# The Synergistic Activation of *FLOWERING LOCUS C* by *FRIGIDA* and a New Flowering Gene *AERIAL ROSETTE 1* Underlies a Novel Morphology in Arabidopsis

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ABSTRACT

The genetic changes underlying the diversification of plant forms represent a key question in understanding plant macroevolution. To understand the mechanisms leading to novel plant morphologies we investigated the *Sy-0* ecotype of Arabidopsis that forms an enlarged basal rosette of leaves, develops aerial rosettes in the axils of cauline leaves, and exhibits inflorescence and floral reversion. Here we show that this heterochronic shift in reproductive development of all shoot meristems requires interaction between dominant alleles at *AERIAL ROSETTE 1* (*ART1*), *FRIGIDA* (*FRI*), and *FLOWERING LOCUS C* (*FLC*) loci. *ART1* is a new flowering gene that maps 14 cM proximal to *FLC* on chromosome V. *ART1* activates *FLC* expression through a novel flowering pathway that is independent of *FRI* and independent of the autonomous and vernalization pathways. Synergistic activation of the floral repressor *FLC* by *ART1* and *FRI* is required for delayed onset of reproductive development of all shoot meristems, leading to the *Sy-0* phenotype. These results demonstrate that modulation in flowering-time genes is one of the mechanisms leading to morphological novelties.

THE establishment of body plan in plants occurs throughout their postembryonic development as a result of the continuous generation of new organs initiated by shoot apical meristems. While plants display a great variability in form, they are composed of a series of repeating body segments, called metamers, that have the same basic structure. Each metamer consists of an internode and a node, which is generally composed of a leaf and its subtended axillary meristem. Variations in metameric structure, like variation of the internodal length, suppression of leaf or axillary meristem development, or transformation of leaves and axillary meristems into specialized structures, lead to variation in plant form. Even metamers formed on a single plant differ in their morphology depending on the phase of the life cycle. For example, Arabidopsis plants initiate leaves with associated secondary shoots during vegetative growth. These metamers differ in leaf shape and trichome density, defining the juvenile and the adult vegetative phases (Telfer *et al.* 1997). A more pronounced morphological alteration of vegetative metamers is elongation of their internodes. While most vegetative metamers have short internodes, metamers that form at the end of the vegetative phase have elongated ones. Reproductive metamers form upon transition to flowering. They consist of an elongated internode, a suppressed leaf, and an axillary meristem that converts to a floral meristem. Therefore, three types of metamers characterize an Arabidopsis body plan (Figure 1A). The vegetative metamers with compressed internodes (V1) form a basal rosette of leaves, the vegetative metamers with elongated internodes (V2) form the bottom of the inflorescence stem, and reproductive metamers (R) form solitary flowers at the top of the inflorescence. The sequence of these metamers is fixed (V1 → V2 → R), which is a reflection of the irreversible transition of Arabidopsis plants to flowering.

A large number of genes that control the timing of the transition to flowering have been identified in Arabidopsis by mutant analysis (Peeters and Koornneef 1996). They were grouped in genetic pathways that mediate responses to multiple environmental and developmental cues (Simpson and Dean 2002). The photoperiod and the vernalization pathways act to promote flowering by mediating environmental responses to light and cold. The autonomous and the gibberellin pathways promote floral transition probably by mediating endogenous cues that reflect the developmental state of the plant. The *FRIGIDA* (*FRI*) gene identifies a floral repression pathway whose input signal is at present unknown. The autonomous and the vernalization pathways promote the floral transition by reducing the level of the floral repressor *FLOWERING LOCUS C* (*FLC*). *FRI* acts as floral repressor by promoting the expression of *FLC*. Thus, the inputs from the autonomous, vernalization, and *FRI* pathways integrate to determine the level of *FLC* floral

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repressor. The inputs from the *FLC* floral-repressing pathway and floral-promoting photoperiod and gibberellin pathways converge to regulate the expression of floral pathway integrator genes (*FT*, *AGL20/SOC1*, and *LFY*). These genes upregulate the function of floral meristem identity genes (*AP1*, *CAL*, *FUL*, and *LFY*), which act as a genetic switch to specify the floral developmental fate.

The timing of flowering depends primarily on inputs from floral-promoting and *FLC*-repressing pathways. It determines the length of the vegetative developmental phase influencing the number of vegetative metamers formed. Late-flowering plants form an enlarged basal rosette of leaves due to the increased number of V1 type metamers. Mutations in meristem identity genes eliminate reproductive metamers: in *lfy ap1* plants, flowers (reproductive metamers) are replaced by shoots with subtending leaves (therefore with V2 metamers; Weigel *et al.* 1992). Overexpression of these genes causes conversion of lateral shoots into flowers, resulting in the modification of V2 metamers. Therefore, modulations of the flowering pathway have a profound effect on plant architecture. However, in most cases these alterations affect the number of metamers formed and not their identity. Only in *35S:LFY* and *35S:AP1* backgrounds do the vegetative metamers (V2) alter their morphology due to the conversion of axillary meristems directly into flowers (Mandel and Yanofsky 1995; Weigel and Nilsson 1995).

In an effort to understand the mechanisms underlying the evolution of novel plant forms, we have initiated the study of the naturally occurring variant of *Arabidopsis thaliana*, *Sy-0*. The body plan of *Sy-0* plants differs significantly from the morphology of most early or late-flowering Arabidopsis strains. The salient morphological feature of *Sy-0* plants is the formation of aerial rosettes in the axils of cauline leaves. Therefore, in *Sy-0* plants V2 vegetative metamers have altered morphology due to the prolonged vegetative development of axillary meristems (Grbic and Bleecker 1996). We previously identified two genes, *ENHANCER OF AERIAL ROSETTE* (*EAR*) and *AERIAL ROSETTE* (*ART*), that are required for the *Sy-0* phenotype. In this report, we show that *EAR* is a dominant allele of *FRI* and that the *ART* locus represents a complex containing two linked genes, *FLC* and *AERIAL ROSETTE 1* (*ART1*). *ART1* is a new flowering gene that identifies a separate floral repression pathway. Its effect on flowering is mediated through activation of the floral repressor *FLC*. We provide evidence that the *Sy-0* phenotype arises due to the synergistic activation of *FLC* by *FRI* and *ART1*, demonstrating that modulation of flowering-time genes can lead to alteration of metamer structure and to morphological novelties.

