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Nonadditive Regulation of *FRI* and *FLC* Loci Mediates Flowering-Time Variation in Arabidopsis Allopolyploids

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ABSTRACT

Allopolyploidy is formed by combining two or more divergent genomes and occurs throughout the evolutionary history of many plants and some animals. Transcriptome analysis indicates that many genes in various biological pathways, including flowering time, are expressed nonadditively (different from the midparent value). However, the mechanisms for nonadditive gene regulation in a biological pathway are unknown. Natural variation of flowering time is largely controlled by two epistatically acting loci, namely *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). *FRI* upregulates *FLC* expression that represses flowering in Arabidopsis. Synthetic Arabidopsis allotetraploids contain two sets of *FLC* and *FRI* genes originating from *Arabidopsis thaliana* and *A. arenosa*, respectively, and flower late. Inhibition of early flowering is caused by upregulation of *A. thaliana FLC* (*AtFLC*) that is *trans*-activated by *A. arenosa FRI* (*AaFRI*). Two duplicate *FLC*s (*AaFLC1* and *AaFLC2*) originating from *A. arenosa* are expressed in some allotetraploids but silenced in other lines. The expression variation in the allotetraploids is associated with deletions in the promoter regions and first introns of *A. arenosa FLC*s. The strong *AtFLC* and *AaFLC* loci are maintained in natural Arabidopsis allotetraploids, leading to extremely late flowering. Furthermore, *FLC* expression correlates positively with histone H3-Lys4 methylation and H3-Lys9 acetylation and negatively with H3-Lys9 methylation, epigenetic marks for gene activation and silencing. We provide evidence for interactive roles of regulatory sequence changes, chromatin modification, and *trans*-acting effects in natural selection of orthologous *FLC* loci, which determines the fate of duplicate genes and adaptation of allopolyploids during evolution.

GENOME sequence analysis indicates that polyploidy can be found throughout the evolutionary history and genetic diversity of all eukaryotes (Ohno 1970), including flowering plants (Masterson 1994). Many important agricultural crops, including wheat, cotton, and Brassica, are allopolyploid, and many, including maize and Arabidopsis (Masterson 1994; Blanc and Wolfe 2004), have identifiable polyploidy in their ancestry. The common occurrence of allopolyploids in nature suggests that the combination of evolutionarily divergent genomes confers selective advantages (Grant 1981; Levin 1983; Ramsey and Schemske 1998; Matzke *et al.* 1999; Wendel 2000; Levy and Feldman 2002; Osborn *et al.* 2003; Comai 2005). Some duplicate loci contribute to a wider range of enzyme and biochemical activity than their parental genotypes (*i.e.*, hybrid vigor), whereas others may become functionally divergent during evolution. Therefore, allopolyploids are generally more adaptive than their progenitors in higher altitudes and latitudes and in broader climates (Grant 1981).

Biologically, allopolyploidy provides a unique system for studying regulatory interactions between evolutionarily divergent loci originating in orthologous genomes. In the early stages of polyploidization, an allopolyploid must reconcile regulatory and transcriptome divergence (Wang *et al.* 2006) between the divergent species that have been separated for millions of years. As a result, many genes involved in various regulatory pathways are expressed nonadditively (or differently from the midparent value) (Wang *et al.* 2004, 2006). However, the underlying molecular mechanisms for nonadditive gene regulation are poorly understood (Osborn *et al.* 2003; Chen and Ni 2006). Flowering time is an important trait for plant evolution and speciation. The pathways affecting the induction of flowering in response to vernalization or autonomous regulation (under short days) have been elucidated in Arabidopsis (Simpson and Dean 2002; Simpson *et al.* 2004; He and Amasino 2005). In the genetic pathway, *FRIGIDA* (*FRI*) acts epistatically on *FLOWERING LOCUS C* (*FLC*) and *FRI* enhances *FLC* expression and inhibits early flowering (Johanson *et al.* 2000). FLC, a MADS-box

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ167446 (*AaFLC1*), DQ167444 (*AaFCL2*), DQ167447 (*AsFLC1*), and DQ167445 (*AaFRI*).

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transcription factor, represses flowering (Michaels and Amasino 1999; Sheldon *et al.* 2002). The *FLC* locus has strong and weak alleles in *A. thaliana* ecotypes (*e.g.*, Columbia *vs.* L*er*) because of transposon insertion in the first intron (Gazzani *et al.* 2003; Michaels *et al.* 2003). FRI is a coiled-coil nuclear protein and exerts positive and epistatic effects on *FLC* expression (Johanson *et al.* 2000). Here we studied the genetic basis and phenotypic outcome of regulatory interactions between *FRI* and *FLC* loci in Arabidopsis allopolyploids. The synthetic allotetraploids contain both *A. thaliana FLC* (*AtFLC*) and *A. arenosa FLC* (*AaFLC*) and only *A. arenosa FRI* (*AaFRI*) because *A. thaliana FRI* (*AtFRI*) is nonfunctional. In natural allotetraploids, *A. suecica* (*AsFLC1* and *-2*) diverged from *A. arenosa FLC* (*AaFLC1* and *-2*), respectively, whereas *AtFLC* originating from *A. thaliana* is identical to that in *A. thaliana*. We demonstrated that genetic interactions between *A. arenosa FRI* and *A. thaliana FLC* and *A. arenosa FLC* loci contribute to late flowering in the synthetic allotetraploids and natural *A. suecica* strains. *AaFRI* complements defective *AtFRI* through *trans*-activation of *AtFLC*, which determines late flowering in the synthetic allotetraploids. Genetic interactions between *AaFRI* and *AtFLC* loci provide a molecular basis for selecting strong *AtFLC* and *AsFLC1* in *A. suecica*, coincident with its natural habitants in cold climates (O'Kane *et al.* 1995; Sall *et al.* 2003). The *trans*-acting effects of *AaFRI* on *AtFLC* are related to histone acetylation and methylation at some specific lysine residues. *AaFLC* and *AtFLC* expression displays allelic variation and correlates with changes in *cis*-regulatory elements. We propose a model that suggests *cis*- and *trans*-regulation and chromatin modification of divergent orthologous loci (*e.g.*, *FRI* and *FLC*) in the progenitors determine the fate of duplicate regulatory pathways during allopolyploid formation and evolution.

MATERIALS AND METHODS

Plant materials: The synthetic *A. suecica* lines were produced by pollinating an autotetraploid *A. thaliana* (L*er*, accession no. CS3900) ($2n = 4x = 20$) with autotetraploid *A. arenosa* (CS3901) ($2n = 4x = 32$). A total of 25 allotetraploid lines (F_1) were produced. Three independent allotetraploid lines, Allo733 (CS3895), Allo738 (CS3896), and Allo745 (CS3897), were selfed to the sixth generation (S6) (Wang *et al.* 2004). *A. suecica* strains were AsLC1 (CS22505), As9502 (CS22509), and As13 (a gift from Luca Comai at the University of Washington). *A. thaliana* Columbia (Col) (CS6673) and San Feliu 2 (SF2) (CS1516) were obtained from the Arabidopsis Biological Resource Center. Seeds harvested from the allotetraploid plants were germinated on Murashige–Skoog medium (Sigma). To mimic a winter/spring transition, we grew plants in a growth chamber with short-day conditions (24°/20° day/night and 8 hr of light/day) for 8 weeks followed by long-day conditions (24°/20° day/night and 16 hr of light/day). The transgenic seedlings (T_0) resistant to kanamycin (50 mg/liter) were selected and grown under the long-day conditions. Except as noted otherwise, seedling leaves were harvested at the vegetative stage (4 weeks in *A. thaliana*, 8 weeks in *A. arenosa*, synthetic allotetraploids, and *A. suecica*), and subjected to DNA and RNA analyses and chromatin immunoprecipitation (ChIP) assays. Flowering time was recorded from the date of seed germination to the development of the first flower. A total of 24 plants (each genotype) in three replications each with 8 plants were used for statistical analysis.

