking gene are comparable between the WT and jmj14 plants and are shown as a control. (C) Heat map of differentially expressed genes in jmj14 and NAC-RNAi plants relative to the WT. (D) Venn diagram showing the overlap of differentially expressed genes in jmj14 and NAC-RNAi plants. (E) Showing is the list of co-upregulated genes that were selected for quantitative RT-PCR assay. Normalized reads in each ecotype and P-values are indicated. (F) Quantitative RT-PCR was performed to confirm the effect of jmj14 and NAC050/052-RNAi on the expression of their target genes as determined by RNA-seq. Two individual NAC050/052-RNAi lines, 8 and 11, were used. The expression of the actin gene was used as an internal control. The results of three technical replicates were shown. Total RNA used for RNA-seq and quantitative RT-PCR was extracted from 2-week-old seedlings.
Hyper H3K4me3 in jmj14, \( N=555 \)

Upregulated genes

jmj14
\( N=938 \)

\( N=1470 \)

Downregulated genes

jmj14
\( N=389 \)

\( N=1559 \)

Figure 5. Effect of jmj14 and NAC050/052-RNAi on H3K4me3 as determined by ChIP-seq. (A and B) Venn diagrams showing the overlap between the H3K4me3 hypermethylated genes in jmj14 and the upregulated (A) or downregulated genes (B) in jmj14 and NAC050/052-RNAi plants. (C) Box plot showing the effect of jmj14 and NAC050/052-RNAi on gene expression for 494 co-upregulated genes in jmj14 and nac050/052-RNAi. (D) H3K4me3 of the 494 overlapping upregulated genes in jmj14 and NAC050/052-RNAi plants was plotted for the transcription regions along with the 1-kb upstream and downstream flanking regions. The y-axis indicates the normalized reads number. (E) H3K4me3 hypermethylated genes identified by ChIP-seq were confirmed by ChIP-PCR in the WT, jmj14 and NAC050/052-RNAi plants. Diagrams show positions of all DNA fragments amplified in the ChIP-PCR assay. The hypermethylated sites include AT1G11540-A, AT2G18193-A and AT5G27940-A. The sites that are adjacent to the H3K4me3 hypermethylated regions were used as negative controls. These sites are AT1G11540-B, AT2G18193-B, AT5G27940-B and AT5G27940-C. Two-week-old seedlings were used for ChIP-seq and ChIP-PCR.

JMJ14 and NAC050/052 directly bind their common target genes and act in transcriptional repression

We performed quantitative ChIP-PCR for JMJ14 to determine whether JMJ14 directly binds its target genes. The construct harboring the native promoter-driven JMJ14–3xFlag transgene was equivalently expressed in WT and NAC050/052-RNAi on H3K4me3 was undervalued in our H3K4me3 ChIP-seq results and that JMJ14 and NAC050/052 are probably required for histone H3K4 demethylation in majority of their common target genes.

tent in NAC050/052-RNAi (Supplementary Figures S13 and S14). The results suggest that the effect of jmj14 and NAC050/052-RNAi on H3K4me3 was undervalued in our H3K4me3 ChIP-seq results and that JMJ14 and NAC050/052 are probably required for histone H3K4 demethylation in majority of their common target genes.

JMJ14 and NAC050/052 directly bind their common target genes and act in transcriptional repression

We performed quantitative ChIP-PCR for JMJ14 to determine whether JMJ14 directly binds its target genes. The construct harboring the native promoter-driven JMJ14–3xFlag transgene was equivalently expressed in WT and NAC050/052-RNAi plants (Supplementary Figure S15A). The occupancy of JMJ14–3xFlag on transcription start sites was determined by ChIP-PCR using anti-Flag antibody-conjugated agarose beads. Genes that were upregulated in both jmj14 and NAC050/052-RNAi were hypothesized to be the common targets of JMJ14 and NAC050/052 and were selected for ChIP-PCR (Figures 4D and 6A; Supplementary Tables S4 and S5). We found that JMJ14 is enriched at the transcription start sites of the common target genes in the WT background, indicating that these genes are direct targets of JMJ14, whereas JMJ14 is not enriched at the transcription start sites of the control genes ACT2 and AT1G02580 whose expression is not affected by jmj14 (Figure 6A; Supplementary Figure S13). Moreover, we found that the enrichment of JMJ14 on these genes is not significantly affected in the NAC050/052-RNAi plants (Figure 6A; Supplementary Figure S13), suggesting that NAC050/052 have no effect on the occupancy of JMJ14 on chromatin.

