Integration of Flowering Signals in Winter-Annual Arabidopsis[1]

Scott D. Michaels[2], Edward Himelblau[2,3], Sang Yeol Kim, Fritz M. Schomburg, and Richard M. Amasino*

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 (E.H., F.M.S., R.M.A.); and Department of Biology, Indiana University, Bloomington, Indiana 47405 (S.D.M., S.Y.K.)

Photoperiod is the primary environmental factor affecting flowering time in rapid-cycling accessions of Arabidopsis (*Arabidopsis thaliana*). Winter-annual Arabidopsis, in contrast, have both a photoperiod and a vernalization requirement for rapid flowering. In winter annuals, high levels of the floral inhibitor *FLC* (*FLOWERING LOCUS C*) suppress flowering prior to vernalization. *FLC* acts to delay flowering, in part, by suppressing expression of the floral promoter *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*). Vernalization leads to a permanent epigenetic suppression of *FLC*. To investigate how winter-annual accessions integrate signals from the photoperiod and vernalization pathways, we have examined activation-tagged alleles of *FT* and the *FT* homolog, *TSF* (*TWIN SISTER OF FT*), in a winter-annual background. Activation of *FT* or *TSF* strongly suppresses the *FLC*-mediated late-flowering phenotype of winter annuals; however, *FT* and *TSF* overexpression does not affect *FLC* mRNA levels. Rather, *FT* and *TSF* bypass the block to flowering created by *FLC* by activating *SOC1* expression. We have also found that *FLC* acts as a dosage-dependent inhibitor of *FT* expression. Thus, the integration of flowering signals from the photoperiod and vernalization pathways occurs, at least in part, through the regulation of *FT*, *TSF*, and *SOC1*.

Flowering time in most plant species is regulated by a combination of endogenous controls and environmental cues (Boss et al., 2004). This combination of signals helps to ensure that flowering takes place at the proper point in the plant's development, as well as at a favorable time of year, thereby maximizing the chances of successful reproduction. Two of the most common environmental factors affecting flowering time are day length (photoperiod) and temperature. In rapid-cycling strains of Arabidopsis (*Arabidopsis thaliana*), photoperiod is the primary environmental signal regulating flowering time; plants flower more rapidly under inductive long days (LD) than short days (SD). The B-box zinc-finger-containing transcription factor CONSTANS (CO) plays a critical role in the regulation of flowering time in response to photoperiod (Putterill et al., 1995). *co* mutants flower late in LD, but flowering is unaffected in SD (Koornneef et al., 1991). Thus, *CO* acts to promote flowering under LD. Recently, the molecular mechanism for the LD-specific promotion of flowering by *CO* has been elucidated. *CO* expression is regulated at both the RNA and protein levels. CO protein level is regulated via the photoreceptors *PHYTOCHROME A* and *CRYPTOCHROME 2* and accumulates only during the light (Valverde et al., 2004). *CO* transcription, in turn, is circadian regulated with expression peaking late in the day (Suarez-Lopez et al., 2001). This peak in mRNA levels takes place at the end of the day in LD but during the night in SD. Thus, only in LD is *CO* transcription coincident with the light required for protein accumulation.

In contrast to rapid-cycling strains of Arabidopsis whose flowering is regulated primarily by photoperiod, flowering in many naturally occurring accessions is also promoted by vernalization. Vernalization is the promotion of flowering by a prolonged exposure to cold temperatures (Chouard, 1960), such as would be experienced during winter in temperate climates. These accessions are delayed in flowering unless vernalized and thus behave as winter annuals. The vernalization-responsive block to flowering is caused by the interaction of two genes, *FLC* (*FLOWERING LOCUS C*) and *FRI* (*FRIGIDA*). *FLC* encodes a MADS-domain-containing transcription factor that acts as a repressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 1999), and *FRI* encodes a protein of unknown biochemical function that is required for *FLC* to be expressed to high levels (Johanson et al., 2000). Thus, both genes are required to block flowering, and loss-of-function mutations in either gene results in early flowering (in the absence of *FRI*, *FLC* is not expressed and in the absence of the floral repressor *FLC*, there is no effect of *FRI* [Michaels and Amasino, 1999, 2001]). It is interesting to note that rapid-cycling accessions of Arabidopsis are naturally occurring mutants in *FRI* or *FLC* (Johanson et al., 2000; Corre et al., 2002; Loudet

[1] This work was supported by the College of Agricultural and Life Sciences and the Graduate School of the University of Wisconsin and by the National Science Foundation (grant nos. 0133663 and 0209786).

[2] These authors contributed equally to the paper.

[3] Present address: Department of Biology, Southampton College, 239 Montauk Hwy, Southampton, NY 11968.

* Corresponding author; e-mail amasino@biochem.wisc.edu; fax 608–262–3453.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.052811.

et al., 2002; Gazzani et al., 2003; Michaels et al., 2003). Vernalization promotes flowering by causing a permanent epigenetic repression of *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). Recent studies have shown that changes in chromatin structure via histone modifications at the *FLC* locus play a critical role in the repression of *FLC* by vernalization (Bastow et al., 2004; Sung and Amasino, 2004).

