CONSTANS Activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to Promote Flowering in Arabidopsis[1][w]

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CONSTANS (*CO*) regulates flowering time by positively regulating expression of two floral integrators, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), in Arabidopsis (*Arabidopsis thaliana*). *FT* and *SOC1* have been proposed to act in parallel pathways downstream of *CO* based on genetic analysis using weak *ft* alleles, since *ft soc1* double mutants showed an additive effect in suppressing the early flowering of *CO* overexpressor plants. However, this genetic analysis was inconsistent with the sequential induction pattern of *FT* and *SOC1* found in inducible *CO* overexpressor plants. Hence, to identify genetic interactions of *CO*, *FT*, and *SOC1*, we carried out genetic and expression analyses with a newly isolated T-DNA allele of *FT*, *ft-10*. We found that *ft-10* almost completely suppressed the early flowering phenotype of *CO* overexpressor plants, whereas *soc1-2* partially suppressed the phenotype, suggesting that *FT* is the major output of *CO*. Expression of *SOC1* was altered in gain- or loss-of-function mutants of *FT*, whereas expression of *FT* remained unchanged in gain- or loss-of-function mutants of *SOC1*, suggesting that *FT* positively regulates *SOC1* to promote flowering. In addition, inactivation of *FT* caused down-regulation of *SOC1* even in plants overexpressing *CO*, indicating that *FT* is required for *SOC1* induction by *CO*. Taken together, these data suggest that *CO* activates *SOC1* through *FT* to promote flowering in Arabidopsis.

The phase transition to flowering in plants is precisely controlled by environmental conditions and endogenous developmental cues so that plants produce their progeny under favorable conditions. The response to multiple factors suggests the existence of a complex network regulating this phase transition in plants (Koornneef et al., 1998). To identify genes that control the transition, mutants that showed accelerated or delayed flowering under different conditions, commonly known as flowering-time mutants, have been isolated (Redei, 1975). These mutants were grouped according to their responses to various physiological conditions and then integrated into genetic pathways to explain the control of flowering time. Four floral promotion pathways have been genetically identified in Arabidopsis (*Arabidopsis thaliana*): the photoperiod, autonomous, vernalization, and GA pathways (Mouradov et al., 2002). Among these pathways, genes within the photoperiod pathway, or the long-day pathway, play an important role in controlling flowering time (Komeda, 2004), since Arabidopsis is a facultative long-day plant.

One of the central regulators in the photoperiod pathway is *CONSTANS* (*CO*), which encodes a nuclear protein that contains a CCT motif and two B-box-type zinc-finger domains (Putterill et al., 1995). Loss of *CO* function delays the phase transition, whereas gain of function of *CO* accelerates it, suggesting that *CO* positively regulates flowering time in Arabidopsis. Furthermore, *CO* mRNA levels show a circadian rhythm under continuous light, such that *CO* mRNA levels peak at night and are reduced during the day (Suarez-Lopez et al., 2001). The circadian pattern is altered in circadian clock mutants, such as *lhy* and *elf3-1*, but constitutive expression of *CO* does not alter the circadian rhythm of the *CHLOROPHYLL A/B-BINDING PROTEIN* gene, suggesting that *CO* acts as a circadian clock output gene to mediate flowering (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002).

CO controls flowering time in Arabidopsis by positively regulating two floral integrators, *FLOWERING LOCUS T* (*FT*; Kardailsky et al., 1999; Kobayashi et al.,

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1999) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*; Lee et al., 2000; Samach et al., 2000). *FT* and *SOC1* encode a protein similar to the RAF kinase inhibitor protein and a MADS box transcription factor, respectively. These two integrators are early targets of *CO* within the photoperiod pathway since up-regulation of *FT* and *SOC1* was detected in inducible *CO* overexpressor plants carrying the *35S*::*CO*:*GR* construct, in the presence of cycloheximide (Samach et al., 2000). *FT* and *SOC1* have been confirmed as downstream mediators of *CO* by a genetic analysis in which *FT* and *SOC1* were identified as strong suppressors of *CO* overexpressor plants (Onouchi et al., 2000). Furthermore, the early flowering phenotype of *CO* overexpressor plants was suppressed, albeit weakly, by either *ft* or *soc1* mutations, suggesting that *FT* and *SOC1* are required for the early flowering of *35S*::*CO* plants. The delay in flowering time in *ft soc1* double mutants was additive, compared to the effects of each single mutant. The phenotype of *ft soc1 35S*::*CO* plants was similar to loss-of-function mutants of *CO* under long-day conditions, implying that inactivation of both *FT* and *SOC1* suppresses signaling from *CO*. These results, together with the observations that no single mutation that completely suppresses the early flowering of *35S*::*CO* plants has been isolated, have been used to conclude that *CO* activates two parallel pathways, which include *FT* and *SOC1*, to promote flowering (Samach et al., 2000).

Although genetic studies have inferred that *FT* and *SOC1* act in parallel pathways downstream of *CO*, up-regulation of *FT* is detected before *SOC1* in *CO* overexpressor plants (Samach et al., 2000). Furthermore, the genetic studies were performed using weaker *ft* alleles (Onouchi et al., 2000). We hypothesized that a null allele of *FT* would produce a stronger suppression of early flowering in *CO* overexpressor plants. To test this hypothesis, we investigated the genetic interactions between *CO* and *FT* with a newly isolated T-DNA-tagged allele of *FT*, *ft-10*, in which *FT* mRNA is almost undetectable. We also examined the expression of *FT* and *SOC1* to determine whether they act independently in parallel pathways.

Here we show that *ft-10* almost completely suppressed the early flowering phenotype of *CO* overexpressor plants, whereas a T-DNA allele of *SOC1* only partially suppressed early flowering. These data suggest that *FT* is the major target of *CO*. In addition, expression of *SOC1* was altered in gain- or loss-of-function mutants of *FT*, whereas expression of *FT* remained unchanged in gain- or loss-of-function mutants of *SOC1*. These data suggest that *FT* and *SOC1* do not act in parallel, but rather that *FT* positively regulates expression of *SOC1*. Furthermore, inactivation of *FT* resulted in down-regulation of *SOC1* even in the presence of *CO* overexpression, suggesting that *FT* is required for *SOC1* induction by *CO*. These results suggest that *FT* is the major output of *CO* and mediates activation of *SOC1* by *CO* to promote flowering in Arabidopsis.