We performed an NAC050 ChIP-PCR assay to determine whether NAC050 directly binds to the target loci
that are shared by JMJ14 and NAC050. The NAC050–3xMyc construct was transformed and expressed in WT and jmj14 plants (Supplementary Figure S15B). The ChIP-PCR results indicate that NAC050 is significantly enriched at the common target genes that are shared by JMJ14 and NAC050/052 but not at ACT2 and AT1G02580 (Figure 6B), suggesting that these common target genes are the direct targets of NAC050. The enrichment of NAC050 at these loci was not decreased in the jmj14 mutant (Figure 6B), implying that JMJ14 is not required for the occupancy of NAC050 on its target loci. These results suggest that JMJ14 and NAC050 are recruited to their common target genes independently. The aforementioned results indicated that the FYRC domain of JMJ14 is responsible for the interaction of JMJ14 with NAC050 (Figure 2B–D). We determined whether the FYRC domain is required for the function of JMJ14 in transcriptional repression by a complementation assay. AT3G18610 and AT3G59250 are two common target genes shared by NAC050/052 and JMJ14. In the jmj14 mutant and the NAC050/052-RNAi lines, the expression of the two genes is significantly upregulated (Supplementary Tables S4 and S5; Figure 4E and F). By the complementation assay, we demonstrated that the expression of the two genes in the jmj14 mutant was restored by the full-length transgene JMJ14-Flag but not by the truncated transgene JMJ14-a-Flag in which the FYRC domain is absent (Supplementary Figure S16). Although the recruitment of JMJ14 to its target genes is independent of NAC050, the interaction of NAC050 with JMJ14 may contribute to the function of JMJ14 on its target loci. However, we cannot absolutely exclude the possibility that the FYRC domain has another function, which is probably responsible for the loss-of-function phenotype. The NAC050/052-RNAi lines exhibit elevated H3K4me3 levels at the common target genes shared by NAC050/NAC052 and JMJ14 (Figure 5D and E; Supplementary Figures S13 and S14).
is difficult to rule out the possibility that the elevation of H3K4me3 is caused by increased transcript levels, but the physical and functional association between NAC050/052 and JMJ14 strongly suggests that NAC050/052 directly regulate the function of the histone demethylase JMJ14 at their common target genes.

JMJ14 acts as a H3K4 demethylase and is involved in transcriptional repression (15–18). NAC050 directly binds the common target genes of NAC050 and JMJ14 and represses the expression of these genes (Figure 6B; Supplementary Table S5), suggesting that NAC050 can act as a transcriptional repressor of these genes. AT2G18720, AT2G21640 and AT5G16020 are the common target genes shared by JMJ14 and NAC050/052 (Supplementary Tables S4 and S5; Figure 6A and B). The promoter sequences of these genes were used to drive the luciferase reporter gene in a transient expression assay in protoplast cells (Figure 6C). While their promoter sequences drive the expression of the luciferase reporter gene, the transformation of the construct harboring the 35S-NAC050 overexpression construct significantly reduces the expression of the reporter gene (Figure 6C). The expression of AT1G02580 was not affected by NAC050/052-RNAi (Supplementary Table S5), and its promoter had no predicted NAC050-binding site and failed to be bound by NAC050 (Figure 6B). Thus, we used the promoter of AT1G02580 as a negative control in the luciferase reporter assay. As expected, we found that the luciferase reporter driven by the promoter of AT1G02580 was not affected by overexpression of NAC050 (Figure 6C). These results suggest that NAC050 acts as a transcriptional repressor on its target genes.

We further performed a reporter gene assay using the luciferase reporter gene driven by a minimum 35S promoter with a GAL4 binding site 5xUAS (Figure 6D). In the effector construct, the full-length or truncated NAC050 coding sequences were ligated in frame with the coding sequence of the GAL4 DBD-VP16 fusion protein, in which the GAL4 DNA binding domain was fused with the VP16 transcriptional activation domain. As previously reported, GAL4 DBD-VP16 by itself activates the expression of the luciferase reporter gene (Figure 6D). When either the full-length NAC050 or the N-terminal half of NAC050 was fused with GAL4 DBD-VP16 in the effector construct, the reporter gene expression was markedly decreased (Figure 6D). However, when the C-terminal half of NAC050 was fused with GAL4 DBD-VP16, the reporter gene expression was only weakly affected (Figure 6D). Thus, NAC050 is a transcription repressor and the transcriptional repression domain is included in the N-terminal region of NAC050. A previous study identified a conserved transcriptional repression domain (NARD) at the N-terminal region of NAC050, which is consistent with the requirement of the N-terminal region of NAC050 for transcriptional repression (Figure 6D).

