siRNAs targeting an intronic transposon in the regulation of natural flowering behavior in *Arabidopsis*

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Allelic variation in *FLOWERING LOCUS C* (*FLC*), a central repressor of flowering, contributes to natural differences in flowering behavior among *Arabidopsis* accessions. The weak nature of the *FLC* allele in the L*er* accession is due to low levels of *FLC* RNA resulting, through an unknown mechanism, from a transposable element inserted in an intron of *FLC*. Here we show that the transposable element renders *FLC*-L*er* subject to repressive chromatin modifications mediated by short interfering RNAs generated from homologous transposable elements in the genome. Our studies have general implications for the role of transposable elements in eukaryotic gene expression and evolution.

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The transition from vegetative to reproductive growth in plants, that is, floral transition or flowering, is controlled by endogenous as well as environmental signals. Longer day length and prolonged cold temperature (known as vernalization) are two of the major environmental signals that induce flowering in *Arabidopsis*. While a complex network of genes controls flowering in *Arabidopsis*, a few genes appear to act as integrators of key flowering pathways (for review, see Boss et al. 2004). One such gene, *FLOWERING LOCUS C* (*FLC*), is a central repressor of flowering and is negatively regulated by vernalization and by genes in the so-called autonomous pathway, and positively regulated by a gene named *FRIGIDA* (*FRI*) (Michaels and Amasino 1999; Sheldon et al. 1999).

Arabidopsis accessions exhibit two major types of flowering behavior. While many are late flowering unless they have been vernalized, a few accessions flower early in the absence of vernalization. The natural variation in flowering behavior is largely conferred by allelic variation at two loci, *FRI* and *FLC* (for review, see Boss et al. 2004). Late-flowering accessions have dominant, functional alleles at both loci, whereas early-flowering accessions have recessive alleles at one or both loci. The two commonly used accessions, Landsberg *erecta* (L*er*) and Columbia (Col), are early flowering and contain *fri*-null alleles (Johanson et al. 2000). The Col *FLC* allele, *FLC*-Col, is functional, and when combined with functional *FRI* (such as *FRI-SF2*) is expressed at high levels and causes extremely late flowering (Michaels and Amasino 1999). The L*er FLC* allele, *FLC*-L*er*, responds minimally to *FRI* (Lee et al. 1993a; Michaels and Amasino 1999), although this allele does not contain missense or nonsense mutations in the *FLC* coding region (Gazzani et al. 2003; Michaels et al. 2003). *FLC*-L*er* has a 1224-bp nonautonomous *Mutator*-like transposable element (TE) inserted in the first intron (Gazzani et al. 2003; Michaels et al. 2003), and this insertion acts in *cis* to cause *FLC*-L*er* to be expressed at low levels (Michaels et al. 2003). However, the underlying mechanism by which the intronic TE insertion affects the steady-state mRNA level of the host gene is unclear.

Small RNAs 21–25 nt long play regulatory roles in a variety of eukaryotic organisms including plants. One class of small RNAs, microRNAs (miRNAs), was first identified in *Caenorhabditis elegans* through genetic analyses (Lee et al. 1993b; Reinhart et al. 2000) and later found in both animals and plants (for review, see Bartel 2004). MiRNAs are generated from longer hairpin precursors by the ribonuclease III-like enzyme Dicer and are incorporated into a protein complex named RISC, in which they guide the sequence-specific regulation of target mRNAs through translation inhibition or transcript cleavage (for review, see Bartel 2004). Another class of small RNAs, siRNAs, was first identified in plants undergoing post-transcriptional gene silencing (PTGS) and subsequently found in metazoans and fungi undergoing RNA silencing (Hamilton and Baulcombe 1999; for review, see Hannon 2002). siRNAs are derived from long double-stranded precursor RNAs by Dicer, and are incorporated into RISC, in which the siRNA mediates the sequence-specific cleavage of the target RNA (for review, see Hannon 2002). SiRNAs corresponding to transposons and repeat sequences have been found in vivo (Hamilton et al. 2002; Reinhart and Bartel 2002; Xie et al. 2004). They lead to DNA methylation and/or histone methylation at the homologous chromatin (Hamilton et al. 2002; Volpe et al. 2002; Zilberman et al. 2003; Xie et al. 2004).

Here we show that the intronic TE in *FLC* in the L*er* accession renders *FLC*-L*er* subject to siRNA-mediated regulation. This results in reduced *FLC*-L*er* expression and vernalization-independent early flowering of L*er*. Our studies demonstrate that an siRNA-based mechanism contributes to the evolution of a fast-cycling accession from a late-flowering accession. In addition, our studies have profound implications in gene expression regulated by intronic TEs in general because eukaryotic genomes contain numerous TEs, many of which are found in introns of genes (Nekrutenko and Li 2001).

