Cell, Vol. 80, 847–857, March 24, 1995, Copyright © 1995 by Cell Press

The *CONSTANS* Gene of Arabidopsis Promotes Flowering and Encodes a Protein Showing Similarities to Zinc Finger Transcription Factors

Joanna Putterill,* Frances Robson, Karen Lee, Rüdiger Simon, and George Coupland
John Innes Centre
Norwich Research Park
Colney
Norwich NR4 7UH
England

Summary

The vegetative and reproductive (flowering) phases of Arabidopsis development are clearly separated. The onset of flowering is promoted by long photoperiods, but the *constans* (*co*) mutant flowers later than wild type under these conditions. The *CO* gene was isolated, and two zinc fingers that show a similar spacing of cysteines, but little direct homology, to members of the GATA1 family were identified in the amino acid sequence. *co* mutations were shown to affect amino acids that are conserved in both fingers. Some transgenic plants containing extra copies of *CO* flowered earlier than wild type, suggesting that *CO* activity is limiting on flowering time. Double mutants were constructed containing *co* and mutations affecting gibberellic acid responses, meristem identity, or phytochrome function, and their phenotypes suggested a model for the role of *CO* in promoting flowering.

Introduction

The initiation of flowering is regulated by a combination of genotype and environmental stimuli such as day length, temperature, and light quality. Flowering of Arabidopsis thaliana is promoted by long photoperiods, and it is therefore classified as a facultative long day (LD) plant (Redei, 1962). In addition, flowering of some varieties is accelerated by low temperatures, a process called vernalization (Napp Zinn, 1957), and by high ratios of far red to red light (Martinez-Zapater and Somerville, 1990; Bagnall, 1993).

Flowers are derived from a population of stem cells called the shoot meristem. During the transition from vegetative growth to flowering, the shoot meristem exists first as a vegetative meristem that gives rise to leaves, and then, in species such as Arabidopsis, as an inflorescence meristem that gives rise both to flowers and to further inflorescence meristems. How environmental cues affect the developmental transition from vegetative growth to flowering is unclear. However, physiological analysis, particularly of species whose flowering is controlled by photoperiod, demonstrated that a graft-transmissable substance, or combination of substances, is produced in the leaves and acts at the shoot apex to control the transition to flowering. This substance has proven elusive, and its identity has not been established (reviewed by Bernier et al., 1993). Moreover, although several genes that are required to determine the identity of floral meristems, and particularly to distinguish these from inflorescence meristems, have been identified in Arabidopsis and Antirrhinum majus, how these genes are activated during the transition from vegetative growth to flowering is not known (Weigel et al., 1992; Gustafson-Brown et al., 1994; Coen et al., 1990; Huijser et al., 1992).

The study of Arabidopsis mutants has begun to establish relationships between physiological processes and the control of flowering by day length (reviewed by Martinez-Zapater et al., 1994). For example, mutations that block, or greatly reduce, biosynthesis of the hormone gibberellic acid (GA) prevent flowering under short day (SD) conditions and delay it under LD conditions (Wilson et al., 1992), suggesting that GA is required to promote flowering. The light receptor phytochrome B, however, is involved in delaying flowering, as mutations in the gene encoding it cause early flowering (Goto et al., 1991).

Genetic approaches were used to identify genes required for the transition from vegetative growth to flowering, and genes that influence the timing of flowering were identified in many species. In Arabidopsis, genes that cause late flowering were identified by crossing different ecotypes (reviewed by Martinez-Zapater et al., 1994; Clarke and Dean, 1994) or by mutagenesis (Redei, 1962; Vetrilova, 1973; Koornneef et al., 1991), and recently, early flowering mutants were isolated (Zagotta et al., 1992; Sung et al., 1992).

Little is known of the function of the genes affected in late-flowering mutants, but their products are thought to be involved in promoting the transition from vegetative growth to flowering, and presumably they activate, directly or indirectly, floral meristem identity genes such as *LEAFY* (*LFY*) (Huala and Sussex, 1992; Weigel et al., 1992). To gain some insight into their function, twelve late-flowering mutants were classified according to their responses to vernalization, day length, and light quality (Redei, 1962; Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Lee et al., 1994). Two of them, *constans* (*co*) and *gigantea* (*gi*), are almost day-neutral, flowering later than wild type under LD conditions and at the same time as wild type under SD conditions. Moreover, these mutants show a much reduced response to vernalization. This suggests that the products of the *CO* and *GI* genes are important in the regulation of flowering in response to environmental conditions.