RESULTS

ft-10 Almost Completely Suppresses the Early Flowering Phenotype of *CO* Overexpressor Plants

To investigate genetic interactions between *CO* and *FT*, we used a new insertional allele of *FT* (290E08) isolated from a T-DNA library generated by GABI-Kat (http://www.gabi-kat.de/), in which a T-DNA is inserted in the first intron of *FT* (Fig. 1A; Rosso et al., 2003; Hanzawa et al., 2005). We obtained and plated seeds of the putative insertional mutant of *FT* and identified a line that was resistant to sulfadiazine. The segregation ratio for sulfadiazine resistance was approximately 3:1, indicating that a single T-DNA is present in the line. This line was PCR genotyped using the JH2295, JH2296, and JH2297 primers to confirm that the line was homozygous for a T-DNA insertion. A single 926-bp PCR product was amplified from the line, confirming homozygosity (Fig. 1B). The line was designated *ft-10*, and this insertional mutant was used in the following experiments.

We first monitored the expression levels of *FT* in *ft-10* plants by reverse transcription (RT)-PCR to examine whether *ft-10* was an RNA null allele. Total RNA extracted from whole seedlings grown for 10 d under long-day conditions was used to synthesize cDNA. We compared *FT* expression levels in wild-type and *ft-10* plants. Since *ft-10* plants were in the Columbia (Col) background, we used an introgressed *ft-1* allele in the Col background (*ft-1* [Col]) as an additional control. *FT* mRNA levels are only slightly reduced in *ft-1* (Col) plants, indicating that transcription of *FT* was largely normal in *ft-1* (Col) plants. In contrast, *FT* expression in *ft-10* plants was greatly reduced (Fig. 1C), but *ft-10* was not completely RNA null.

We measured the flowering time of *ft-10* plants under long-day conditions to determine whether a decrease in *FT* mRNA delayed flowering time. *ft-10* plants flowered with 40.6 leaves, whereas wild-type plants flowered with 15.0 leaves, demonstrating that *ft-10* plants showed a late-flowering phenotype. In contrast, *ft-1* (Col) plants flowered with 33.5 leaves under the same conditions, showing that the T-DNA insertion in *ft-10* caused a stronger effect than the point mutation in *ft-1* (Fig. 1D).

Since *ft-10* was identified as a strong allele showing a late-flowering phenotype, we used *ft-10* plants to determine the genetic interactions between *FT* and *CO*. To facilitate genetic studies with *ft-10* plants, *35S*::*CO* plants were generated in the Col background. The *35S*::*CO* plants showed early flowering (6.6 leaves) with formation of a determinate inflorescence under long-day conditions (Fig. 1E). The *35S*::*CO* plants were crossed with *ft-10* plants, and flowering time of the resulting double mutants under long-day conditions was measured. The *35S*::*CO ft-10* plants flowered with 37.6 leaves, and *ft-10* plants flowered with 40.6 leaves. The flowering times were not significantly different (Fig. 1D). In contrast, *35S*::*CO soc1-2* plants flowered

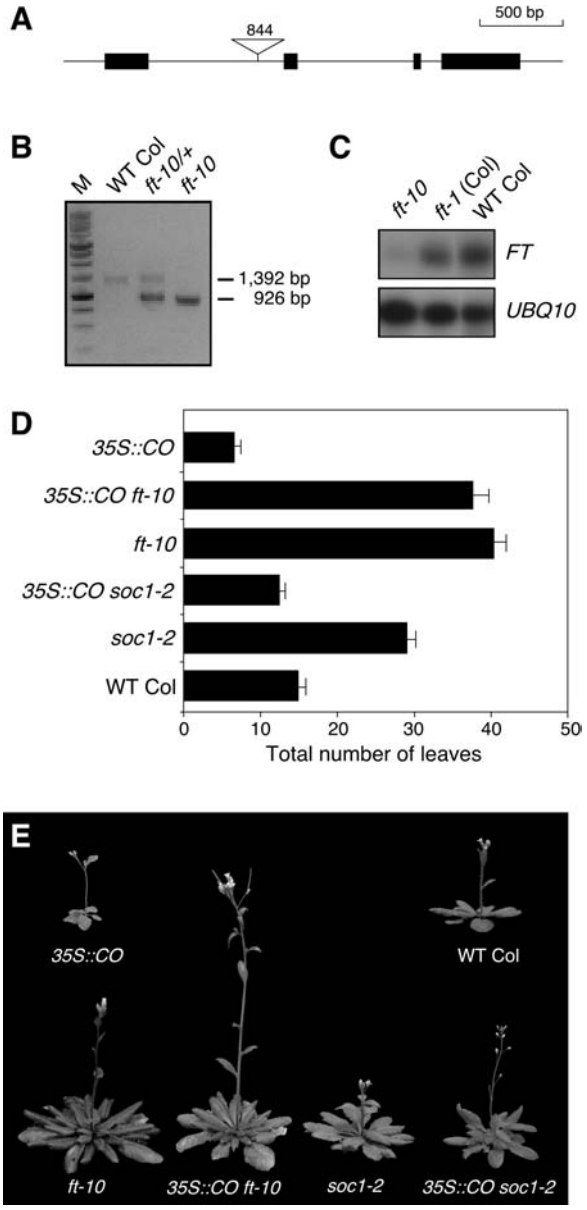

Figure 1. Flowering time of an *ft-10* allele and its genetic interaction with *35S*::*CO* plants. A, Map of a T-DNA insertion 844 bases from the A of the start codon of *FT*; the allele was named *ft-10*. Black boxes indicate the four exons of *FT*. B, PCR genotyping of the *ft-10* allele. Genotyping primer sets (JH2295, JH2296, and JH2297) were used to identify a homozygous line. Homozygous *ft-10* plants produced a single band of 926 bp in size, whereas wild-type plants produced a band of 1,392 bp in size. M, One-kilobase ladder. C, An RT-PCR experiment showing that *ft-10* was a strong knock-down allele. *FT* mRNA abundance in *ft-10* plants was compared to *ft-1* (Col), a point mutation allele of *FT*, and to wild-type Col plants. D and E, Flowering time and phenotypes of *35S*::*CO*, *ft-10*, *35S*::*CO ft-10*, *soc1-2*, *35S*::*CO soc1-2*, and wild-type Columbia (WT Col) plants under long-day conditions. *ft-10* almost completely suppressed the early flowering phenotype of *35S*::*CO* plants, whereas *soc1-2* partially suppressed the early flowering.

with 12.3 leaves, whereas *soc1-2* plants flowered with 29.4 leaves, suggesting that inactivation of *SOC1* slightly suppresses the early flowering of *CO* overexpressor plants. This analysis shows that *ft-10* strongly suppresses the early flowering of *35S*::*CO* plants, suggesting that *FT* is the primary downstream target of *CO* to promote flowering.