Molecular cloning and sequencing analysis of *A.arenosa FLC* and *FRI* genes in *A. arenosa* and *A. suecica*: Total RNA was isolated using the Trizol reagent (Invitrogen). Full-length *AaFLC* cDNA fragments were amplified from *A. arenosa* and a natural *A. suecica* line (As9502) using the forward and reverse primers AaFLC-F: 5′-AAATTAGGGCACAAAGCCCTCTCGG-3′ and AaFLC-R: 5′-CAACCGCCGATTTAAGGTGGCTA-3′. To clone *AaFRI*, we used 5′-RACE kit (Invitrogen) to clone and sequence the 5′-*AaFRI* region in *A. arenosa*. The full-length *AaFRI* cDNA was then amplified using the primer pair AaFRI-F: 5′-CGCTTTCTCATGGCCAATTAT-3′ and AaFRI-R: 5′-CG CGGATCCTGCATTCTTAAGCCCCAAAC-3′. *A. arenosa* and *A. suecica FLC* promoter regions were amplified using the primer pair pFLC-F: 5′-ATGGCGAAGGTGAAATGCATAC-3′, located in At5g10150, upstream of *AtFLC*, and pFLC-R: 5′-AGCT TTCTCGATGAGACCGT-3′, located in the 5′ end of the first exon in *AtFLC*. The first intron regions of *AaFLC* and *AsFLC* were amplified by PCR using primers designed from the exons 1 and 2, respectively, InFLC-F: 5′-AGCCCTCTCGGAGACAG AAG-3′ and InFCL-R: 5′-CAGGCTGGAGAGATGACAAA-3′.

We cloned the PCR-amplified full-length cDNA fragments into pGEM-T vector (Promega) and sequenced 5–20 individual inserts from each cloning event. DNA sequence data were analyzed using DNAStar LASERGENE programs, version 5.05. Multiple alignments were performed using Megalign ClustalW alignment of DNAStar, and percent amino acid identity indicates the percentage of identical residues between complete full-length cDNA sequences. Phylogenetic trees were constructed using ClustalW protein alignments and PAUP 4.0 (Swofford 2003), using maximum parsimony analysis with heuristic search and stepwise addition options, and were confirmed using bootstrap analysis with heuristic search and 1,000 replicates.

For protein domain analysis, we used the translated amino acid sequence of *AaFRI* as query in the InterPro database (http://www.ebi.ac.uk/interpro/index.html) and SWISS-PROT (http://us.expasy.org/ExpasyHunt/).

RT–PCR and cleaved amplified polymorphic sequence analyses: For each reaction, 10 μg of total RNA was treated with DNase I, and the first-strand cDNA was synthesized using RT superscript II enzyme (Invitrogen). An aliquot (1/100) of cDNA was used as template in the PCR reaction with one cycle of 94° for 2 min followed by 30 cycles of amplification at 94° for 30 sec, 53° for 30 sec, and 72° for 90 sec. *Act2* was used as internal control (Wang *et al.* 2004). *AtFLC*, *AaFLC*, or *AsFLC* transcripts from the position 223 to 491 (+1 ATG codon) were amplified using the primer pair shown in supplemental Figure 1 (http://www.genetics.org/supplemental/). The amplified products were digested with *Cla*I to distinguish the transcripts between *AtFLC* and *AaFLC1/AaFCL2* or *AsFLC1/AsFLC2*.

Allele gene specific expression with *Taq*Man detection system: To measure quantitative levels of allele-specific expression, quantitative PCR was performed in an ABI7500 thermal cycler (ABI Biosystems) with the *Taq*Man probe detection system. Briefly, we used the primer pair TM-FLC-F: 5′-GAAACA (A/G)CATGCTGATCTTAAA-3′ and TM-FLC-R: 5′-CAT(A/G) GTGTG(A/G)ACCATAGTTCGAGCTT-3′ to amplify both *AtFLC* and *AaFLC*, which were detected specifically and quantified simultaneously by two *Taq*Man probes, namely, *AtFLC*-probe: FAMCTTGGATCATCAGTCAAMGBNFQ and *AaFLC*-probe: VICCCTTGGATATTCAGTCAAMGBNFQ. The data were normalized to the expression level of an internal control

Act2 (At5g09810), which was quantified by the PCR products amplified using primer pair: 5′-GTCTGTGACAATGGAACTG GAA-3′ and 5′-CTTTCTGACCCATACCAACCAT-3′.

Single-strand conformation polymorphism analysis: Single-strand conformation polymorphism (SSCP) analysis was performed using the 0.5× mutation detection enhancement (MDE, Cambrex Bio Science) gel containing 7.5% (w/v) urea. After electrophoresis at room temperature for 18 hr with constant watts (6 W), the gel was fixed in 10% acetic acid for 30 min followed by washing three times 5 min each in deionized water. The gel was stained using silver nitrate solution (0.5 g silver nitrate in 500 ml H_2O) for 30 min and quickly rinsed in H_2O and transferred to an ice-cold sodium carbonate solution [45 g sodium carbonate in 1500 ml H_2O plus 450 μl thiosulfate solution (10 mg/ml) and 1.5 ml formaldehyde]. When clear bands appeared, 10% acetic acid was added to stop development. Gel images were taken using a CCD camera.

To determine allelic variation in allotetraploids and their progenitors, we cloned and sequenced every fragment present in the SSCP gel (Figure 6A). The sequence data were used to designate specific allele/locus names.

Plasmid construction and plant transformation: The plasmid pART27-35S-*AaFRI* was constructed as follows. The *Eco*RI/*Bam*HI fragment in pGEM-T Easy vector (Promega) consisting of full-length *AaFRI* cDNA was cloned into compatible sites of pKANNIBAL vector, resulting in a 35S-*AaFRI* cassette, which was subcloned into binary vector pART27. The vectors were generously supplied by Peter Waterhouse of the CSRIO Plant Industry, Canberra, Australia. Arabidopsis (Columbia and L*er*) was transformed with Agrobacterium-mediated transformation (floral dipping method). No transformants were obtained from L*er* plants.