Recently the gene affected in another late-flowering mutant, *luminidependens* (*ld*), was isolated. This mutant retains sensitivity to environmental stimuli, flowering later than wild type under both LD and SD conditions and responding to vernalization. The sequence of the *LD* cDNA shows no direct homology to any previously isolated gene, but contains nuclear targeting sequences and a high glutamine content reminiscent of some transcription factors (Lee et al., 1994).

*Present address: School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand.

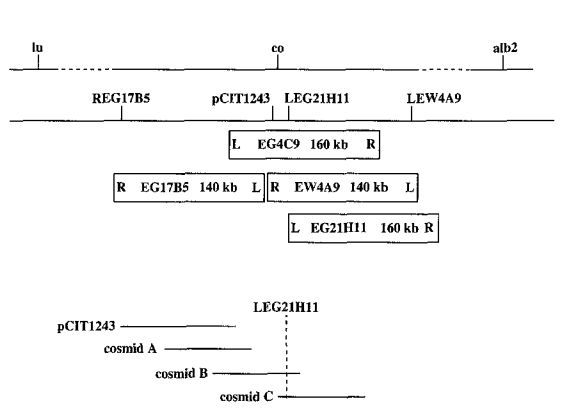

Figure 1. Molecular and Genetic Markers in the Vicinity of *CO*

(A) The upper line shows the phenotypic markers flanking *co-2*. The second line shows the RFLP markers in the region; *CO* was previously shown to be located in the 300 kb region between REG17B5 and LEW4A9. The rectangles represent yeast artificial chromosomes (YACs) that span this region. The left (L) and right (R) ends of each YAC are marked, as are their sizes and colony numbers. These data are described in more detail in the work by Putterill et al. (1993).

(B) Four overlapping cosmid clones in the region of *CO*. RFLP mapping located *CO* between an RFLP detected by cosmid A and one detected by cosmid C. The positions of pCIT1243 and LEG21H11 locate this contig in relation to the markers shown in (A).

We are studying the function of *CO* in regulating flowering in response to day length. We describe the isolation of the gene and demonstrate that its protein product is similar to zinc finger transcription factors. We also report the construction and analysis of double mutants carrying *co* as well as mutations affecting phytochrome structure, the gibberellic acid pathway, and a floral meristem identity gene.

Results

Location of the *CO* Gene as Determined by Detailed Restriction Fragment Length Polymorphism Mapping and Molecular Complementation

A map-based cloning strategy was used to isolate *CO*. Previously, we identified a 300 kb region containing the gene (Figure 1; Putterill et al., 1993), and further restriction fragment length polymorphism (RFLP) mapping was used to determine its position more accurately. First, 68 crossovers were identified in the 1.6 cM interval between *co* and a proximal phenotypic marker, *alb2*, and 128 were identified in the 5.3 cM interval between *co* and the distal phenotypic marker *lu*. These recombinant chromosomes were then analyzed with DNA markers located within the 300 kb region known to contain *CO*, and the markers pCIT1243 and LEW4A9 were shown to flank the gene (Figure 1). From an amplified cosmid library (Olszewski and Ausubel, 1988), 20,000 colonies were screened with a pool of short probes located between, or within, pCIT1243 and LEW4A9. In this way, twelve cosmids within the region of interest were identified, and three of these overlapped to form a short contiguous segment of DNA approximately 48 kb long (Figure 1). Use of cosmids B and C as RFLP

Table 1. Flowering Time and Segregation of Kanamycin Resistance in Progeny of *co-2* and Wild-Type Plants Carrying Cosmids B or C