FT Positively Regulates Expression Levels of *SOC1*

Parallel roles of *FT* and *SOC1* downstream of *CO* were proposed using *ft soc1* double mutants, which largely suppressed the phenotype of the *35S*::*CO* plants (Samach et al., 2000). However, other data have suggested that *FT* may act earlier than *SOC1* in response to induction of *CO* (Samach et al., 2000). Therefore, we examined whether inactivation or up-regulation of one gene affected expression of the other gene. We used *35S*::*FT* and *35S*::*SOC1* plants for gain-of-function mutants of *FT* and *SOC1*, respectively, and *ft-10* and *soc1-2* plants (Lee et al., 2000) for loss-of-function mutants of *FT* and *SOC1*. All these mutants were in the same genetic background (Col). We extracted total RNA from 6-, 8-, 10-, and 12-d-old plants and examined the time course of *SOC1* expression by RT-PCR.

Activation or inactivation of *FT* altered expression of *SOC1*; however, gain or loss of *SOC1* function did not alter expression of *FT* (Fig. 2). Expression of *SOC1* is greatly up-regulated in *35S*::*FT* plants, such that expression levels of *SOC1* in *35S*::*FT* plants were similar to those of *35S*::*SOC1* plants, as previously suggested (Moon et al., 2005). Up-regulation of *SOC1* was delayed in *ft-10* plants, indicating that changes in *FT* expression affected expression of *SOC1*. In contrast, expression levels of *FT* in *35S*::*SOC1* plants did not increase over the time tested; rather, the levels were similar to those of wild-type plants. Furthermore, expression levels of *FT* in *soc1-2* plants were comparable to those of wild-type plants. These results demonstrate that expression of *FT* is not affected by *SOC1* and that *FT* is likely to be upstream of *SOC1*. This experiment further suggests that *FT* and *SOC1* are not independent, but rather that *FT* positively regulates *SOC1*.

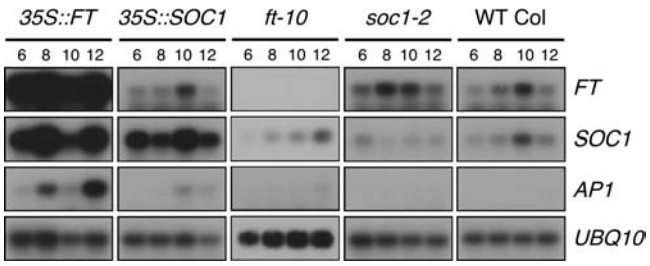

Figure 2. Time-course expression of *FT*, *SOC1*, and *AP1* in *35S*::*FT*, *35S*::*SOC1*, *ft-10*, *soc1-2*, and wild-type plants grown under long-day conditions. *soc1-2* is a T-DNA-tagged allele of *SOC1* (Lee et al., 2000). Gene expression levels of *FT* and *SOC1* in 6-, 8-, 10-, and 12-d-old seedlings were measured. *AP1* and *UBQ10* were used as a molecular marker of floral transition and an internal positive control, respectively.

These data contradict a recent model suggesting parallel roles for *FT* and *SOC1* (Onouchi et al., 2000; Samach et al., 2000). To confirm whether *FT* positively regulates *SOC1*, we employed a β-glucuronidase (GUS) reporter assay. We used *FT*::*GUS* (Takada and Goto, 2003) and *SOC1*::*GUS* (I. Lee, personal communication) plants to visualize expression of *FT* and *SOC1*, respectively. We generated *35S*::*FT*/+ *SOC1*::*GUS*/+ plants to examine whether *SOC1* promoter-driven expression of GUS was affected by overexpression of *FT*. The *35S*::*SOC1*/+ *FT*::*GUS*/+ plants were generated to test whether *FT* promoter-driven expression of GUS was altered by overexpression of *SOC1*.

A histochemical GUS assay showed that expression of *SOC1* was dependent on the mRNA level of *FT*. In *SOC1*::*GUS* plants, GUS staining was faintly observed in the shoot apex and vascular bundles in cotyledons (Fig. 3A). In contrast, strong GUS staining was observed in the shoot apex and roots in *35S*::*FT*/+ *SOC1*::*GUS*/+ plants (Fig. 3, B and E). In addition, increased GUS staining was seen in vascular bundles of cotyledons (Fig. 3C). This suggests that *SOC1* expression is induced by *FT* and that the strong *SOC1* expression at the apical region during the vegetative phase is associated with the early flowering of *35S*::*FT* plants. In contrast, similar localization patterns of GUS staining with similar staining intensities were observed in *35S*::*SOC1*/+ *FT*::*GUS*/+ plants (Fig. 3F). The vascular tissue-specific GUS expression of *FT*::*GUS* was not altered by activation of *SOC1* (Fig. 3H). Furthermore, overexpression of *SOC1* did not cause ectopic staining in hypocotyls and roots of *35S*::*SOC1*/+ *FT*::*GUS*/+ plants (Fig. 3, I and J). This indicates that expression of *FT* was not affected by *SOC1*. Taken together, the results from the GUS reporter assays were consistent with those of the RT-PCR expression analysis (Fig. 2), suggesting that expression of *SOC1* is positively regulated by *FT*, which is consistent with the previous findings (Michaels et al., 2005).