Results and Discussion

In previous studies, we isolated recessive mutations in a gene named *HEN1* (Chen et al. 2002), which was later shown to act in microRNA (miRNA) biogenesis, in the

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accumulation of siRNAs in sense-transgene post-transcriptional gene silencing (S-PTGS), and in the accumulation of siRNAs from transposons in *Arabidopsis* (Park et al. 2002; Boutet et al. 2003; Xie et al. 2004). The two *hen1* alleles in the L*er* accession, *hen1-1* and *hen1-2*, have different flowering time phenotypes. *hen1-1* mutants exhibit delayed flowering, whereas plants with the weaker *hen1-2* allele are normal in flowering time (Fig. 1a,e; Chen et al. 2002). In F2 populations of a *hen1-1* × L*er* cross, the late-flowering phenotype cosegregates with the *hen1-1* mutation. In contrast to the large delay of flowering in L*er* by *hen1-1*, a strong *hen1* allele, *hen1-4*, causes only a slight delay in the floral transition in the Col accession (Fig. 1e). These findings implicate the role of small RNAs in the regulation of the floral transition in *Arabidopsis*, particularly in the L*er* accession.

To incorporate *HEN1* into the regulatory networks controlling flowering in *Arabidopsis* (Boss et al. 2004), we examined the flowering behavior of *hen1-1* plants under various physiological conditions (Supplementary Fig. S1). These studies place *HEN1* into the broad category of the so-called autonomous pathway (Boss et al. 2004), whose primary target is *FLC* (Michaels and Amasino 2001). We thus examined the expression of *FLC* at the RNA level. Indeed, *hen1-1*, but not *hen1-2*, exhibits elevated accumulation of *FLC* mRNA relative to L*er* (Fig. 2a). This elevated *FLC* expression underlies the late-flowering behavior of *hen1-1* plants, since *flc-3*, an *flc*-null allele (Michaels and Amasino 1999), suppresses the late-flowering phenotype of *hen1-1* plants (Fig. 1b,c,f).

A larger *FLC* transcript of ~2200 nt is detectable by filter hybridization in *hen1-1*, but not *hen1-2*, *dcl1-9*, or *dcl1-7* plants in the L*er* accession (arrow in Fig. 2a). This transcript is not detectable in *hen1-4* in the Col accession (Fig. 2a). This RNA species can hybridize to probes corresponding to *FLC* exons 4–7 (probe A), exons 1–3 (probe B), and the TE (probe C) (Fig. 2a,c,d; Supplementary Fig. S2), but not to probes corresponding to sequences 5′ to the TE in intron 1, intron 6, or sequences upstream or downstream of the *FLC* mRNA (data not shown). Sequences of cloned RT–PCR products from the TE-containing RNAs indicate the presence of two major RNA species containing exon 1, the TE, intron 1 sequences 3′ to the TE, and exons 2–7 (Fig. 2e). One species contains the entire intron 1 sequence 3′ to the insertion, while the other species has part of the intron 1 sequence 3′ to the insertion. The second species is apparently generated by an alternative splicing event using a cryptic splice donor site in the TE and the normal intron 1 splice acceptor site. Therefore, these RNAs are aberrantly spliced products that retain the TE and partial intron 1 sequences and will be referred to as FLC^{TE} RNAs. They would potentially generate a severely truncated, presumably nonfunctional protein. FLC^{TE} RNAs can be detected, at much lower levels, in L*er* plants by RT–PCR (Supplementary Fig. S3), indicating that they can be generated independently of the *hen1-1* mutation. This suggests that the generation of FLC^{TE} RNAs is intrinsic to the presence of the TE in *FLC*-L*er*, which would be consistent with the well-documented ability of plant TEs to affect RNA processing (for review, see Weil and Wessler 1990).

Is the regulation of *FLC* RNA levels by *HEN1* mediated by miRNAs or siRNAs? miRNA biogenesis in *Arabidopsis* requires *DCL1*, a Dicer homolog (Park et al. 2002; Reinhart et al. 2002). *dcl1-7* and *dcl1-9*, two mutations in *DCL1* (in the L*er* accession), strongly affect the accumulation of miRNAs but have little effect on the accumulation of endogenous siRNAs (Xie et al. 2004) or siRNAs from transgenes undergoing S-PTGS (Finnegan et al. 2003). While *dcl1-7* and *dcl1-9*, like *hen1-1*, cause delayed floral transition (Fig. 1e; Ray et al. 1996), *FLC* mRNA abundance is only slightly increased in these plants (Fig. 2a). Therefore, an miRNA pathway plays a small role in the regulation of *FLC* RNA abundance. The Col *FLC* allele (*FLC*-Col) does not contain the TE. There is only a small increase in *FLC* RNA abundance in *hen1-4* relative to Col (0.7-fold increase), indicating that it is the intronic TE that renders *FLC*-L*er* subject to regulation by *HEN1*. Given the role of RNA silencing and siRNAs in the regulation of transposable elements (for review, see Plasterk 2002) and the established role of *HEN1* in siRNA metabolism (Boutet et al. 2003; Xie et al. 2004), we evaluated whether an siRNA-based mechanism regulates the TE-containing *FLC*-L*er* gene.