## MATERIALS AND METHODS

**Plant material and growth conditions:** The seed for *Sy-0*, accession 1204, was obtained from the Arabidopsis Information Service Stock Center (Frankfurt, Main, Germany). Seeds for Landsberg *erecta* (L*er*) and lines homozygous for morphological markers *lutescence* (*lu*), *male sterile 1* (*ms1*), and *transparent testa glabra* (*ttg*) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. *FRI-Sf2*, *FLC-Col*, and *FRI-Sf2/FLC-Col* homozygous lines in L*er* background were kindly provided by Richard Amasino (University of Wisconsin, Madison). Monogenic lines containing *Sy-0* alleles of *FRI*, *FLC*, and *ART1* loci were backcrossed to L*er* at least five times.

All plants were grown under 100–150 μE $m^{-2}$ $sec^{-1}$ cool-white fluorescent light at 22° under long-day conditions consisting of 16 hr of light followed by 8 hr of darkness. As variability in flowering time has been observed between experiments, control plants (parents used in the cross) were always grown in parallel to the progeny that had been tested. Seedlings for the RNA analysis were grown on half strength Murashige and Skoog medium (Sigma, Irvine, UK) under 75 μE $m^{-2}$ $sec^{-1}$ cool-white fluorescent light at 22° and were harvested when the first true leaves were 1–2 mm in length.

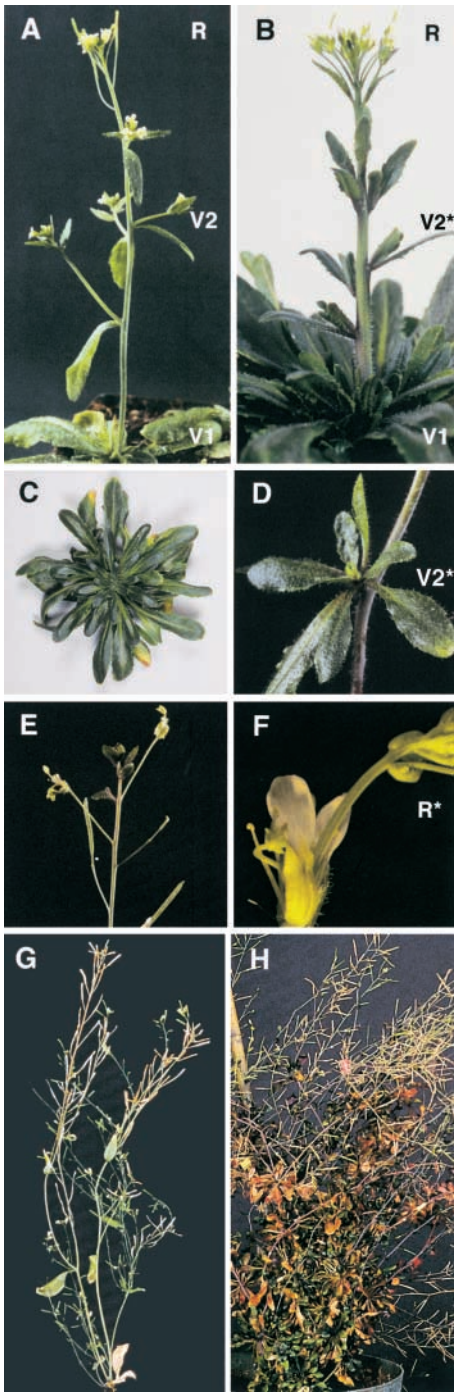
**Mapping and the analysis of the *FRI-Sy-0* allele:** Cleaved amplified polymorphic markers generated from left borders of MUG13, MAC12, MWD9, MDJ22, K5A21, MRN17, MYJ24, and K19M13, as well as NIT4, were used for mapping the *ART1* and the upstream modifier of *ART1* present in *lu Ms1 ART1 Ttg* recombinant lines. The chromosomal positions of these clones can be found at http://www.arabidopsis.org/servlets/mapper. Analysis of the *FRI-Sy-0* allele was done by PCR amplification of genomic fragments containing two identified deletion sites within the *FRI* sequence. The primers used and PCR conditions were as described in Johanson *et al.* (2000).

**RNA gel blot analysis:** Total RNA was extracted using the RNAwiz RNA isolation reagent (Ambion, Austin, TX) according to the manufacturer's instructions. Ten micrograms of total RNA was run on a 1× TBE agarose gel and transferred to a nylon membrane. A partial *FLC* cDNA fragment that was kindly provided by Richard Amasino (University of Wisconsin, Madison) and described in Michaels and Amasino (1999) was used as an *FLC* probe. A partial cDNA *FRI* fragment was synthesized by RT-PCR using the following primers: 5′-GATTTGCTG GATTTGATAAGG-3′ and 5′-TTCAATGACCACCGTAAAGG-3′. Products were cloned into pGEMT-Easy (Promega, Madison, WI). The identity of the FRI cDNA fragment was initially confirmed with diagnostic restriction digests and was subsequently sequenced. It was used as a *FRI* probe. Probes were labeled using the Rediprime II labeling kit (Amersham, Uppsala, Sweden).