In winter-annual Arabidopsis, the decision to flower is influenced by environmental information from the photoperiod and vernalization pathways. The molecular confluence of these pathways takes place at the level of two floral integrators, *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS*)/*AGAMOUS-LIKE 20* (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). Both *FT* and *SOC1* act as strong floral promoters; overexpression of either gene leads to a dramatic early-flowering phenotype. The expression of *FT* appears to be controlled primarily by photoperiod; *CO* promotes flowering by activating *FT* expression (Kardailsky et al., 1999; Kobayashi et al., 1999). *SOC1* expression, in contrast, is strongly regulated by vernalization (Lee et al., 2000). In the absence of vernalization, *SOC1* is repressed by *FLC*. Following cold treatment, however, *FLC* expression is suppressed and *SOC1* is expressed at high levels. Although *FT* and *SOC1* are most strongly regulated by photoperiod and vernalization, respectively, crosstalk between pathways does occur. *CO* overexpression increases *SOC1* levels and elevated levels of *FLC* expression decrease *FT* levels (Samach et al., 2000). To further characterize the integration of flowering signals, we examined activation-tagged mutants of *FT* and the *FT* homolog, *TSF* (*TWIN SISTER OF FT*; Kardailsky et al., 1999; Kobayashi et al., 1999) in a winter-annual background.

RESULTS

Identification of Activation Alleles of *FT* and *TSF*

Two T-DNA-mutagenized populations were generated in either wild-type Wassilewskija (Ws) or a late-flowering vernalization-responsive line containing *FRI*-SF2 in the Columbia (Col) background (*FRI*-Col) (Lee et al., 1994). Plants were transformed with the activation-tagging vector pSKI015 (Weigel et al., 2000), which carries 4 copies of the 35S cauliflower mosaic virus enhancer element. Several early-flowering plants were isolated from the T1 generations, suggesting that they contained dominant mutations due to gene activation. To determine the site of T-DNA integration in these mutants, genomic DNA flanking the site of T-DNA insertion was isolated using Thermal Asymmetric Interlaced PCR (Liu et al., 1995) and sequenced.

An early-flowering mutant from the Ws population was found to contain a T-DNA insertion 0.4 kb downstream of the 3′ end of the floral promoter *FT*, suggesting that the early-flowering phenotype of this mutant is due to the activation of *FT* (Fig. 1, A and B). This model is supported by the fact that the position and orientation of the T-DNA relative to *FT* is nearly identical to that in a previously described activation-tagged *FT* mutant and the two mutants exhibit similar early-flowering phenotypes (Kardailsky et al., 1999). A second mutant from the *FRI*-Col population contained a T-DNA 1.6 kb upstream of the translational start site of *TSF* (Fig. 1, A and B). Like *FT*, *TSF* overexpression also causes early flowering, suggesting that the early-flowering phenotype of this mutant is due to an activation of *TSF* (Kobayashi et al., 1999). Indeed, reverse transcription (RT)-coupled PCR showed that *FT* and *TSF* levels are elevated in these mutants (Fig. 1C). Interestingly, *TSF* mRNA levels are reduced in the *FT* activation mutant; similarly, *FT* levels are reduced in the *TSF* activation, indicating that negative feedback may play a role in the regulation of *FT* and *TSF*.

FT and *TSF* Activation Mutants Flower More Rapidly under Short Days

To determine the effects of *FT* and *TSF* activation on flowering time in a non-*FRI*-containing background, the *TSF* activation-tagged line was crossed to Col and plants were identified in the F_2 generation that were homozygous for the T-DNA and the Col allele of *fri* (which is a naturally occurring null). Genotypes were verified using PCR-based markers.

Previous studies have shown that overexpression of *FT* or *TSF* driven by the constitutive 35S promoter results in a strong early-flowering phenotype and the formation of terminal flowers (Kardailsky et al., 1999; Kobayashi et al., 1999). Consistent with these overexpression studies, the *FT* and *TSF* activation-tagged mutants flowered much earlier than the corresponding wild types in both LD and SD (Fig. 1D), and terminal flowers were observed in a small fraction of the plants (data not shown). An interesting distinction between the activation-tagged mutants and previously described *FT* overexpression lines is that the activation-tagged mutants flowered with fewer leaves in SD than in LD. This phenotype was not restricted to the *fri*-null background; in all genetic situations tested, the *FT* and *TSF* activation alleles caused earlier flowering in SD than in LD (see below).

FT and *TSF* Activation Alleles Strongly Suppress the Late-Flowering Phenotype of *FRI* and *FLC*

The photoperiod and vernalization pathways monitor the two primary environmental factors controlling flowering time in Arabidopsis. A major component of the photoperiodic regulation of flowering is the regulation of *FT* levels by *CO*, whereas the regulation of flowering by vernalization is achieved largely through the regulation of *FLC*. To investigate the interaction between these two pathways, the effects of *FT* and *TSF*