Identification of NAC050-binding DNA elements

We have demonstrated that NAC050 is recruited to its target genes independently of JMJ14 (Figure 6B). It is interesting to identify the DNA cis-element that is bound by NAC050/052. As described above, UGT74E2 (AT1G05680) is a common target gene that is shared by NAC050/052 and JMJ14 (Figure 6A and B; Supplementary Tables S4 and S5). An EMSA indicated that the bacterially expressed GST-NAC050 and GST-NAC052 can bind the ∼300-bp promoter sequence (P1) of UGT74E2 (Supplementary Figure S17A and B). In NAC050, the N-terminal NAC domain but not the C-terminal regulatory domain is responsible for the DNA-binding ability (Supplementary Figure S17C). Therefore, we used the NAC domain protein in EMSA to determine the NAC050-binding DNA element in the UGT74E2 promoter sequence. The NAC domain of NAC050 not only binds to the ∼300-bp UGT74E2 promoter sequence (P1), but also binds to the truncated sequence (P2) from −194 to +13 (Figure 7A; Supplementary Figure S17A). Seven individual oligonucleotides (P3–P9) covering the full-length P2 sequence were used in the EMSA to identify the fragments that are required for NAC050 binding (Figure 7A; Supplementary Figure S17). The results indicate that only the oligonucleotides P8 and P9 can be bound by NAC050 (Figure 7A).

To determine the NAC050-binding DNA element in the P9 sequence of the UGT74E2 promoter, we generated a series of mutant P9 oligonucleotides harboring mutations in three residues and performed EMSA (Figure 7B). The binding of NAC050 to the mutant P9 sequences was normalized by the binding of NAC050 to the WT P9 sequence. The results indicate that the binding of NAC050 to the mutant P9 oligonucleotide P9-M7 was significantly reduced compared to the binding of NAC050 to the WT P9 oligonucleotide (Figure 7B), suggesting that the mutation site in P9-M7 is required for the binding of NAC050. We thereafter generated a series of mutant P9 oligonucleotides harboring mononucleotide mutations covering the mutant site of P9-M7 (Figure 7C). The EMSA demonstrated that the CACG motif in P9 is sufficient for the binding of NAC050 (Figure 7C). In the CACG motif, the first and third cytosines can be substituted by thymine and adenine, respectively, without affecting the binding of NAC050, while other substitutions reduce the binding (Figure 7C). Thus, the optimal NAC050-binding motif is (C/T)(T/C)/G. This motif is absent in the five other tested oligonucleotides P3, P4, P5, P6 and P7, which is consistent with the defect in the binding of NAC050 to these oligonucleotides (Figure 7A; Supplementary Figure S17A). However, although the canonical NAC050-binding motif is absent in P8, NAC050 can still bind to P8 (Figure 7A; Supplementary Figure S17A). The sequence of P8 is _CAATTTTGGATTGAGTCCAAATGG_ and its reverse-complement sequence is CTCAATTGATCGACTCAACTCGAAATTTG (Supplementary Figure S17A). The underlined DNA sequences are weak NAC050-binding motifs as determined by our EMSA experiment (Figure 7C). There are totally seven weak NAC050-binding motifs in P8, suggesting that the presence of a high number of weak NAC050-binding motifs is probably sufficient for binding.

To confirm that the identified NAC050-binding element is functional, we integrated five repeats of the NAC050-binding element into the promoter of the luciferase reporter
gene, which contains a 35S minimal promoter and a GAL4 binding site (Figure 7D). The luciferase reporter gene was transiently expressed in protoplast cells, whereas NAC050 overexpression inhibited ~50% of the luciferase reporter gene expression (Figure 7D). However, when the NAC050-binding element repeats were mutated, NAC050 overexpression failed to inhibit the expression of the reporter gene (Figure 7D). The results demonstrate that NAC050 directly binds to the NAC050-binding element repeats and acts as a transcriptional repressor.