We first searched for siRNAs corresponding to *FLC*-L*er*. Indeed, small RNAs corresponding to the TE (referred to as $siRNA^{TE}$s), but not other regions in *FLC*-L*er* (data not shown), were detected with both antisense and sense riboprobes in L*er* (Fig. 3a,b). The TE in *FLC*-L*er* is present in several copies elsewhere in the *Arabidopsis* genome (Gazzani et al. 2003; Michaels et al. 2003). $siRNA^{TE}$s also accumulate in Col (Fig. 3a–c), which does not have the TE in the *FLC* gene. This suggests that $siRNA^{TE}$s are generated from homologous TEs elsewhere in the genome. $siRNA^{TE}$s are present in *dcl1-9* and *hen1-2* but are barely detectable in *hen1-1* (Fig. 3a,b). The accumulation of siRNAs corresponding to another transposon, AtSN1 (Hamilton et al. 2002), is also much reduced in *hen1-1* but not affected by *dcl1-9* (Fig. 3d; Xie et al. 2004). *ago4-1* and *sde4-1* mutations are also known to affect the accumulation of siRNAs from AtSN1 (Hamilton et al. 2002; Zilberman et al. 2003). We found that $siRNA^{TE}$s are reduced in abundance in *ago4-1* and are not detectable in *sde4-1* plants

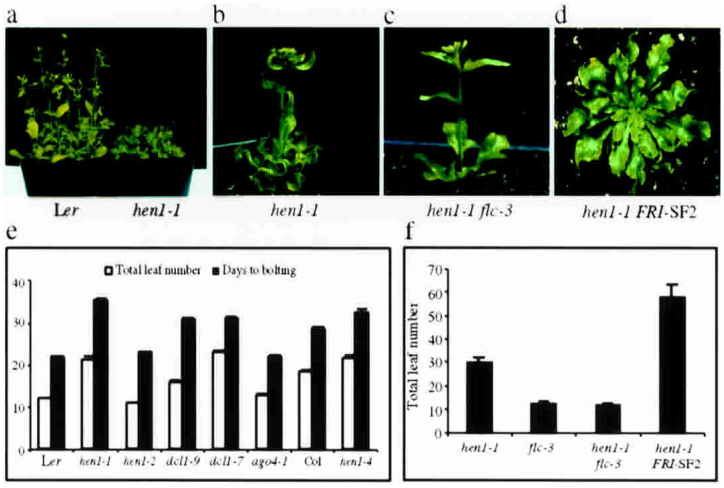

Figure 1. Flowering time of various genotypes under long-day (LD) conditions. (*a*) One-month-old L*er* and *hen1-1* plants. (*b*) A 50-d *hen1-1* plant. (*c*) A 30-d *hen1-1 flc-3* plant. (*d*) A 50-d *hen1-1 FRI*-SF2 plant. (*e*,*f*) Flowering time (total leaf number and/or days to bolting) of the indicated genotypes. $n = 90$ (*e*) or 20–30 (*f*).