## RESULTS

**The morphology of *Sy-0* plants:** *Sy-0* is a late-flowering accession, which results in the formation of an enlarged basal rosette common to all Arabidopsis strains that have delayed flowering (Figure 1, B and C). The unique aspect of the *Sy-0* body plan is the formation of aerial rosettes in the axils of cauline (stem) leaves and reversion of inflorescence and floral meristems (Figure 1, D–F). In Arabidopsis, the primary shoot apical meristem usually irreversibly switches from vegetative to reproductive development, giving rise to leaf-bearing nodes at the bottom of the inflorescence and flower-bearing nodes at the top. In *Sy-0*, leaf-bearing nodes occasionally form when the plant has already initiated ≥10 flowers (Figure 1E). This suggests that after switching to reproductive development, the primary shoot apical meristem has reverted

to vegetative development. In addition, early flowers of *Sy-0* plants regularly show reversion of the floral to an inflorescence meristem, which is seen as formation of a branch from the middle of the flower (Figure 1F). If *Sy-0* morphology is viewed in terms of metamer structure, it can be described as V1 → V2* ↔ R* → R, where V2* corresponds to the aerial-rosette-bearing nodes, R* to reverting flowers, and the double-headed arrow to the reversion of an inflorescence meristem. Therefore, in *Sy-0*, all shoot apical meristems—the primary shoot apical, axillary, and floral meristems—have delayed establishment of reproductive development leading to modulation of either number or identity of Arabidopsis metamers.




The heterochronic shift in shoot meristem development has a profound effect on plant morphology (Figure 1, A, B, G, and H). A consequence of this morphological change is the extension of vegetative development beyond the transition to flowering. This change in the life history strategy increases the life span of the plant to >1.5 years compared to the three-month life span of the L*er* plants (Figure 1, G and H). It also enables *Sy-0* plants to form a greater number of secondary branches as a greater number of leaves and axillary meristems form. This affects overall plant fitness, since *Sy-0* plants produce more seed per plant than many other Arabidopsis strains, giving *Sy-0* an adaptive advantage at least under some environmental conditions.

**Identification of genes underlying changes in morphology of *Sy-0* plants:** *The EAR locus corresponds to the FRI-Sy-0 allele:* In our previous work, we determined that *EAR*, a gene required for the aerial rosette phenotype of *Sy-0*, maps on chromosome IV in the vicinity of the *FRI* gene (Grbic and Bleecker 1996). This led to the possibility that a dominant allele at the *FRI* locus might be involved in specification of the *Sy-0* phenotype. To test this hypothesis, we investigated the nature of the *FRI-Sy-0* allele. A survey of 40 Arabidopsis accessions by Johanson *et al.* (2000) established a correlation between the early flowering phenotype and loss of *FRI* function due to the two independent deletion events. When the *FRI-Sy-0* allele was tested for the presence of these deletions, neither was found, indicating that *FRI-Sy-0* could be an active allele (Figure 2A). In addition, Northern blot analysis revealed that *FRI-Sy-0* RNA is expressed, further suggesting that the *FRI-Sy-0* allele may be functional (Figure 2B).

*FRI* is a putative transcription factor that activates the floral repressor *FLC*, leading to delayed flowering only if functional alleles at both loci are present (Michaels and Amasino 1999; Sheldon *et al.* 1999). Therefore, we tested the ability of the *FRI-Sy-0* allele to delay flowering in the presence of a reference *FLC-Col* allele. $F_1$ plants derived from a cross between *FRI-Sy-0* and *FLC-Col* lines flowered much later than either of the parents,

Figure 1.—Morphology of L*er* and *Sy-0* plants. (A) A 30-day-old L*er* plant. Its body plan can be described as V1 → V2 → R, where V1 and V2 vegetative metamers form a basal rosette and the bottom of the inflorescence stem and reproductive metamers (R) designate solitary flowers at the top of the inflorescence stem. (B) An 80-day-old *Sy-0* plant that can be described as V1 → V2* ↔ R* → R. V1 metamers form a basal rosette of leaves, V2* marks aerial-rosette-bearing nodes at the bottom of the inflorescence stem, and R represents solitary flowers at the top of the inflorescence. (C) Vegetative rosette of a 55-day-old *Sy-0* plant. (D) Detail of an aerial rosette borne on an *Sy-0* plant illustrating V2* metamer. (E) Detail of the apex of the *Sy-0* primary shoot that has reverted to vegetative development. (F) Detail of the flower from *Sy-0* that has reverted to the inflorescence meristem, an R* metamer. (G) A 50-day-old L*er* plant. (H) A 1.5-year-old old *Sy-0* plant.

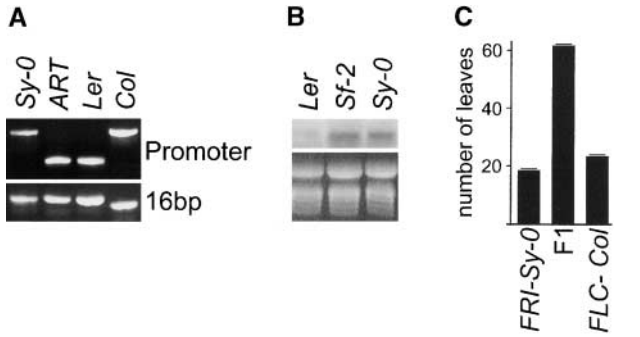



FIGURE 2.—Characterization of the *FRI-Sy-0* allele. (A) An analysis of the promoter and 16-bp deletions in *Sy-0*, *ART* line, L*er*, and *Col*. The *FRI*-L*er* allele has a promoter deletion and the *FLC-Col* allele has a 16-bp deletion. (B) Expression of *FRI* in L*er*, *Sf-2*, and *Sy-0*. (C) Flowering time of *FRI-Sy-0*, *FLC-Col*, and their $F_1$ progeny.

