

Mutagenesis of Plants Overexpressing *CONSTANS* Demonstrates Novel Interactions among Arabidopsis Flowering-Time Genes

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CONSTANS (*CO*) promotes flowering of Arabidopsis in response to long photoperiods. Transgenic plants carrying *CO* fused with the cauliflower mosaic virus 35S promoter (35S::*CO*) flowered earlier than did the wild type and were almost completely insensitive to length of day. Genes required for *CO* to promote flowering were identified by screening for mutations that suppress the effect of 35S::*CO*. Four mutations were identified that partially suppressed the early-flowering phenotype caused by 35S::*CO*. One of these mutations, *suppressor of overexpression of CO 1* (*soc1*), defines a new locus, demonstrating that the mutagenesis approach is effective in identifying novel flowering-time mutations. The other three suppressor mutations are allelic with previously described mutations that cause late flowering. Two of them are alleles of *ft*, indicating that *FT* is required for *CO* to promote early flowering and most likely acts after *CO* in the hierarchy of flowering-time genes. The fourth suppressor mutation is an allele of *fwa*, and *fwa soc1* 35S::*CO* plants flowered at approximately the same time as *co* mutants, suggesting that a combination of *fwa* and *soc1* abolishes the promotion of flowering by *CO*. Besides delaying flowering, *fwa* acted synergistically with 35S::*CO* to repress floral development after bolting. The latter phenotype was not shown by any of the progenitors and was most probably caused by a reduction in the function of *LEAFY*. These genetic interactions suggest models for how *CO*, *FWA*, *FT*, and *SOC1* interact during the transition to flowering.

INTRODUCTION

Flowering time of Arabidopsis is influenced by both environmental conditions, which include length of day, temperature, light quality, and nutrient deprivation, and developmental factors associated with the age of the plant (Martinez-Zapater et al., 1994). This complexity is mediated by several genetic pathways that control the response to different environmental signals (Koorneef et al., 1998a; Levy and Dean, 1998). These pathways were originally demonstrated by analyzing the phenotypes of late-flowering mutants (Martinez-Zapater and Somerville, 1990; Koorneef et al., 1991). The mutations were classified on the basis of their effects on environmental responses, particularly those involving temperature and photoperiod, and their genetic interactions. These experiments led to the proposal that three general pathways promote flowering of Arabidopsis.

One of these pathways, called the long-day pathway, controls the promotion of flowering by day length. Arabidop-

sis flowers rapidly under long-day conditions consisting of 16 hr of light but flowers later during short days with 8 or 10 hr of light. The mutations that define the long-day pathway (*constans* [*co*], *gigantea* [*gl*], *lhy*, *ft*, *fwa*, and *fha*) delay flowering under long-day but not short-day conditions (Redei, 1962; Koorneef et al., 1991).

Mutants of the second class (e.g., *fca*, *fve*, *luminidependens* [*ld*], and *fy*) flower later than those of the wild type under both long and short days and respond strongly to vernalization—an extended exposure to low temperatures soon after germination (Martinez-Zapater and Somerville, 1990; Koorneef et al., 1991). This class of mutations is assigned to the autonomous flowering pathway, which is active under all day lengths. This pathway was recently shown to act, at least in part, by reducing the abundance of the mRNA encoded by the *FLOWERING LOCUS C* (*FLC*) gene (Michaels and Amasino, 1999; Sheldon et al., 1999).

Mutations of the third class reduce synthesis or response to the growth regulator gibberellin (GA). These have their most severe effect under short-day conditions, and mutations that have a strong effect on GA biosynthesis can prevent flowering in plants growing under short days (Wilson et al., 1992).

Further evidence that these phenotypic classes represent

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genetic pathways resulted from analyses of double mutants. Generally, double mutants containing two mutations assigned to one of these classes do not flower later than the more extreme parental mutant, whereas combining mutations from different classes causes a greater delay in flowering than does either single mutation (Koornneef et al., 1998b). Because the three pathways are partially redundant, inactivation of two pathways results in a more severe phenotype than inactivation of one.

Several of these flowering-time genes have now been cloned and generally encode regulatory proteins. *CO*, *LD*, *FLC*, *LHY*, and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* encode putative transcription factors (Lee et al., 1994; Putterill et al., 1995; Schaffer et al., 1998; Wang and Tobin, 1998; Michaels and Amasino, 1999; Sheldon et al., 1999), whereas *FCA* encodes a probable RNA binding protein (Macknight et al., 1997). The *FT* gene encodes a protein with similarity to phosphatidylethanolamine binding proteins (Kardailsky et al., 1999; Kobayashi et al., 1999) and is a homolog of *TERMINAL FLOWER 1* (Bradley et al., 1997; Oshima et al., 1997), whereas the *GI* gene encodes a putative membrane protein of unknown function (Fowler et al., 1999; Park et al., 1999).

The order in which genes act within a single flowering-time pathway has been difficult to assess genetically because each mutation causes a similar phenotype. The order of gene action was determined genetically in other plant signaling pathways, such as the ethylene signaling pathway in which repressor and promotive steps occur within the same pathway, and mutations affecting these distinct functions have opposite phenotypes (Roman et al., 1995). Some information on the order in which flowering-time genes act has come from analyzing the expression of cloned genes in mutant backgrounds. Such analyses have demonstrated, for example, that the autonomous pathway genes and genes for vernalization act upstream of *FLC* to repress its expression (Michaels and Amasino, 1999; Sheldon et al., 1999). Similarly, in the long-day pathway, *CO* expression is reduced in a *cryptochrome 2 (cry2)* mutant, and *FT* expression is reduced by *co* mutations, suggesting that these genes act in the order *CRY2-CO-FT* (Guo et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999).

The long-day, autonomous, and GA pathways must eventually converge to regulate the expression and function of the same floral meristem identity genes that control flower development (Simon et al., 1996; Blazquez et al., 1998; Nilsson et al., 1998; reviewed in Piñeiro and Coupland, 1998), and the effects of flowering-time genes on the expression or function of floral meristem identity genes have also been informative. Inducing the function of *CO* by using a steroid-inducible construct increased *LEAFY (LFY)* expression within 24 hr after steroids were applied (Simon et al., 1996). These results suggest that *LFY* acts downstream of *CO*.

The relationship between flowering time and floral meristem identity genes has also been examined by using trans-

genic plants in which the *LFY* gene was overexpressed from the cauliflower mosaic virus (CaMV) 35S promoter. Broadly, the effect of the 35S::*LFY* transgene on flowering time was not greatly affected by most flowering-time mutations—again suggesting that the flowering-time genes act to promote *LFY* transcription (Nilsson et al., 1998). However, *ft* and *fwa* were exceptions among the flowering-time mutations because they did delay flowering of 35S::*LFY* plants (Nilsson et al., 1998). These mutations may therefore act to influence the activity of LFY protein. Ruiz-Garcia et al. (1997) came to a similar conclusion by examining the effects of combining *ft* and *fwa* mutations with *lfy* and concluded that these flowering-time genes do not act to regulate *LFY* transcription but rather regulate transcription of another floral meristem identity gene, *APETALA1 (AP1)*. Overexpression of *FT*, however, did cause premature expression of *LFY* (Kardailsky et al., 1999), probably as an indirect effect of activation of *AP1* by *FT*.

Our interest is in the long-day pathway, and we have previously described the *CO* gene (Putterill et al., 1995). Transgenic plants containing increased copy numbers of *CO* flower earlier than the wild type under long- and short-day conditions, suggesting that the amount of *CO* expression in wild-type plants limits flowering time. This was confirmed by expressing the steroid-inducible *CO* fusion protein from the strong CaMV 35S promoter (Simon et al., 1996). In the presence of the steroid dexamethasone, overexpression of the fusion protein was sufficient to promote very early flowering under both long and short days. Transcriptional regulation of *CO* is therefore an important determinant of the photoperiodic regulation of flowering time. Here, we describe transgenic plants expressing the wild-type *CO* protein from the CaMV 35S promoter. The transgene causes early flowering and therefore provides a phenotype opposite to that of the *co* loss-of-function mutations. Suppressor mutagenesis of these plants was used to identify genes that are required for *CO* function. This approach identified mutations in the previously identified flowering-time genes *ft* and *fwa* as well as in a previously unidentified gene that we designate *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)*. The roles of these genes in the control of flowering time are discussed.

RESULTS

Effects of Expression of the *CO* Gene from the CaMV 35S Promoter

The CaMV 35S promoter was fused to genomic DNA encoding the *CO* open reading frame (see Methods). The gene fusion (35S::*CO*) was introduced into a Landsberg *erecta* (*Ler*) line homozygous for the *co-2* mutation. Plants homozygous for the transgene at a single locus were identified from among the progeny of five independent transformants. RNA was extracted from all five lines, and gel blots were made

and hybridized with a *CO* probe. As expected from previous experiments, no hybridization was detected in wild-type plants because of the very low expression of *CO* (Putterill et al., 1995). However, mRNA of the expected size was recognized in all five transformants (Figures 1A and 1B), indicating that transcription of the gene was considerably increased in all of the transgenic lines. The phenotypes of these five lines were then studied in detail.