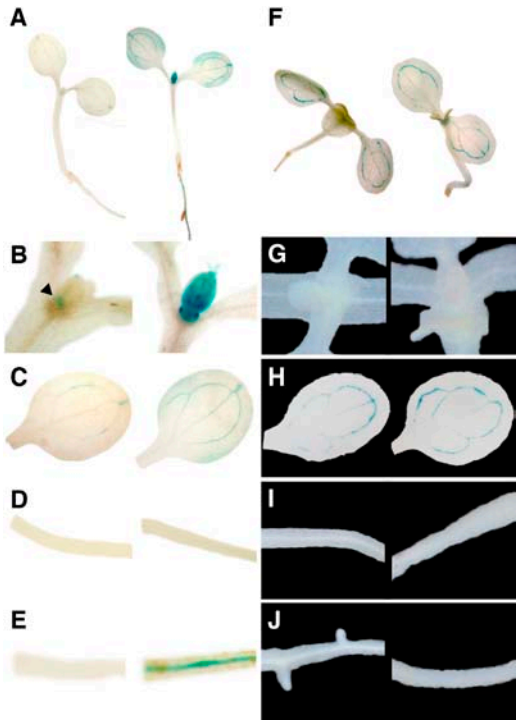

Figure 3. Histochemical GUS analysis to examine genetic interactions between *FT* and *SOC1*. A, Comparison of GUS-staining pattern of 5-d-old seedlings of *SOC1*::*GUS* (left) and *35S*::*FT*/+ *SOC1*::*GUS*/+ plants (right). B to E, Close-up views of GUS-staining patterns in different tissues of *SOC1*::*GUS* (left) and *35S*::*FT*/+ *SOC1*::*GUS*/+ plants (right): apical region (B), cotyledon (C), hypocotyl (D), and root (E). An arrowhead indicates faint staining in the shoot apex of a *SOC1*::*GUS*/+ seedling. F, Comparison of the staining pattern of 10-d-old seedlings of *FT*::*GUS* (left) and *35S*::*SOC1*/+ *FT*::*GUS*/+ plants (right). G to J, Close-up views of staining patterns observed in different tissues of *FT*::*GUS* (left) and *35S*::*SOC1*/+ *FT*::*GUS*/+ plants (right); apical region (G), cotyledon (H), hypocotyl (I), and root (J).

Double Overexpression of *FT* and *SOC1* Does Not Have an Additive Effect

To confirm the results obtained from the RT-PCR analysis and GUS reporter assay, we investigated the genetic interactions between *FT* and *SOC1*. We measured the flowering time of plants in which both *FT* and *SOC1* are overexpressed to examine whether the mutations had an additive effect. The single overexpressor plants, *35S*::*FT* and *35S*::*SOC1*, flowered with 4.0 and 8.6 leaves under long-day conditions (Fig. 4A), respectively. Homozygous *35S*::*FT* *35S*::*SOC1* plants flowered with 3.9 leaves under the same conditions, indicating that the phenotype of the double-overexpressor plants was similar to the *35S*::*FT* plants, implying that *FT* and *SOC1* do not act in parallel. This result is not consistent with previous genetic results that showed an additive effect of *FT* and *SOC1* overexpression (Moon et al., 2005). This raises the possibility that the difference in flowering time was due to the 35S promoter, which may cause overexpression of *SOC1* not reflective of the normal role of *SOC1*. Thus, to confirm our genetic data, we generated additional double-overexpressor plants using an activation-tagged allele of *SOC1*, *soc1-101D* (Lee et al., 2000). Flowering time of the resulting *35S*::*FT*/+ *soc1-101D*/+ plants, in which both *FT* and *SOC1* were activation tagged, was measured. The *35S*::*FT*/+ and *soc1-101D*/+ plants that were used for controls flowered with 4.2 and 5.5 leaves, respectively. In contrast, *35S*::*FT*/+ *soc1-101D*/+ plants exhibited an intermediate flowering time (5.0 leaves; Fig. 4). These results using the activation-tagged lines and the 35S promoter-driven overexpressor lines indicate that overexpression of *FT* and *SOC1* did not have an additive effect. However, *soc1-2* only partially suppressed the early flowering of *35S*::*FT* plants (8.4 leaves), suggesting that *SOC1* is not the only gene downstream of *FT*. Interestingly, the *ft-10 soc1-2* double mutant flowered with 52.0 leaves, showing an additive delay in flowering time.

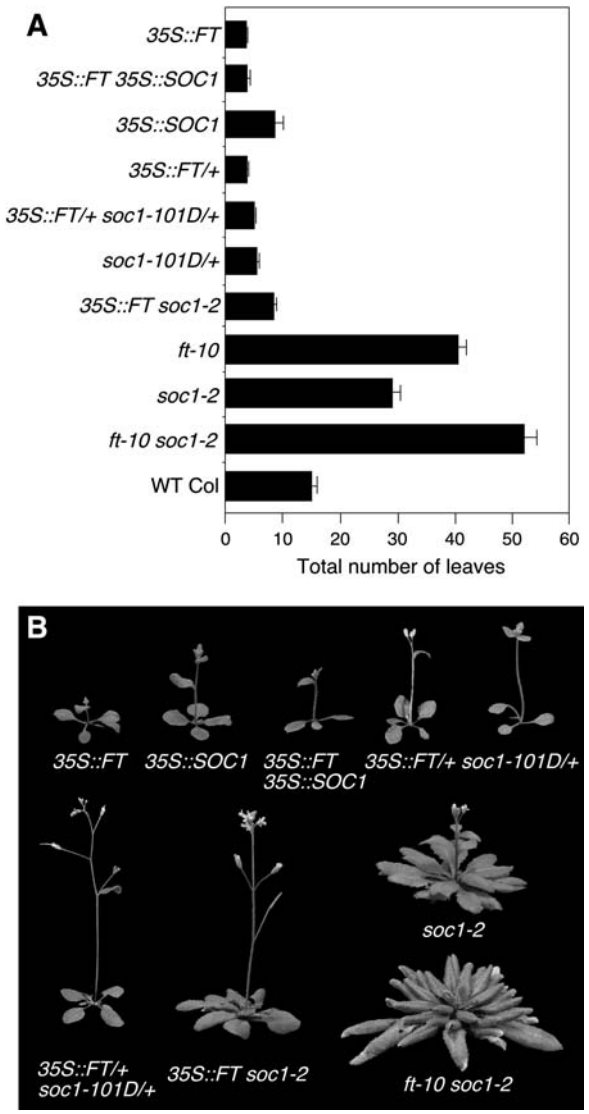

Figure 4. Genetic interactions of *FT* and *SOC1*. Flowering time (A) and phenotypes (B) of single overexpressors, double overexpressors, and double mutants under long-day conditions are shown. All plants were in the Col background. *soc1-101D* is an activation-tagged line of *SOC1* (Lee et al., 2000). The total number of primary leaves formed was scored in at least 10 plants to measure flowering time. The horizontal T-bars indicate the SD.

