A Matrix Protein Silences Transposons and Repeats through Interaction with Retinoblastoma-Associated Proteins

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Summary

Epigenetic regulation helps to maintain genomic integrity by suppressing transposable elements (TEs) and also controls key developmental processes, such as flowering time [1–3]. To prevent TEs from causing rearrangements and mutations, TE and TE-like repetitive DNA sequences are usually methylated, whereas histones are hypoacetylated and methylated on specific residues (e.g., H3 lysine 9 dimethylation [H3K9me2]) [4, 5]. TEs and repeats can also attenuate gene expression [2, 6–8]. However, how various histone modifiers are recruited to target loci is not well understood. Here we show that knockdown of the nuclear matrix protein with AT-hook DNA binding motifs [9–11] TRANSPOSABLE ELEMENT SILENCING VIA AT-HOOK (TEK) in Arabidopsis results in robust activation of various TEs, the TE-like repeat-containing floral repressor genes FLOWERING LOCUS C (FLC) and FWA [1, 2, 12]. This derepression is associated with chromatin conformational changes, increased histone acetylation, reduced H3K9me2, and even TE transposition. TEK directly binds to an FLC-repressive regulatory region and the silencing repeats of FWA and associates with Arabidopsis homologs of the Retinoblastoma-associated protein 46/48, FVE and MSIS5, which mediate histone deacetylation [13, 14]. We propose that the nuclear matrix protein TEK acts in the maintenance of genome integrity by silencing TE and repeat-containing genes.

Results and Discussion

TEK Knockdown Leads to Late Flowering

Matrix proteins with AT-hook DNA binding motifs bind to the AT-rich nuclear matrix attachment regions, possibly affecting the epigenetic state of target chromatin in animals and plants [15–17]. Some AT-hook proteins are known to regulate tissue-specific gene expression [15, 16] but very little is known about their functional mechanisms, especially in gene silencing. Most of the Arabidopsis AT-hook DNA binding proteins are dominantly expressed in root, but AHL16 (At2g42940, we renamed it TEK) is preferentially expressed in the inflorescence meristem and young floral buds, as well as in seedling-stage vegetative meristems (the Arabidopsis eFP browser http://bcb.botany.utoronto.ca/eFP; Figures 1A–1D and see Figure S1 available online). To study TEK functions, we created two artificial microRNA (amiRNA) constructs [18], 35S::amiTEKa and 35S::amiTEKb, to target the TEK coding and 3’ untranslated regions, respectively (Figure 1E). Transgenic lines for both constructs with strongly reduced TEK levels (20%–40% compared to the wild-type) showed late-flowering phenotypes in the Landsberg erecta (Ler) ecotype background (Figures 1F–1I and S2A and S2B). About 40% of T1 35S::amiTEKb plants showed extremely late flowering (Figures 1F). RNA levels of control genes including the closest homolog AHL28 were unaffected (Figure S2C). Many T1 amiTEK transgenic plants also showed altered phyllotaxis, extra cauline leaves of larger size, and reduced fertility, in agreement with the expression pattern of TEK in the inflorescence meristem and reproductive organs (Figures 1D and S2C; data not shown) [19]. It is notable that none of the 35S::amiTEK plants in the Colombia (Col) background showed late flowering (data not shown), suggesting that TEK knockdown may have accession-specific effects. We also identified T-DNA insertion mutants (tek-1, tek-2, and tek-3) in Col or Wassilewskija (Ws) (Figure S2D). These mutants were sterile, but they showed no obvious defects in flowering time (data not shown). Next, we confirmed that the late-flowering phenotype is accession dependent by backcrossing tek-1 (in the Col background) into Ler. Some homozygous plants started to exhibit late flowering after the fourth backcross (Figure S2E).

TEK Directly Controls FLC Expression and Chromatin Conformation

To examine what genes cause the delayed flowering in the TEK knockdown plants, we examined transcript levels of flowering regulators in stable and fertile amiTEK lines (they are called “amiTEK”) (Figures 1K–1M and S2F–S2J). We found that FLC, a central flowering repressor (reviewed in [20]), was dramatically upregulated (Figure 1K), whereas the levels of FLC downstream flowering integrators, including AGL24, SOC1, and FT [20], were moderately decreased (Figures 1L–1M and S2F). Furthermore, transcript levels of the FLC upstream factors such as the putative histone H3 lysine 4 demethylase FLD, FVE, and FRI LIKE 1 (FL1) were not significantly changed (Figures S2G–S2J), indicating direct regulation of FLC by TEK.