All five lines flowered earlier than did wild-type plants under both long- and short-day conditions (Figure 2 and Table 1). Earlier flowering was apparent both in the time between sowing until floral buds were visible in the center of the rosette and in the fewer leaves formed by the shoot meristem before the initiation of flower development. Overexpression of *CO* in these lines, therefore, causes early flowering under long- and short-day conditions, although the effect is most dramatic under short-day conditions (Table 1).

The 35S::CO plants were almost insensitive to the length of day, and their total leaf number was the same under both long- and short-day conditions (Table 1). In contrast, wild-type plants flowered ~30 days earlier under long-day conditions than under short days (Table 1). Occasionally, 35S::CO plants grown during short days formed more rosette leaves and fewer cauline leaves than did long-day-grown plants, suggesting that in these individuals, day length affected the node at which internode elongation first occurred.

In wild-type plants, the distribution of trichomes alters around the time of the transition to flowering (Chien and Sussex, 1996; Telfer et al., 1997); therefore, we tested

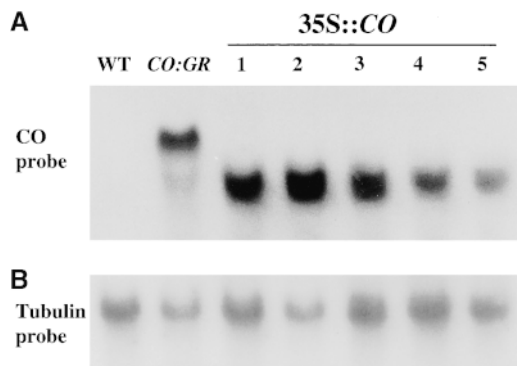


Figure 1. Detection of *CO* mRNA in Wild-Type and Transgenic Plants.

(A) Total RNA extracted from wild-type Ler (WT) and transgenic plants carrying 35S::CO:GR (*CO:GR*) or five transformants carrying 35S::CO (lanes 1 to 5) was transferred to a filter and hybridized with the *CO* cDNA.
 (B) As a control for loading, the filter was stripped and hybridized with a fragment derived from the tubulin gene (see Methods).

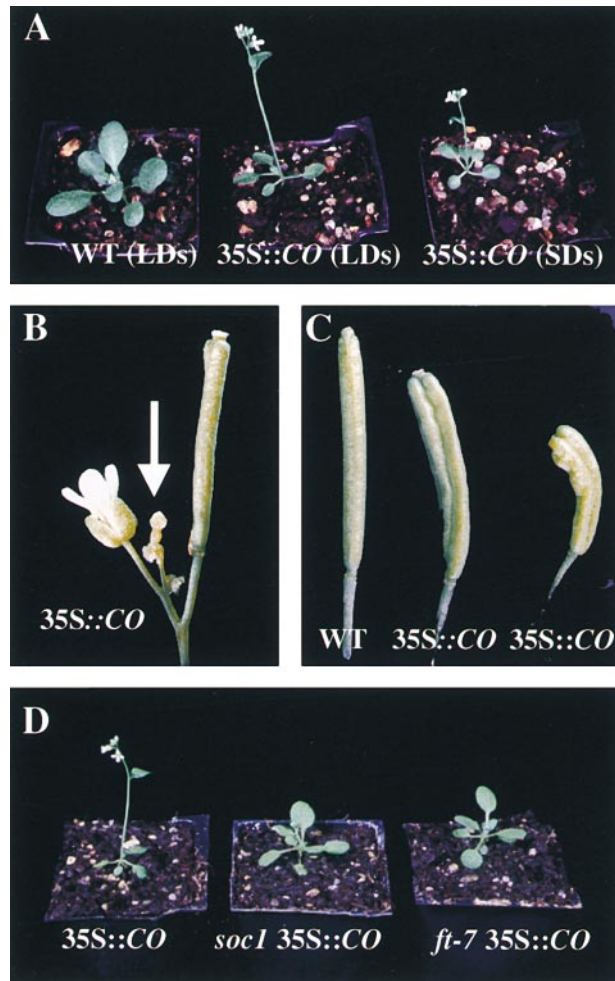


Figure 2. Phenotype of 35S::CO and 35S::CO Plants Carrying Suppressor Mutations.

(A) A 3-week-old wild-type (WT) plant grown under long days (LDs) (left), a 35S::CO plant grown under long days (center), and a 35S::CO plant grown under short days (SDs) (right).
 (B) The inflorescence apex and the pistil-like structure formed at the apex of the shoot of a 35S::CO plant (arrow).
 (C) Siliques of wild-type (left) and 35S::CO (center and right) plants.
 (D) A 3-week-old 35S::CO plant (left), a *soc1* 35S::CO plant (center), and a *ft-7* 35S::CO plant (originally designated *suppressor mutant 1*) (right) grown under long-day conditions.

whether 35S::CO caused trichome distribution to change earlier than it did in wild-type plants. The rosette leaves that form early during the development of wild-type plants form numerous trichomes on their upper (adaxial) sides and no trichomes on their lower (abaxial) sides, whereas leaves that develop later form fewer adaxial trichomes and many abaxial trichomes. This effect is influenced by day length, because

Table 1. Effect of the Suppressor Mutations and *fca-1* on Flowering Time in the Presence of 35S::CO

Genotype	No. of Rosette Leaves	No. of Cauline Leaves	Total No. of Leaves	No. of Days to Flowering
<i>ft-7</i> 35S::CO ^a				
Long day	5.7 ± 0.6	3.1 ± 0.8	8.8 ± 0.8	16.5 ± 0.8
Short day	8.9 ± 0.9	3.2 ± 0.5	12.1 ± 1.1	22.0 ± 1.3
<i>fwa-100</i> 35S::CO ^b				
Long day	7.6 ± 0.5	7.2 ± 0.9	14.8 ± 1.0	ND ^c
Short day	8.8 ± 1.1	5.9 ± 1.1	14.7 ± 1.7	ND
<i>soc1</i> 35S::CO ^d				
Long day	4.4 ± 0.6	2.5 ± 0.5	6.9 ± 0.6	15.4 ± 1.5
Short day	5.0 ± 0.4	1.5 ± 0.5	6.5 ± 0.8	18.0 ± 0.7
<i>ft-8</i> 35S::CO ^e				
Long day	6.3 ± 0.6	3.2 ± 0.6	9.6 ± 0.8	ND
Short day	10.4 ± 0.7	2.9 ± 0.4	13.3 ± 0.7	ND
<i>fca-1</i> 35S::CO				
Long day	4.8 ± 0.4	2.1 ± 0.4	6.8 ± 0.5	ND
<i>ft-8 fca-1</i> 35S::CO ^e				
Long day	15.4 ± 1.2	5.7 ± 0.7	21.1 ± 1.3	ND
<i>ft-1</i> 35S::CO				
Long day	4.1 ± 0.1	2.5 ± 0.3	6.6 ± 0.3	ND
Short day	7.1 ± 0.2	3.2 ± 0.3	10.2 ± 0.5	ND
35S::CO				
Long day	2.5 ± 0.5	2.0 ± 0.4	4.5 ± 0.8	13.5 ± 0.9
Short day	2.4 ± 0.5	2.0 ± 0.6	4.5 ± 0.5	15.0 ± 1.2
<i>Ler</i>				
Long day	5.5 ± 0.5	3.0 ± 0.4	8.5 ± 0.7	17.0 ± 0.7
Short day	31.4 ± 1.8	12.2 ± 1.0	43.6 ± 2.0	46.7 ± 1.6

^a *ft-7* was originally designated suppressor mutation 1.

^b *fwa-100* was originally designated suppressor mutation 2.

^c ND, not determined.

^d *soc1* was originally designated suppressor mutation 3.

^e *ft-8* was originally designated suppressor mutation 4.

abaxial trichomes are formed later in shoot development under short-day conditions (Chien and Sussex, 1996; Telfer et al., 1997). Wild-type plants growing under our long-day conditions usually produced their first abaxial trichomes on leaf 5, which was often the first cauline leaf, although ~20% of the plants formed a few abaxial trichomes on leaf 4. After their initial appearance, abaxial trichomes were always more frequent on successive leaves. Abaxial trichomes appeared earlier on 35S::CO plants growing under the same conditions: they were present on leaf 3 of ~60% of the plants and on leaf 4 of all plants. Under short-day conditions, 35S::CO plants always had abaxial trichomes on leaf 4, but they were present on the third leaf of only ~20% of plants. Abaxial trichomes, therefore, appear earlier on 35S::CO plants than on the wild type, and their distribution is slightly influenced by day length.

35S::CO plants also showed characteristic modifications in flower and shoot morphology. The transformants formed fewer flowers than did the wild type. For example, under long-day conditions, 35S::CO plants formed 11 ± 1.5 flowers compared with 29.3 ± 1.6 for *Ler*. Also, the shoot of 35S::CO plants terminated in the formation of a pistil-like structure (Figure 2). The shoot of 35S::CO plants is therefore

determinate, in contrast to the indeterminate shoot of wild-type plants. The flowers of 35S::CO plants also show alterations in morphology, particularly to the gynoecium, which often comprises three or four carpels rather than the two that are present in wild-type flowers (Figure 2).