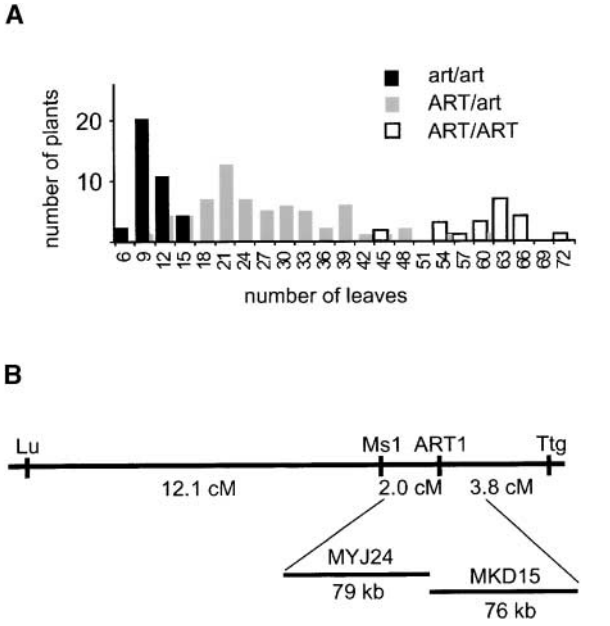



FIGURE 3.—(A) Frequency distribution of flowering time of $F_2$ segregating population of cross between *ART* line and L*er*. (B) The location of *ART1* on chromosome V. Distances were calculated using the Map Maker program.

indicating that the *FRI-Sy-0* allele is active and that the previously identified *EAR* locus specifies an allele of the *FRI* gene (Figure 2C).

*ART1 is a novel flowering locus identified in the ART line:* The *ART* locus has been identified as another factor required for the late-flowering aerial-rosette-bearing phenotype of *Sy-0* plants (GRBIC and BLEECKER 1996). The *ART*-containing line (*ART* line) flowers late, after initiating 76 rosette leaves. The late-flowering phenotype segregates as a single semidominant gene (36 *art/art*; 56 *ART/art*; 23 *ART/ART*, chi-square = 3.02, $P > 0.1$) with the heterozygous genotypic class overlapping both early and late-flowering homozygous plants (Figure 3A).

*ART* has been mapped to the short arm of chromosome V in a region that contains several other genes implicated in transition to flowering (GRBIC and BLEECKER 1996). To further refine the position of *ART*, recombinants around it were selected from the cross between the *ART* line (*ART/ART*) and a line homozygous for the morphological markers *lu*, *ms1*, and *ttg*. *ART* was initially located between *Ms1* and *Ttg* markers and later positioned on a 150-kb genomic fragment contained within the MYJ24 and MKD15 bacterial artificial chromosome clones (Figure 3B). As this region does not contain any genes previously implicated in flowering, *ART* identifies a novel flowering locus designated *ART1*.

*The ART1-induced delay in flowering requires the presence of additional gene(s):* During the course of mapping the *ART1* gene, we identified numerous recombinant lines. Their phenotypic classification and flowering time are shown in Figure 4. Plants that had the *Lu Ms1 ttg* phenotype (Figure 4A), and therefore having recombination between *Ms1* and *Ttg* markers, were genotyped for the *ART1* locus using the NIT4 PCR marker. Plants heterozygous at the *ART1* locus displayed a range of flowering times, from early to late, in the same pattern as seen in the $F_2$ segregating population shown in Figure 3A. Similarly, *art1/art1* plants were early flowering.

However, plants that had the *lu Ms1 Ttg* phenotype (Figure 4B), which were heterozygous at the *ART1* locus, flowered early (like the *art1/art1* genotypic class in Figure 4A) instead of displaying a range of flowering times. This indicated that *ART1* is not sufficient for the delayed flowering seen in the *ART* line and that some other factor(s), uncoupled from *ART1* in this recombinant class, must be present for late flowering.

To identify such additional factor(s), we examined the genetic organization of the *lu Ms1 ART1 Ttg* plants. The genomic region upstream of *Ms1* was homozygous for L*er* alleles (Figure 4B). Upon genotyping the *ART* line with markers spanning the *Ms1-Lu* region, we found that it contained an *Sy-0* genomic segment despite the five backcrosses of the *ART* line to L*er*. This indicated that an *Sy-0* region upstream of *Ms1* may contain a factor required for the *ART1*-induced late flowering. Within *lu Ms1 ART1 Ttg* plants, 25 out of 379 showed varying degrees of delayed flowering characteristic for the *ART1/art1* heterozygotes. To test the possibility that these plants flowered late due to the presence of an upstream factor, we genotyped them by using a MUG13 PCR-based marker that maps upstream of the *Lu* locus. In these plants a double crossover had occurred (plants marked with the asterisk in Figure 4B), making them heterozygous for the upstream *Sy-0* chromosomal fragment. This suggests that the region upstream of *Ms1* harbors a factor(s) from *Sy-0* that is required for the *ART1*-induced delay in flowering.

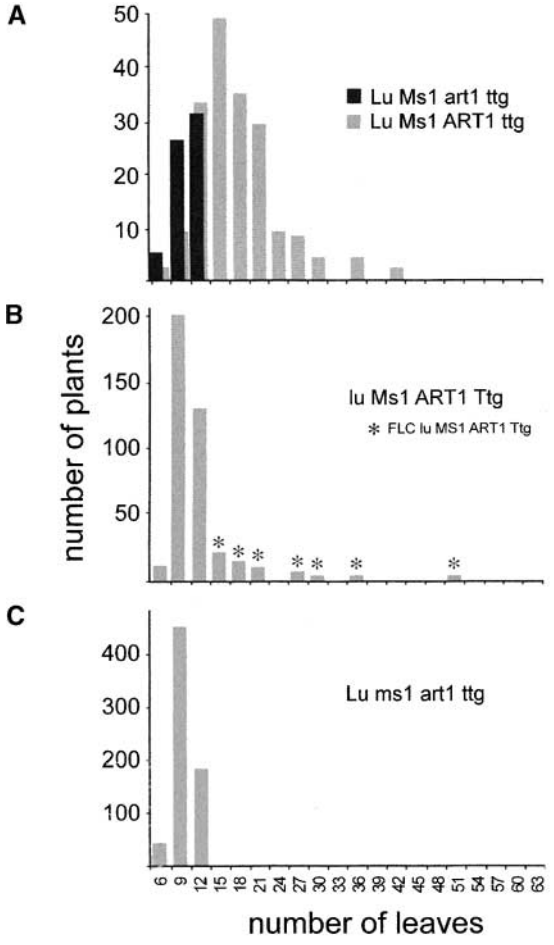



FIGURE 4.—Frequency distribution of flowering time of recombinant classes derived from the cross between homozygous lines *Lu Ms1 ART1 Ttg* (*Sy-0*) and *lu ms1 art1 ttg* (L*er*). (A) *Lu Ms1 ttg* recombinant class that segregates for *ART1/art1* and *art1/art1* plants. (B) *lu Ms1 ART1 Ttg* recombinant class; double crossing over late-flowering plants is marked with an asterisk. (C) *Lu ms1 art1 ttg* recombinant class.