Inactivation of *FT* Causes Down-Regulation of *SOC1* in the Presence of *CO* Overexpression

Genetic interaction studies using *ft-10* together with expression analysis indicated that *FT*, which primarily mediates signaling inputs from *CO*, positively regulates *SOC1*. These results strongly suggest that *FT* acts downstream of *CO* and *SOC1* in turn acts downstream of *FT*. Therefore, to determine the signaling network downstream of *CO*, we monitored the expression level of *SOC1* in 6-, 8-, 10-, and 12-d-old *35S*::*CO*, *ft-10*, *35S*::*CO ft-10*, and wild-type plants. If *FT* mediates *SOC1* activation by *CO*, inactivation of *FT* would cause down-regulation of *SOC1* even in the presence of abundant *CO* mRNA.

Indeed, inactivation of *FT* caused down-regulation of *SOC1* in *35S*::*CO ft-10* plants. Although the *CO* mRNA level was greatly up-regulated in *35S*::*CO ft-10* plants, expression of *SOC1* was significantly down-regulated over the time tested (Fig. 5). The *SOC1* expression level in *35S*::*CO ft-10* plants was similar to that of wild-type plants. Considering that expression of *SOC1* was greatly up-regulated in *35S*::*CO* plants, these data suggest that loss of *FT* function altered expression of *SOC1* even in the presence of abundant *CO* mRNA, which is a strong activator of *SOC1* in wild-type plants. This result suggests that *FT* is required for *SOC1* activation by *CO* to promote flowering.

DISCUSSION

FT Is the Major Target of *CO* in the Photoperiod Pathway

FT is one of the early targets of *CO* because inactivation of *FT* delayed early flowering resulting from the constitutive expression of *CO* (Onouchi et al., 2000). However, since previously identified *ft* alleles only partially suppressed the early flowering phenotype of *35S*::*CO* plants, the existence of additional genes downstream of *CO* was inferred and led to the identification of *SOC1* (Samach et al., 2000). Indeed, *ft soc1* double mutants proposed an additive effect in suppressing the early flowering phenotype of *35S*::*CO* plants. Therefore, parallel flowering-time pathways downstream of *CO* have been proposed (Samach et al., 2000). However, our data suggest that *FT* is the major target of *CO*, as suggested by observation that *ft-10* was almost completely epistatic to *35S*::*CO* (Fig. 1).

This genetic result revisits the molecular data obtained by Samach et al. (2000) using a dexamethasone (DEX)-inducible *CO* construct. The authors showed that *FT* is rapidly up-regulated within 2 h of DEX treatment, whereas *SOC1* was up-regulated 4 h after DEX induction. According to this sequential induction pattern, it appeared that *FT* is an earlier acting gene in response to *CO* activation. However, since this sequential induction pattern is largely incompatible with the genetic analysis, in which preexisting *ft soc1* double mutants showed additive effects on flowering time (Onouchi et al., 2000), a model of parallel

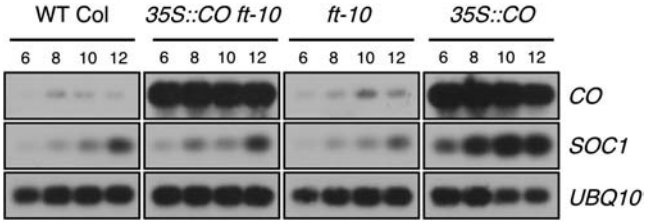

Figure 5. Expression of *SOC1* in 6-, 8-, 10-, and 12-d-old *35S*::*CO ft-10*, *ft-10*, *35S*::*CO*, and wild-type Col plants grown under long-day conditions. Semiquantitative RT-PCR was used to measure expression of *SOC1*. *UBQ10* and *CO* were used for internal positive controls.

pathways was proposed instead. In contrast, the almost complete suppression of the early flowering of *35S*::*CO* plants by *ft-10* (Fig. 1) is consistent with the sequential induction model. In addition, incomplete suppression by *soc1-2* also supports the interpretation that *FT* is the major target of *CO*. Our interpretation is also consistent with the previous finding that showed the genomic response of *co* and *ft* mutants using microarray analysis (Schmid et al., 2003). The authors showed that *co* and *ft* had very similar expression profiles and, therefore, suggested that *FT* is the major output of *CO* at the shoot apex. Our genetic interaction data using *ft-10* strongly supports this interpretation. Considering that *CO* is a B-type zinc-finger protein, it remains to be determined whether CO directly binds to *FT* or recruits additional components to promote flowering, as previously suggested (Hepworth et al., 2002).

An important question is why previously identified *ft* alleles, for example, *ft-1* and *ft-7*, did not suppress the early flowering phenotype of *35S*::*CO* plants. The partial suppression of the *CO* overexpressor phenotype by *ft-1* (Onouchi et al., 2000) can be explained by different levels of *FT* transcripts between *ft-1* and *ft-10*. Unlike *ft-10*, transcription of *FT* is largely normal in *ft-1* plants (Fig. 1); thus, a mutant form of FT protein is likely produced. This mutant *ft-1* protein appeared to be partially functional since *ft-1* showed a weaker phenotype than did *ft-10*. Moreover, the mutant *ft-1* protein is probably overproduced in *35S*::*CO ft-1* plants because *ft-1* mRNA was up-regulated by overexpression of *CO* (K.S. Chung and J.H. Ahn, unpublished data). The overproduced *ft-1* proteins in *35S*::*CO ft-1* plants may weakly promote flowering, for instance, by increasing expression of *SOC1*. The *ft-7* (=*ft-2*) mutant, in which a point mutation changes Trp-138 to a stop codon (TGA), most likely produced a truncated FT protein, which is predicted to show a stronger effect. Indeed, *ft-7* plants showed a later flowering phenotype than did *ft-1* plants, indicating that the *ft-7* mutation has a more severe effect. The difference between *ft-1* and *ft-7* is seemingly comparable to that between *ft-1* and *ft-10*, suggesting that *ft-7* may also be a null or almost null. However, *ft-7* partially suppressed the early flowering phenotype of *35S*::*CO* plants (Onouchi et al., 2000). There are two possibilities to explain the discrepancy. First, the truncated FT protein produced in *ft-7* plants is likely to be partially functional. This is inferred by the fact that the truncated FT protein contains a ligand-binding pocket that is important for *FT* function (Banfield and Brady, 2000; Hanzawa et al., 2005) and an important domain in the C terminus that determines the functional specificity of *FT* (J.H. Ahn and D. Weigel, unpublished data). Secondly, using different genetic backgrounds for *ft-7* may lead to discrepancies in phenotype. Previous genetic analysis with *35S*::*CO ft-7* plants used the ecotype Landsberg *erecta* background (Onouchi et al., 2000), whereas our genetic studies with *35S*::*CO ft-10* plants used the Col background. The functional *FLOWERING LOCUS C* (*FLC*), which encodes a MADS domain-containing transcription factor that acts as a floral repressor (Michaels and Amasino, 1999; Sheldon et al., 1999), presented in the Col ecotype may further suppress expression of *SOC1*, causing an additional delay in flowering time in *35S*::*CO ft-10* plants. Genetic analysis using an introgressed allele of *ft-7* in the Col background may provide a clue to explain this discrepancy.