Isolation of Mutations That Suppress the Extreme Early-Flowering Phenotype of 35S::CO Plants

To identify mutations that suppress the effect of overexpression of *CO*, we performed two screens for mutations that delay the extreme early-flowering phenotype of 35S::CO plants. In the first experiment, seeds carrying 35S::CO were mutagenized with ethyl methanesulfonate (EMS), and individuals that flowered later than the 35S::CO progenitor were identified in an M₂ population of ~20,000 plants grown under short-day conditions (see Methods). Five M₂ plants that flowered substantially later than 35S::CO were identified. Three of these mutations were reliably transmitted to subsequent generations and were designated suppressor mutations 1 to 3.

In the second screen, *fca-1* 35S::CO seeds were mutagenized with EMS. The flowering-time gene *FCA* acts in the autonomous flowering-time pathway (Koornneef et al., 1998b; Levy and Dean, 1998), which is distinct from the long-day pathway in which *CO* acts. The *fca-1* mutation strongly delays flowering under long-day conditions, whereas 35S::CO almost entirely overcomes this effect, although *fca-1* 35S::CO plants flower slightly later than do 35S::CO plants (Table 1). To reduce the likelihood of isolating mutations, such as *fca-1*, that affect the autonomous flowering-time pathway and therefore to focus the screen on genes more closely related to *CO*, *fca-1* 35S::CO plants in which this pathway was already inactivated were used for the mutagenesis. An M₂ population of ~5000 plants, generated by EMS mutagenesis, was screened under long-day conditions, and one plant flowering later than the *fca-1* 35S::CO progenitor was identified. The mutation in this plant was designated suppressor mutation 4.

Genetic Characterization of the Suppressor Mutations

All four suppressor mutations in the 35S::CO background were crossed to wild-type Ler, and F₂ populations were generated. Individuals that flowered as early as 35S::CO were present in each F₂ population, indicating that all of the suppressor mutations are extragenic suppressors of 35S::CO and none is closely linked to 35S::CO. Furthermore, in these

populations, plants that carried each of the suppressor mutations but lacked 35S::CO were identified. All of the suppressor mutations caused a late-flowering phenotype under long days in the absence of 35S::CO (Table 2).

Suppressor Mutations 1 and 4 Are Alleles of *ft*, and Suppressor Mutation 2 Is an Allele of *fwa*

Suppressor mutations 1 and 4 were mapped between the simple sequence length polymorphism markers nga280 and nga111 on chromosome 1 (see Methods), in the vicinity of the *ft* locus. To test whether these mutations are alleles of *ft*, we crossed them to *ft-1* (Koornneef et al., 1991), which causes a less severe delay in flowering time than do both suppressor mutations. The F₁ progeny of a cross between suppressor 1 or 4 and *ft-1* flowered later than did wild-type plants and *ft-1* mutants but earlier than did the parental suppressor mutant. The late-flowering phenotype of the F₁ plants suggested that both suppressor mutations 1 and 4 are alleles of *ft*. *FT* was cloned recently (Kardailsky et al., 1999; Kobayashi et al., 1999). The *FT* gene from suppressor mutants 1 and 4 was therefore sequenced, and in both cases, mutations were identified. Suppressor mutation 1 is predicted to introduce a premature stop codon that results in a 137-amino acid protein rather than the 175-amino acid wild-type protein (see Methods). The truncated 137-amino acid protein is identical to that predicted to be formed from the *ft-2* mutant allele

Table 2. Effect of the Suppressor Mutations and *fca-1* on Flowering Time in Wild-Type Background

Genotype	No. of Rosette Leaves	No. of Cauline Leaves	Total No. of Leaves	No. of Days to Flowering
<i>ft-7</i> ^a				
Long day	16.4 ± 1.0	7.1 ± 0.7	23.5 ± 1.6	31.5 ± 0.9
Short day	31.7 ± 1.8	12.8 ± 1.0	44.5 ± 1.7	54.6 ± 3.1
<i>fwa-100</i> ^b				
Long day	16.5 ± 0.7	11.6 ± 0.8	28.1 ± 1.1	29.9 ± 1.4
Short day	36.5 ± 2.4	18.8 ± 1.4	55.3 ± 2.5	53.7 ± 2.2
<i>soc1</i> ^c				
Long day	9.2 ± 0.4	3.5 ± 0.5	12.7 ± 0.6	19.1 ± 0.5
Short day	38.5 ± 2.9	15.3 ± 1.6	53.9 ± 2.7	56.3 ± 1.6
<i>ft-8</i> ^d				
Long day	15.6 ± 0.8	7.5 ± 0.7	23.1 ± 1.0	ND ^e
<i>ft-1</i>				
Long day	9.8 ± 0.5	4.1 ± 0.5	13.8 ± 0.4	ND
Short day	32.1 ± 1.6	15.4 ± 0.7	47.4 ± 1.8	51.3 ± 1.9
<i>fca-1</i>				
Long day	21.2 ± 2.7	6.8 ± 0.9	28.0 ± 3.4	ND
Ler				
Long day	5.5 ± 0.5	3.0 ± 0.4	8.5 ± 0.7	17.0 ± 0.7
Short day	31.4 ± 1.8	12.2 ± 1.0	43.6 ± 2.0	46.7 ± 1.6

^a *ft-7* was originally designated suppressor mutation 1.

^b *fwa-100* was originally designated suppressor mutation 2.

^c *soc1* was originally designated suppressor mutation 3.

^d *ft-8* was originally designated suppressor mutation 4.

^e ND, not determined.

(Kardailsky et al., 1999; Kobayashi et al., 1999). In suppressor mutant 4, a mutation was found in the 3' splice acceptor site of the third intron of the *FT* gene (see Methods). Suppressor mutations 1 and 4 are therefore alleles of *ft* and are referred to as *ft-7* and *ft-8*, respectively.

Suppressor mutation 2 showed genetic linkage to the cleaved amplified polymorphic sequence marker g8300 on chromosome 4. The marker mapped at ~17.4 centimorgans (cM) from suppressor mutation 2 (see Methods). The *fwa* mutation, which causes late flowering and, like suppressor mutation 2, is semidominant (see below), is also located on chromosome 4 around the position of suppressor mutation 2. To examine whether suppressor mutation 2 is an allele of *fwa*, we performed segregation analysis. All 694 F₂ progeny from a cross between suppressor mutant 2 and *fwa-1* showed late flowering, indicating strong linkage between them. These observations, together with the effects of *fwa* on floral meristem identity described below, indicate that suppressor mutant 2 is an allele of *fwa*, and we refer to it as *fwa-100*.

Demonstration That Suppressor Mutation 3 Defines a Novel Flowering-Time Gene

Suppressor mutation 3 is recessive and is located in a region ~16 cM from simple sequence length polymorphism marker nga168 on chromosome 2 (see Methods). Further mapping demonstrated that the mutation cosegregated with marker 40E1T7, ~10 cM from marker AthBio2 (see Methods). The *fpa* mutation, which causes late flowering, is the only flowering-time locus previously shown to be located in this region. In an allelism test between suppressor mutation 3 and *fpa-2*, the F₁ progeny flowered at a time similar to that of the wild type, demonstrating that suppressor mutation 3 is not an allele of *fpa*. These experiments demonstrate that suppressor mutation 3 defines a novel flowering-time gene, which we designated *SOC1*.

The *soc1* mutation caused 35S::CO plants to produce an extra two or three leaves under both long and short days (Table 1). The *soc1* 35S::CO mutants were insensitive to day length, flowering at the same time under long and short days; therefore, the *SOC1* gene seems to be required equally under both conditions for the phenotype of the 35S::CO plants. In the absence of 35S::CO, the *soc1* mutation also delayed flowering under both long and short days (Table 2).

The Delay in Flowering of 35S::CO Caused by *ft* and Its Enhancement by *fca-1*

Unlike the 35S::CO progenitor, the *ft-7* 35S::CO plants responded to length of day, flowering later under short-day conditions. This suggests that the *FT* gene is more strongly required for 35S::CO function under short days. In the absence of 35S::CO, however, the *ft-7* and *ft-1* mutations had

no effect or caused only a slight increase in leaf number under short-day conditions. This confirms previous observations indicating that *FT* is required only weakly for flowering under short-day conditions (Koornneef et al., 1991, 1998b).

The *fca-1* mutation strongly enhances the effect of *ft-8*. For example, *fca-1 ft-8* 35S::CO produced ~21 leaves, compared with 10 for *ft-8* 35S::CO and seven for *fca-1* 35S::CO (Table 1). Similarly, in greenhouse-grown plants, *ft-8 fca-1* produced 53 leaves compared with ~30 for both *ft-8* and *fca-1*. These experiments suggest that *fca-1* and *ft-8* have additive effects on flowering time, which is consistent with their acting in different flowering-time pathways.