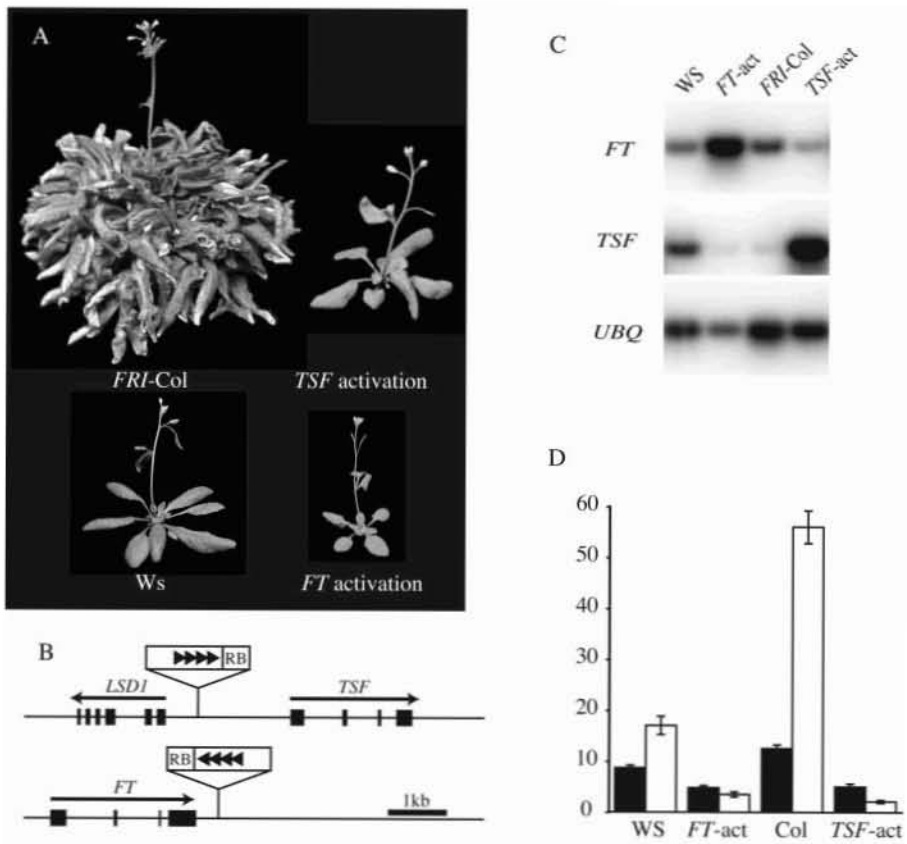

Figure 1. Activation-tagged alleles of *TSF* and *FT*. A, The early-flowering phenotype of the *TSF* activation mutant in the *FRI*-Col background and of the *FT* activation mutant in the Ws background. Plants were grown under LD. B, Location and orientation of the four 35S enhancer elements (black triangles) and T-DNA right border (RB) relative to *TSF* and *FT*. C, RT-PCR analysis of *FT* and *TSF* expression in wild type and activation-tagged mutants. RNA was extracted from 7-d-old seedlings. D, Flowering time of *FT* and *TSF* activation mutants in *fri*-null backgrounds (*FT*-act in the Ws background and *TSF*-act in the Col background). Black and white bars represent plants grown in LD and SD, respectively. Flowering time is expressed as the number of rosette leaves formed by the primary shoot apical meristem prior to the initiation of flowering. Error bars indicate 1 SD.

activation alleles were evaluated in a *FRI*-containing background.

In a *FRI*-containing background, *TSF* activation completely suppressed the delayed flowering effect of *FRI* in LD (Fig. 2A). Moreover, the early-flowering phenotype exceeded that of a *fri* null; the *TSF* activation in the *FRI* background flowered with approximately five fewer leaves than Col, a *fri* null (Col data shown in Fig. 1D). The effect of *TSF* activation under SD was even more dramatic. The *TSF* activation mutant in the *FRI* background flowered after forming approximately 4 leaves, whereas *FRI*-Col did not flower even after forming >100 leaves (Fig. 2A). As in LD, the *TSF* activation in the *FRI* background flowered much earlier than Col, which flowered with approximately 55 leaves in SD (Fig. 1D). Because the early-flowering phenotype of *TSF* activation is much stronger than a loss of *FRI* activity, the activation of *TSF* clearly does more to promote flowering than to simply counteract the effects of *FRI* and *FLC*. To determine the dosage dependence of the *TSF*-activation allele, the *TSF*-activation mutant was crossed to the *FRI*-Col. The flowering time of the F_1 plants was indistinguishable from the homozygous *TSF*-activation mutant (Fig. 2A), indicating that the *TSF*-activation allele behaves dominantly.

To determine the effect of *FT* activation in a *FRI*-containing background, the *FT*-activation mutant and Ws as a control were crossed to *FRI*-Col and the resulting F_1 plants were grown under LD and SD (*FRI* is completely dominant). Similar to the results with the *TSF*-activation allele, the *FT*-activation allele also strongly suppressed the effects of *FRI* (Fig. 2A). Under LD, the F_1 plants from the *FT*-activation/*FRI*-Col cross flowered similarly to wild-type Ws (Fig. 1D), which is a *fri*-null. Under SD, the early-flowering phenotype was even stronger, with the F_1 plants flowering with 12 fewer leaves than Ws (Figs. 1D and 2A).

FT and *TSF* Activation Suppresses the Late-Flowering Phenotype of *FLC* Overexpression

FT- and *TSF*-activation alleles strongly suppress the late-flowering phenotype caused by *FRI* and *FLC*. One possible model to explain this early-flowering phenotype is that *FT* and *TSF* activation may directly suppress *FLC* expression. Alternatively, the activation of *FT* and *TSF* may bypass the block to flowering created by *FRI* and *FLC*. To differentiate between these models, the activation-tagged alleles of *FT* and *TSF* were crossed to a line containing *FLC* under control of the constitutive 35S promoter (Odell et al., 1985) in an *flc-3* mutant background. The flowering time of F_1 plants resulting from crosses between the activation-tagged mutants and 35S::*FLC* were similar to that of crosses between the mutants and *FRI*-Col (Fig. 2, A and B). Given that *FT* and *TSF* activation alleles cause a similar early-flowering phenotype in *FRI*-containing or *FLC*-overexpression backgrounds, *FT* and *TSF* are not likely to affect