Given that *FT* is the major mediator of floral inductive inputs from *CO*, an important question is whether two other genes induced in *35S*::*CO*:*GR* plants, *ACS10* and *AtP5CS2* (Samach et al., 2000), play a redundant role in flower development. Some evidence suggests that *ACS10* and *AtP5CS2* do not play a role in reproductive development. First, a null mutation of *ACS10* does not have a phenotype associated with flowering time, but the gravitropic curvature of hypocotyls, which is related to auxin distribution and ethylene synthesis, is increased in *ACS10* T-DNA mutants (Porter and Harrison, 2003). Second, expression of *ACS10* is not affected in *co-2* mutants (Schmid et al., 2003) or in other flowering-time mutants such as *leafy* (*lfy*) and *ft*. Third, *ACS10* is located between the EMB173 and FHA markers on chromosome 1, which have not been reported to contain flowering-time mutants. Rather, *ACS10* encodes an aminotransferase with broad specificity for Asp and aromatic amino acids (Bernier, 2004). Another gene activated in *35S*::*CO*:*GR* plants is *AtP5CS2*, but *AtP5CS2* does not appear to be involved in regulation of flowering time. To the best of our knowledge, no loss-of-function mutant of *AtP5CS2* has been reported. The loss-of-function phenotype of *AtP5CS2* may be inferred by transgenic plants expressing antisense *AtP5CS1* (Nanjo et al., 1999), since *AtP5CS1* and *AtP5CS2* share 85% sequence homology. Transgenic plants expressing antisense *AtP5CS1* exhibited severe defects in vegetative tissues, but flowering time was largely normal. Thus, it is likely that down-regulation of *AtP5CS2* does not have a flowering-time phenotype. Rather, *AtP5CS2* is likely to be involved in stress signaling and low-temperature regulation of gene expression (Gilmour et al., 2000; Fabro et al., 2004).

Nonetheless, why *ACS10* and *AtP5CS2* are induced in *35S*::*CO*:*GR* plants remains unanswered. One possibility is that the inducible version of *CO* does not precisely duplicate the normal role of *CO*. For instance, 35S promoter-driven expression of *SEP3*, a floral organ identity gene, caused early flowering (Honma and Goto, 2001), and overexpression of *CO* resulted in pleiotropic carpel defects (Jeong and Clark, 2005), suggesting that overexpressor phenotypes are not necessarily correlated with the wild-type function of the genes tested. An alternative explanation is that *CO* may play a role in vegetative growth. Microarray results showed that *CO* expression is also detected in vegetative tissues, and both spatial and developmental expression patterns of *ACS10* and *AtP5CS2* mRNA abundance largely overlap with *CO* during vegetative development (Schmid et al., 2003). Colocalization of

ACS10 and *AtP5CS2* with *CO* suggests that vegetative tissue-specific functions of *CO* may require *ACS10* and *AtP5CS2*.

Genetic Framework of Flowering-Time Pathways Downstream of *CO*

Expression analyses in this study showed that *FT* positively regulates expression of *SOC1* (Figs. 2 and 3). Our results are consistent with a previous result obtained from an experiment using an activation-tagged mutant of *FT* (Michaels et al., 2005). The authors showed that activation of *FT* or *TWIN SISTER OF FT*, which acts as a floral pathway integrator redundantly with *FT* (Yamaguchi et al., 2005), leads to up-regulation of *SOC1*, even in the presence of functional *FRIGIDA* and *FLC*. They further showed that *FT* activation causes increased *SOC1*::*GUS* expression throughout the plant, similar to our results. In this study, we additionally showed that *SOC1*::*GUS* expression is strongly induced in the shoot apex during vegetative phase by *FT* activation and, furthermore, that loss of *FT* function delays expression of *SOC1*. All these results suggest that *SOC1* expression is regulated by *FT* in the photoperiod pathway.

If *FT* positively regulates *SOC1*, then the question of how *CO* regulates expression of *SOC1* arises. Two possible scenarios have been suggested, based on the expression patterns of *FT* and *SOC1* (Michaels et al., 2005). *CO* may positively regulate *SOC1* through indirect mechanisms, such that *CO* promotes expression of *FT*, which in turn promotes expression of *SOC1*. Alternatively, *CO* could regulate *SOC1* by binding to the *SOC1* promoter as part of a regulatory complex, as suggested in previous binding assays (Hepworth et al., 2002). Our results support the indirect mechanism of *CO* function to activate *SOC1*, since *FT* is the major target of *CO* and *FT* is required for *SOC1* activation by *CO* (Fig. 5). However, it is still possible that genes other than *FT* contribute, albeit weakly, to mediate floral inductive inputs from *CO*, since *FT* is not completely epistatic to *CO* (Fig. 1).

Although we suggest that *FT* and *SOC1* do not act in parallel, our results with the *ft-10 soc1-2* double mutants (Fig. 4) are not consistent with a linear relationship since *ft-10 soc1-2* double mutants showed an additive delay. One reason for this discrepancy may be that the additive delay in flowering time is the result of repression of multiple pathways by the *soc1* mutation. Although *FT* most likely mediates flowering signals in the photoperiod pathway, *SOC1* is downstream of multiple pathways including the autonomous, GA, and vernalization pathways (Moon et al., 2003). These data suggest that inactivation of *SOC1* inhibits signaling in each of the floral promotion pathways (Mouradov et al., 2002). Thus, although *FT* is sufficient to induce expression of *SOC1*, suppression of signaling inputs from multiple pathways, due to inactivation of *SOC1*, may cause an additional delay under long-day conditions. An alternative explanation is that the synergistic delay simply resulted from greater suppression of *SOC1* expression in *ft-10* plants. Expression of *SOC1* is detectable, albeit down-regulated, in *ft-10* plants. However, expression of *SOC1* is further down-regulated in *ft-10 soc1-2* plants, due to a T-DNA insertion in *SOC1*. The discrepancy can be resolved by a genetic study using null alleles of both *FT* and *SOC1*. Nevertheless, we favor the first explanation since *SOC1* is involved in more floral promotion pathways than *FT* (Mouradov et al., 2002; Michaels et al., 2005).