The Delay in Flowering of 35S::CO Caused by *fwa*

The *fwa-100* 35S::CO mutants formed ~10 more leaves than did 35S::CO plants (Table 1). However, approximately half of the extra leaves were cauline leaves, and given that *fwa-100* 35S::CO also shows severe defects in floral development, the increase in cauline leaf number may reflect in part a loss of floral identity rather than a delay in the transition to reproductive development. Nevertheless, the increase in rosette leaf number caused by *fwa-100* in a 35S::CO background under long days is more than that caused by *soc1* or *ft* mutations, suggesting that *fwa* has the most severe effect on flowering time of 35S::CO under these conditions (Table 1).

The Combination of *soc1* and *fwa-1* Strongly Suppresses the Promotion of Flowering by 35S::CO

None of the suppressor mutations completely suppressed the early-flowering phenotype of 35S::CO plants. This may reflect genetic redundancy between the genes that act after CO in the long-day pathway. The effects of combining *ft-1* and *fwa-1* on flowering were tested previously, and the double mutants flowered at a time similar to the single mutants, suggesting that *FT* and *FWA* act in the same pathway (Koornneef et al., 1998a). To address whether *SOC1* acts in the same pathway as *FT* and *FWA* or in a different pathway, the flowering time of an F₂ population made from a cross between *soc1* 35S::CO and *fwa-1/+* 35S::CO was examined. Eight of 138 F₂ plants produced markedly more rosette leaves than did *fwa-1* 35S::CO or *soc1* 35S::CO (Figure 3). These plants showed severe floral meristem identity defects, as seen in *fwa/fwa* 35S::CO plants (see below), but their rosette leaf number was twice the average leaf number of *fwa-1/fwa-1* 35S::CO plants and similar to that of *co-2* mutants (Figure 3). This is a greater difference than was expected from a simply additive interaction between *soc1* and *fwa-1* and suggests that they affect separate flowering pathways downstream of CO.

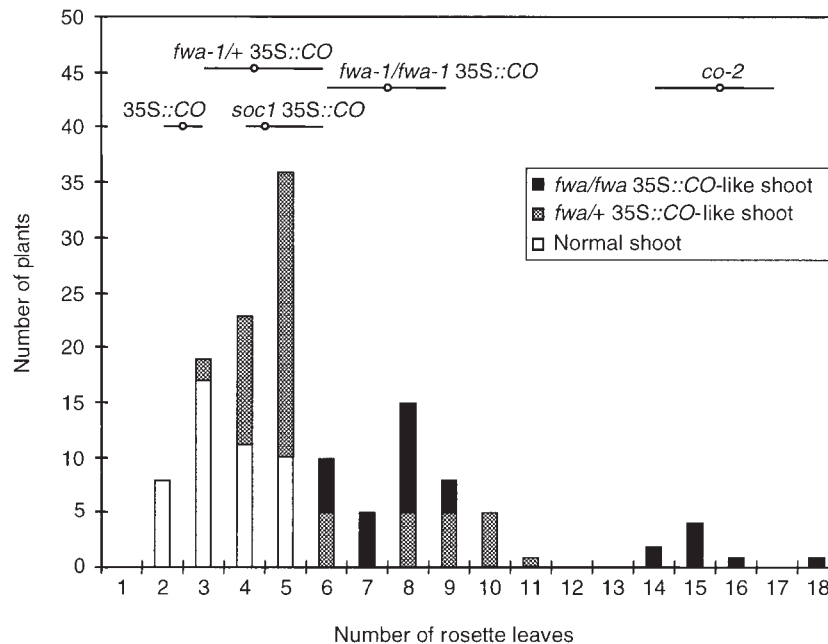


Figure 3. Segregation of the Late-Flowering Phenotype in the F₂ Population of a Cross between *soc1* 35S::CO and *fwa-1/+* 35S::CO Plants.

Plants were grown under long-day conditions. Plants showing severe (*fwa-1/fwa-1* 35S::CO-like) and weak (*fwa-1/+* 35S::CO-like) floral meristem identity defect segregated in the population and are indicated by the black and cross-hatched bars, respectively. Plants showing a normal 35S::CO-like shoot are indicated by the open bars. The open circles and horizontal lines indicate the average and the range, respectively, of rosette leaf numbers of defined genotypes grown under the same conditions. The plants flowering with between 14 and 18 rosette leaves are presumed to have the genotype *soc1/soc1 fwa-1/fwa-1* 35S::CO and represent approximately one in 17 of the total population.

Analysis of the Floral Meristem Identity Phenotype of *fwa* 35S::CO

The *fwa-100* mutation is semidominant, and *fwa-100* 35S::CO plants showed a defect in floral meristem identity (see below) in addition to later flowering. The progeny derived from self-pollination of plants heterozygous for *fwa-100* and homozygous for 35S::CO segregated in a ratio of ~1:2:1; that is, 24.7% showed the normal 35S::CO phenotype and had not inherited *fwa-100*; 51.3% showed a phenotype similar to that of the parental plants; and 24.0% showed an extreme loss of floral meristem identity and flowered later than did the parents. The flower and shoot morphology phenotype and the late-flowering phenotype cosegregated, suggesting that both result from a single mutation. The *fwa-100* mutation is therefore semidominant, and in the presence of 35S::CO it affects flowering time and floral meristem identity.

The floral meristem identity phenotype observed in *fwa-100* 35S::CO plants was not present in *fwa-100* single mutants, suggesting that it occurs only in the presence of 35S::CO. To confirm this notion, the *fwa-100* mutant was backcrossed to 35S::CO plants; in the F₁ progeny, the floral meristem identity phenotype reappeared. Therefore, this

phenotype is present only in plants that carry both *fwa-100* and 35S::CO.

To test whether *fwa-1* caused the same floral meristem identity phenotype in the presence of 35S::CO that was observed in *fwa-100* 35S::CO plants, we crossed *fwa-1* to 35S::CO plants. The *fwa-1* 35S::CO plants produced in the F₁ and F₂ generations of this cross showed flowering-time and floral meristem identity phenotypes similar to those observed for *fwa-100* 35S::CO (Table 3).

Under long days, *fwa-100* 35S::CO double homozygotes never formed floral organs, except for carpel-like structures observed at the apex of some lateral shoots after 2 months of growth. They produced more lateral shoots subtended by cauline leaves than did wild-type plants and then formed >30 lateral shoots that were not subtended by cauline leaves (Table 3) until shoot growth terminated in a cluster of leaves (Figure 4). Under short days, these plants produced flowers at the apex of some lateral shoots, and these flowers contained carpels, stamens, petals, and leaflike sepals (Figure 4). They also formed fewer cauline leaves than they did under long-day conditions (Table 1). These observations suggest that the floral meristem identity defect of *fwa-100* 35S::CO plants is influenced by day length and is weaker under short-day conditions.

Table 3. Effects of *fwa* on Vegetative and Floral Organ Number in a 35S::*CO* Background

Genotype	Rosette Leaf	Cauline Leaf	Secondary Shoot	Whorl 1		Whorl 1 or 2 Sepaloid/Petaloid Organ ^a		Whorl 2 Petal ^a	Secondary Flower ^a	Whorl 3 Stamen ^a	Whorl 4 Carpel ^a
				Leaflike Sepal ^a	Sepal ^a	Sepaloid	Petaloid				
<i>Ler</i>	5.5 ± 0.5	3.0 ± 0.4	3.0 ± 0.4	0 ± 0	4.0 ± 0	0 ± 0	4.0 ± 0	0 ± 0	5.8 ± 0.4	2.0 ± 0	
35S:: <i>CO co-2</i>	2.5 ± 0.5	2.0 ± 0.4	2.0 ± 0.4	0 ± 0	4.0 ± 0	0 ± 0	4.0 ± 0	0 ± 0	6.1 ± 0.4	2.6 ± 0.4	
<i>fwa-100</i>	16.5 ± 0.7	12.5 ± 0.9	12.7 ± 1.0	0 ± 0	4.9 ± 0.8	0.8 ± 0.9	2.6 ± 1.0	0 ± 0.1	6.0 ± 0.2	2.0 ± 0	
<i>fwa-1</i>	12.9 ± 0.7	8.9 ± 0.8	9.0 ± 0.7	0 ± 0	4.3 ± 0.6	0 ± 0.2	4.0 ± 0.4	0 ± 0	6.0 ± 0.1	2.0 ± 0.1	
<i>fwa-100/+</i> 35S:: <i>CO co-2</i>	4.2 ± 0.5	3.1 ± 0.7	6.4 ± 2.3	1.3 ± 1.6	2.4 ± 1.7	0.4 ± 0.9	2.8 ± 1.7	0.4 ± 0.8	5.0 ± 0.9	2.0 ± 0	
<i>fwa-1/+</i> 35S:: <i>CO co-2</i>	3.8 ± 0.7	3.2 ± 0.7	6.6 ± 1.7	1.1 ± 1.3	2.2 ± 1.6	0.5 ± 0.9	2.9 ± 1.5	0.3 ± 0.6	6.4 ± 1.3	2.2 ± 0.4	
<i>fwa-1/+</i> 35S:: <i>CO/+</i> <i>co-2/+</i>	5.0 ± 0.6	3.1 ± 1.0	4.8 ± 1.7	1.1 ± 1.4	2.8 ± 1.6	0.4 ± 0.9	3.5 ± 1.2	0.2 ± 0.5	5.8 ± 0.4	2.0 ± 0.2	
<i>fwa-1/+</i> 35S:: <i>CO/+</i> 35S:: <i>AP1/+</i> <i>co-2/+</i>	4.1 ± 0.7 ^b	3.4 ± 1.3 ^b	4.2 ± 1.4 ^b	1.2 ± 1.5	2.3 ± 1.5	0.4 ± 0.9	3.3 ± 1.3	0.1 ± 0.3	5.7 ± 0.6	2.0 ± 0	
<i>fwa-100/fwa-100</i> 35S:: <i>CO co-2</i>	7.6 ± 0.5	7.2 ± 0.9	>30 ^c	ND ^d	ND	ND	ND	ND	ND	ND	
<i>fwa-1/fwa-1</i> 35S:: <i>CO co-2</i>	6.7 ± 0.5 ^e	6.1 ± 0.6 ^e	>30 ^{c,e}	ND	ND	ND	ND	ND	ND	ND	

In each case, >15 plants were analyzed, except where indicated.