Bisulfite DNA sequencing: Genomic DNA (~2 μg) of At2L*er*, At4L*er*, *A. arenosa*, F_1-12, -19, -22, Allo733, Allo738, and As9502 was digested using *Eco*RI and purified (QIAGEN). The purified DNA was treated in a bisulfite solution as previously described (Johnson *et al.* 2002). Genomic fragments (~300 bp) upstream of the ATG codon were amplified with the primers designed using the web-based software MethPrimer (http://www.urogene.org/methprimer/index1.html) (supplemental Figure 1, http://www.genetics.org/supplemental/). The *AaFLC* fragments amplified from *A. arenosa*, F_1-12, -19, -22, Allo733, Allo738, and As9502 were subject to direct sequencing. The *AtFLC* fragments were cloned into pGEM-T vector (Promega), and 8–10 individual inserts were sequenced in each line (At2L*er*, At4L*er*, Allo733, and As9502).

Chromatin immunoprecipitation assays: The ChIP assays were performed using a protocol modified from previously published methods (Bastow *et al.* 2004; He *et al.* 2004; Tian *et al.* 2005). For each assay, fresh leaves (~3 g) were subjected to vacuum infiltration in a formaldehyde (1%) solution for crosslinking the chromatin proteins to DNA. Chromatin was extracted and sonicated (Fisher, Model 60 sonicator) at half maximal power for five 10-sec pulses with chilling on ice for 3 min after each pulse. The average size of the resultant DNA fragments produced was ~0.3–1.0 kb. We used an aliquot of chromatin solution (1/10 of total volume) as input DNA to determine the DNA fragment sizes. The remaining chromatin solution was diluted 10-fold and divided into two aliquots: one was incubated using 10 μl of antibodies (anti-dimethyl-histone H3-Lys4, anti-dimethyl-H3-Lys9, or anti-acetyl-H3-Lys9; Upstate Biotechnology) and the other incubated without antibodies (mock). The immunoprecipitated DNA was amplified by semiquantitative PCR using the primers designed from upstream sequences of the *FLC* ATG codon (supplemental Figure 1, http://www.genetics.org/supplemental/). Two independent experiments were performed in each assay.

RESULTS

Genetic dominance of late flowering in synthetic Arabidopsis allotetraploids and natural *A. suecica*: Genetically stable Arabidopsis allotetraploids were generated by interspecific hybridization between *A. thaliana* (L*er*) autotetraploids and *A. arenosa* (Figure 1A) that diverged ~6 million years ago (Koch *et al.* 2000). Under a combination of short- (8 weeks) and long-day conditions, *A. thaliana* (L*er*) autotetraploids flowered in 57 ± 10 days (corresponding to 8–10 true leaves), and *A. arenosa* flowered in 102 ± 12 days (Figure 1C). All 25 synthetic allotetraploids (F_1) flowered later (119–142 days) than tetraploid parents, indicating nonadditive effects (overdominance in this case) and immediate changes in flowering time after allopolyploidization. Although the F_1 lines varied in flowering time, their offspring flowered at similar times after five generations of selfing. Notably, Allo745, a synthetic allotetraploid that was outcrossed to *A. suecica* (LC1) (Wang *et al.* 2004), flowered later than Allo733 and -738 but earlier than *A. suecica* strains. *A. suecica* is a natural allotetraploid or amphidiploid containing the genomes derived from *A. thaliana* and *A. arenosa* ancestors (O'Kane *et al.* 1995) (Figure 1B). The formation of *A. suecica* is estimated to have occurred from ~20,000 years (on the basis of chloroplast DNA) (Sall *et al.* 2003) to ~1.5 million years (Koch *et al.* 2000). *A. suecica* strains did not flower until 150–220 days after seed germination. It is likely that the genetic interactions between *A. arenosa* and *A. thaliana* loci contribute to late flowering in the synthetic allotetraploids and natural *A. suecica* strains.

Sequence analysis of *FLCs* in *A.thaliana*, *A. arenosa*, and *A. suecica*: Microarray analysis indicated that *FLC* is upregulated (Wang *et al.* 2006) in the synthetic allotetraploids that are late flowering. To determine the molecular basis of *FLC* upregulation and flowering-time variation in Arabidopsis allotetraploids, we characterized *FLC* full-length cDNAs in *A. arenosa* and *A. suecica* (Figure 2A). *A. arenosa* contains *AaFLC1* and *AaFLC2* that have 96 and 98% identities in nucleotide and amino acid sequences. Interestingly, among nine independently isolated BACs, each contained two *AaFLC* fragments within a 10-kb region (J. Wang and Z. J. Chen, unpublished data), suggesting that *AaFLC1* and *-2* are tandem duplicate genes. Compared to *AtFLC*, *AaFCL1* and *AaFLC2* have 95.4 and 94.8% nucleotide-sequence identities, respectively.

In self-pollinating *A. suecica* (As9502), *AaFLC1*-like locus (designated *AsFLC1*) diverged from *AaFLC1* (Figure 2B), whereas *AtFLC* in *A. suecica* is identical to *FLC* in *A. thaliana*, suggesting *AtFLC* sequence is highly conserved during polyploid evolution. *AsFLC1* has 99, 98.5, and 94.9% nucleotide-sequence identities to *AaFLC1*, *AaFLC2*, and *AtFLC*, respectively. *AsFLC2* corresponding to *AaFLC2* was not identified by sequencing >20 cDNA fragments amplified in *A. suecica*, but a partial genomic

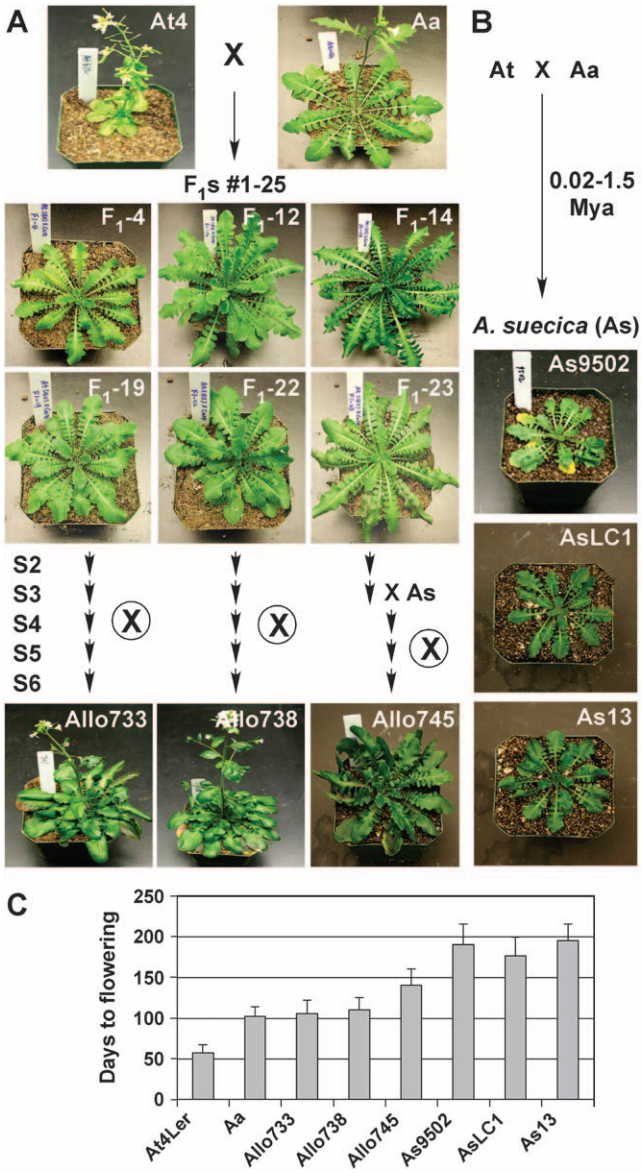

FIGURE 1.—Flowering-time variation correlated with *FLC* expression in Arabidopsis allotetraploids and their progenitors. (A) A total of 25 nascent allotetraploids (F_1) were produced by direct hybridization between *A. thaliana* (L*er*) autotetraploid (At4) and *A. arenosa* autotetraploid (Aa). Allo733, -738, and -745 were produced by self-pollination, and Allo745 was outcrossed to *A. suecica* in S3. (B) Natural *A. suecica* (As) was formed by combining *A. thaliana* and *A. arenosa* ancestral genomes. Three strains are shown. All photos were taken at 100 days after seed germination. (C) Flowering-time variation in *A. thaliana* (L*er*) autotetraploid (At4), *A. arenosa* (Aa), Allo733, -738, -745, and natural *A. suecica* strains (As9502, AsLC1, and As13).