The partial suppressions of the phenotype of *35S*::*FT* plants by *soc1-2* (Fig. 4) and the phenotype of *ft-1* plants by *35S*::*SOC1* (S.K. Yoo and J.H. Ahn, unpublished data) are not consistent with a simple linear-pathway model. The partial suppression suggests that the floral promotion signal from *FT* is not solely mediated by *SOC1*, but that *FT* activates several downstream mediators. One such candidate is *APETALA1* (*AP1*), which encodes a MADS box protein and functions both as a flowering-time gene and a floral meristem identity gene. *AP1* is an early target of *FT* since the *AP1* level is precociously elevated before *LFY* up-regulation in *FT* overexpressor plants (Kardailsky et al., 1999). Additionally, *ft lfy* double mutants have a phenotype associated with loss of function of *AP1* (Ruiz-Garcia et al., 1997). Thus, *AP1* may be required to promote flowering in response to *FT* activation. However, it is not clear why *ap1* loss-of-function mutants did not suppress early flowering of *FT* overexpressor plants (Kardailsky et al., 1999). One possibility is that *AP1* and *SOC1* have redundant roles. We are currently investigating whether *ap1-10 soc1-2* double mutants can completely block the accelerated flowering phenotype of *35S*::*FT* plants. Another candidate for a downstream mediator of *FT* is *FD*, a bZIP transcription factor expressed in the shoot apex (Abe et al., 2005;

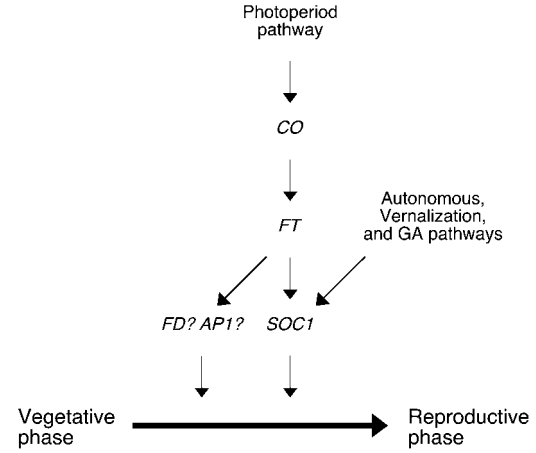

Figure 6. Model for genetic interactions of *CO*, *FT*, and *SOC1* in determination of flowering time in Arabidopsis. *FT* primarily mediates floral inductive inputs from *CO*. Although *FT* positively regulates *SOC1*, *FT* appears to have other downstream mediators in parallel with *SOC1*. Putative candidates for downstream mediators are *FD* and *AP1*.

Wigge et al., 2005). Some previous studies suggest that *FD* is downstream of *FT*. First, *fd* loss-of-function mutants strongly repressed the early flowering of *35S*::*FT* plants, although the loss-of-function phenotype on its own is very weak (Abe et al., 2005; Wigge et al., 2005). Secondly, the FT protein interacts with FD protein by two-hybrid analysis in yeast, as well as genetically (Abe et al., 2005; Wigge et al., 2005). These results indicate that floral promotion by *FT* requires the activity of *FD* and suggest that *FD* is downstream of *FT*. However, *FD* does not simply act downstream of *FT*; rather, *FT* and *FD* are interdependent, since overexpression of *FD* did not rescue the late-flowering phenotype of *ft* mutants (T. Araki, personal communication) and activation of *AP1* by *FD* correlates with the level of *FT* mRNA (P.A. Wigge, M.C. Kim, and D. Weigel, personal communication).

A model of the roles of *CO*, *FT*, and *SOC1* in the regulation of flowering is presented in Figure 6. It is likely that *FT* is the major downstream target of *CO* and *SOC1* is in turn activated downstream of *FT*. Although *FT* is sufficient to induce expression of *SOC1*, it appears that these proteins integrate different floral promotion pathways. For instance, *SOC1* is mainly regulated by the autonomous pathway through *FLC* (Moon et al., 2005), whereas *FT* is strongly dependent on the photoperiod pathway through *CO*. The effects of photoperiod and vernalization are thus additive and overlap on these floral integrators. *FT* most likely has additional downstream mediators other than *SOC1*. Possible candidates for downstream mediators of *FT* include *AP1* and *FD*.

The Spatial Regulation of *CO*, *FT*, and *SOC1*

The spatial pattern of flowering-time gene activities has recently been studied, since little is known about how a flowering stimulus is transmitted to the shoot apex. Classical grafting experiments showed that day length is perceived in leaves, which generate a systemic, graft-transmissible signal. Recently, *CO* was shown to act noncell autonomously in leaves to induce flowering (An et al., 2004), since phloem-specific expression of *CO* is sufficient to induce early flowering. *FT*, the major target of *CO*, is also expressed in vascular bundles of leaves. Interestingly, phloem-specific expression of *CO* causes increased *FT* expression, specifically in the phloem, suggesting that *CO* activates *FT* in a cell-autonomous manner in leaves (An et al., 2004). However, *SOC1* is expressed in the shoot apex and leaves (Lee et al., 2000; Samach et al., 2000). Thus, to activate *SOC1* in the shoot apex to induce flowering, the flowering stimulus generated by *FT* must be transmitted to the shoot apex. One possibility is that *FT* itself, either the *FT* RNA or the FT protein, physically moves to the apex to trigger flowering. FT protein movement is the more likely hypothesis, since microarray results have shown that *FT* RNA is not detected at the shoot apex (Schmid et al., 2003). The possibility of FT protein movement is further supported by the molecular size of the FT protein (23 kD), which is below the size exclusion limit of plasmodesmata (Imlau et al., 1999). The FT protein may thus freely move to the shoot apex. However, this FT protein movement should be precisely coordinated to induce flowering since the size exclusion limit can change during organ development (Imlau et al., 1999). Nevertheless, we cannot exclude the movement of *FT* RNA since *FT* RNA may be present at the shoot apex below the level of detection. An alternative explanation is that *FT* signaling requires an intermediate component, which generates a downstream mobile signal. A possible candidate for this component is a gene that is genetically downstream of *FT* but is not expressed in the shoot apex. However, it is still possible that our interpretation on the spatial regulation of *CO*, *FT*, and *SOC1* based on our expression studies can be misleading because we used the constitutive 35S promoter for overexpression. Taken together, the intercellular signaling processes to transmit a flowering stimulus are largely unknown and await further investigation.