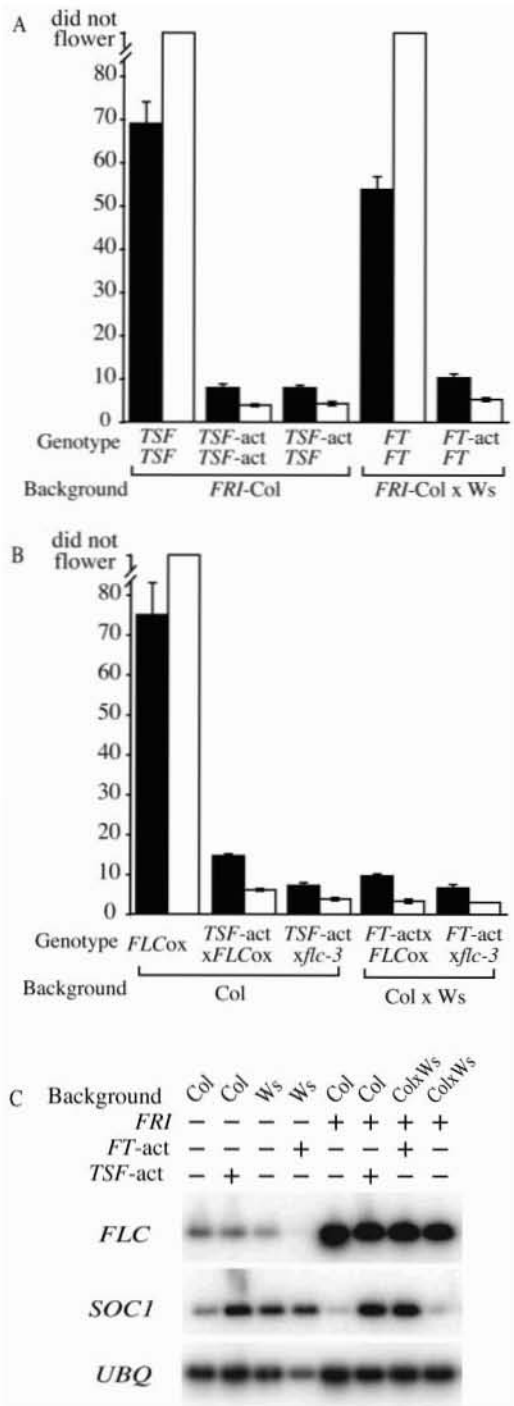

Figure 2. Activation alleles of *TSF* and *FT* suppress the late-flowering phenotype of *FRI* and *FLC* overexpression. Black and white bars represent plants grown in LD and SD, respectively. Flowering time is expressed as the number of rosette leaves formed by the primary shoot apical meristem prior to the initiation of flowering. Error bars indicate 1 SD. A, Flowering time of *TSF* and *FT* activation alleles in a *FRI*-containing background. B, Effect of *TSF* and *FT* activation on flowering time in a 35S::*FLC* background (*FLCox*). C, RT-PCR analysis of the effect of *TSF* and *FT* activation alleles on the expression of *FLC* and *SOC1*. *UBIQUITIN* (*UBQ*) was included as a control for loading.

FLC expression, but rather the elevated expression of *FT* or *TSF* appears to bypass the block to flowering created by *FLC*. Indeed, *FT* or *TSF* overexpression does not affect *FLC* mRNA levels (see below).

FT and *TSF* Act As Positive Regulators of *SOC1*, a Downstream Target of *FLC*

To gain molecular insight into the strong suppression of the late-flowering phenotype of *FRI* or 35S::*FLC* by activation alleles of *FT* or *TSF*, we investigated the effects of *FT* and *TSF* activation on *FLC* and *SOC1* expression. *SOC1* is a floral promoter that is negatively regulated by *FLC*, and *SOC1* overexpression has been shown to suppress the late-flowering phenotype of *FRI*-containing lines (Lee et al., 2000). Thus, a possible model for the suppression of *FLC*-mediated late flowering by *FT* and *TSF* activation is that *SOC1* is up-regulated by *FT* and *TSF*. To test this model, RT-PCR analysis was performed using total RNA isolated from 7-d-old seedlings of *FRI*-containing lines with or without *FT* or *TSF* activation alleles (Fig. 2C). All lines showed high levels of *FLC* expression regardless of the presence or absence of *FT*- or *TSF*-activation alleles. *SOC1* expression, however, was up-regulated by *FT* or *TSF* activation. Thus, it appears that *FT* and *TSF* suppress the late-flowering phenotype of lines containing high levels of *FLC*, at least in part, through the up-regulation of *SOC1*.

Histochemical Analysis of Gene Expression

FLC and *SOC1* are expressed at the highest levels in shoot and root apicies (Lee et al., 2000; Michaels and Amasino, 2000; Samach et al., 2000; Hepworth et al., 2002), whereas *FT* is most strongly expressed in the vasculature of the leaves (Takada and Goto, 2003). Because of these differences in spatial expression patterns, β-glucuronidase (GUS) fusions were used to further investigate the interactions between *FLC*, *SOC1*, *FT*, and *TSF*. Because *FRI*, *FLC*, and the *FT* and *TSF* activation alleles all behave dominantly, gene expression patterns were determined in F_1 plants resulting from crosses to lines containing *SOC1*::*GUS*, *FLC*::*GUS*, and *FT*::*GUS*.