Transgenic Lines Scored	Ratio of Kanamycin-Resistant Seedlings in T2[a]	Average Leaf Number at Flowering of T3 Individuals under LDs[b]	Average Leaf Number at Flowering of T3 Individuals under SDs[b]	Ratio of Kanamycin-Resistant Seedlings in T3
co-2 transformants				
Cosmid B, line 1	3.0:1	4.6 ± 0.4	14.0 ± 2.5[c]	1:0
Cosmid B, line 2	3.7:1	4.2 ± 0.3	18.5 ± 1.1	1:0
Cosmid B, line 3	2.9:1	4.6 ± 0.8	13.5 ± 4.1	1:0
Cosmid B, line 4	2.4:1	4.6 ± 0.8	16.4 ± 2.2	1:0
Cosmid B, line 5	3.0:1	5.1 ± 0.5	18.5 ± 1.1[d]	1:0
Cosmid C, line 1	2.9:1	4.6 ± 0.6	20.6 ± 3.8	1:0
Cosmid C, line 2	3.4:1	3.9 ± 0.4	11.7 ± 3.2	1:0
Cosmid C, line 3	3.3:1	4.0 ± 0.4	20.4 ± 1.2	1:0
Cosmid C, line 4	4.9:1	3.7 ± 0.3	7.6 ± 5.3[e]	1:0
Cosmid C, line 5	3.8:1	3.5 ± 0.5	6.6 ± 1.4	1:0
Landsberg erecta transformants				
Cosmid B, line 1	3.4:1	4.4 ± 1.0	18.1 ± 2.1	1:0
Cosmid B, line 2	5.9:1	3.2 ± 0.6	10.1 ± 2.2	1:0
Cosmid B, line 3	2.3:1	4.0 ± 0.5	19.6 ± 2.2[f]	1:0
Controls				
Landsberg erecta	–	5.1 ± 0.8	18.9 ± 2.4	–
co-2	–	12.4 ± 1.0	18.1 ± 3.4	–

Flowering time was measured by counting the number of leaves present at the time that the flower bud appeared in the center of the rosette (Koornneef et al., 1991; Experimental Procedures).

[a] Over 80 plants were tested in each family, except for cosmid B, line 3, in which 35 plants were used.

[b] In most cases, ten plants from each family were tested.

[c] n = 4.

[d] n = 5.

[e] The large standard error in this population was due to two plants that flowered with 18 leaves, while the other eight had a leaf number of 5.1 ± 1 at flowering. Southern blot analysis of this line using a T-DNA fragment as probe identified six hybridizing fragments. The variation in flowering time could therefore be due to the segregation of one T-DNA copy that is required for early flowering, or to the occurrence of cosuppression-repressing activity of the transgenes in some individuals.

[f] n = 5.

markers demonstrated that *CO* is located within the 48 kb region.

To position the gene within the cosmids, each of them was introduced into *co* mutants and the resulting plants examined to determine which of the cosmids corrected the *co* mutant phenotype. Roots of plants homozygous for *co-2* were cocultivated with Agrobacterium tumefaciens strains containing each cosmid, and kanamycin-resistant plants regenerated. The regenerants (T1 generation) were self-fertilized and their progeny sown on medium containing kanamycin to confirm that they contained the T-DNA. The flowering time of 20–40 plants from each of these T2 families was measured. The progeny of five independent transformants made with cosmid A flowered as late as the *co-2* mutants, suggesting that this cosmid did not contain the gene. However, several of the families derived from plants containing cosmids B and C included early-flowering individuals. In total, 6 of the 9 families derived from plants harboring cosmid B, and 12 of the 13 derived from those carrying cosmid C, contained plants that flowered as early as wild type.

Further experiments were carried out in the T3 generation to confirm that cosmids B and C were able to complement *co*. A total of five T2 plants derived from cosmid B and six from cosmid C were self fertilized and studied further in the T3 generation. Each of the T2 plants chosen for this analysis was derived from a different transformant, was the earliest-flowering plant in the T2 family, and was a member of a family that had shown a ratio of three kanamycin-resistant seedlings for each kanamycin sensitive and therefore probably contained the transgene at only one locus (Table 1). All of the seedlings in these T3 families were resistant to kanamycin, demonstrating that the parental T2 plants were homozygous for the T-DNA, and therefore in all cases examined, the earliest-flowering T2 plants were homozygous for the *CO* transgene. All of the T3 transgenic plants flowered at least as early as wild type, and some individuals flowered earlier than wild type under these conditions (Table 1). This analysis confirmed that cosmids B and C can correct the effect of the *co-2* mutation.