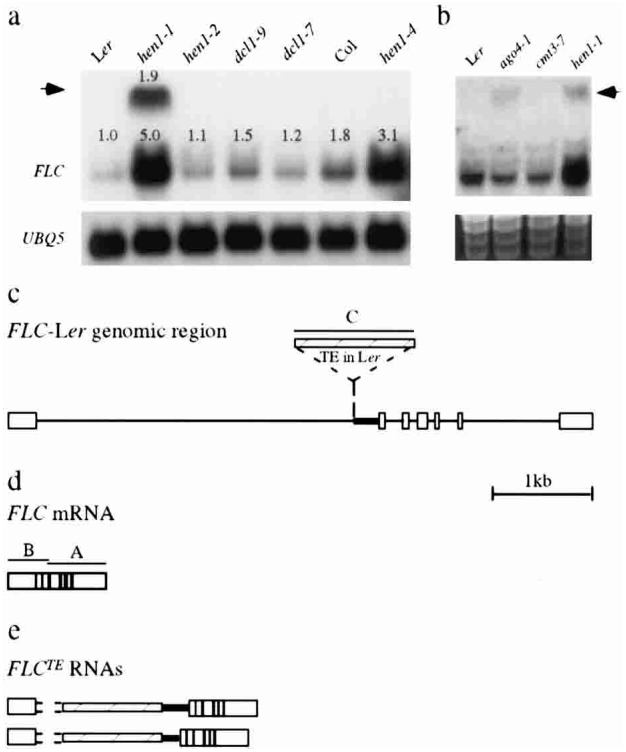

Figure 2. Analysis of *FLC* expression. (*a*,*b*) *FLC* RNA filter hybridization with probe A (*d*). The numbers in *a* indicate the abundance of the RNAs relative to *FLC* mRNA in L*er*. An image of a stained RNA gel in *b* indicates the amount of RNAs in each lane. (*c*) A diagram of *FLC*-L*er* genomic region, with white boxes representing exons, lines representing introns, and the TE represented by the striped box. The thick, black line represents the intron 1 sequence downstream of the TE. (*d*) *FLC* mRNA. (*e*) Two FLC^{TE} RNAs that differ in intron 1 sequences 3′ to the TE. The sequence at the junction of exon 1 and the TE was not determined, but RT–PCR experiments indicate the presence of exon 1 and the lack of intron 1 sequence 5′ to the TE. A–C indicate probes for RNA filter hybridization.

(Fig. 3c). In addition, $rdr6^{sgs2\text{-}1}$ and *sgs3-1*, mutations known to affect siRNA accumulation from transgenes undergoing S-PTGS (Dalmay et al. 2000; Mourrain et al. 2000; Boutet et al. 2003), do not abolish the accumulation of $siRNA^{TE}$s (Fig. 3c). Therefore, the accumulation of $siRNA^{TE}$s requires *SDE4*, *HEN1*, and to some extent *AGO4*.

The levels of *FLC* mRNA and FLC^{TE} RNAs are low in L*er*, *hen1-2*, *dcl1-9*, *ago4-1* (in L*er*) (Fig. 2a,b), genotypes that accumulate $siRNA^{TE}$s. Conversely, the levels of *FLC* mRNA and FLC^{TE} RNAs are high in *hen1-1*, a genotype that does not accumulate $siRNA^{TE}$s (Fig. 3a,b). The large increase in FLC^{TE} RNAs in a *hen1* mutant in L*er*, together with the fact that in *hen1-4* in the Col background there is very little effect on the mRNA levels of the TE-free *FLC*-Col allele (Fig. 2a), support a model in which $siRNA^{TE}$s target *FLC*-L*er* for silencing. Because $siRNA^{TE}$s are present in both Col and L*er*, the $siRNA^{TE}$s can be generated from other copies of the TE in the genome and are likely to target *FLC*-L*er* due to the presence of a TE in *FLC*-L*er*.

Two hypotheses can best explain the concomitant increase in abundance of *FLC* mRNA and FLC^{TE} RNAs in *hen1-1*. First, *FLC* is transcribed at a higher rate in *hen1-1*, resulting in a higher abundance of *FLC* pre-mRNA, which undergoes normal splicing to give rise to *FLC* mRNA and aberrant splicing to produce FLC^{TE} RNAs. Second, the *hen1-1* mutation leads to increased stability of the *FLC* pre-mRNA or both *FLC* mRNA and FLC^{TE} RNAs. Notably, $siRNA^{TE}$s belong to the 24–25-nt size class that has been correlated with histone H3-K9 methylation and DNA methylation (Hamilton et al. 2002; Zilberman et al. 2003; Xie et al. 2004), hallmarks of transcriptionally repressive chromatin (Hall et al. 2002; Volpe et al. 2002), suggesting that $siRNA^{TE}$s may lead to transcriptional silencing of *FLC*-L*er*. We first examined DNA methylation at *FLC*-L*er* by Southern hybridization. A probe flanking the TE in the *FLC*-L*er* genomic region was used to detect the TE in *FLC* and its surrounding DNA (Fig. 3e). While the methylation-insensitive enzyme HindIII results in identical patterns in L*er* and *hen1-1*, enzymes sensitive to methylation at CG sites (AciI, ClaI, and HhaI) result in patterns of digestion consistent with the presence of DNA methylation in the TE in *FLC*-L*er* but not in its surrounding genomic DNA