Consistent with RNA expression data, the *SOC1*::*GUS* fusion is negatively regulated by *FRI* and *FLC* (Fig. 3A). In the absence of *FRI*, *SOC1*::*GUS* is expressed broadly in seedlings with the highest staining in the shoot and root tips. When the *SOC1*::*GUS* line is crossed to *FRI*-Col, GUS staining is strongly reduced in the shoot apex and the cotyledons (Fig. 3A). *SOC1*::*GUS* expression in the root tip, however, is relatively unaffected by *FRI* (Fig. 3B). This result is surprising because *FLC* is expressed at high levels in both the root tip and shoot tip in a *FRI* background (Fig. 3, C and D). One possible explanation for this result is that, in the root tip, *FLC* is not expressed in the necessary cell types or is not expressed to sufficient level to suppress *SOC1*. Another possibility is that *FLC*

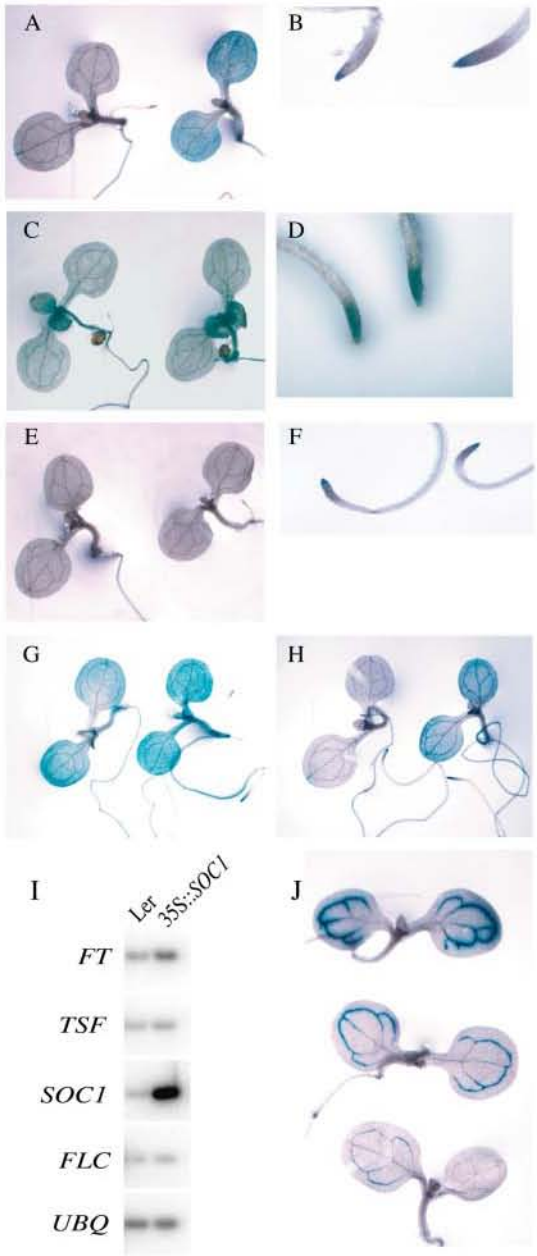

Figure 3. Histochemical analysis of the interactions between *FLC*, *SOC1*, *TSF*, and *FT*. To minimize variation in GUS staining, plants that appear in the same section were grown, fixed, and stained in parallel. A and B, Effect of *FRI* on *SOC1*::*GUS* expression. F_1 seedlings and root tips resulting from the cross of *SOC1*::*GUS* to *FRI*-Col (left) or Ws (right). C and D, *FLC*::*GUS* expression in seedlings and root tips in a *FRI*-containing background. E and F, Comparison of the effects of *FRI* and 35S::*FLC* on *SOC1*::*GUS* expression. F_1 seedlings and root tips resulting from the cross of *SOC1*::*GUS* to *FRI*-Col (left) or 35S::*FLC* (right). G, Effect of *FT* activation on *SOC1*::*GUS* expression. F_1 seedlings resulting from the cross of *SOC1*::*GUS* to Ws (left) and the *FT* activation mutant (right). Plants are in a *fri*-null background. H, Effect of *TSF* activation on *SOC1*::*GUS* expression. F_1 seedlings resulting from the cross of *SOC1*::*GUS* to *FRI*-Col (left) and to the *TSF* activation mutant (right). Plants are in a *FRI*-containing background. I, Effect of *SOC1* overexpression. RT-PCR analysis of *FT*, *TSF*, *SOC1*, and *FLC* expression in wild-type and *35S*::*SOC1* lines. *UBIQUITIN* (*UBQ*) was included as a control for loading. Plants are in the Landsberg *erecta* (L*er*) background. J, Inhibition of *FT* expression by *FRI* and *FLC*. F_1 seedlings resulting from the cross of *FT*::*GUS* in a Col background to Col (top), *FRI*-Col (middle), or 35S::*FLC* (bottom).

requires the presence of other factors to suppress *SOC1* and that one or more of these factors are not expressed in roots. To differentiate between these models, *SOC1*::*GUS* expression was also determined in a 35S::*FLC* background (Fig. 3, E and F). The pattern of *SOC1*::*GUS* expression in the *FLC* overexpression background was similar to that in a *FRI*-containing background; expression was suppressed in the shoot, but was unaffected in the root tip. Thus, it seems that *FLC* expression alone is insufficient to suppress *SOC1* expression in roots, suggesting that other factors, which are present in the shoot, may be lacking in roots.

SOC1::*GUS* expression is up-regulated by both the *FT* and *TSF* activation alleles in the presence or absence of *FRI* (Fig. 3, G and H). Comparison of crosses of *SOC1*::*GUS* to wild type and the *FT* activation mutant revealed that *SOC1*::*GUS* expression is increased throughout the plant by *FT* activation (Fig. 3G). Likewise, *TSF* activation increased *SOC1*::*GUS* expression in a *FRI* background (Fig. 3H). Although both the *FT* and *TSF* activation alleles increased *SOC1*::*GUS* expression, it is interesting to note that the expression patterns of *SOC1*::*GUS* differ slightly. In the *FT*-activation background, *SOC1*::*GUS* expression is relatively uniform throughout the plant, whereas in the *TSF*-activation background expression is highest in the vasculature of the cotyledons. One possible explanation for the concentration of *SOC1*::*GUS* expression in the vasculature is that the *TSF*-activation allele may be expressed to highest levels in the vasculature, similar to *FT*::*GUS*. (In some cases activation alleles maintain aspects of endogenous gene regulation [Weigel et al., 2000].)