Flowering Time under SD Conditions of Plants Carrying *CO* Transgenes

Under SD conditions, wild-type plants and *co-2* homozygotes both flower at approximately the same time (Table 1), suggesting that the product of the *CO* gene is not required for flowering under these conditions. However, under SD conditions, several of the *co-2* families carrying the T-DNAs derived from cosmids B and C flowered earlier than both the parental *co-2* line and wild type (Table 1). In particular, three lines (2, 4, and 5) carrying cosmid C flowered much earlier than wild type. This suggested that in some families, transgenic copies of *CO* caused early flowering under SDs, and therefore that the level of the product of *CO* is limiting under these conditions.

Cosmid B was also introduced into wild-type plants, and T2 plants homozygous for the transgene at a single locus were identified in the same way as described above (Table 1). Of the three independent transformants analyzed in the

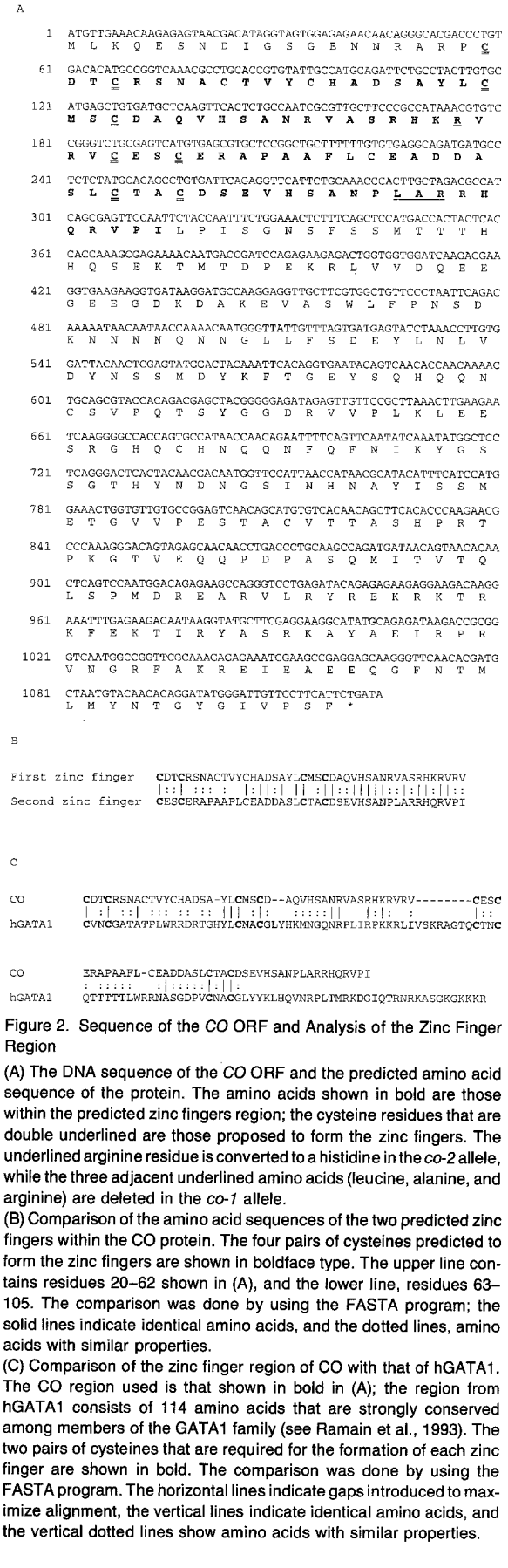

Figure 2. Sequence of the *CO* ORF and Analysis of the Zinc Finger Region

(A) The DNA sequence of the *CO* ORF and the predicted amino acid sequence of the protein. The amino acids shown in bold are those within the predicted zinc fingers region; the cysteine residues that are double underlined are those proposed to form the zinc fingers. The underlined arginine residue is converted to a histidine in the *co-2* allele, while the three adjacent underlined amino acids (leucine, alanine, and arginine) are deleted in the *co-1* allele.