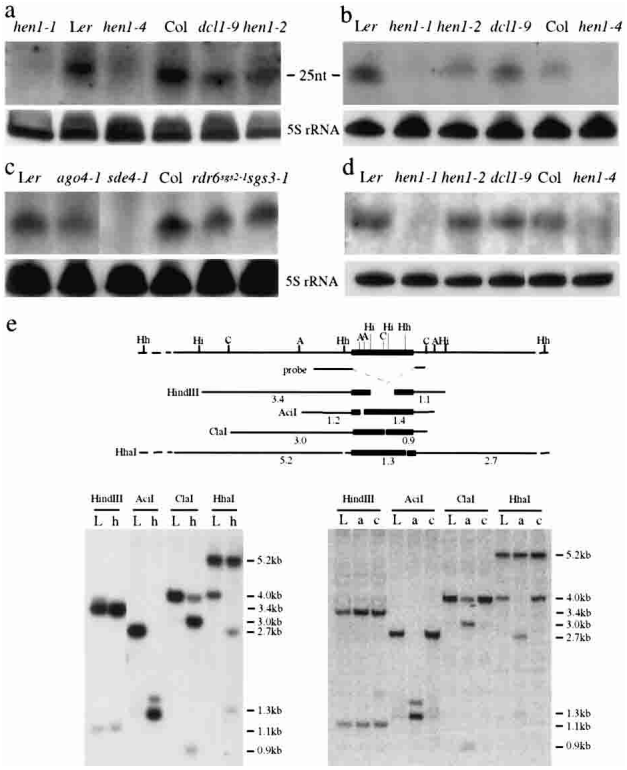

Figure 3. Transposon siRNA accumulation and DNA methylation in various genotypes. Sense (*a*,*c*) or antisense (*b*) probes both detected $siRNA^{TE}$s. (*d*) Detection of siRNAs corresponding to AtSN1 in various genotypes. The 5S rRNA served as an internal control. $rdr6^{sgs2\text{-}1}$, *sgs3-1*, and *hen1-4* are in the Col accession; *sde4-1* is in the C24 accession; and all other genotypes are in the L*er* accession. The size of the $siRNA^{TE}$s was estimated with a 10-nt RNA ladder (Ambion) and by probing the same blot to visualize miR173 of known size (22 nt). (*e*) Determination of DNA methylation status at *FLC*-L*er* in L*er* (L), *hen1-1* (h), *ago4-1* (a), and *cmt3-7* (c). A diagram of *FLC*-L*er* genomic region is shown on *top* with the positions of restriction sites as indicated: (Hh) HhaI; (Hi) HindIII; (C) ClaI; (A) AciI; (Hh) HhaI. The probe corresponds to sequences flanking the TE (black box). The fragments that can hybridize to the probe in various digests are diagramed. The size of the fragments is indicated by numbers (in kilobases) underneath the fragments.

and with the release of TE methylation in *hen1-1* (Fig. 3e). Additional probes in *FLC*-L*er* in combination with these and other restriction enzymes (HphI, BstBI, Fnu4HI, and MspI) did not detect DNA methylation in *FLC*-L*er* outside the TE (including 1.5 kb of the *FLC* promoter). Consistent with previous findings that *CMT3* is primarily responsible for CNG methylation (Bartee et al. 2001; Lindroth et al. 2001), *cmt3-7* (in L*er*) does not affect TE methylation at these sites (Fig. 3e). DNA methylation of the TE is also reduced or released in *ago4-1* (Fig. 3e). Because *ago4-1* has low levels of *FLC* mRNA (Fig. 2b) and flowers early (Fig. 1e), loss of DNA methylation at the TE cannot explain the elevated levels of *FLC* RNA or the lateness to flowering in *hen1-1*. However, it is possible that DNA methylation at other sites within the TE causes transcriptional silencing of *FLC*-L*er*.

Next, we performed chromatin immunoprecipitation (ChIP) assays to determine the status of histone H3-K9 dimethylation at the *FLC*-L*er* locus in L*er*, *hen1-1*, *ago4-1*, and *cmt3-7* plants. The level of H3-K9 dimethylation in a region of *FLC*-L*er* chromatin including the TE (region IV) and a region downstream of the TE (region V) was elevated in L*er* (Fig. 4). The level of H3-K9 dimethylation in this region was much reduced in the *hen1-1* mutant (Fig. 4). However, there was no reproducible difference in the level of H3-K9 dimethylation between L*er* and *cmt3-7* (Fig. 4c) and only a slight difference between L*er* and *ago4-1* (Fig. 4b). The levels of histone H3-K9 methylation at the *FLC*-L*er* locus in these four genotypes inversely correlate with the levels of *FLC* mRNA (Fig. 2a,b). Therefore, it is likely that the TE in *FLC*-L*er* results in transcriptional silencing of *FLC*-L*er* through histone H3-K9 methylation triggered by siRNA^{TE}s. Consistent with transcriptional rather than post-transcriptional gene silencing in L*er*, the TE affects *FLC*-L*er* in *cis* in an *FLC*-L*er*/*FLC*-Col heterozygous plant (Michaels et al. 2003). Interestingly, the relatively high level of histone H3-K9 dimethylation at the *FLC*-L*er* locus is confined to the TE and a region around the TE. At least two other regions of the first intron, which contains multiple regulatory elements (Sheldon et al. 2002; He et al. 2003), are not affected (Fig. 4). Thus, the presence of the TE appears to create an island of heterochromatin that attenuates, but does not prevent, gene expression. This probably permits expression of *FLC*-L*er* to be modulated by other regulators: *FLC*-L*er* can still respond to genes in the autonomous flowering pathway, vernalization, and, to some degree, *FRI* (Lee et al. 1993a; Rouse et al. 2002).