We also investigated whether there might be reciprocal regulation of *FT* or *TSF* by *SOC1*. *FT* and *TSF* expression were examined in wild type and a 35S::*SOC1* line by RT-PCR. *TSF* expression was similar in both backgrounds (Fig. 3I); thus, *SOC1* does not appear to regulate *TSF*. *FT* expression was slightly higher in the *35S*::*SOC1* line, suggesting that *SOC1* may regulate *FT*. It should be noted, however, that the extreme early-flowering phenotype of the *35S*::*SOC1* line (approximately 2 leaves) makes it difficult to ensure that wild type and *35S*::*SOC1* are at the same developmental stage (i.e. the *35S*::*SOC1* seedlings may have initiated flowering, while wild type remained vegetative). Thus, the increase in *FT* expression caused by *35S*::*SOC1* may reflect a difference in developmental stage of the plants rather than a direct effect of *SOC1* overexpression.

The *FT*::*GUS* line was crossed to both Col and *FRI*-Col to determine the effect of *FRI* and *FLC* on *FT*::*GUS* expression (Fig. 3J). When crossed to Col, strong *FT*::*GUS* expression was observed in the vasculature of the cotyledons. The level of *FT*::*GUS* expression

was reduced in the *FRI*-containing line, which has higher levels of *FLC*. Thus, *FLC* negatively regulates *FT* expression. The *FT*::*GUS* line was also crossed to *35S*::*FLC* to determine the effect of increased *FLC* levels in the cotyledons. This line showed an even greater reduction in *FT*::*GUS* expression. Thus, *FLC* acts as a dosage-dependent suppressor of *FT*.

FT and *TSF* Have Overlapping Roles in the Promotion of Flowering

The similar phenotypes of activation or overexpression of *FT* and *TSF* suggest that they have similar functions in the regulation of flowering. Loss-of-function mutations in *ft* were identified on the basis of their late-flowering phenotype in LD. To determine if *tsf* loss-of-function mutations affect flowering, we obtained a line containing a T-DNA insertion in *TSF* from the SALK collection (Alonso et al., 2003). Homozygous *tsf*-mutant plants were identified using PCR-based markers and grown in both LD and SD. Under both conditions, the *tsf* mutant flowered similarly to wild type (Fig. 4A). A possible explanation for the lack of a flowering-time phenotype in the *tsf* mutant is that *FT* and *TSF* have overlapping or partially redundant roles in the promotion of flowering and that *FT* activity can compensate for the lack of *TSF*. Therefore, we investigated the effect of the *tsf* mutant in an *ft*-mutant background by crossing *ft* and *tsf* and isolating the *ft tsf* double mutant in the F_2 generation. Genotypes were verified using PCR-based markers. In LD, the *ft tsf* double mutant formed approximately 12 more leaves than the *ft* single mutant before flowering. Thus, *FT* and *TSF* have overlapping roles in the promotion of flowering, with *FT* playing the dominant role.

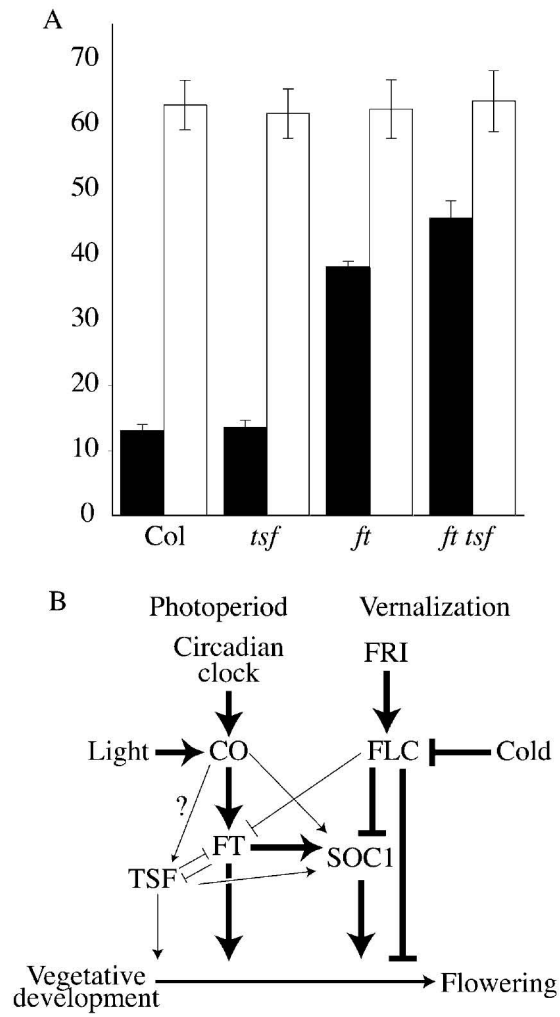

Figure 4. Interactions between flowering time genes. A, Flowering phenotype of *tsf* loss-of-function mutations in wild-type and *ft*-mutant backgrounds. Black and white bars represent plants grown in LD and SD, respectively. Flowering time is expressed as the number of rosette leaves formed by the primary shoot apical meristem prior to the initiation of flowering. Error bars indicate 1 SD. B, A model for the regulatory relationships between flowering genes. Line thickness is intended as a speculative measure of the strength of promotion or inhibition.