We performed an informatic analysis to search for over-presented DNA elements at the promoters of the 494 common target genes shared by JM14 and NAC050 (Supplementary Tables S4 and S5; Figure 4D), and found that the DNA element containing CACG is the most enriched DNA element at the promoters of these genes (Supplementary Figure S18). These results suggest that the NAC050-binding DNA element identified in this study is predictive and is a functional NAC050-binding DNA element in vivo.
DISCUSSION

The physical and functional links between JmjC histone demethylases and transcription factors have been well studied in mammals (22–24). It is important to determine whether and how JmjC histone demethylases associate with transcription factors in plants. NAC transcription factors form one of the largest transcription factor families in plants and are involved in diverse biological processes (26,27). We demonstrate that JMJ14 is involved in flowering time regulation by associating with two previously uncharacterized NAC transcription repressors NAC050 and NAC052. The NAC family proteins contain a conserved NAC domain that is responsible for DNA binding (40). Although the NAC domains are conserved in different subgroups of the NAC family proteins, their DNA recognition sequences are diverse (28,40–42). We identified a NAC050-binding DNA element in the promoter of the NAC050/052 target gene (AT1G05680), demonstrating that (C/T)A(C/A)G is the optimal NAC050 recognition motif (Figure 7), which is similar to the previously identifiedCACG motif that is bound by NAC019, NAC055 and NAC072 (40). We propose that in addition to the NAC050/052-binding element, other characteristics of chromatin are probably required for NAC050/052 recognition of chromatin.

JMJ14 is a histone H3K4 demethylase that is involved in the demethylation of H3K4 concomitantly with repression of gene transcription (15–18). We demonstrate that JMJ14 is physically associated in vivo with the two NAC proteins NAC050 and NAC052 (Figures 1 and 2), which is consistent with the finding of the interaction between JMJ14 and NAC050 in a proteome-wide yeast two-hybrid assay (43). More importantly, we confirm the functional association between JMJ14 and NAC050/052 in flowering time control (Figure 3A–G). RNA-seq identified a number of genes that are increased in either jmj14 or NAC050/052-RNAi, with more than half of the increased genes in jmj14 overlapping with those in NAC050/052-RNAi (Figure 4C and D). These results demonstrate a functional link between JMJ14 and NAC050/052. Previous studies demonstrated that the early flowering phenotype of jmj14 is correlated with increased expression of floral integrator genes (13,16,17), which are consistent with our study (Figure 3E). We found that the expression of the floral integrator genes FT, LFY and PI is also increased in the two individual NAC050/052-RNAi lines (Figure 3E), which is consistent with the fact that the expression of a large number of genes is increased by both jmj14 and NAC050/052-RNAi in our RNA-seq results (Figure 4C and D; Supplementary Tables S4 and S5). Moreover, the expression of the floral integrator genes FT and PI is decreased in the NAC050 overexpression plants (Figure 3E), confirming that NAC050 is involved in the repression of floral integrator genes. The early flowering phenotype that is shared by jmj14 and NAC050/052-RNAi is related to the repression of their common target floral integrator genes.

Histone H3K4 methylation is predominantly enriched at the transcription start sites of two-thirds of the protein-coding genes in the Arabidopsis genome (33,34). H3K4 methylation is usually correlated with actively transcribed genes (33). The SET domain-containing histone H3K4 methyltransferases ATX1, EFS/SDG8 and ATXR7/SDG25 are responsible for H3K4 methylation at the flowering repressor gene FLC. The atx1, efs/sgd8 and atxr7/sgd25 mutants present an early flowering phenotype that is accompanied by decreased H3K4 trimethylation at FLC (44–46). FLD, a homolog of the human histone H3K4 demethylase LSD1, is involved in the H3K4 demethylation of FLC and is therefore responsible for the repression of FLC (47–49). The histone H3K4 methylation activity of EFS/SDG8 is antagonized by the H3K4 demethylation activity of FLD at FLC (14). The JmjC-type histone demethylases JMJ15 and JMJ18 are also involved in H3K4 demethylation at FLC (14,20,21). Thus, histone H3K4 methylation and demethylation at FLC play important roles in flowering time regulation.