To test whether TEK directly binds to FLC chromatin, we performed chromatin immunoprecipitation (ChIP) using the lines expressing the functional TEK with epitope tags driven by a ubiquitous promoter or the native TEK promoter. Enrichment with a primer set spanning 5’ end of the first intron (region c), essential for FLC silencing [21], was detected (Figures 2A, 2B, S3A, and S3B). Because TEK encodes an AT-hook DNA binding matrix protein, we next tested whether nuclear matrix association of FLC is affected in amiTEK. DNA attached to nuclear matrix is enriched by washing purified nuclei with high salt buffer [16]. We detected a dramatic increase in the ratio of free DNA to nuclear matrix-attached DNA in amiTEK compared to wild-type (Figures 2A and 2C). These results

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suggest that TEK is necessary for the association of FLC chromatin with the nuclear matrix for silencing.

**TEK Knockdown Leads to Reduced H3K9me2 and Increased H3 Acetylation**

Next, we compared histone modifications in wild-type and amiTEK by ChIP. We found that in wild-type seedlings, the repressive mark H3K9me2 was enriched in FLC, especially at the TEK binding site (region c) and 3’ region of the intron 1 (region e) (Figures 2A and 2D). This enrichment was abolished in amiTEK (Figures 2D and S3C). In contrast, the activation mark H3 acetylation (H3Ac) was increased in amiTEK compared with wild-type (Figures 2E, S3D, and S3E). Notably, another repressive mark, histone H3 lysine 27 trimethylation (H3K27me3), was not primarily changed in amiTEK (Figure 2F and S3F). Together, these data suggest that TEK may function through H3K9 dimethylation and histone deacetylation.

**TEK Knockdown Leads to Robust Derepression of TEs and Even Transposition of the Mutator-like TE from Ler FLC**

We performed global transcriptional analyses in amiTEK using the Arabidopsis oligonucleotide microarray (NimbleGen, Roche). We detected 1,209 genes including FLC upregulated and 416 genes downregulated in amiTEK seedlings compared with wild-type (p < 0.05) (Figure 3A, Table S1, NCBI Gene Expression Omnibus GSE39158). Among the upregulated genes, around 69% were TEs. Drastic upregulation of various retrotransposons and DNA transposons [22–24] was confirmed in two independent amiTEK lines (Figure 3B). To further confirm TE activation upon the loss of TEK function, we performed RNA-sequencing using the tek-1 inflorescences showing late-flowering phenotypes. It showed that 75 TEs were upregulated over 4-fold compared to the wild-type Ler (p < 0.05 cutoff) (Table S2). We also detected an overlap of upregulated protein-coding genes between amiTEK and the introgressed tek-1 (Figure S4A, Table S3). Moderate activation of several representative TEs as well as FLC was further confirmed in the seedling of introgressed tek-1 homozygous lines (Figure S4B) but not in the original Col background (data not shown). These show that TEK is essential for TE silencing in Ler.

The Ler FLC allele contains a Mutator-like insertion in the first intron, which silences FLC expression [2, 6] (Figure 3C). The significant derepression of Ler FLC in amiTEK prompted us to check whether the TE insertion was still present. Strikingly, it was lost in four randomly picked T1 lines with continuous light growth condition is shown by the number of rosette leaves (RL) (F), wild-type Ler plant (G), a 3SS:amiTEK plant (H), and 3SS:amiTEK plants (I) and (J). (K-M) Expression analyses of FLC (K), SOC1 (L), and FT (M) relative to TUB2 in DAG 5, 7, 9, 11, 13, and 15 in wild-type Ler (WT) and amiTEK plants. Error bars represent SD based on three biological replicates.

![Image](image.png)

**Figure 1. TEK Knockdown Leads to Significant FLC Derepression and Late Flowering**

(A) The relative expression of TEK to TUBULIN2 (TUB2) in root, leaf, stem, inflorescence meristem, and young flower bud (IM&FB), flower, and siliqule. (B and C) In situ hybridization of TEK in the vegetative shoot apical meristem (VSAM) 10 days after germination (DAG 10) (B), the inflorescence meristem (IM) (C), and young floral buds (inset). Scale bars represent 25 μm for (B) and 50 μm for (C). (D) Spatial expression pattern of pTEK::TEK-GUS ([γ-glucuronidase] in the reproductive tissues. TEK is highly expressed in the whole inflorescence and ovules and anthers of a developing flower (inset). (E) The locations and target sequences of the artificial TEK knockdown constructs a and b. (F–J) Knockdown of TEK by amiTEKa and amiTEKb caused late flowering. Distribution of late-flowering phenotypes of 80 T1 lines with amiTEKb under continuous light growth condition is shown by the number of rosette leaves (RL) (F), wild-type Ler plant (G), a 3SS:amiTEK plant (H), and 3SS:amiTEK plants (I) and (J). (K-M) Expression analyses of FLC (K), SOC1 (L), and FT (M) relative to TUB2 in DAG 5, 7, 9, 11, 13, and 15 in wild-type Ler (WT) and amiTEK plants. Error bars represent SD based on three biological replicates.
DNA methylation was almost abolished \cite{12}. At great as those found in the epi with loss of DNA methylation \cite{12}, we used bisulfite sequencing-tissues and derepression of TEs are reportedly associated is related to the Mutator but still flowered later than wild-type (Figure S4 D). These suggest that both FLC and WFA contribute to late flowering in amiTEK.