DISCUSSION

Much is now known of the molecular mechanisms by which the photoperiod and vernalization pathways regulate flowering time in Arabidopsis (e.g. Boss et al., 2004). Less is known, however, about how signals from these two pathways are integrated to control the transition to flowering. Rapid-cycling strains of Arabidopsis do not have a vernalization requirement for early flowering; thus, photoperiod is the major environmental factor regulating flowering time in these accessions. Flowering in winter-annual accessions, in contrast, is controlled by both photoperiod and vernalization, and therefore winter-annual Arabidopsis is an attractive system in which to study the integration of the photoperiod and vernalization pathways.

In the course of screening for early-flowering mutants in *fri*-mutant and *FRI*-containing backgrounds, we identified activation-tagged alleles of *FT* and *TSF*. The function of *FT* in the regulation of flowering time is most closely associated with the promotion of flowering in response to inductive photoperiods. *ft* mutants flower relatively normally in SD, but are late flowering in LD (Koornneef et al., 1991). Subsequent studies have shown that *FT* expression is positively regulated by *CO* (Kardailsky et al., 1999; Kobayashi et al., 1999), which is regulated by light and the circadian clock (Suarez-Lopez et al., 2001; Valverde et al., 2004). The activation mutants have elevated steady-state RNA levels of *FT* and *TSF* and exhibit a strong early-flowering phenotype in *fri*-null backgrounds consistent with previous overexpression studies (Kardailsky et al., 1999; Kobayashi et al., 1999). Interestingly, the activation-tagged alleles of *FT* and *TSF* also strongly suppressed the late-flowering phenotype caused by *FRI* and *FLC* in a winter-annual strain. These winter-annual strains containing activation alleles of *FT* and *TSF* were used to investigate the integration of flowering signals between the photoperiod and vernalization pathways.

The late-flowering vernalization-responsive phenotype of winter-annual Arabidopsis is caused by the up-regulation of *FLC* by *FRI* (Michaels and Amasino, 1999; Sheldon et al., 1999). Although the activation alleles of *FT* and *TSF* effectively suppress the late-flowering phenotype of winter annuals, *FLC* expression remains high. Expression of *SOC1*, a promoter of flowering that is negatively regulated by *FLC*, however, is up-regulated by *FT* and *TSF* activation. Thus, the regulation *SOC1* by both *FLC* and *FT* represents a mechanism for the integration of signals from the photoperiod and vernalization pathways.

It should be noted, however, that previous work suggests that positive regulation of *SOC1* by the photoperiod pathway is not solely accomplished through *FT* and *TSF*. Studies have shown that *CO* and *FLC* antagonistically regulate *SOC1* via separate promoter elements (Hepworth et al., 2002). In those experiments, FLC was shown to bind directly to *SOC1* promoter sequences in gel shift assays. CO was not shown to bind *SOC1* promoter sequences, but other experiments using a translational fusion of CO to the ligand-binding domain of the glucocorticoid receptor in cycloheximide-treated plants indicate the CO can up-regulate *SOC1* in the absence of the translation of new proteins (Samach et al., 2000), suggesting that CO is a direct regulator of *SOC1*. In total, these experiments indicate that CO may positively regulate *SOC1* through both indirect mechanisms (i.e. CO promotes *FT* expression, which in turn promotes *SOC1* expression) and direct mechanisms (e.g. CO can bind to the *SOC1* promoter as part of a complex).

Experiments using a *SOC1::GUS* fusion also demonstrated the regulation of *SOC1* by *FT* and *TSF* and revealed an interesting aspect of the regulation of *SOC1* by *FLC*. Despite the fact that both *FLC* and *SOC1* are expressed in the shoot and root tips, *FLC* is only effective in suppressing *SOC1* in the shoot. In lines containing *FRI* or *35S::FLC*, *SOC1::GUS* expression is suppressed in the shoot but is unchanged in the root, suggesting that additional shoot-specific factors may be required for the suppression of *SOC1* by *FLC*. It is interesting to note that differences in the expression requirements for root and shoot expression of *FLC* have also been observed; in a *pie1* mutant background, *FLC* expression is suppressed in the shoot but is not affected in roots (Noh and Amasino, 2003).

An interesting phenotype of the *FT* and *TSF* activation mutants is that, in all genetic backgrounds tested (*fri*-null, *FRI*-containing, and *35S::FLC* backgrounds), plants flowered after forming fewer leaves in SD than LD. The reason for earlier flowering in SD is not clear. One possibility is that the slower overall growth in SD provides additional time for *FT* and *TSF* to act. This SD-plant phenotype was not observed in plants containing *35S::FT* or *35S::TSF* constructs, which flowered with a similar number of leaves in LD or SD (Kardailsky et al., 1999; Kobayashi et al., 1999). These overexpression lines, however, flower after forming four or fewer leaves in LD or SD. (In our laboratory, transgenic plants containing *35S::FT* in a *FRI*-containing background flowered with approximately three leaves in either photoperiod.) Thus, the transition to flowering may occur so rapidly in these lines as to obscure any effect of photoperiod.

In summary, the results of these and previous experiments show that the integration of flowering signals from the photoperiod and vernalization pathways occurs, at least in part, through the regulation of *FT*, *TSF*, and *SOC1* (Fig. 4B). *CO* and *FLC* are regulated by the photoperiod and vernalization pathways, respectively. Both pathways, however, regulate the floral integrators *FT*, *TSF*, and *SOC1*; *FT* is positively regulated by *CO* and negatively regulated by *FLC*, whereas *SOC1* is negatively regulated by *FLC* and positively regulated by *CO*, *FT*, and *TSF*. In species such as Arabidopsis that have a quantitative response to both photoperiod and vernalization (i.e. plants will eventually flower even in the absence of inductive photoperiods or vernalization), it is possible that the levels of these integrators may provide a composite picture of the favorableness of the environment for flowering.