Although JMJ14 is a histone H3K4 demethylase that shows a high degree of similarity to JMJ15 and JMJ18 (15), JMJ14 can specifically repress floral integrator genes, but not the floral repressor gene FLC, that are targeted by JMJ15 and JMJ18. Thus, JMJ14 acts as a floral repressor (15–18), which is in contrast to the floral integrator role of JMJ15 and JMJ18 (20,21). It is necessary to understand how histone demethylases act on their specific target genes.

We demonstrate that JMJ14 specifically cooperates with NAC050/052 and is involved in the repression of a number of common target genes (Figures 1 and 4C and D; Supplementary Tables S4 and S5). Unlike JMJ14, JMJ15 and JMJ18 fail to interact with NAC050/052 (Supplementary Figure S5). The C-terminal FYRC domain of JMJ14 is necessary for the interaction between JMJ14 and NAC050/052 (Figure 2B–D). We found that although the N-terminal region of JMJ14 is highly similar with that of JMJ15 and JMJ18, the C-terminal FYRC domain of JMJ14 is much different from that of JMJ15 and JMJ18 (Supplementary Figure S4). The absence of the FYRC domain in JMJ15 and JMJ18 is consistent with the finding that JMJ15 and JMJ18 fail to interact with NAC050/052 as determined by yeast two-hybrid. JMJ14 and NAC050/052 directly bind their common target genes and act as transcription repressors of these genes (Figure 6A–D; Supplementary Tables S4 and S5). Thus, the functional specificity of JMJ14 in flowering time control is consistent with the interaction specificity of JMJ14 with NAC050/052 on their common target genes. Further studies are required to understand whether the interaction of JMJ14 with NAC050/052 is necessary for the function of JMJ14 in gene regulation and flowering time control.

Our results suggest that the full length of NAC050 is required for the interaction of NAC050 with JMJ14 (Supplementary Figure S6). The N-terminal NAC domain of NAC transcription factors is conserved and is required for NAC dimerization as well as for DNA binding (25). NAC050 and NAC052 not only form dimers but also associate with JMJ14 (Figures 1A–C and 2A). The NAC transcription factors have been demonstrated to bind DNA in the form of dimers (50,51). JMJ14 may interact with NAC050/052 dimers or higher molecular weight oligomers but not with NAC050/052 monomers at their common target genes. Thus, the NAC domain-mediated oligomerization of NAC050/052 is most likely necessary for the inter-
action between NAC050/052 with JMJ14. The C-terminal transcription regulatory domain is diversified in the NAC transcription factors and is necessary for functional specificities of distinct NAC subgroups (25,52). It is possible that the C-terminal transcription regulatory domain is required for the formation of an integral NAC050/052 oligomer structure, thereby facilitating the interaction between NAC050/052 and JMJ14.

It is interesting to understand how JMJ14 and NAC050/052 collaborate in the JMJ14-NAC050/052 complex to transcriptionally repress their common target genes. The H3K4me3 level for co-upregulated genes in jmj14 and NAC050/052-RNAi is not only increased in jmj14 but also in NAC050/052-RNAi, suggesting that NAC050/052 are involved in JMJ14-mediated H3K4 demethylation (Figure 5D and E; Supplementary Figure S14). However, the effect of NAC050/052-RNAi on H3K4me3 is much less than that of jmj14 for co-upregulated genes shared in jmj14 and NAC050/052-RNAi even though the effect of jmj14 and NAC050/052-RNAi on gene expression is comparable (Figure 5C and D). The results suggest that NAC050/052 are involved in transcriptional repression by both H3K4 demethylation-dependent and demethylation-independent pathways. Moreover, we identified a large number of genes that are exclusively upregulated in NAC050/052-RNAi but not in jmj14, suggesting that NAC050/052 act as a transcription repressor even without JMJ14 (Figure 4C and D; Supplementary Tables S4 and S5). For common target genes shared by NAC050/052 and JMJ14, we propose that NAC050/052 not only acts as a transcription repressor to directly repress Pol II-dependent transcription but also associates with JMJ14 and is probably responsible for enhancing the H3K4 demethylation activity of JMJ14 (Supplementary Figure S19). The integrity of the JMJ14-NAC050/052 complex is necessary for transcriptional repression of these genes. Moreover, JMJ14 represses the expression of NAC050/052 (Figure 3A; Supplementary Table S4), facilitating a feedback loop between NAC050/052 and JMJ14 in transcriptional repression control (Supplementary Figure S19).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement.

REFERENCE