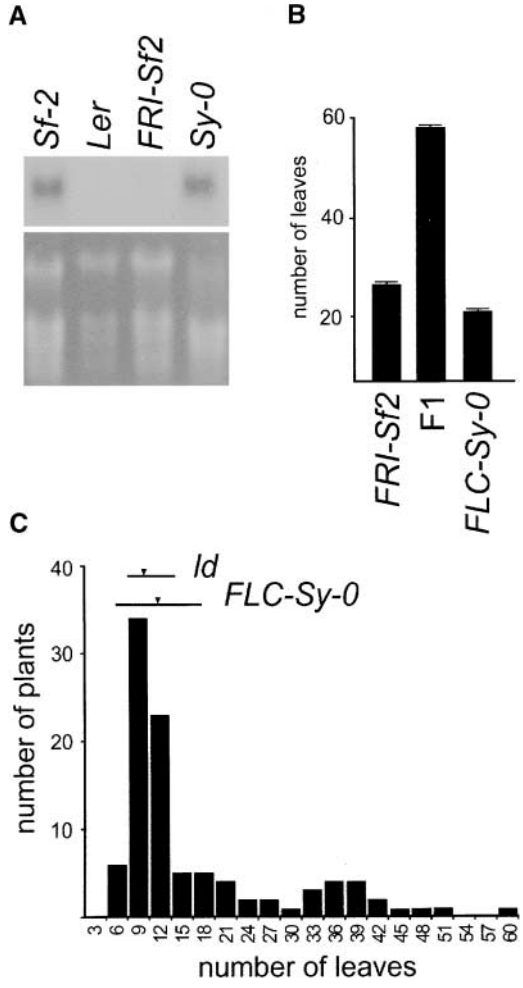



FIGURE 5.—Characterization of the *FLC-Sy-0* allele. (A) Expression of *FLC* in *Sf-2,* L*er, FRI-Sf2* in L*er*, and *Sy-0.* (B) Flowering time of *FLC-Sy-0, FRI-Sf2*, and their $F_1$ progeny. (C) Frequency distribution of flowering time of $F_2$ segregating population of cross between *FLC-Sy-0* and *ld.*

The *Lu ms1 art1 ttg* recombinant class contains only the upstream region from *Sy-0* (Figure 4C). All of these plants flowered early, demonstrating that the upstream factor(s) alone has no effect on flowering.

*FLC could be the upstream factor required for the ART1-mediated delay in flowering: FLC* is a known repressor of flowering that maps 2 cM upstream of *Lu* (LEE *et al.* 1994; KOORNNEEF *et al.* 1994). Therefore, there is a possibility that *Sy-0* contains a functional *FLC* allele, which may be the upstream factor required for the *ART1*-induced late flowering.

Three independent lines of evidence suggest that the *FLC-Sy-0* allele is functional. First, *FLC* is expressed in *Sy-0* (Figure 5A). *Sy-0* accumulates approximately the same level of *FLC* transcript as San Feliu-2 (*Sf2*), an Arabidopsis accession from which the reference *FLC-Sf2* allele has been isolated (LEE *et al.* 1994). Second, a cross between early flowering *FLC-Sy-0* and *FRI-Sf2* monogenic lines yielded late-flowering $F_1$ progeny, indicating that a typical synergistic interaction exists between active *FRI* and *FLC* alleles (Figure 5B). Finally, the $F_2$ population of a cross between the *FLC-Sy-0* and *ld* segregated plants that flowered significantly later than either of the parents (Figure 5C). Since *LD* acts to suppress the function of *FLC* (MICHAELS and AMASINO 1999), in *FLC ld* plants the functional *FLC* allele is derepressed and thus capable of delaying flowering in these plants. These results indicate that the *FLC-Sy-0* allele is functional and therefore *FLC* could be the upstream factor required for the *ART1*-mediated delay in flowering.

**Identification of the genetic interactions underlying morphological changes in *Sy-0* plants:** The genetic analysis of the late-flowering aerial-rosette-bearing phenotype indicated that *Sy-0* alleles at *FRI, FLC*, and *ART1* loci are required for the morphology of *Sy-0* plants. Monogenic lines of each of these genes are all early flowering (Table 1, Figure 6). Thus, none of the *Sy-0* alleles of these loci

**TABLE 1**

**Flowering times of lines used in this study**

| Line | Rosette leaf number at flowering[a] | Description |
|---|---|---|
| | Wild types | |
| L*er* | 8.1 ± 0.2 | *fri-Ler/fri-Ler; flc-Ler/flc-Ler; art1-Ler/art1-Ler* |
| *Sy-0* | 87.8 ± 1.0 | *FRI-Sy-0/FRI-Sy-0; FLC-Sy-0/FLC-Sy-0; ART1-Sy-0/ART1-Sy-0* |
| | Derived homozygous lines | |
| *FRI-Sf2* in L*er* | 13.6 ± 0.2 | *FRI-Sf2/FRI-Sf2; flc-Ler/flc-Ler; art1-Ler/art1-Ler* |
| *FLC-Col* in L*er* | 14.7 ± 0.3 | *fri-Ler/fri-Ler; FLC-Col/FLC-Col; art1-Ler/art1-Ler* |
| | Derived homozygous lines from *Sy-0* | |
| *EAR, FRI* | 16.0 ± 0.3 | *FRI-Sy-0/FRI-Sy-0; flc-Ler/flc-Ler; art1-Ler/art1-Ler* |
| *ART1* | 13.3 ± 0.5 | *fri-Ler/fri-Ler; flc-Ler/flc-Ler; ART1-Sy-0/ART1-Sy-0* |
| *FLC* | 12.0 ± 0.3 | *fri-Ler/fri-Ler; FLC-Sy-0/FLC-Sy-0; art1-Ler/art1-Ler* |
| *ART, ART1 FLC* | 76.4 ± 1.7 | *fri-Ler/fri-Ler; FLC-Sy-0/FLC-Sy-0; ART1-Sy-0/ART1-Sy-0* |

[a] Each value represents the average of at least 20 plants ± standard error. As variability in flowering time has been observed between experiments, these values represent results from the typical population analyzed.

act alone in their effect on flowering. To identify allelic interactions leading to the *Sy-0* morphology, we combined some of these alleles in crosses and determined the phenotype of their progeny.