^aOnly the first to 10th flowers produced by the main shoot were scored in each genotype.

^bSeven plants were analyzed.

^cExact number was difficult to determine because the main shoot of these plants produced secondary shoots indefinitely.

^dND, not determined. Floral organ numbers for these plants were not scored because the main shoot never produced flowers.

^eTen plants were analyzed.

Plants heterozygous for *fwa-100* and homozygous for 35S::*CO* exhibited weaker floral meristem identity defects than did the double homozygotes. After production of several coflorescences, the main stems formed flowers that often had inflorescence-like characters, such as leaflike sepals, leaflike or sepaloid petals, and secondary flowers that formed within flowers (Table 3). Although flower phenotypes were highly variable, the first and second whorls seemed to be more affected than the third and fourth whorls (Table 3). These plants also often formed coflorescences on the main inflorescence after nodes at which solitary flowers had formed.

In *fwa-100* single mutants, severe effects on floral structure were observed very rarely (Table 3), although chimeric organs containing both sepal and petal tissue were often observed and occasionally secondary flowers were formed within flowers. 35S::*CO* plants never showed such floral meristem identity phenotypes (Table 3).

The Floral Meristem Identity Phenotype of *fwa-1* 35S::*CO* Plants Can Be Corrected by 35S::*LFY* but Not by 35S::*AP1*

The *lfy fwa* double mutant exhibits an extreme floral meristem identity defect, suggesting that *fwa* controls floral

meristem identity genes redundantly with *LFY* (Ruiz-Garcia et al., 1997). This enhancement is similar to that observed when *fwa* is combined with 35S::*CO*. However, this phenotypic similarity cannot be readily explained, because 35S::*CO* is expected to activate *LFY* expression (Simon et al. 1996; Nilsson et al., 1998) and therefore would not be expected to have the same effect on *fwa* as an *lfy* mutation would.

To investigate the cause of the floral meristem identity defect of *fwa* 35S::*CO*, we tested whether overexpression of the floral meristem identity genes *LFY* and *AP1* could suppress the phenotype of *fwa* 35S::*CO*. Plants homozygous for 35S::*LFY* were crossed to *fwa-1/+* 35S::*CO* plants. All of the 40 F₁ progeny showed the characteristic 35S::*LFY*-like shoot phenotype and formed a terminal flower (Figure 5). Half of the F₁ progeny were *fwa-1/+* 35S::*CO/+* 35S::*LFY/+*, but they did not show the floral meristem identity phenotype described for *fwa-1/+* 35S::*CO/+* plants. Therefore, overexpression of *LFY* overcomes the floral meristem identity defect of *fwa-1* 35S::*CO* plants. However, 35S::*LFY* did not affect the delay in flowering time of 35S::*CO* caused by *fwa-1*, which is consistent with previous reports that *fwa* substantially delayed flowering of 35S::*LFY* plants (Nilsson et al., 1998).

In contrast, when plants heterozygous for 35S::*AP1* were crossed to a *fwa-1/+* 35S::*CO*, 18 of 38 F₁ progeny showed

a shoot and flower phenotype similar to that of *fwa-1/+ 35S::CO* plants (Figure 5). The presence of 35S::AP1 in seven of these plants was confirmed by polymerase chain reaction (PCR); despite the presence of 35S::AP1, those plants were indistinguishable from *fwa-1/+ 35S::CO/+* plants (Table 3). Therefore, unlike 35S::LFY, the 35S::AP1 transgene did not suppress the floral meristem identity defect of *fwa-1 35S::CO* plants.

In an *fwa* Background, the Effect of 35S::CO on Floral Meristem Identity Can Be Separated from Its Effect on Flowering Time

The floral meristem identity defect shown by 35S::CO *fwa* plants may be an indirect consequence of 35S::CO driving early flowering in the presence of *fwa* or of a more direct interaction between 35S::CO and *fwa* that occurs indepen-

dent of flowering time. To distinguish between these possibilities, we used a fusion of CO to the ligand binding domain of the rat glucocorticoid receptor (35S::CO:GR). This fusion enables CO overexpression to be induced by application of the steroid dexamethasone (Simon et al., 1996). The 35S::CO:GR transgenic plants were crossed to *fwa-1* mutants, and the F₁ plants were treated with dexamethasone at various times after germination. When plants were treated repeatedly with dexamethasone from germination onwards, the phenotype was very similar to that of *fwa-1/+ 35S::CO/+* plants, demonstrating that when continually induced, CO:GR has an effect similar to that of 35S::CO.

When plants were treated once with dexamethasone at 7, 14, or 21 days after sowing, flowering occurred in response to the application of dexamethasone, and floral defects similar to those described for *fwa-1/+ 35S::CO* plants (Figure 5) were present. The plants treated 21 days after sowing produced as many leaves as *fwa-1/+ 35S::CO:GR/+ co-2/+*

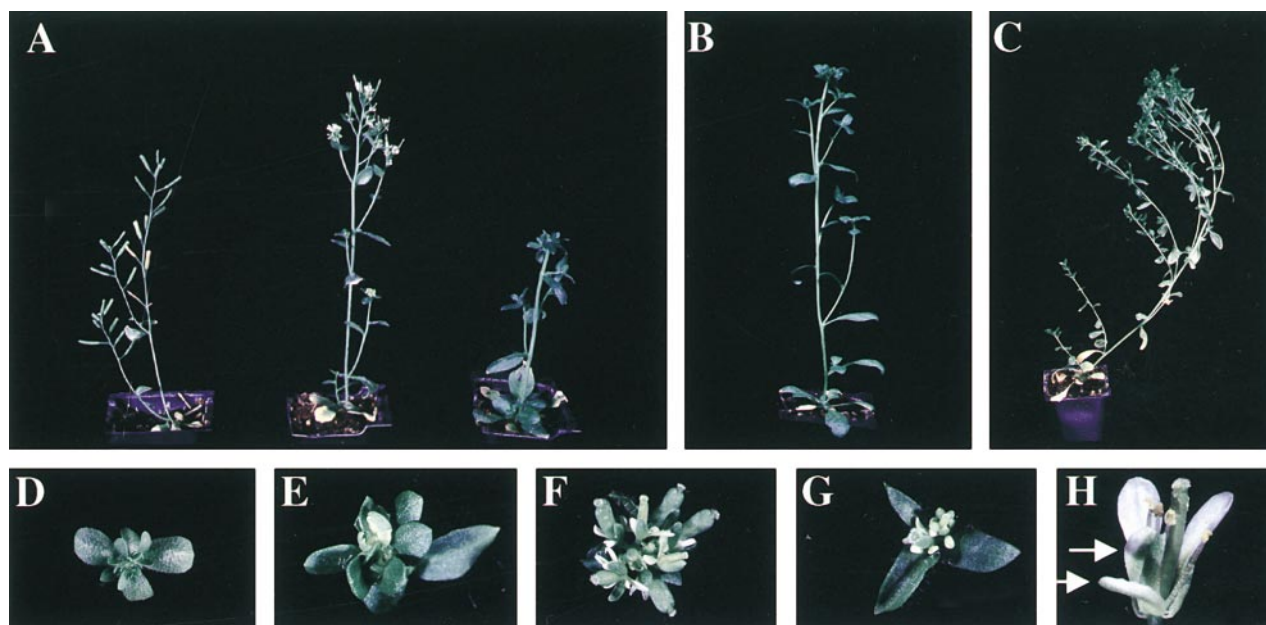


Figure 4. Shoot and Flower Phenotype of *fwa 35S::CO* Plants.

All plants were grown under long-day conditions except for the plant shown in (F).

(A) A 35S::CO plant (left), an *fwa-100/+ 35S::CO* plant (center), and an *fwa-100/fwa-100 35S::CO* plant (right). All plants are 1 month old.

(B) A 5-week-old *fwa-1/fwa-1 35S::CO* plant.

(C) A 2-month-old *fwa-1/fwa-1 35S::CO* plant showing no obvious floral organs, except occasional carpelloid structures at the apex of lateral shoots.