sequence of *AsFLC2* was 99.6% identical to *AaFLC2*, suggesting that *AsFLC2* is likely silenced in *A. suecica*. In the phylogenetic tree, *FLC* loci in *A. thaliana*, *A. arenosa*, and *A. suecica* are closely related. Many Brassica *FLC* loci, except *BnFLC1*, diverged from *AtFLC* with ~86% nucleotide-sequence identity. *BnFLC1* in *B. napus* is rooted in the clade next to the Arabidopsis locus, suggesting that it is an ancient homolog that might have existed before the species divergence between Arabidopsis and Brassica. Other members of the MAF/MADS-box gene family, including *MAFs* (RATCLIFFE *et al.* 2001), are distantly related to *FLC*.

We further examined the *cis*-regulatory changes in *FLC* orthologs by cloning promoters and first introns of all possible *FLC* loci in *A. thaliana* (Col and L*er*), *A. arenosa*, and *A. suecica*. Without exception, *AaFLC* and *AsFLC* had an ~1.0-kbp deletion (Figure 3A and supplemental Figure 1, http://www.genetics.org/supplemental/) located 253 bp upstream of the ATG, giving rise to a short promoter compared with the *AtFLC* promoter. As a result, the upstream regulatory elements were deleted in the *AaFLC* and *AsFLC* promoters but present in the *AtFLC* promoter region. Coincidentally, this region was identified as a minimal promoter (~250 bp) in a previous study (SHELDON *et al.* 2002).

The first intron of *FLC* loci displayed a high degree of sequence changes (Figure 3A). A major deletion (~560 bp) was identified in the upstream region (from +855 to +1412) of the intron in *AaFLC1*, *AaFLC2*, and *AsFLC2* but not in *AsFLC1*. A *MUTATOR*-like transposon (~1.3 kbp) was inserted in *A. thaliana* (L*er*) (GAZZANI *et al.* 2003; MICHAELS *et al.* 2003), but absent in *AaFLC1/2*, *AsFLC1/2*, or *AtFLC(Col)*. Sequence length in that region varied in *AtFLC(Col)*, *AaFLC1/2*, and *AsFLC1/2*. *AsFLC1* showed sequence features different from other loci (dashed line).

Using the primers flanking the 1.3-kbp transposon insertion, we genotyped *FLC* loci in Arabidopsis allotetraploids and their progenitors. In the synthetic allotetraploids, all lines (F_1) each possessed one *AtFLC(Ler)* and two *A. arenosa* loci (Figure 3B). *AaFLC1* was absent in one allotetraploid (no. 22) due to allelic variation in the outcrossing *A. arenosa* strains, and the allele was amplified using a new primer pair (data not shown). In the selfing progeny (S6) of Allo733 and -738, each had three *FLC* fragments corresponding to *AtFLC*, *AaFLC1*, and *AaFLC2*, respectively. Similarly, the self-pollinating natural *A. suecica* strains had three loci, *AtFLC*, *AsFLC1*, and *AsFLC2*, of which *AtFLC* was derived from a Col-like strain as determined by sequencing the fragment (data not shown).

Upregulation of *AtFLC* and downregulation of *AaFLC* in synthetic allotetraploids: Using the *FLC* sequences, we designed *Taq*Man probes to discriminate the transcripts between *AtFLC* and *AaFLC1/2* or *AsFLC1/2* (Figure 2B). Alternatively, the amplified RT–PCR products were distinguishable using *Cla*I that cleaves *AtFLC*. Allelic variation between *AaFLC1* and *-2* or *AsFLC1* and *-2* was indistinguishable. We found that *AtFLC* was immediately reactivated in *de novo* allotetraploid lines (F_1) and expressed at constantly high levels in selfing progeny and *A. suecica* (Figure 4A), whereas *AaFLC1/2* was downregulated in the synthetic allotetraploids and