Because that ectopic expression of FWA in vegetative tissues and derepression of TEs are reportedly associated with loss of DNA methylation \cite{12}, we used bisulfite sequencing to examine DNA methylation of FWA. Methylation at CG, CNG, and CHH was reduced in the tandem repeats of amiTEK, but not FWA (Figures S4E and S4F) \cite{26}. The methylation-treated amiTEK flowered earlier than those without treatment but still flowered later than wild-type (Figure S4D). These suggest that both FLC and WFA contribute to late flowering in amiTEK.

Next, we examined the histone modification state at FWA. The level of H3K9me2 was reduced in the tandem repeats (region b) of FWA in amiTEK (Figures S4H and S4I). The acetylation level was increased significantly in region b and moderately in region a (Figures 3G and 3H), whereas the H3K27me3 level did not show any obvious change (Figure S4J). The change in histone acetylation at FWA appears greater than the change in DNA methylation in amiTEK, suggesting that histone deacetylation may play a greater role in FWA silencing. The ChIP assays using pTEK::TEK-YFP confirmed that TEK directly binds to the repeated sequences of FWA (Figure S3), suggesting that FWA is a direct TEK target.

TEK Associates with FVE and Its Homolog MSI5, Components of HDAC Corepressor Complexes

To understand the causal link between TEK and histone modifications, we performed yeast two-hybrid assays to examine the interaction of TEK with various histone-modification factors, including HDAC, FVE, MSI5, and Polycomb group proteins. We found that TEK interacted with FVE and MSI5, but not with others (Figure 4A and data not shown). The interactions were further confirmed in plant cells by bimolecular fluorescence complementation (BiFC). Fluorescence was observed in the nuclei of onion epidermal cells only when TEK and FVE or TEK and MSI5 constructs were coinjected (Figure 4B). Moreover, we carried out coimmuno-precipitation experiments to confirm the in vivo association of TEK with FVE/MSI5 using the seedlings expressing the TEK-YFP and FVE-FLAG/MSI5-FLAG. We found that anti-FLAG (recognizing FVE-FLAG and MSI5-FLAG) coinjected (Figure 4B). Moreover, we carried out coimmuno-precipitation experiments to confirm the in vivo association of TEK with FVE/MSI5 using the seedlings expressing the TEK-YFP and FVE-FLAG/MSI5-FLAG. We found that anti-FLAG (recognizing FVE-FLAG and MSI5-FLAG) coimmuno-precipitated TEK-YFP from the seedlings (Figure 4C). Together, these show that TEK is in a complex with FVE/MSI5 in Arabidopsis.

Because both FVE and MSI5, Arabidopsis homologs of the mammalian Retinoblastoma-associated protein 46/48, are components of histone deacetylation complexes that silence FLC, TEs (e.g., AtMu1) and repetitive sequence-containing loci (e.g., FWA) \cite{14}, the TEK-FVE/MSI5 complex is likely to bind to TEs. However, we were unable to detect significant ChIP enrichments of TEK at TEs (data not shown). This might be due to weak binding abilities of AT-hook proteins. In Brassica, it has been shown that SINE-like repeats are highly associated with nuclear matrix \cite{27}. Taken together, we propose that the nuclear matrix protein TEK associates with the FVE/MSI5 complex and binds to various target sites of...
TEs and repeat-containing genes, leading to histone deacetylation and, thus, gene silencing (Figure 4D).

We compared the upregulated genes in amiTEK with those in the mutants for the DNA methyltransferase MET1 and the histone deacetylase HDA6 [28–30]. Significant overlap of genes silenced by TEK, MET1, and HDA6 was detected, including some of siRNA-directed DNA methylation targets (Figures S4K and S4L). These indicate the cooperative action of DNA methylation, histone deacetylation, and TEK. In addition, HDA6 can directly interact with MET1 [31], indicating that TEK may act as a part of large protein complexes including histone deacetylase as well as DNA methyltransferase.