(D) Top view of the apex of the primary inflorescence of a 1-month-old *fwa-1/fwa-1 35S::CO* plant.

(E) Structure of the apex of a lateral branch of a 2-month-old *fwa-100/fwa-100 35S::CO* plant. A few carpel-like structures are visible among the leaves.

(F) Structure of the apex of a lateral branch of a 2-month-old *fwa-100/fwa-100 35S::CO* plant grown under short-day conditions.

(G) A *fwa-100/+ 35S::CO* flower with leaflike sepals and a secondary flower.

(H) A *fwa-100/fwa-100* flower with sepal/petal chimeric organs (arrows).

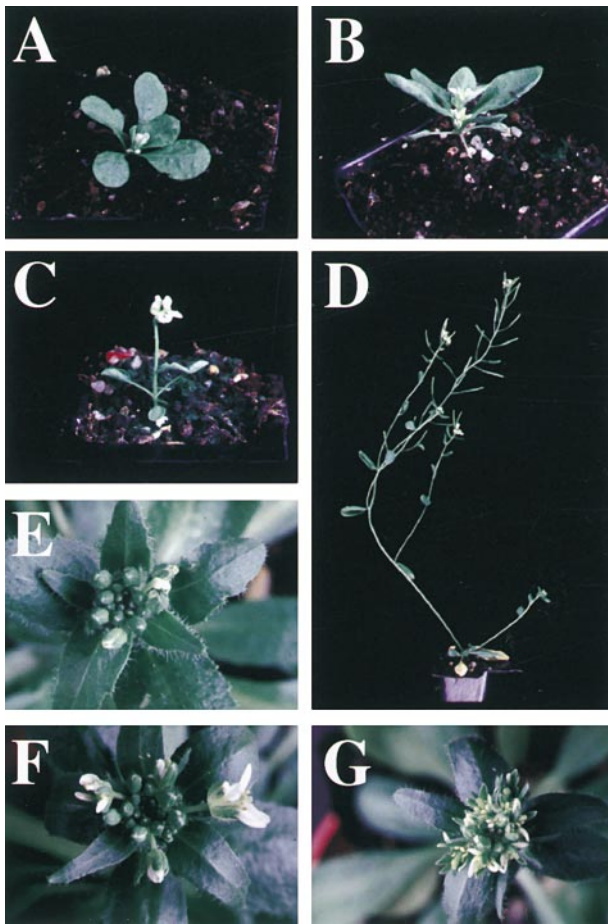


Figure 5. Effects of 35S::LFY and 35S::AP1 on the *fwa* 35S::CO Phenotype and the Effects of Dexamethasone Applications on 35S::CO:GR *fwa* Plants.

All plants were grown under long-day conditions.

(A) An 18-day-old 35S::LFY plant showing a terminal flower phenotype.

(B) An 18-day-old *fwa-1/+* 35S::CO/+ 35S::LFY/+ plant showing a terminal flower phenotype.

(C) An 18-day-old 35S::AP1 plant showing a terminal flower phenotype.

(D) A 5-week-old *fwa-1/+* 35S::CO/+ 35S::AP1/+ plant.

(E) Top view of a 6-week-old *fwa-1/+* *co-2/+* plant treated with dexamethasone 3 weeks after sowing.

(F) Top view of a 6-week-old *fwa-1/+* 35S::CO:GR *co-2/+* plant not treated with dexamethasone.

(G) Top view of a 6-week-old *fwa-1/+* 35S::CO:GR *co-2/+* plant treated with dexamethasone 3 weeks after sowing.

plants not treated with dexamethasone or as *fwa-1/+* *co-2/+* plants; however, these plants still had floral defects. In contrast, such flower defects were not observed in *fwa-1/+* 35S::CO:GR/+ *co-2/+* plants not treated with dexamethasone or in dexamethasone-treated *fwa-1/+* *co-2/+* plants

(Figure 5). This result suggests that the floral meristem identity defect in 35S::CO *fwa* plants indicates a direct effect on floral development and not an indirect consequence of their delayed flowering time.

DISCUSSION

Effect of 35S::CO on Flowering Time

35S::CO caused early flowering in both long- and short-day conditions, so that the transgenic plants were almost completely insensitive to day length. A similar phenotype was reported previously for 35S::CO:GR plants treated with dexamethasone early in development (Simon et al., 1996). Together with previous observations that showed a correlation between copy number of the *CO* gene and flowering time (Koorneef et al., 1991; Putterill et al., 1995), this suggests that transcriptional control of *CO* expression is an important determinant in the photoperiodic control of flowering. All five 35S::CO lines contained the *CO* mRNA in much greater abundance than did wild-type plants, but the amount of the mRNA varied between lines. Nevertheless, all five lines showed similar phenotypes, suggesting that beyond the expression level of the weakest line, increasing the abundance of *CO* mRNA does not have a further effect on phenotype. Above that amount, therefore, other factors may limit *CO* function.

35S::CO Can Overcome the Effect of Mutations in the Autonomous Pathway

The *fca-1* mutation strongly delays flowering in an *Ler* background but only slightly delays flowering of 35S::CO plants. *FCA* acts in the autonomous flowering-time pathway, whereas *CO* acts in the long-day pathway (Koorneef et al., 1998a, 1998b; Levy and Dean, 1998). Nevertheless, increasing the activity of the long-day pathway by overexpressing the *CO* gene can almost completely overcome the effect of strong mutations in the autonomous pathway. This suggests that flowering time is controlled by the balance of activity of these two pathways and that when the long-day pathway is superactivated, the autonomous pathway is not required for early flowering.

Nevertheless, the *fca-1* mutation does slightly delay flowering of 35S::CO plants. This effect is strongly enhanced in the presence of the *ft-8* mutation so that *ft-8 fca-1* 35S::CO plants flower much later than either *fca-1* 35S::CO or *ft-8* 35S::CO plants. These observations suggest that the autonomous pathway is required for 35S::CO to have its full effect. The marked enhancement of the effect of *fca-1* by *ft-8* suggests that the contribution of the autonomous pathway is partially redundant with *FT*.

Identification of Suppressor Mutations of 35S::CO and the Role of the Novel Flowering-Time Gene *SOC1*

Suppressor mutagenesis of a gain-of-function allele has been used in model organisms such as *Drosophila* and *Caenorhabditis elegans* to identify factors that act downstream of a gene of interest (Stern and DeVore, 1994; Karim et al., 1996). In plants, screens for extragenic suppressors of a loss-of-function allele have been described (e.g., Pepper and Chory, 1997; Reed et al., 1998), as have mutations that suppress the effect of constitutive activation of ethylene signaling (Roman et al., 1995). To identify genes that act downstream of *CO* to control flowering, we performed suppressor mutagenesis of 35S::CO.

Three loci were identified as suppressors of the early-flowering phenotype of 35S::CO. One of them, *soc1*, defines a novel flower-promoting gene; its position on chromosome 2 distinguishes it from all other flowering-time loci described to date. The *soc1* mutation had not been previously identified by mutagenesis of wild-type Arabidopsis, demonstrating that using suppressor mutagenesis of transgenic Arabidopsis plants overexpressing a gene is an effective way to identify novel loci likely to be functionally associated with the gene of interest. The other three suppressor mutations were alleles of *ft* and *fwa*.

The *soc1* mutation delays flowering under both long- and short-day conditions. This is a characteristic of mutations that affect the autonomous flowering pathway rather than the long-day pathway in which *CO* acts. If *SOC1* acts in the autonomous pathway, then the delay in flowering of 35S::CO plants caused by *soc1* would be similar to the effect of *fca-1*. Alternatively, *SOC1* may act both downstream of *CO* in the long-day pathway and in the autonomous pathway. In this case, *soc1* mutations may show effects similar to those of mutations in the autonomous pathway but nevertheless act in the long-day pathway downstream of *CO*. We favor this latter possibility because of the relative severity of the effect of *soc1* on 35S::CO. The delay caused by *soc1* on the flowering time of wild-type plants is very much weaker than that of *ft*, *fwa*, and *fca* (Table 2), but its effect on 35S::CO is at least as strong as that of *fca-1* (Table 1). The relatively strong effect of *soc1* on the phenotype of 35S::CO may argue that *SOC1* acts more directly downstream of *CO* than does *FCA*. Furthermore, *soc1* has an effect similar to that of *ft-1* in both wild-type and 35S::CO backgrounds (Tables 1 and 2), and extensive genetic and molecular data now place *FT* downstream of *CO* (see below).