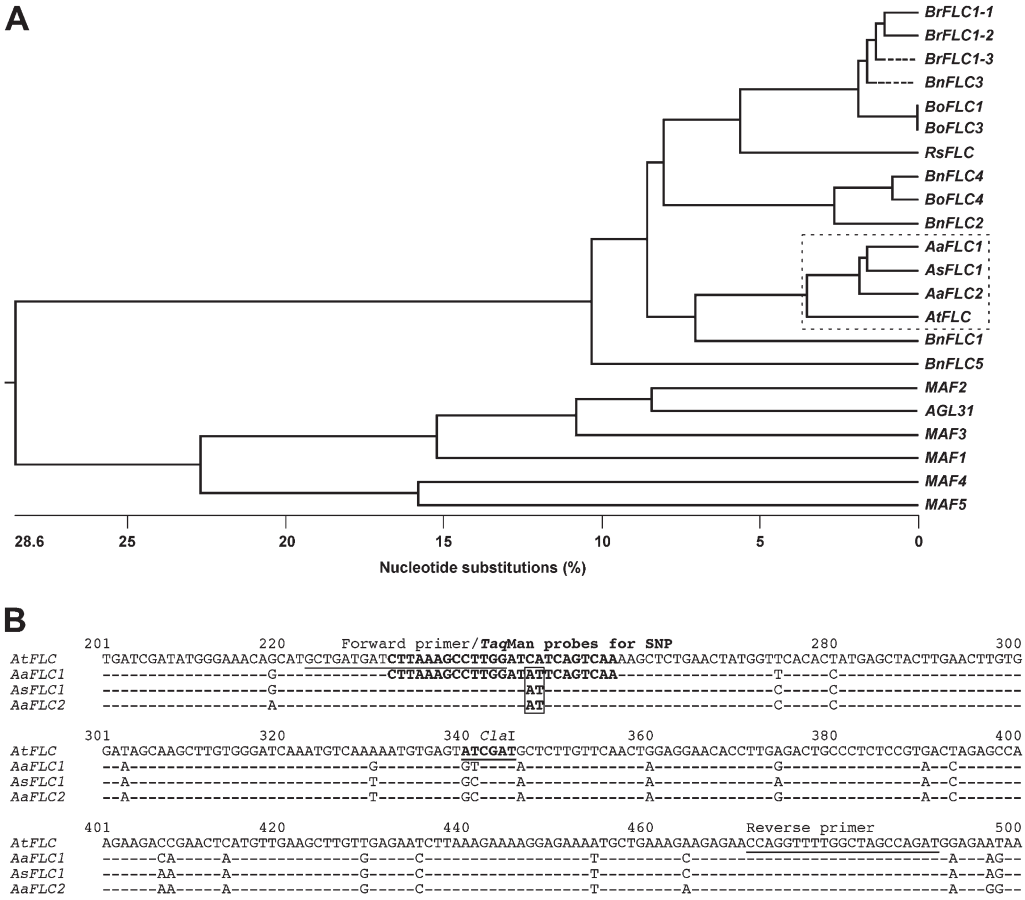

FIGURE 2.—Phylogeny of FLC/MADS box gene family in Arabidopsis and Brassica. (A) Phylogenetic tree of *FLC* loci constructed using the maximum parsimony method (see MATERIALS AND METHODS). The box with dashed lines indicates the phylogenetic location of three new *FLC* alleles detected in *A. thaliana*-related species. The GenBank accession numbers are provided in the supplemental data (http://www.genetics.org/supplemental/). (B) Partial sequence alignment of *AtFLC*, *AaFLC1*, *AaFLC2*, and *AsFLC1*. Letters and – indicate changes and no changes, respectively, in *AaFLC1*, *AaFLC2*, and *AsFLC1* compared to *AtFLC*. The forward and reverse primers used in RT–PCR analysis and the *Cla*I site (ATCGAT) in *AtFLC* are underlined. Allele-specific *Taq*Man probes (boldface type) contain the dinucleotide polymorphism (boxed) in quantitative RT–PCR analysis. The GenBank accession numbers of *FLC* loci are as follows: *AtFLC* (At5G10140), *AaFLC1* (DQ167446), *AsFLC1* (DQ167447), *AaFLC2* (DQ167444), *BrFLC1-1* (AY364013), *BrFLC1-2* (AY273165), *BrFLC1-3* (AY273164), *BnFLC3* (AY036890), *BoFLC1* (AY273161), *BoFLC3* (AY306123), *RsFLC* (AY273160), *BnFLC4* (AY036891), *BoFLC4* (AY306122), *BnFLC2* (AY036889), *BnFLC1* (AY036888), *BnFLC5* (AY036892), *MAF2* (At5g65050), *AGL31* (AF312667), *MAF3* (At5g65060), *MAF1* (AF342808), *MAF4* (At5g65070), and *MAF5* (AY231455).

AsFLC1/2 was highly expressed in *A. suecica*. Using quantitative RT–PCR and Pearson correlation analyses, we found that the flowering-time variation (days to flowering) in *A. thaliana*, *A. arenosa*, synthetic allotetraploids, and *A. suecica* (Figure 1C) is correlated with the cumulative levels of *AtFLC* and *AaFLC1/2* and */AsFLC* transcripts (Figure 4B) ($r^2 = 0.94$). Allo745 displayed increased levels of *AaFLC1/2* and *AsFLC1/2* expression and late flowering because of outcrossing to *A. suecica* (WANG *et al.* 2004).

***AaFRI* complements *AtFRI* and likely *trans*-activates *AtFLC* in Arabidopsis allotetraploids:** A winter-annual ecotype, such as *A. thaliana* San Feliu 2 (SF2), contains both *AtFRI* and *AtFLC* loci (LEE *et al.* 1993). Moreover, *FRI* from SF2 has moderate effects on *FLC* expression in L*er* background (GAZZANI *et al.* 2003; MICHAELS *et al.* 2003). However, it is unknown whether *AaFRI* and *AtFLC* originating in two divergent species are genetically compatible. To test this, we cloned a full-length *AaFRI* cDNA (supplemental Figure 3, http://www.genetics.org/supplemental/) and another partial *AaFRI*-like fragment (data not shown) in *A. arenosa*. Putative *AaFRI* and *AtFRI* (At4g00650) had 92 and 89% identities in nucleotide and amino acid sequences, respectively. Like *AtFRI*, *AaFRI* is a plant-specific gene and encodes a predicted nuclear coiled-coil protein containing a FRIGIDA domain (JOHANSON *et al.* 2000).

To test *trans*-acting effects of *AaFRI* on *AtFLC* activation, we overexpressed *AaFRI* in *A. thaliana* Col plants (Figure 5A). Among eight independent transgenic lines examined, all flowered late. The control (At2Col) flowered in ~40 days under long-day conditions, and SF2 flowered in ~85 days. The severity of abnormal phenotypes in the transgenic plants was correlated with the high levels of *AaFRI* overexpression, which may induce deleterious effects on other biological pathways. *AaFRI* was highly expressed in the transgenic lines, but *AtFLC* was expressed at a high level similar to that in S2F (Figure 5B). The correlation between the levels of *AaFRI* expression and *AtFLC* upregulation was not proportional, suggesting that other regulators in vernalization, autonomous, and photoperiod pathways affect *FLC* expression (SIMPSON and DEAN 2002; BASTOW *et al.* 2004; HE *et al.* 2004; SUNG and AMASINO 2004; HE and AMASINO 2005). Compared to relatively small *trans*-acting effects between ecotypes (MICHAELS *et al.* 2003), *AaFRI* has

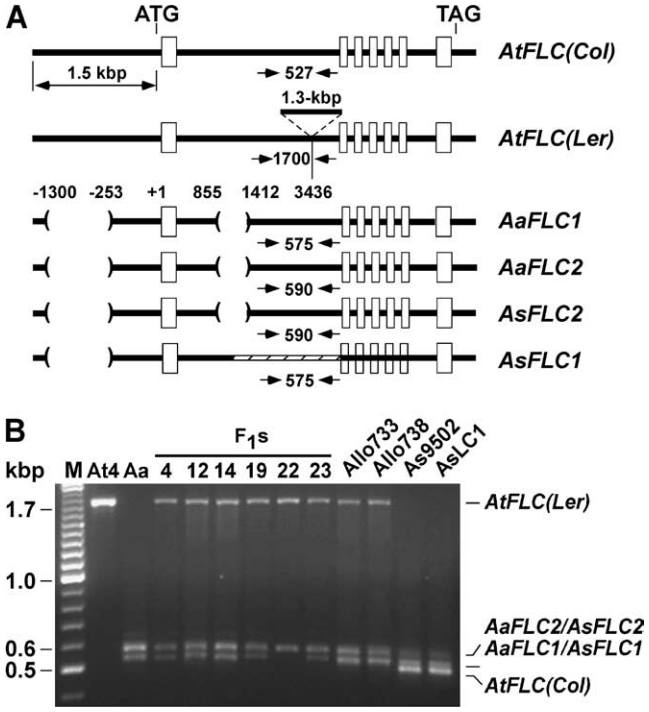

FIGURE 3.—Sequence changes in the promoter regions and first introns of *FLC* loci. (A) Diagrams showing sequence variation in the promoters and first introns of *FLC* in *A. thaliana*, *A. arenosa*, and *A. suecica*. Open boxes and solid lines indicate exons and genomic fragments, respectively. ATG and TAG codons are shown. *AaFLC/AsFLC* had a 253-bp promoter fragment, whereas *AtFLC* had a 1.5-kbp fragment. Transposon insertion in the first intron (1.3 kbp) was detected in *AtFLC* (L*er*) but not in *A. thaliana* (Col), *A. arenosa*, or *A. suecica*. Locations of the PCR primers (arrows and expected size of the fragments) in the first intron are indicted below each diagram. A 557-bp deletion in the first intron of *AaFLC1*, *-2*, and *AsFLC2* is shown. Dashed line in *AsFLC1* indicates sequence variation compared to other *FLC* loci. (B) PCR amplification of the intron fragments using the primers indicated above. The corresponding *FLC* loci are indicated on the right. The same strains as in A were used for genotyping. M, molecular size markers.