Figure 3. TEK Knockdown Causes TE Derepression and TE Transposition from Lpr FLC

(A) Pie chart of microarray results comparing amiTEK and wild-type Ler plants at DAG 5. (B) The relative ratio of TE transcript levels in amiTEK and wild-type representative. TEs: Copia-like (AT5G43800), gypsy-like (AT5G33050), Athila-like (AT5G32306), LINE-like (AT3G43436), Mutator-like (AT1G33460), CACTA-like (AT5G45082), and nAT-like (AT2G35700). (C and D) The Mutator-like element in the intron 1 of Lpr FLC was lost in amiTEK. Black and white boxes, untranslated and coding regions, respectively. Arrowheads, the 30 bp repeat in the intron 1 (one in Ler and two in Col). In four randomly picked late-flowering plants in the Ler background (1–4), the 1.2 kb TE was absent (D). (E) A genomic Southern blot showed that the Mutator-like TE in FLC (white arrow) was excised and translocated in amiTEK (lines 2 and 21, black arrows). (F) Bisulfite sequencing showed that cytosine methylation levels of CG, CHG, and CHH at FWA were decreased in amiTEK. (G) Schematic structure of FWA. Arrowheads, the SINE-like direct repeats. Letters a and b show regions tested in (H) and (I). (H) H3 acetylation levels were increased in amiTEK. Asterisk indicates statistically significant difference (paired Student’s t test, p < 0.05) between samples. (I) Binding of TEK to FWA by ChIP using pTEK: TEK-YFP inforesences. Error bars represent SD based on three biological replicates.

Figure 4. TEK Directly Associates with FVE and MSi5

(A) Interaction of TEK with FVE and MSi5 analyzed by yeast two-hybrid assay. Full-length TEK, FVE, or MSi5 was fused to GAL4 activation (AD) and/or DNA binding domains (BDs), respectively. Yeast colonies harboring these fusion constructs and/or empty vectors, as indicated, were grown on selective media. Yeast growth was detected only when the combinations of TEK and FVE or TEK and MSi5 were cotransformed. (B) Biotinylation of TEK association with FVE and MSi5 in onion epidermal cells. Green signal indicates the binding of TEK with FVE or MSi5 in the nuclei. DAPI (4',6-diamidino-2-phenylindole) staining indicates nuclei. Scale bar represents 20 μm. (C) Coimmunoprecipitation of TEK with FVE or MSi5 in the nuclei. DAPI (4',6-diamidino-2-phenylindole) staining indicates nuclei. Scale bar represents 20 μm. (D) A model of TEK function. TEK binds to specific targets and forms a protein complex with FVE/MSi5, which participates in histone deacetylation. Deacetylation of the target locus leads to transcriptional silencing.
Silencing of Transposons and Repeat Sequences

In summary, our data show that TEK acts in the maintenance of genomic integrity by silencing TEs and repeat-containing genes through epigenetic machinery. In tek insertion mutants in the Col and WS backgrounds, FLC was not derepressed, in contrast to FLC derepression in the fve mutant in the Col background [13]. The different ecotypic effects of tek mutations and amiTEK in Col and Ler suggest that these two ecotypes may have different susceptibility to the loss of TEK activities. This may be due to presence of redundant genes that can substitute for TEK, downstream effectors of TEK or a parallel pathway for silencing in Col and WS (but not in Ler). Such accession-specific effects have been reported in epigenetic regulation including differentially expressed small RNAs and genome imprinting in Arabidopsis [23, 32]. Further studies are needed to identify the genetic modifier(s) in different ecotypes.

Supplemental Information

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.030.

Acknowledgments

We thank Arabidopsis TAIR (http://arabidopsis.org) for information and materials and also H.Y. and F. Berger for valuable comments on the manuscript. This work was supported by research grants to T.I. from Temasek Life Sciences Laboratory (TLL), the National Research Foundation Singapore under its Competitive Research Programme (CRP Award NRF-CRP001-108), and PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan. The work in Y.H.’s laboratory was supported by a grant from the Singapore Ministry of Education (AcRF Tier 2, T207B3105). CRP Award NRF-CRP001-108, and TLL. The research in the S.E.J. laboratory was supported by National Science Foundation Grant MCB-1121245. Illumina sequencing was performed at the UCLA BSCR BioSequencing Core Facility. H.S. was supported by a Fred Eiserling and Judith Lengyel Graduate Doctorate Fellowship. S.E.J. is an Investigator of the Howard Hughes Medical Institute.

Received: August 4, 2012
Revised: December 12, 2012
Accepted: January 11, 2013
Published: February 7, 2013

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