MATERIALS AND METHODS

Plant Materials and Measurement of Flowering Time

Wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Col) was used to generate transgenic plants. Plants were grown in Sunshine Mix 5 (Sungro Horticulture) under long-day conditions (16/8-h photoperiod at 120 μmol m^{-2} s^{-1}) at 23°C. *SKI083* plants (Kardailsky et al., 1999), in which transcription of *FT* is increased by four copies of the 35S enhancers, are referred to as *35S*::*FT* plants in this study. *SK231* and *pJA1148* plants were generated using the 35S promoter-fused *SOC1* cDNA and *CO* cDNA constructs, respectively, which were kindly provided by Dr. George Coupland (Max Planck Institute, Germany). These *SK231* plants and *pJA1148* plants were referred to as *35S*::*SOC1* and *35S*::*CO* plants, respectively, in this study. The original *ft-1* allele, which is in the ecotype Landsberg *erecta* background, was introgressed seven times into the Col background to generate the *ft-1* (Col) plants used in this study. *FT*::*GUS*, *LFY*::*GUS*, and *SOC1*::*GUS* seeds were kind gifts from Dr. Goji Koto (Research Institute for Biological Science, Japan), Dr. Detlef Weigel (Max Planck Institute), and Dr. Ilha Lee (Seoul National University, Korea), respectively. *soc1-2* is a T-DNA insertional mutant of *SOC1* that was described previously (Lee et al., 2000). The flowering time of the plants used in this study was measured by scoring the total number of primary leaves of at least 10 plants grown under long-day conditions.

RT-PCR and PCR-Genotyping

Sequence information of the oligonucleotides used in this study is available in Supplemental Table I. The reverse transcriptase-mediated PCR procedure has been described previously (Kardailsky et al., 1999). Total RNA was isolated from whole seedlings using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA treated with DNaseI. The expression levels were normalized against *UBQ10*. The JH1061 and JH1063 primers were used to detect *FT* mRNA. The JH1145 and JH1146 primers were used to measure transcript levels of *SOC1*. The JH1015 and JH1016 primers were used to monitor the mRNA levels of *CO*. To genotype *soc1-2* plants, a new set of primers, JH2537, JH2538, and JH2539, was used. The JH2295, JH2296, and JH2297 primers were used to genotype *ft-10* plants.

Histochemical GUS Assay and Microscopy

GUS staining of whole seedlings and tissue sections were performed as described previously (Sessions et al., 2000). GUS-stained seedlings were photographed using a Nikon SMZ1000 dissection microscope. Stained primary inflorescences were fixed and embedded in paraffin. After deparaffinization,

sections of primary inflorescences were examined using a Nikon Optiphot-2 microscope.

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LITERATURE CITED

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309:** 1052–1056

An H, Roussot C, Suarez-Lopez P, Corbesier L, Vincent C, Pineiro M, Hepworth S, Mouradov A, Justin S, Turnbull C, et al (2004) CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131:** 3615–3626

Banfield MJ, Brady RL (2000) The structure of Antirrhinum centroradialis protein (CEN) suggests a role as a kinase regulator. J Mol Biol **297:** 1159–1170

Bernier G (2004) Increased ethylene production in long days does not arise from activation of the ACS10 gene. Flowering Newsletter **38:** 49–50

Fabro G, Kovacs I, Pavet V, Szabados L, Alvarez ME (2004) Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. Mol Plant Microbe Interact **17:** 343–350

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854–1865

Hanzawa Y, Money T, Bradley D (2005) A single amino acid converts a repressor to an activator of flowering. Proc Natl Acad Sci USA **102:** 7748–7753

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

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409:** 525–529

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11:** 309–322

Jeong S, Clark SE (2005) Photoperiod regulates flower meristem development in Arabidopsis thaliana. Genetics **169:** 907–915

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

Komeda Y (2004) Genetic regulation of time to flower in Arabidopsis thaliana. Annu Rev Plant Biol **55:** 521–535

Koornneef M, Alonso-Blanco C, Peeters AJ, Soppe W (1998) Genetic control of flowering time in Arabidopsis. Annu Rev Plant Physiol Plant Mol Biol **49:** 345–370

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

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

Michaels SD, Himelblau E, Kim SY, Schomburg FM, Amasino RM (2005) Integration of flowering signals in winter-annual Arabidopsis. Plant Physiol **137:** 149–156

Moon J, Lee H, Kim M, Lee I (2005) Analysis of flowering pathway integrators in Arabidopsis. Plant Cell Physiol **46:** 292–299

Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, Kim SG, Lee I (2003) The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J **35:** 613–623

Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: interacting pathways as a basis for diversity. Plant Cell (Suppl) **14:** S111–S130

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in Arabidopsis thaliana. FEBS Lett **461:** 205–210

Onouchi H, Igeno MI, Perilleux C, Graves K, Coupland G (2000) Mutagenesis of plants overexpressing CONSTANS demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell **12:** 885–900

Porter JE, Harrison M (2003) Role of ethylene biosynthesis in hypocotyl gravitropism. *In* 14th International Conference on Arabidopsis Research, June 20–24, 2003, Madison, WI

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

Redei GP (1975) Arabidopsis as a genetic tool. Annu Rev Genet **9:** 111–127

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An Arabidopsis thaliana T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol **53:** 247–259

Ruiz-Garcia L, Madueno F, Wilkinson M, Haughn G, Salinas J, Martinez-Zapater JM (1997) Different roles of flowering-time genes in the activation of floral initiation genes in Arabidopsis. Plant Cell **9:** 1921–1934

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

Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D, Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. Development **130:** 6001–6012

Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. Science **289:** 779–782

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

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

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309:** 1056–1059

Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T (2005) *TWIN SISTER OF FT* (*TSF*) acts as a floral pathway integrator redundantly with *FT*. Plant Cell Physiol **46:** 1175–1189

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in Arabidopsis. Nature **419:** 308–312