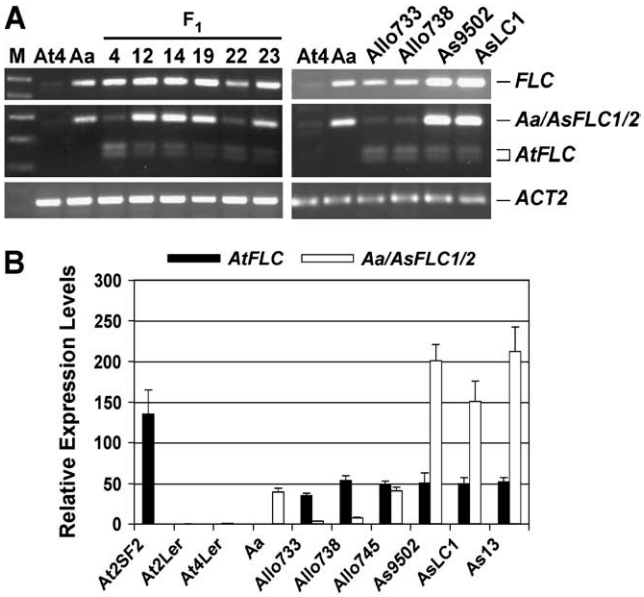

FIGURE 4.—Expression of *AaFLC* and *AtFLC* in *A. thaliana* tetraploid, *A. arenosa*, synthetic allotetraploids, and *A. suecica*. (A) RT–PCR and cleaved amplified polymorphic sequence analysis of allele-specific expression of *FLC*. *FLC* was amplified using the primers indicated in Figure 2B (top), and the amplified fragments were digested with *Cla*I (middle). *ACT2* was amplified as a control for mRNA and PCR amplification (bottom). (B) Quantitative RT–PCR (qRT–PCR) analysis of *AtFLC* and *AaFLC/AsFLC* expression in *A. suecica* strains, allotetraploids, and their progenitors using allele-specific probes (Figure 2B). At2SF2, *A. thaliana* SF2, a late-flowering ecotype. Solid and open bars indicate relative expression levels of *AtFLC* and *AaFLC/AsFLC*, respectively.

large *trans*-activating effects on *AtFLC* expression in the allotetraploids.

***AtFLC*, *AaFLC*, and *AsFLC* expression variation contributes to late flowering in Arabidopsis allotetraploids:** Do changes in *cis*-regulatory elements affect *FLC* expression and flowering-time variation in synthetic allotetraploids and *A. suecica*? *AtFLC* was instantaneously upregulated in all synthetic and natural allotetraploids lines tested (Figure 4), suggesting that despite ~6 million years of divergence between *A. thaliana* and *A. arenosa* (KOCH *et al.* 2000), *AtFLC* was *trans*-activated by *A. arenosa FRI* in the synthetic allotetraploids, probably because it has an intact promoter and intron (Figure 3A). *AaFLC1* and *AaFLC2* have short promoter regions (Figure 3A) and their expression levels were highly variable (Figure 4). To distinguish allelic expression patterns, we used SSCP analysis followed by cloning and sequencing individual fragments in each locus. The data (Figure 6A) indicated that *AaFLC2* expression was barely detectable in *A. arenosa* and undetectable in F_1 lines, Allo733, and -738, leading to the silencing of *AsFLC2* in *A. suecica*. Therefore, the contribution of *AaFLC2* and *AsFLC2* to flowering-time variation in *A. arenosa* and *A. suecica* is negligible. The fate of *AaFLC1* is unpredictable: it was highly expressed in *A. arenosa*, F_1-12, -14, and -19 but poorly expressed in F_1-4, -22, and -23, Allo733, and -738, suggesting epigenetic variation of *AaFLC1* expression in the synthetic allotetraploids. Low levels of *AaFLC2* and *AsFLC2* expression were probably associated with sequence deletions and mutations in the first introns. Deletions in the same upstream region (supplemental Figure 1, http://www.genetics.org/supplemental/) were shown to cause downregulation of *FLC* in *A. thaliana* (SHELDON *et al.* 2002). Collectively, the data suggest sequence divergence in the promoters and introns (Figure 3A) provides a molecular basis for differential regulation of *FLC* orthologs in Arabidopsis allotetraploids (Figure 6A). This does not preclude a possibility that other genes in the flowering pathways (SIMPSON and DEAN 2002; HE and AMASINO 2005) contribute to late flowering during evolution. Indeed, *AsMAF1*, an *AtMAF1* homolog (RATCLIFFE *et al.* 2001; SCORTECCI

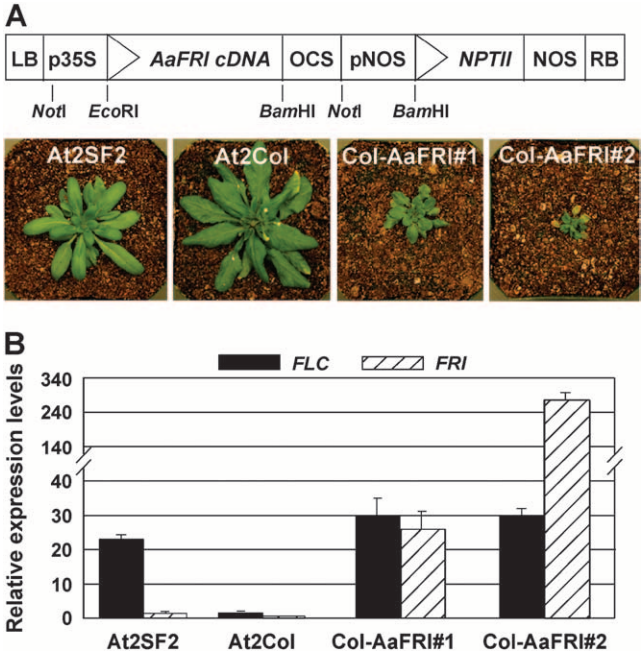

FIGURE 5.—(A) *AaFRI* confers late-flowering phenotype in rapid cycling *A. thaliana* Col. The construct for transgenic plants is shown at the top. LB, left border; p35S, 35S CaMV promoter; *AaFRI*, full-length *AaFRI* cDNA; OCS, octopine gene; pNOS, NOS promoter; NPTII, selective marker; NOS, terminator; RB, right border. Restriction sites for cloning are shown. The transgenic plants (Col-AaFR1#1 and -#2, T1) grew slowly. A late-flowering SF2 and an early-flowering Col are shown. (B) Activation of *AtFLC* in the transgenic plants. Relative expression levels of *FLC* and *FRI* were determined by qRT–PCR analysis in SF2, Col, and transgenic plants.