While a release of transcriptional silencing of *FLC*-L*er* is probably responsible for the elevated levels of *FLC* mRNA in *hen1-1*, it likely only partially accounts for the elevated levels of *FLC*^{TE} RNAs in this genotype. If transcriptional silencing of *FLC*-L*er* were the only silencing mechanism in L*er*, it would be expected that the increase in abundance for both *FLC*^{TE} RNAs and *FLC* mRNA in *hen1-1* relative to L*er* be comparable. However, the increase in *FLC*^{TE} RNAs in *hen1-1* relative to L*er* is much greater than that of *FLC* mRNA (Fig. 2a; Supplementary Fig. S3), suggesting that an additional mechanism reduces the levels of *FLC*^{TE} RNAs in L*er* and this mechanism is released in *hen1-1*. Perhaps siRNA^{TE}s also guide the degradation of *FLC*^{TE} RNAs. Intriguingly, *FLC*^{TE} RNAs can be detected by filter hybridization in *ago4-1* (Fig. 2a), although *FLC* mRNA does not accumulate to high levels in this genotype. This also supports the notion that *FLC*^{TE} RNAs are regulated by an additional, perhaps PTGS, mechanism.

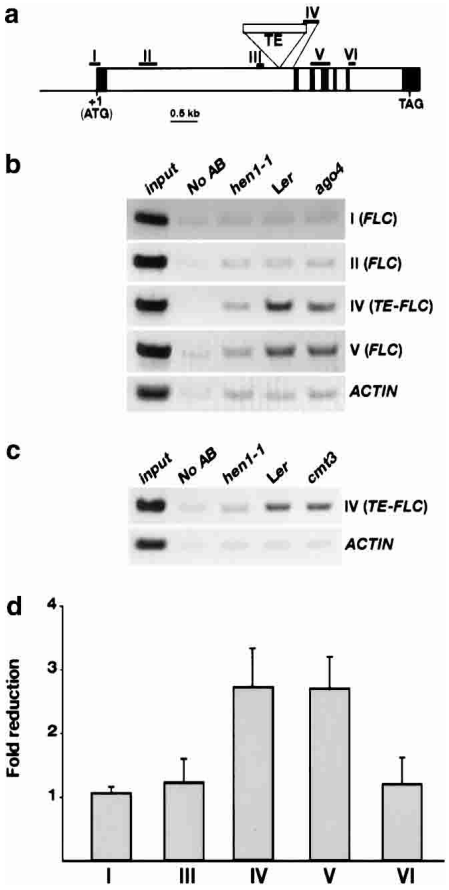

Figure 4. Chromatin immunoprecipitation (ChIP) to detect histone H3-K9 dimethylation at the *FLC*-L*er* locus. (*a*) Schematic structure of the genomic *FLC*-L*er* region. I–VI represent the regions for which H3-K9 dimethylation states were examined by ChIP. The translation initiation point is +1. The filled boxes represent exons, and open boxes represent introns. (*b*) ChIP analyses of the H3-K9 dimethylation state of various genomic *FLC* regions. The input is L*er* chromatin before immunoprecipitation. "No AB" refers to the control sample lacking antibody. *ACTIN* served as an internal control. (*c*) ChIP analyses of the H3-K9 dimethylation state of *FLC* chromatin in L*er*, *hen1-1*, and *cmt3-7*. (*d*) The fold reduction of dimethylated H3-K9 of *FLC* chromatin in *hen1-1* compared to L*er* at the indicated regions. The fold reduction of *hen1-1* compared to L*er* was calculated as follows: *FLC* in L*er* was first normalized to *ACTIN*; the normalized *FLC* in L*er* was then divided by the normalized *FLC* in *hen1-1* to obtain the fold reduction in the mutant. The error bars represent standard deviations of three independent immunoprecipitations.

Consistent with the conclusion that siRNA-mediated silencing of *FLC*-L*er* is the primary contributor to the weak nature of the *FLC*-L*er* allele, relieving *FLC*-L*er* from silencing converts the *FLC*-L*er* allele into a functional allele such that it is highly responsive to *FRI* regulation: *hen1-1 FRI*-SF2 plants are extremely late flowering (Fig. 1d,f). Interestingly, modulation of *FLC* expression by intronic TE insertion appears to have occurred at least twice in evolution to accelerate flowering (Michaels et al. 2003). Two summer annual *Arabidopsis* accessions, L*er* and Da (1)-12, harbor independent TE insertions in *FLC* intron 1 (Michaels et al. 2003). In fact, an H3mK9 island is also present in *FLC*-Da (1)-12 at the

transposon (Supplementary Fig. S4). Therefore, an siRNA-based silencing mechanism has been used as a way to evolve new developmental traits.