FT and *FWA* Act Downstream of *CO*

The suppressor mutations 1 and 4 are alleles of *ft*, and suppressor mutation 2 is an allele of *fwa*. *FT* and *FWA* were proposed to act in the same pathway as *CO*, based on analyses of double mutants and their response to environmental conditions (Koornneef et al., 1998b). These observations are

extended by our findings that the *ft* and *fwa* mutations partially suppress 35S::CO, which suggests that *FT* and *FWA* act downstream of *CO* (Figure 6A). Furthermore, Kardailsky et al. (1999) and Kobayashi et al. (1999) have recently cloned the *FT* gene and found that not only is *FT* mRNA abundance reduced in the *co-2* mutant background and rapidly increased by activation of *CO* but also that 35S::FT suppresses *co-2* mutations. Together with our genetic data, this strongly suggests that *FT* acts downstream of *CO* to promote flowering; however, because strong mutant alleles of *ft* only partially suppress 35S::CO (Table 1), *CO* must also activate other downstream genes (see below). In addition, *fwa* largely suppresses the early-flowering phenotype caused by the overexpression of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999), which would again be consistent with the idea that *fwa* affects the long-day pathway downstream of *CO*.

FT is most strongly required for the phenotype of 35S::CO plants under short days. Whereas 35S::CO plants flowered at the same time under long and short days, *ft* 35S::CO plants flowered later under short days than under long days. *FT* is either not required or only minimally required for flowering of wild-type plants under short days (Table 2; see also Koornneef et al., 1998a), and it is probably strongly required under short days in 35S::CO plants because 35S::CO activates transcription of *FT* under these conditions. The weaker effect of *ft* on 35S::CO under long days suggests that under these conditions, other genes may be activated that can partially compensate for *ft*. This could occur through post-translational activation of *CO* under long-day conditions, or it might occur independently of *CO*.

Genetic Evidence for Parallel Flowering-Time Pathways Downstream of *CO*

No single mutation that completely suppresses early flowering of 35S::CO was identified from our screens. 35S::CO could still accelerate flowering of any of the suppressor mutants. Such partial suppression could be explained by genetic redundancy between genes that act downstream of *CO*. Of the three suppressor mutations, *ft* and *fwa* were previously proposed to affect the same pathway and not to act redundantly (Koornneef et al., 1998a). Therefore, 35S::CO must promote flowering of the *ft/fwa* mutants by way of another pathway. Analysis of an F₂ population generated by intercrossing *soc1* 35S::CO and *fwa-1* 35S::CO suggested that *SOC1* may define such a pathway (Figure 6A), because combining both *soc1* and *fwa-1* in a 35S::CO background largely suppressed early flowering of 35S::CO, and the number of rosette leaves produced by these plants was similar to that produced by *co-2* mutants. Previous genetic and molecular experiments suggested that *CO* does not promote flowering by way of *FT* in a simple linear pathway (Simon et al., 1996; Kardailsky et al., 1999; Kobayashi et al., 1999), and our data indicate that *CO* promotes flowering via

at least two genetic pathways, one involving *SOC1* and a second involving *FT/FWA*.

Effect of *fwa* on Floral Meristem Identity

Both *fwa-100* 35S::*CO* and *fwa-1* 35S::*CO* showed an extreme floral meristem identity defect that was not shown by any of the parents (Figure 4) and was similar to that of *lfy fwa* double mutants (Ruiz-Garcia et al., 1997). *CO* was also overexpressed late in the development of *fwa* mutants using 35S::*CO:GR*, and the floral meristem identity defect was in-

duced irrespective of flowering time. Therefore, the effect of 35S::*CO* on floral meristem identity in *fwa* is not an indirect effect of early flowering. Suppression of this defect by 35S::*LFY* suggests that *LFY* expression or activity may be decreased in *fwa* 35S::*CO* plants. Also petal/sepal chimeric organs observed in *fwa-100* single mutant flowers are reminiscent of the flower phenotype caused by weak *lfy* alleles (Figure 4; Weigel et al., 1992). These observations suggested that *fwa* may slightly affect *LFY* and that 35S::*CO* may strongly enhance the effect. No difference between *fwa* mutants and wild-type plants regarding *LFY* mRNA abundance was detected in previous experiments (Ruiz-Garcia et al., 1997; Nilsson et al., 1998), indicating that the slight effect of *fwa* may not be easily detectable or that the repression of *LFY* does not occur by decreasing the amount of its mRNA.

In contrast to 35S::*LFY*, 35S::*AP1* did not suppress the floral meristem identity defect of *fwa* 35S::*CO*. The *AP1* gene is directly activated by *LFY* (Parcy et al., 1998; Wagner et al., 1999), and 35S::*AP1* can largely but not completely suppress *lfy* (Mandel and Yanofsky, 1995; Liljegren et al., 1999). The observation that 35S::*LFY* suppresses *fwa-1* 35S::*CO* but 35S::*AP1* does not suggests that the floral meristem identity phenotype of *fwa-1* 35S::*CO* plants is largely caused by reductions in *LFY* activity affecting the expression of genes other than *AP1*. In addition to its effect on *AP1*, *LFY* has also been proposed to activate the *AGAMOUS* and *AP3* genes by interaction with different coactivators (Parcy et al., 1998). Similarly, *LFY* may activate other floral meristem identity genes, which could be repressed by the combination of *fwa* and 35S::*CO*. Consistent with this idea, Ruiz-Garcia et al. (1997) suggested that *LFY* and *FWA* could control other floral meristem identity genes as well as *AP1*, because the floral meristem identity defect in *lfy fwa* was more severe than in *ap1 fwa*, *ap1 lfy*, or *ap1 cauliflower lfy*.

The *fwa* mutant alleles may be gain-of-function mutations caused by hypomethylation, because all known *fwa* mutations are dominant, and Kakutani (1997) reported that a dominant late-flowering mutation induced in a *decreased DNA methylation 1* background was likely to be an allele of *fwa*. In addition, some *fwa* mutants showed other epigenetic features. For example, an epigenetic allele of *superman* (*clark kent-1*) was identified from the *fwa-1* mutant line (Jacobsen and Meyerowitz, 1997). The *fwa-100* mutant line that we identified as suppressor mutation 2 showed phenotypic instability, such as variation in the severity of the floral meristem identity defect between branches (data not shown).

If the *fwa* mutant allele results from increased expression of the *FWA* gene, then overexpression of *CO* can somehow be correlated with increased expression of *FWA* to inhibit *LFY* activity. This appears to be specific to the *fwa* 35S::*CO* interaction and was not shown by *fwa* 35S::*FT* plants (Kardailsky et al., 1999; Kobayashi et al., 1999). The effect on floral meristem identity of combining 35S::*CO* and *fwa* may be explained by two general models. The first model is based on that of Ruiz-Garcia et al. (1997), who proposed

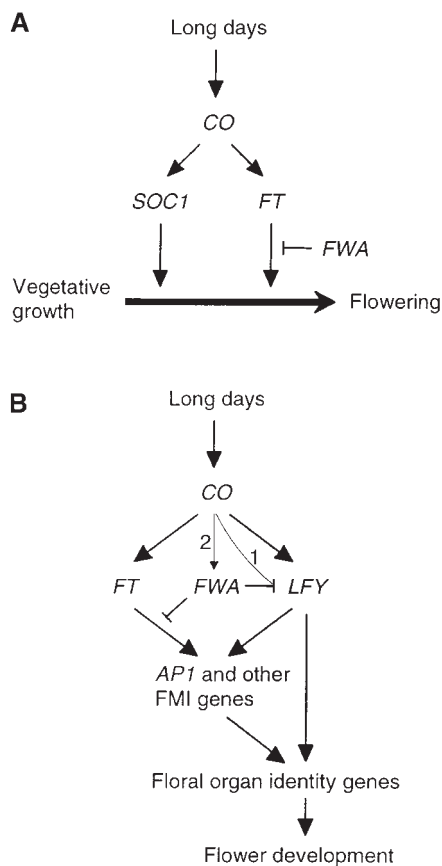


Figure 6. Schematic Model for the Interaction of Genes in the Control of Flowering Time and Floral Meristem Identity.

(A) Model for control of flowering time.

(B) Model for control of floral meristem identity (FMI). The thin numbered lines represent two possibilities, described in the text, for how the interaction between 35S::*CO* and *fwa* might inhibit *LFY* function. In both **(A)** and **(B)**, arrows represent promotive interactions and T-bars represent inhibitory interactions. The proposed inhibitory function of *FWA* is based on the assumption that *fwa* mutations are gain-of-function mutations.

that the severe enhancement of the phenotype of *lfy* mutants caused by *fwa* occurs because the *fwa* mutation represses a floral meristem identity gene, the function of which is redundant with *LFY*. The *fwa* 35S::CO phenotype can be explained by this model if the overexpression of *CO* in combination with *fwa* also acts to repress the function of *LFY* or of a gene downstream of *LFY*. That *CO* may activate genes that repress flowering was shown previously for the activation of *TFL1* by 35S::CO:GR (Simon et al., 1996) and proposed because *co-1* 35S::FT plants flowered slightly earlier than did 35S::FT (Kobayashi et al., 1999), although a similar effect was not seen with *co-2* (Kardailsky et al., 1999). A second model proposes that the FWA product is present at increased amounts in the *fwa* mutant and that 35S::CO causes the amount of overexpression to be increased further or increases the activity of FWA protein. In this case, if FWA represses floral meristem identity gene expression, and the severity of this effect is proportional to the degree of overexpression of FWA, then 35S::CO would enhance the effect of *fwa* on floral meristem identity.