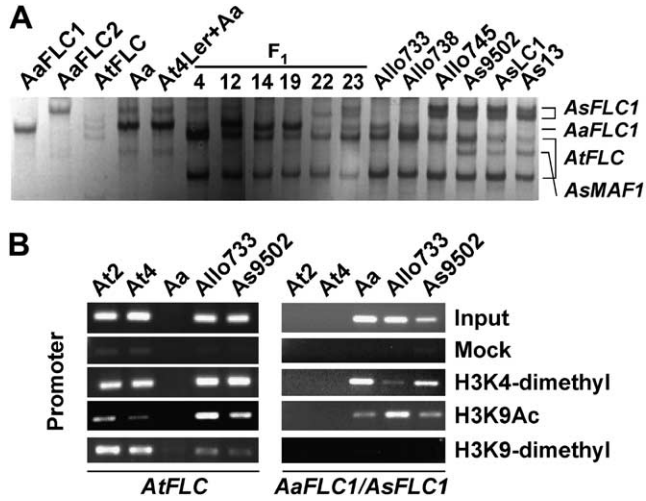

FIGURE 6.—(A) Expression variation detected by semiquantitative RT–PCR and SSCP analyses using primers shown in Figure 2B. Products in lanes (*AaFLC1* and *AaFLC2*) were amplified from inserts in plasmid DNA as molecular size markers. The corresponding loci indicated on the right were determined by cloning and sequencing individual cDNA fragments (data not shown). Note that two different-size bands in *AtFLC* or *AsFLC1* represented the same allele. (B) ChIP analysis of promoter regions of *AtFLC* (left) and *AaFLC/AsFLC* (right). *FLC* expression is associated positively with histone H3-Lys4 dimethylation and H3-Lys9 acetylation and negatively with H3-Lys9 dimethylation. The immunoprecipitates were heated to reverse the crosslinks and amplified by PCR using the *A. thaliana* and *A. arenosa* promoter-specific primers (supplemental Figure 1, http://www.genetics.org/supplemental/) so that only the *AtFLC* promoter was amplified in the left and the *AaFLC* in the right. The controls were the amplified DNA, respectively, from the chromatin fractions prior to antibody incubation (Input) and from those that were precipitated without antibodies (mock).

et al. 2001), was upregulated in three natural *A. suecica* strains (Figure 6A).

***FLC* expression variation is mediated by histone H3-Lys9 acetylation, H3-Lys4 methylation, and H3-Ly9 methylation:** To determine the cause of *AtFLC* upregulation and *AaFLC1* and *AsFLC1* expression variation in the synthetic and natural allotetraploids, we investigated the levels of H3-Lys9 acetylation and H3-Lys4 and H3-Lys9 methylation using ChIP assays (He *et al.* 2003, 2004; Bastow *et al.* 2004) and semiquantitative PCR amplification with the locus-specific primers designed for *A. thaliana* (L*er*) and *A. arenosa*, respectively (supplemental Figure 1, http://www.genetics.org/supplemental/). *AtFLC* reactivation was associated with the increased levels of histone H3-Lys9 acetylation and H3-Lys4 dimethylation (Figure 6B), two epigenetic marks for gene activation. Similarly, *AsFLC1* upregulation in *A. suecica* correlated with the increased levels of H3-Lys9 acetylation and H3-Lys4 dimethylation. A low level of H3-Lys4 dimethylation was detected in Allo733, which correlates with the relatively low level of *AaFLC1* expression in this synthetic allotetraploid (Figures 4 and 6A). This is because *AaFLC* is reactivated only in a subset of cells or gene expression changes may be reversible (Bastow *et al.* 2004) in the synthetic allotetraploids. Indeed, a high level of H3-Lys9 acetylation was detected, which may be related to variable and unstable levels of *AaFLC1* expression in Allo733. Using antibodies against H3-Lys9 dimethylation, an epigenetic mark for gene repression, we found H3-Lys9 dimethylation was dramatically reduced in the *AaFLC1/AsFLC1* promoter and moderately reduced in the *AtFLC* promoter. The data suggest that *AtFLC* activation and *AsFLC1* expression are mediated by histone acetylation and methylation at H3-Lys4 and -Lys9 sites. It is notable that H3-Lys4 dimethylation and Lys9 dimethylation levels may vary in some assays because H3-Lys9 dimethylation levels do not correlate with *FLC* repression in nonvernalized plants (Bastow *et al.* 2004). Residual levels of H3-Lys9 dimethylation detected in *AtFLC* and low levels of H3-Lys4 dimethylation and high levels of H3-Lys9 acetylation detected in *AaFLC1* may also suggest that other regions, such as the first intron, are important to *FLC* expression (Sheldon *et al.* 2002; Bastow *et al.* 2004). Alternatively, other genes in the vernalization and photoperiod pathways may contribute to the *FLC* repression (Levy and Dean 1998; Gendall *et al.* 2001; Simpson and Dean

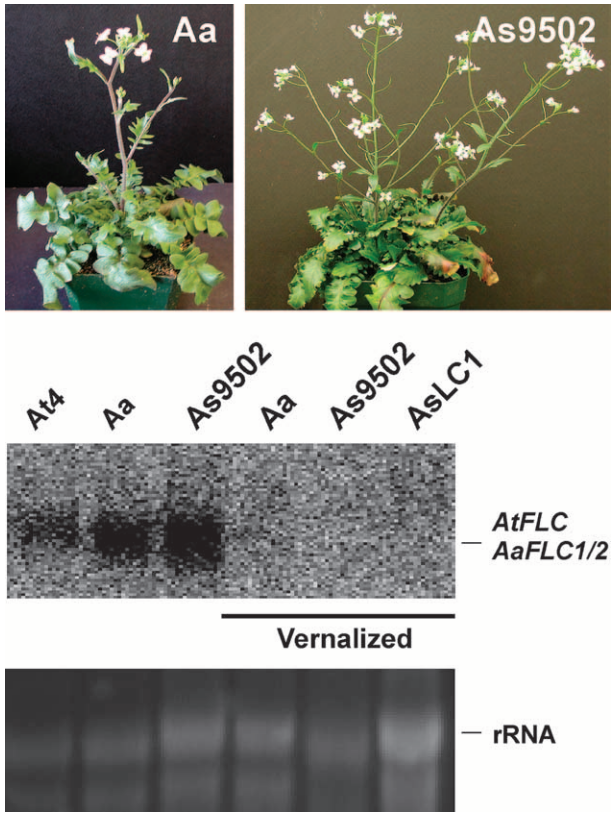

FIGURE 7.—Vernalization reduces *FLC* expression (RNA blot analysis, middle) and induces early flowering in *A. arenosa* (Aa) and *A. suecica* 9502 (As9502) (top). Agarose gel showing total RNA as a loading control for RNA blot analysis (bottom).

2002; He *et al.* 2003; Sung and Amasino 2004; He and Amasino 2005).