Although TEs inserted in introns of genes are known to affect RNA processing, we demonstrate here that an intronic TE can render its host gene susceptible to siRNA-mediated transcriptional gene silencing and that this mechanism has been adopted to evolve new developmental traits. A recent study showed that transposons affect the expression of nearby genes at the genome scale in *Arabidopsis* (Lippman et al. 2004). These studies confirm McClintock's view that transposons can serve as regulators of gene expression by introducing heterochromatin into genes in which they insert (McClintock 1950).

Most eukaryotic genomes are littered with repeat sequences and transposable elements, and many TEs or remnants of TEs are in introns of genes or have been recruited into exons of genes during evolution (Nekrutenko and Li 2001). For example, the majority of all known genes in the human genome have Alu insertions in their introns and/or UTRs (Grover et al. 2004). Given the universality of siRNA-based regulation, we propose that a TE integrated in the transcribed region of a gene may confer siRNA-based regulation to its host gene if the TE itself or its homologs in the genome are actively repressed by siRNA-mediated gene silencing.

Materials and methods

RNA filter hybridization

Forty micrograms of total RNA from 20-d-old plants was resolved on denaturing formaldehyde gels, transferred to nylon membranes, and hybridized with randomly labeled probes corresponding to different portions of *FLC* genomic DNA or cDNA. The probes and primers used were as follows: Probe A was amplified by PCR from *FLC* cDNA using primers p4 (5′-CTGAACTATGGTTCACACTATGAGCTA-3′) and p5 (5′-AGATCTCGATGCAATTCTCACACGAA-3′). Probe B was amplified by PCR from *FLC* cDNA with primers p1 (5′-CGCTTAGTATCTCCGGCGACTTGAAC-3′) and p41 (5′-GTTCAAGTAGCTCATAGTGTGAACCA-3′). Probe C was amplified by PCR from L*er* genomic DNA with primers p52 (5′-GGGTGAATTGCAAAAATAACCTATTTGGAA-3′) and p53 (5′-GGGAAATTTGGAAAACTAACCTACAAA-3′).

RNA filter hybridization was also carried out with RNAs isolated from 10-d-old plants of the genotypes used in the chromatin immunoprecipitation experiments. The relative levels of *FLC* RNAs among the genotypes are similar to those of 20-d-old plants.

siRNA detection

Small RNA enrichment, gel electrophoresis, blotting, and hybridization were carried out as described (Hamilton et al. 2002). The RNAs were isolated from 20-d-old plants. The TE DNA was amplified by PCR with p52 and p53, cloned into pGEMTE (Promega), and used as templates for in vitro transcription to generate sense and antisense probes. The pCR4-AtSN1 plasmid was a gift from Steve Jacobsen (University of California, Los Angeles).

Chromatin Immunoprecipitation (ChIP) Assays

The chromatin immunoprecipitation experiments were performed as described by Johnson et al. (2002) using 10-d-old seedlings. The anti-dimethyl-histone H3-K9 and ChIP assay kit were from Upstate Biotechnology. Each of the immunoprecipitations was replicated three times. The amounts of genomic DNA immunoprecipitated were determined by quantitative PCR. The primer pair HL1 (5′-CAAGTTGAAGGAGTCTGTGGTCT-3′) and HL2 (5′-GGATCTTGACCAGGCTGGAGAG-3′) was used to amplify the junction between the 3′-end of the *Mutator*-like transposon and *FLC*-L*er* (region IV); the primer pair HL3 (5′-TTCATGGGCAGGATCATCAGTCAA-3′) and HL4 (5′-GCTTCAACATGAGTTCGGTCTGCA-3′) was used to amplify region V of *FLC*-L*er*. The primer pair HD1 (5′-GAGTCGTGTCTGTTACACAGTTGT-3′) and HD2 (5′-GTTATGAAAGAAGAACAGAGTAAGAACGA-3′) was used to amplify the retrotransposon in *FLC*-Da. HD1 and HD3 (5′-AAATATAGGAAATTGGAACCTCACAGT-3′) were used to amplify the junction between the 3′-end of the retrotransposon and *FLC*-Da. Aliquots of the PCR reactions were resolved by electrophoresis in 1.6% agarose gels, and quantified with ImageQuant software (Molecular Dynamics, Inc).

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References

Bartee, L., Malagnac, F., and Bender, J. 2001. *Arabidopsis* cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes & Dev.* **15:** 1753–1758.