In conclusion, the genetic approach that we have used to identify genes required for *CO* function demonstrated that *CO* controls several distinct activities that regulate flowering. One of these somehow acts together with FWA to repress flower development. In addition, two parallel pathways act downstream of *CO* to promote flowering; one of these pathways involves *FT/FWA*, and the other includes *SOC1*. The isolation of *SOC1* will shed further light on the function of this second pathway.

METHODS

Plant Materials and Growth Conditions

Wild-type plants were *Arabidopsis thaliana* in the wild-type Landsberg *erecta* (*Ler*) ecotype. All mutant and transgenic lines were in the *Ler* background. *constans-2* (*co-2*) mutants carried the *transparent testa4* (*tt4*) mutation (Putterill et al., 1995), which causes a white seed color phenotype and was used as a visible marker to indicate the presence of the *co-2* mutation. Seeds of late-flowering mutants *fca-1*, *ft-1*, *fpa-2*, and *fwa-1* were kindly provided by M. Koornneef (Koornneef et al., 1991). 35S::LEAFY (*LFY*) and 35S::APETALA1 (*AP1*) transgenic seeds were gifts of D. Weigel (Weigel and Nilsson, 1995) and M. Yanofsky (Mandel and Yanofsky, 1995), respectively. The fusion of *CO* to the ligand binding domain of the rat glucocorticoid receptor (35S::CO:GR) was as described previously (Simon et al., 1996). Seeds were placed on moist filter paper at 4°C for 4 or 5 days, planted in soil, and germinated in growth cabinets or greenhouses.

Measurement of Flowering Time and Floral Organ Number

Flowering time and floral organ analyses were performed with plants grown in controlled-environment cabinets under either short-day (10-hr-light/14-hr-dark) or long-day (10-hr-light/6-hr-day extension/8-hr-dark) conditions as described in Putterill et al. (1995). Flowering

time was measured by scoring the number of leaves on the main stems or the number of days from sowing until flower buds were visible by eye at the center of the rosette. Floral organ number was scored under a stereoscopic microscope. Data from one experiment are presented as mean \pm SD, where $n = \geq 15$ plants or 50 to 80 flowers, except where indicated.

Construction of 35S::CO

To make the 35S::CO fusion, we inserted the 35S promoter upstream of a genomic clone of the *CO* gene. The entire *CO* gene had previously been inserted into the EcoRV site of pBluescript (Stratagene, La Jolla, CA) as a 4.2-kb EcoRV-PvuII fragment in plasmid d178. The EcoRV site is located \sim 2450 bp upstream of the translational start site of *CO*, and the PvuII site is located \sim 400 bp downstream of the translational stop codon. The fragment was inserted into the EcoRV site of pBluescript such that the EcoRV site of the EcoRV-PvuII fragment was adjacent to the HindIII site of the polylinker. The 35S promoter was removed from vector pJIT62 (P. Mullineaux, John Innes Centre) as a 400-bp KpnI-HindIII fragment. Plasmid d178 was cleaved with KpnI and HindIII, and the largest fragment generated was ligated to the KpnI-HindIII fragment containing the 35S promoter. This ligation generated a fusion of the 35S promoter to the *CO* gene at a HindIII site 60 bp upstream of the *CO* translational start codon. To enable easy transfer of this fragment to the binary vector, the KpnI site was converted to a ClaI site by using the adapter 5'-TATCGATAGTAC-3'. The fusion was then removed as a ClaI-BamHI fragment and inserted into vector SLJ1711 (Jones et al., 1992). The 35S::CO construct was introduced into *co-2 tt4* mutants by the root transformation procedure (Valvekens et al., 1988). Transgenic line 5 was used for further study.

RNA Gel Blot Analysis

RNA gel blot analysis was performed as previously described (Schaffer et al., 1998). RNA was extracted from soil-grown plants with 3 to 4 visible leaves.

Mutagenesis and Phenotypic Screens for Mutations That Delay Flowering of 35S::CO

Approximately 20,000 35S::CO *co-2* seeds in three independent batches and 10,000 35S::CO *fca-1* seeds were mutagenized by imbibition in 0.3% ethyl methanesulfonate (EMS; Sigma) for 8 to 9 hr, followed by washing with 0.1 M Na₂SO₃ and distilled water (five times). M₂ seeds were collected in pools, with each pool containing seeds of \sim 50 M₁ plants. Approximately 20,000 M₂ seeds representing \sim 4000 M₁ plants after mutagenesis of 35S::CO *co-2* seeds were sown on soil and screened for late-flowering mutants under short-day conditions in a greenhouse. Approximately 5000 M₂ seeds representing \sim 500 M₁ plants from the mutagenesis of 35S::CO *fca-1* seeds were sown on soil and screened for a late-flowering phenotype under long-day conditions in a greenhouse.

Genetic Analysis

The suppressor mutations in 35S::CO *co-2* background were backcrossed to 35S::CO *co-2* plants twice before phenotypic analysis.

Detailed information on the construction of lines and genetic segregation ratios is available from the authors.

Molecular Determination of the Presence of *co-2*, 35S::*CO*, and 35S::*AP1*

For detection of the *co-2* mutation, the primers CO41 (5'-GGT-CCCAACGAAGAAGTGC-3') and CO52 (5'-GGAACAGCCACGAAGCAA-3') were used. After amplification, the polymerase chain reaction (PCR) products were digested with BspLU111 (Boehringer Mannheim), which produces 324-, 292-, and 114-bp fragments for the wild-type allele and 616- and 114-bp fragments for the *co-2* allele.

For detection of the 35S::*CO* transgene, a set of primers 35S01 5'-TATCCTTCGCAAGACCCTTC-3' and CO52 was used. This primer pair amplifies an ~600-bp fragment.

For detection of the 35S::*AP1* transgene, the primers 5'-GAA-GAGGATAGAGAACAAGAT-3' and 5'-ATTGACGTCGACTCAGG-TG-3' were used. This primer pair amplifies a 241-bp fragment from the *AP1* cDNA and a 1583-bp from the genomic copy.

Template DNA for PCR reactions was extracted from leaves of each genotype, as described by Sawa et al. (1997).

Genetic Mapping

Suppressor mutants 1, 3, or 4 were crossed to wild-type Columbia to generate mapping populations. For mapping suppressor 1, 3, or 4, DNA was isolated (Sawa et al., 1997) from 32, 30, and 48 F₂ plants that showed a late-flowering phenotype, respectively, and analyzed with simple sequence length polymorphism (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993). *soc1* showed strong linkage to a CAPS marker 40E1T7 (no recombinants were found in analysis of 58 chromosomes) and weaker linkage to a simple sequence length polymorphism marker, AthBIO2 (seven recombinants were found in analysis of 60 chromosomes), on chromosome 2.

To facilitate restriction fragment length polymorphism mapping of the suppressor mutations that were recovered in the 35S::*CO co-2* plants in the *Ler* background, 35S::*CO* was introgressed into Columbia. The introgression was performed by crossing 35S::*CO co-2 Ler* to wild-type Columbia four times. Plants inheriting 35S::*CO* were selected by their early-flowering phenotype and the results of a PCR test (see above) performed after each cross. Plants homozygous for 35S::*CO* were selected after the fourth backcross. 35S::*CO* Columbia plants were then used to map suppressor 2 by crossing 35S::*CO Ler* carrying this mutation to 35S::*CO* Columbia plants, and DNA was isolated from plants homozygous for suppressor mutant 2 and carrying 35S::*CO* (Sawa et al., 1997). A total of 15 F₂ plants showing the severe floral meristem identity defect phenotype were analyzed with simple sequence length polymorphism and CAPS markers. Suppressor mutation 2 showed linkage to CAPS marker g8300 (five recombinants in analysis of 30 chromosomes) on chromosome 4.

Map distance between the markers and the suppressor mutations was determined by using the Kosambi mapping function (Koonneef and Stam, 1992).

DNA Sequencing

The *FT* gene was amplified by PCR from genomic DNA of suppressor mutants 1 and 4 with two sets of primers: 5'-CAGAAACAATCAACA-

CAGAGAAACCACCTG-3' and 5'-TCAATTTAGCTTGGGTGG-3', and 5'-TTATGTTGTGGTGCCATAGC-3' and 5'-ACTATAGGCATC-ATCACCGTTCGTTCTACTCG-3'. These pairs of primers were used to amplify 1406- and 571-bp fragments, respectively, that covered all of the *FT* coding region. PCR products were purified with the Quiaquick PCR purification kit (Qiagen, Chatsworth, CA) and were sequenced on one strand with a DNA sequencing kit (Perkin Elmer) by using the same primers used to amplify the *FT* coding regions. Once mutations were found, another strand of an independently amplified PCR product was sequenced to verify that the DNA sequence alteration was not a PCR-derived error or a sequencing error. A mutation in *ft-7* changed the 138th amino acid residue Trp (TGG) to a stop codon (TGA). *ft-8* contained a mutation in the 3' splice acceptor site of the third intron (AG changed to AA).

Application of Dexamethasone

Dexamethasone (10 μM; Sigma) was prepared and applied to plants as described previously (Simon et al., 1996).

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