MATERIALS AND METHODS

Plant Material and Mutagenesis

FRI-SF2 in the Col background (Lee et al., 1994) and *flc-3* (Michaels and Amasino, 1999) have been described previously. *ft-1* introgressed into the Col background (Kardailsky et al., 1999) was kindly provided by D. Weigel (Max Planck Institute for Developmental Biology, Tubingen, Germany). The *tsf* mutant was obtained from the Salk collection (SALK 087522; Alonso et al., 2003). T-DNA and fast-neutron mutagenized populations have been described previously (Michaels and Amasino, 1999).

Growth Conditions

All plants were grown under 120 μE m^{-2} s^{-1} of cool-white fluorescent light at 22°C. LD conditions consisted of 16 h of light followed by 8 h of darkness; SD consisted of 8 h of light followed by 16 h of darkness. Plants used for RNA analysis were grown under constant light for 7 d prior to tissue harvest.

Gene Expression Analysis

For RT-PCR analysis, RNA isolation, RT, and PCR were performed as described previously (Michaels et al., 2004). Primers used for the detection of *SOC1*, *UBIQUITIN*, and *FLC* have been described previously (Michaels et al., 2004). For the detection of *FT* (5′-ACCTCAGGAACTTCTATACTTTGG-3′ and 5′-TACTATAGGCATCATCACCGTTCG-3′) and *TSF* (5′-ATGTCTTTAAGTCGTAGAGATCCTCTTGTGGT-3′ and 5′-CTACGTTCTTCTTCCCCCACAGCCATTC-3′), the indicated primers were used.

GUS Constructs

FT::GUS (Takada and Goto, 2003) and *SOC1::GUS* (Hepworth et al., 2002) constructs have been described previously. The *FLC::GUS* fusion was created by inserting the *GUS* gene into an *Nhe*I site located in the sixth exon of a 16-kb genomic clone containing 5.4 kb upstream of the *FLC* start codon and 5 kb downstream of the stop codon.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third part owners of all or parts of

the material. Obtaining any permissions will be the responsibility of the requestor.

ACKNOWLEDGMENTS

We thank Yannick Jacob for critical reading of this paper and Ann Marie DeGruccio for assistance in plant culture.

Received September 3, 2004; returned for revision November 15, 2004; accepted November 23, 2004.

LITERATURE CITED

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature **427:** 164–167

Borner R, Kampmann G, Chandler J, Gleissner R, Wisman E, Apel K, Melzer S (2000) A MADS domain gene involved in the transition to flowering in Arabidopsis. Plant J **24:** 591–599

Boss PK, Bastow RM, Mylne JS, Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell (Suppl) **16:** S18–S31

Chouard P (1960) Vernalization and its relations to dormancy. Annu Rev Plant Physiol **11:** 191–238

Corre VL, Roux F, Reboud X (2002) DNA polymorphism at the *FRIGIDA* gene in Arabidopsis thaliana: extensive nonsynonymous variation is consistent with local selection for flowering time. Mol Biol Evol **19:** 1261–1271

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

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G (2002) Antagonistic regulation of flowering-time gene *SOC1* by *CONSTANS* and *FLC* via separate promoter motifs. EMBO J **21:** 4327–4337

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

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer *FT.* Science **286:** 1962–1965

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. Science **286:** 1960–1962

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late-flowering mutants in *Arabidopsis thaliana.* Mol Gen Genet **229:** 57–66

Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim SG, Lee JS, Kwon YM, Lee I (2000) The *AGAMOUS-LIKE 20* MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev **14:** 2366–2376

Lee I, Michaels SD, Masshardt AS, Amasino RM (1994) The late-flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of Arabidopsis. Plant J **6:** 903–909

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J **8:** 457–463

Loudet O, Chaillou S, Camilleri C, Bouchez D, Daniel-Vedele F (2002) Bay-0 x Shahdara recombinant inbred line population: a powerful tool for the genetic dissection of complex traits in Arabidopsis. Theor Appl Genet **104:** 1173–1184

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

Michaels S, Amasino R (2000) Memories of winter: vernalization and the competence to flower. Plant Cell Environ **23:** 1145–1154

Michaels SD, Amasino RM (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:** 935–942

Michaels SD, Bezerra IC, Amasino RM (2004) *FRIGIDA*-related genes are required for the winter-annual habit in Arabidopsis. Proc Natl Acad Sci USA **101:** 3281–3285

Michaels SD, He Y, Scortecci KC, Amasino RM (2003) Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. Proc Natl Acad Sci USA **100:** 10102–10107

Noh YS, Amasino RM (2003) *PIE1,* an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell **15:** 1671–1682

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810–812

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80:** 847–857

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G (2000) Distinct roles of *CONSTANS* target genes in reproductive development of Arabidopsis. Science **288:** 1613–1616

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The *FLF* MADS box gene. A repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11:** 445–458

Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410:** 1116–1120

Sung S, Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein *VIN3.* Nature **427:** 159–164

Takada S, Goto K (2003) *TERMINAL FLOWER2,* an Arabidopsis homolog of *HETEROCHROMATIN PROTEIN1,* counteracts the activation of *FLOWERING LOCUS T* by *CONSTANS* in the vascular tissues of leaves to regulate flowering time. Plant Cell **15:** 2856–2865

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004) Photoreceptor regulation of *CONSTANS* protein in photoperiodic flowering. Science **303:** 1003–1006

Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrandiz C, Kardailsky I, Malancharuvil EJ, Neff MM, et al (2000) Activation tagging in Arabidopsis. Plant Physiol **122:** 1003–1013