*ART1 activates FLC independently of FRI:* The $F_1$ progeny of a cross between *ART1* and *FLC* flowered later than either of the parents, indicating synergistic interaction between the alleles examined (Figure 7A). This indicates cooperative action between genes that may be in the same pathway or that act through parallel pathways to produce the same outcome. To test if *ART1* acts in the same pathway as *FLC* and upstream of it, we analyzed the *FLC* mRNA levels in the *ART1*-containing lines. As seen in Figure 7B, *FLC* mRNA cannot be detected in L*er* plants. Therefore, detectable *FLC* transcripts in any genotype signify the activation of *FLC* expression.

*FLC* mRNA can be detected in the *ART1* line, indicating that *ART1* activates *FLC* (Figure 7B). The effect of *ART1* on *FLC* expression is allele specific, as *FLC-Col* mRNA accumulates at a higher level than *FLC*-L*er* when combined with *ART1*. Given that the transcripts encoded by these two alleles are the same (Sheldon *et al.* 2000), this difference in mRNA levels can be attributed to the differential capacity of these *FLC* alleles to be upregulated by *ART1*.

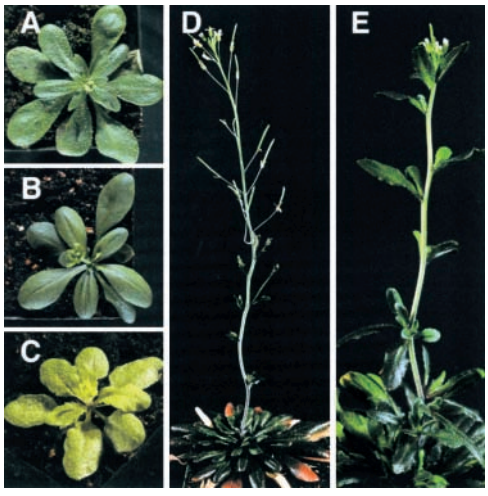



Figure 6.—Flowering phenotype of lines in L*er* background derived from *Sy-0*. (A) *FRI-Sy-0*. (B) *FLC-Sy-0*. (C) *ART1*. (D) *ART1 FLC*. (E) *ART1 FLC FRI*.

Activation of *FLC* by *ART1* occurs in the *FRI*-L*er* background and therefore in the absence of a functional *FRI* allele. This indicates that *ART1* acts through a *FRI*-independent pathway to activate *FLC* expression. In addition, these results confirm that *FLC* is the downstream factor required for the *ART1*-mediated delay in flowering previously seen in the *ART* line.

*ART1 acts cooperatively with FRI to activate FLC expression:* A cross between early flowering lines homozygous for *ART1* and *FRI-Sf2* yielded $F_1$ progeny that flowered late (Figure 7C). The synergistic interaction between these loci indicates that they act on a common factor. Since this interaction occurs in the presence of the *FLC*-L*er* allele, which has a reduced capacity to be upregulated, it was necessary to determine whether these genes interact through *FLC* or some other downstream factor. If they act through *FLC*, then one would expect that *ART1* and *FRI* could activate the *FLC*-L*er* allele.

Northern analysis of *FLC* expression shows that *ART1* and *FRI-Sf2* alone have a limited capacity to induce the *FLC*-L*er* allele (Figure 7D). However, they cooperatively induce *FLC*-L*er* to high levels, similar to those seen for the *FRI-Sf2 FLC-Sy-0* line. This suggests that *FRI*- and *ART1*-specific pathways converge at or upstream of *FLC* to activate its expression.

*ART1 acts independently of the autonomous flowering pathway:* Several flowering pathways integrate to affect *FLC* expression. The results described above establish that *ART1* activates *FLC* expression through a *FRI*-indepen-

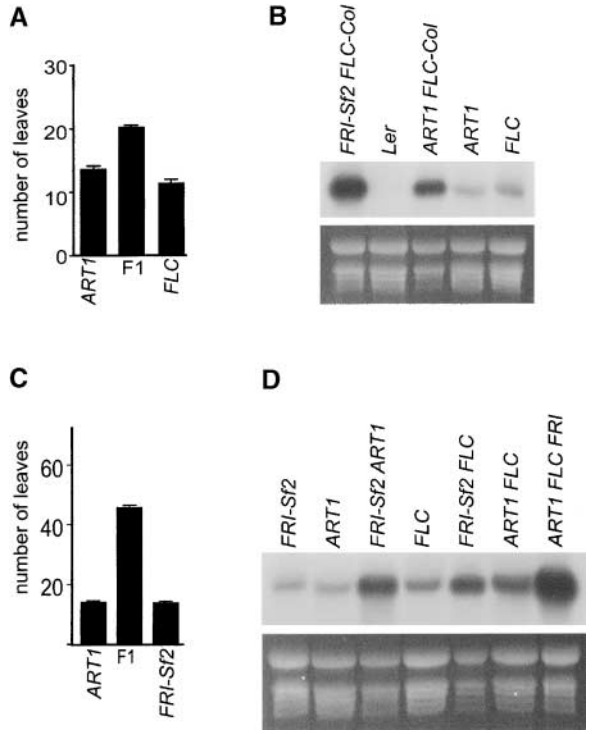



FIGURE 7.—Analysis of genetic interactions between *ART1*, *FRI*, and *FLC*. (A) Flowering time of *ART1*, *FLC-Sy-0*, and their $F_1$ progeny. (B) Expression pattern of *FLC* in various genotypes. (C) Analysis of genetic interactions between *ART1* and *FRI*. (D) Expression pattern of *FLC* in various genotypes. *Sy-0* alleles of corresponding loci are marked by capital letters, L*er* alleles are not shown, and *Col* and *Sf2* alleles are marked.