Bartel, D.P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116:** 281–297.

Boss, P.K., Bastow, R.M., Mylne, J.S., and Dean, C. 2004. Multiple pathways in the decision to flower: Enabling, promoting, and resetting. *Plant Cell* **16 Suppl:** S18–S31.

Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J.B., Crete, P., Chen, X., and Vaucheret, H. 2003. *Arabidopsis HEN1*. A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.* **13:** 843–848.

Chen, X., Liu, J., Cheng, Y., and Jia, D. 2002. *HEN1* functions pleiotropically in *Arabidopsis* development and acts in C function in the flower. *Development* **129:** 1085–1094.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. 2000. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101:** 543–553.

Finnegan, E.J., Margis, R., and Waterhouse, P.M. 2003. Posttranscriptional gene silencing is not compromised in the *Arabidopsis carpel factory* (*dicer-like1*) mutant, a homolog of Dicer-1 from *Drosophila*. *Curr. Biol.* **13:** 236–240.

Gazzani, S., Gendall, A.R., Lister, C., and Dean, C. 2003. Analysis of the molecular basis of flowering time variation in *Arabidopsis* accessions. *Plant Physiol.* **132:** 1107–1114.

Grover, D., Mukerji, M., Bhatnagar, P., Kannan, K., and Brahmachari, S.K. 2004. Alu repeat analysis in the complete human genome: Trends and variations with respect to genomic composition. *Bioinformatics* **20:** 813–817.

Hall, I.M., Shankaranarayana, G.D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S.I. 2002. Establishment and maintenance of a heterochromatin domain. *Science* **297:** 2232–2237.

Hamilton, A.J. and Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286:** 950–952.

Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21:** 4671–4679.

Hannon, G.J. 2002. RNA interference. *Nature* **418:** 244–251.

He, Y., Michaels, S., and Amasino, R. 2003. Regulation of flowering time by histone acetylation in *Arabidopsis*. *Science* **302:** 1751–1754.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. 2000. Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* **290:** 344–347.

Johnson, L., Cao, X., and Jacobsen, S. 2002. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.* **12:** 1360–1367.

Lee, I., Bleecker, A., and Amasino, R.M. 1993a. Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **237:** 171–176.

Lee, R.C., Feinbaum, R.L., and Ambros, V. 1993b. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75:** 843–854.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. 2001. Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* **292:** 2077–2080.

Lippman, Z., Gendrel, A.V., Black, M., Vaughn, M.W., Dedhia, N., McCombie, W.R., Lavine, K., Mittal, V., May, B., Kasschau, K.D., et al. 2004. Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430:** 471–476.

McClintock, B. 1950. The origin and behavior of mutable loci in maize. *Proc. Natl. Acad. Sci.* **36:** 344–355.

Michaels, S.D. and Amasino, R.M. 1999. *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11:** 949–956.

———. 2001. Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13:** 935–941.

Michaels, S., He, Y., Scortecci, K.C., and Amasino, R. 2003. Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*. *Proc. Natl. Acad. Sci.* **100:** 10102–10107.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., et al. 2000. *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101:** 533–542.

Nekrutenko, A. and Li, W.-H. 2001. Transposable elements are found in a large number of human protein-coding genes. *Trends Genet.* **17:** 619–621.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* **12:** 1484–1495.

Plasterk, R.H. 2002. RNA silencing: The genome's immune system. *Science* **296:** 1263–1265.

Ray, A., Lang, J.D., Golden, T., and Ray, S. 1996. *SHORT INTEGUMENT* (*SIN1*), a gene required for ovule development in *Arabidopsis*, also controls flowering time. *Development* **122:** 2631–2638.

Reinhart, B.J. and Bartel, D.P. 2002. Small RNAs correspond to centromere heterochromatic repeats. *Science* **297:** 1831.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. 2000. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403:** 901–906.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. 2002. MicroRNAs in plants. *Genes & Dev.* **16:** 1616–1626.

Rouse, D.T., Sheldon, C.C., Bagnall, D.J., Peacock, W.J., and Dennis, E.S. 2002. *FLC*, a repressor of flowering, is regulated by genes in different inductive pathways. *Plant J.* **29:** 183–191.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. 1999. The *FLF* MADS box gene: A repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11:** 445–458.

Sheldon, C.C., Conn, A.B., Dennis, E.S., and Peacock, W.J. 2002. Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *Plant Cell* **14:** 2527–2537.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297:** 1833–1837.

Weil, C.F. and Wessler, S.R. 1990. The effects of plant transposable element insertion on transcription initiation and RNA processing. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41:** 527–552.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2:** e107.

Zilberman, D., Cao, X., and Jacobsen, S.E. 2003. *ARGONAUTE4* control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299:** 716–719.