Indeed, vernalization reduced the expression of *AtFLC* and *Aa/AsFLC1/2* and reversed late-flowering phenotype in *A. arenosa* and *A. suecica* strains to early flowering (Figure 7), suggesting that the first intron contains regulatory elements required for vernalization (Sheldon *et al.* 2002). Consistent with the previous study (Finnegan *et al.* 2005), no changes in DNA methylation were detected in the *AtFLC* and *AaFLC* promoter regions using the bisulfite sequencing method (supplemental Figure 1, http://www.genetics.org/supplemental/). The data indicate functional *AaFRI* and a selective combination of *AaFLC1* and *AtFLC* expression contribute to the natural variation of flowering time in response to vernalization (extended winter-like cold temperatures) in Arabidopsis allopolyploids as in the *A. thaliana* ecotypes.

DISCUSSION

Flowering-time variation in Arabidopsis allopolyploids: Allotetraploids may provide a unique genetic system to test the effects of sequence divergence on the expression of orthologous genes originating in different species (Chen and Ni 2006). Arabidopsis allotetraploids are late flowering mainly because of *trans*-activation of *AtFLC* by *AaFRI*. The data suggest that *AaFRI* and *AtFLC*, the two genes responsible for natural variation of flowering time, are conserved between *A. thaliana* and *A. arenosa* after ~6 million years of evolution (Koch *et al.* 2000). The *trans*-acting effect of *AaFRI* on *AtFLC* reflects a strategy of allopolyploids using the best combination of orthologous loci in a genetic pathway. This selective epistatic interaction may be determined by an intact promoter and the first intron of *AtFLC*. *AaFLC* loci possess deletions both in the promoter and the first intron. Therefore, compared to *AtFLC*, *AaFLC* loci are relatively weak. Between two *AaFLC* loci, *AaFLC2* expression is repressed immediately in the synthetic allotetraploids, whereas *AaFLC1* expression levels are variable in the synthetic allotetraploids, which may correspond to epigenetic transient expression states during selfing (Wang *et al.* 2004). A strong *AaFLC1* is selected in *A. suecica*. The selection likely acts on the first intron of *AaFLC1*, leading to the high level of *AsFLC1* expression because no sequence divergence was detected in the *AsFLC1* and *AaFLC1/2* promoters (Figure 3 and supplemental Figure 1, http://www.genetics.org/supplemental/). It is intriguing that although *AaFLC1* and *AaFLC2* share similar promoter and intron sequences, *AaFRI trans*-activates *AtFLC* and maintains the expression of *AaFLC1* but not *AaFLC2* in the new allotetraploids and natural *A. suecica* strains. Sequence variation between the first introns of *AsFLC1* and *AaFLC1* (Figure 2) may also suggest that a different *A. arenosa* strain is the genome donor of *A. suecica*.

In addition to *AaFRI* effects on *AtFLC* expression, flowering-time variability in the synthetic allotetraploids may be related to the allelic variation in the first intron of *FLC*. For example, the low levels of *FLC* RNA accumulation in *A. thaliana* L*er* result from a transposable element inserted in the intron, which induces repressive chromatin modifications mediated by short interfering RNAs generated from homologous transposable elements in the genome (Liu *et al.* 2004). *AtFLC* in *A. suecica* is derived from a Col-like ecotype and does not contain the insertion, which is associated with high level of *AtFLC* expression in *A. suecica*. In contrast, the first intron of *AaFLC2* may have little effect on changes in *FLC* expression in the synthetic and natural allotetraploids. Despite absence of transposable element in the first intron, the *AaFLC2* expression level is very low in the synthetic allotetraploids and undetectable in *A. suecica*.

Note that >80 genes regulate flowering time in Arabidopsis (Levy and Dean 1998). Within *FRI* and *FLC* loci, there are numerous allelic variations detected among various ecotypes (Lempe *et al.* 2005; Shindo *et al.* 2005). Therefore, the molecular basis for this complex flowering-time trait remains to be carefully dissected in the allotetraploid plants that combine two interactive regulatory pathways inherited from two divergent progenitors.

A model for nonadditive gene regulation in allotetraploids: Our data suggest a model for genetic interactions between orthologous loci in a genetic pathway (Chen and Ni 2006) that mediates flowering time variation in Arabidopsis allotetraploids, which explains how new allopolyploid species assemble a functionally compatible pathway by selecting and modifying the expression of orthologous loci originating from divergent species. During ~6 million years of evolution (Koch *et al.* 2000), *A. arenosa* and *A. thaliana* (L*er*) diverged in flowering habits probably because of selective adaptation to the cold and warm climates (O'Kane *et al.* 1995; Sall *et al.* 2003), respectively. Sequence evolution of *FRI* and *FLC* loci leads to a nonfunctional *AtFRI* in *A. thaliana* (Johanson *et al.* 2000) and *cis*-regulatory changes in *A. thaliana* and *A. arenosa FLC* loci. In synthetic allotetraploids, *A. arenosa FRI* interacts in *trans* with the downstream gene, *AtFLC*, making the synthetic allotetraploids winter annual in a dosage-dependent manner. Interestingly, it was *trans*-activation of *AtFLC* that determined genetic dominance of late flowering in the synthetic allotetraploids, despite evolutionary divergence between *A. thaliana* and *A. arenosa*. Low levels of *AaFLC* expression may be associated with *cis*-regulatory changes in *A. arenosa* loci. *AtFLC1* and *AsFLC1* with intact *cis*-regulatory elements (promoters and/or introns) are selectively associated with a strong winter-annual habit in natural *A. suecica* strains, whereas *AaFLC2* and *AsFLC2* expression appears to be dispensable. The effects of *AaFRI* on *AtFLC* and *AaFLC1/AsFLC1* upregulation are mediated by histone acetylation and methylation. FRI is in the same pathway as FRIGIDA-LIKE 1 (FRL1) (Michaels *et al.* 2004) and FRIGIDA-ESSENTIAL 1 (FES) (Schmitz *et al.* 2005). Although there is no direct evidence for FRI interacting with these proteins, FRI may interact with protein complexes such as PAF1 (He *et al.* 2004) responsible for locus-specific chromatin modifications.

The current model may be generalized to explain the fate of duplicate genes being involved in biological pathways during allopolyploidization. Many orthologous genes in the progenitors might have evolved to possess divergent *cis*-regulatory elements that confer strong or weak, dominant or recessive alleles, tissue-specific expression, and/or developmental regulation. Evidently, the regulatory networks may be reset by chromatin modification immediately after allopolyploidization, leading to novel variation and increased fitness (Grant 1981; Wendel 2000; Osborn *et al.* 2003; Comai 2005). We provided the mechanistic evidence that altered regulatory networks (Birchler 2001; Osborn *et al.* 2003) and *cis*- and *trans*-regulation (Wittkopp *et al.* 2004) between the divergent biological pathways lead to epigenetic reprogramming of a biological (flowering) pathway after polyploidization. A similar mechanism may be responsible for the functional diversification of duplicate genes in developmental regulation of gene expression, a phenomenon known as subfunctionalization of duplicate genes (Lynch and Force 2000; Adams *et al.* 2003). It is notable that flowering time directly affects plant reproduction and adaptation. Therefore, sequence evolution and epigenetic regulation play interactive and pervasive roles in reconciling the regulatory incompatibilities between divergent genomes, leading to natural variation and selective adaptation during allopolyploid evolution.

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