E

| cross | number of plants w/o aerial rosettes | number of plants w/ aerial rosettes | tested ratio | Chi square test | mode of gene's action |
|---|---|---|---|---|---|
| *fcafca x ARTART* | 61 | 11 | 13:3 | 0.36 | recessive |
| *fvefve x ARTART* | 32 | 62 | 7:9 | 3.20 | dominant |
| *fpafpa x ARTART* | 43 | 46 | 7:9 | 0.58 | dominant |
| *ldld x ARTART* | 81 | 19 | 13:3 | 0.01 | recessive |

FIGURE 8.—Late-flowering aerial-rosette-bearing plants from the $F_2$ segregating population of a cross between the *ART1 FLC* line and *fca* (A), *fve* (B), *fpa* (C), and *ld* (D) homozygous lines. (E) Chi-square test analysis of aerial rosette phenotype in crosses shown in A–D.

dent pathway. Likewise, this activation occurs despite the functional alleles at *LD*, *FPA*, *FVE*, and *FCA* loci that act to repress the *FLC* expression through the autonomous pathway, suggesting that *ART1* acts to activate *FLC* independently of these genes. If *ART1* acts independently of the autonomous pathway, then one would expect a synergistic interaction between *ART1* and mutant alleles that eliminate the *FLC* repression imposed by the autonomous pathway.

This hypothesis was tested by analyzing the flowering time of an $F_2$ segregating population derived from a cross between the *ART1 FLC* line and lines homozygous for *ld*, *fve*, *fpa*, or *fca* recessive alleles. The effect of *ART1 FLC* and mutations that disrupt the autonomous pathway was additive regarding the flowering time. The latest-flowering $F_2$ plants flowered after producing an additional 10–15 leaves relative to the *ART1 FLC* line. However, the latest flowering plants also formed aerial rosettes phenocopying the *Sy-0* phenotype (Figure 8, A–E), indicating synergistic interaction between these loci in respect to the aerial rosette phenotype. These interactions indicate that *ART1* acts independently of the autonomous pathway to affect timing of flowering and aerial rosette formation. In addition, the formation of aerial rosettes in a fraction of $F_2$ plants suggests that modulation of *FLC* expression underlies this phenotype.

## DISCUSSION

**Genetic bases of *Sy-0* morphology:** *Sy-0* plants form an enlarged basal rosette and develop aerial rosettes in the axils of cauline leaves. The aerial rosette formation was inseparable from the late-flowering phenotype. In addition, their inflorescence meristem infrequently displays reversion of flowering. Also, several floral meristems, the first ones to form upon transition of the plant to reproductive development, show floral reversion. Therefore, in *Sy-0*, *all* shoot meristems—primary, axillary, and floral—have delayed establishment of reproductive development. However, once the reproductive development is established, both inflorescence and floral meristems develop normally.

In this article we establish that *Sy-0* alleles at *FRI*, *FLC*, and *ART1* loci are required for the *Sy-0* phenotype. *FRI* and *FLC* are repressors of flowering previously identified from late-flowering Arabidopsis accessions. *FRI* is an activator of *FLC*, which acts to repress the onset of reproductive development (SIMPSON and DEAN 2002; Figure 9). *ART1* is another locus required for the *Sy-0* phenotype. It is a novel flowering locus that activates *FLC* expression. It maps 14 cM proximal to *FLC* on chromosome V. Thus, *ART1* and *FLC* form a set of floral repressors that behave genetically as a single gene. Linked floral repressors on chromosome V, named *FLF* and *FLG*, have also been identified in the early flowering *Cvi* accession of Arabidopsis. These genes may correspond to late-flowering alleles of *FLC* and *ART1* (ALONSO-BLANCO *et al.* 1998). In this context, it is interesting that *Cvi* flowers

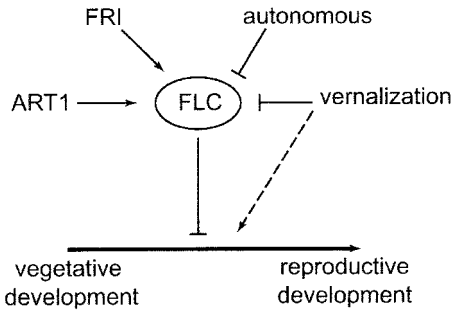



FIGURE 9.—Model for the interactions of *FLC*, *FRI*, and *ART1* in the regulation of flowering time.

early, which may be attributed to two other quantitative trait loci, *EDI* and *FLH*, that promote flowering in such a way as to suppress the effect of *FLF* and *FLG*.

Monogenic lines homozygous for *Sy-0* alleles at *FLC*, *FRI*, and *ART1* loci introgressed into L*er* background are all early flowering (Table 1 and Figure 6). However, each flowers slightly later then L*er*, which correlates with the detectable expression of *FLC* in these lines (Figure 7). Early flowering of monogenic lines derived from *Sy-0* indicates the importance of genetic interactions among these loci for establishment of the *Sy-0* phenotype. We have shown that *ART1* activates *FLC* through a *FRI*-independent pathway. As predicted from such a model, *ART1* acts cooperatively with *FRI* to activate *FLC* expression. The interactions between *Sy-0* alleles at these loci are required for the late-flowering aerial-rosette-bearing *Sy-0* phenotype.

Vernalization suppresses the effect of *FLC* on flowering (SHELDON *et al.* 2000). It also suppresses the delayed flowering of *Sy-0* plants (GRBIC and BLEECKER 1996), indicating that the effect of *ART1* on flowering can be abolished by vernalization. Two possibilities may account for the effect of vernalization on flowering time in *Sy-0*. Vernalization can decrease the expression or the activity of *ART1*, in which case *ART1* is a member of the vernalization pathway acting to increase *FLC* expression. Alternatively, *ART1* activates *FLC* expression through a vernalization-independent pathway, in which case the interaction between these pathways regulates the level of *FLC* expression. We favor the latter possibility as *ART1-Sy-0* and *ART1-Ler* alleles confer different *FLC* responses in the absence of vernalization (Figure 7), yet the presence of these alleles does not affect the plants' ability to respond to cold. In addition, we have provided evidence that *ART1* activates *FLC* expression independently of genes in the autonomous *FLC*-repressing pathway. Therefore, we propose that *ART1* identifies a novel *FLC*-activation flowering pathway (Figure 9).

**Implication of *Sy-0* morphology:** Upregulation of *FLC* expression underlies the late-flowering phenotype of many Arabidopsis accessions (JOHANSON *et al.* 2000). The common phenotypic feature among these accessions is formation of an enlarged basal rosette of leaves, which forms due to the prolonged vegetative development of the primary shoot apical meristem. However, these accessions still follow the basic body plan V1 → V2 → R, characteristic for the majority of Arabidopsis strains.

The *Sy-0* morphology can be described as V1 → V2* ↔ R* → R, indicating formation of two new types of metamers, V2* and R*. The formation of aerial-rosette-bearing nodes (V2*) has also been observed in some short-day-grown *35S:TFL* plants (RATCLIFFE *et al.* 1998). It has been proposed that *TFL* regulates the shoot apical phase transitions in such a way that its overexpression retards progression through phase transitions. In some *35S:TFL* plants it resulted in prolonged vegetative development of the primary and axillary meristems leading to a late-flowering aerial-rosette-bearing phenotype. Floral reversion (marked by R* metamers) is another hallmark of the *Sy-0* phenotype. This aspect of the *Sy-0* phenotype has also been described for heterozygous *lfy* and homozygous *ag* plants grown in a short-day photoperiod. These plants have flowers that display the same heterochronic transformation of flowers into inflorescence meristems (OKAMURO *et al.* 1996). Recently, this phenotype has been attributed to the reduced floral meristem identity maintenance function provided by *LFY* (PARCY *et al.* 2002).

A phenotype similar to *Sy-0* has been described for *indeterminate1* (*id1*) maize mutant (COLASANTI *et al.* 1998). It has been proposed that *ID1* regulates the synthesis of a floral-promoting signal or its transmission from leaves to shoot meristems, resulting in its absence at shoot meristems and their delayed conversion to reproductive development in *id1* plants. Reversion of flowering in *Impatiens balsamina* provides an additional example. In this case, the reversion of flowering occurs due to the depletion of the leaf-borne flower-promoting signal (POUTEAU *et al.* 1997).

We have previously proposed a model to explain the heterochronic shift common to all shoot meristems in *Sy-0* (GRBIC and BLEECKER 1996). According to the model, the *Sy-0* phenotype arises due to either the deficiency of floral-promoting signals at shoot meristems or the lack of competence of shoot meristems to respond to these signals. This model can explain the phenotypes of short-day-grown *lfy*/+ and *ag* plants, the *id1* maize mutant, and reverting Impatiens plants, in which presumably floral-promoting signals are lacking at shoot meristems that display reversion. Likewise, short-day-grown *35S:TFL* plants may lack a competence to respond to floral-promoting signals, resulting in the extension of developmental phases.

Our data indicate that the late-flowering aerial-rosette-bearing *Sy-0* phenotype requires synergistic activation of *FLC* by *ART1* and *FRI*. Interactions between *ART1* and mutations that disrupt the autonomous *FLC*-repression pathway also lead to formation of aerial rosettes, sug-

gesting that modulation of *FLC* expression in these genotypes underlies the establishment of the late-flowering aerial-rosette-bearing phenotype. How can modulation of *FLC* expression be integrated into the above model?

The mechanism by which *FLC* specifies the *Sy-0* phenotype is at present unknown. However, upregulation of *FLC* expression may not be sufficient for its establishment. *Sf-2* plants have comparable *FLC* expression levels and flower at approximately the same time as *Sy-0* plants, but do not form aerial rosettes (Figure 5A and data not shown). Moreover, while aerial-rosette-bearing plants were among the latest flowering ones in the segregating populations, there was an overlap in flowering time between plants that did and did not form aerial rosettes (data not shown). This implies that there is specific modulation of *FLC* expression that leads to *Sy-0* phenotype.

The *FLC* pathway represses the expression of floral pathway integrators (Simpson and Dean 2002). Its expression pattern has not been studied in great detail, but it has been shown that it localizes to the shoot and root tips (Michaels and Amasino 1999, 2000). This suggests that synergistic activation of *FLC* expression by *ART1* and *FRI* in *Sy-0* plants could act to decrease the expression of floral pathway integrators at shoot meristems. Therefore, the *Sy-0* phenotype would arise due to the lack of floral-promoting signals at all shoot apical meristems, leading to their delayed conversion to reproductive development.

Modulated expression of flowering genes can lead to variability in plant form. However, in most cases it results in an altered number of metamers formed and not in the change of their identity. Experimental manipulations of expression of *LFY*, *AP1*, and *TFL* can cause novel metamer morphology, indicating that these genes could be targets for selection leading to novel plant form. Moreover, variations in the temporal or spatial expression of *LFY* and *TFL* may account for many of the different inflorescence types (Coen and Nugent 1994). However, it is still unclear whether these genes were targets for the natural selection to derive new forms. Our analysis of the *Sy-0* ecotype demonstrates that naturally selected alteration in *FLC* expression underlies the evolution of novel morphological form. In this case, the morphological novelty arose by upregulation of the floral repressor *FLC* by *ART1* and *FRI*, demonstrating that modulation of flowering-time genes has been employed to produce novel plant morphology. Future molecular characterization of the new flowering gene *ART1*, which underlies specific modulation of *FLC* expression, should shed more light on the mechanism of plant morphological evolution.

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