(B) Comparison of the amino acid sequences of the two predicted zinc fingers within the CO protein. The four pairs of cysteines predicted to form the zinc fingers are shown in boldface type. The upper line contains residues 20–62 shown in (A), and the lower line, residues 63–105. The comparison was done by using the FASTA program; the solid lines indicate identical amino acids, and the dotted lines, amino acids with similar properties.

(C) Comparison of the zinc finger region of CO with that of hGATA1. The CO region used is that shown in bold in (A); the region from hGATA1 consists of 114 amino acids that are strongly conserved among members of the GATA1 family (see Ramain et al., 1993). The two pairs of cysteines that are required for the formation of each zinc finger are shown in bold. The comparison was done by using the FASTA program. The horizontal lines indicate gaps introduced to maximize alignment, the vertical lines indicate identical amino acids, and the vertical dotted lines show amino acids with similar properties.

T3 generation, one flowered slightly earlier than wild-type plants under LD and significantly earlier under SD conditions (line 2, Table 1). This again indicated that at least at some chromosomal locations, extra copies of the *CO* gene can cause early flowering.

Identification and Analysis of the *CO* Gene Sequence

The observation that both cosmids B and C were able to correct the co phenotype suggested that the gene was located in the 6.4 kb region of overlap between the two cosmids. No cDNAs that were completely contained within the region were identified (Experimental Procedures). The sequence of the 6.4 kb region was therefore determined.

To identify exons within this region, the sequence was analyzed by use of the GRAIL program (Uberbacher and Mural, 1991). The two longest open reading frames (ORFs) (792 bp and 474 bp) identified were both on the same strand and separated by only 51 bp, suggesting that these two ORFs might be exons of one gene. We reasoned that if these ORFs are part of *CO*, then their sequence might be altered in *co* mutants. This region was then sequenced in the *co-1* and *co-2* lines. A deletion of 9 bp that would precisely remove three amino acids from the predicted protein was identified in *co-1*. This mutation was originally isolated by Redei (1962) after X-ray mutagenesis, which is consistent with it being a deletion allele. A G→A transition was detected in *co-2*, and this is predicted to cause a change of an arginine to a histidine. The occurrence of these mutations strongly suggested that at least part of the 792 bp ORF was an exon of the *CO* gene.

To determine the gene structure, a *CO* cDNA for the gene was identified by using reverse transcription–polymerase chain reaction (RT–PCR). This contains a 1122 bp ORF that is derived from both ORFs identified in the genomic sequence by removal of a 233 bp intron. Translation of the ORF is predicted to form a protein containing 373 amino acids with a molecular mass of 42 kDa (Figure 2). An in-frame translation termination codon is located three codons upstream of the ATG, indicating that the entire translated region was identified. The 3′ end of the transcript was located by sequencing four fragments produced by 3′ rapid amplification of cDNA ends (RACE). They all contained the poly(A) tail at different positions within 5 bases of each other.

Available data bases were searched for proteins sharing homology with the predicted translation product of the *CO* gene. Searching the PROSITE directory detected no motifs within the CO protein, and a FASTA search comparing the CO protein sequence with those in GenBank detected no significant homologies. Direct comparison of the *CO* sequence with that of *LD*, the other flowering time gene cloned from Arabidopsis (Lee et al., 1994), detected no homology. However, analysis of the protein sequence by eye identified a striking arrangement of cysteine residues that is present in two regions near the amino terminus of the CO protein. Each of these regions contains four cysteines in a $C\text{-}X_2\text{–}C\text{-}X_{16}\text{–}C\text{-}X_2\text{–}C$ arrangement, which is similar to the zinc finger domains of GATA-1 transcription factors ($C\text{-}X_2\text{–}C\text{-}X_{17}\text{–}C\text{-}X_2\text{–}C$). Comparison of two 43 amino acid stretches that are directly adjacent to each other within the predicted CO protein sequence and each of which contains one of the proposed zinc fingers indicates striking homology: 46% of the amino acids are identical, and 86% are either identical or related (Figure 2B). The conservation is most apparent on the carboxyl side of each finger, which is again reminiscent of GATA1 transcription factors, in which this region is a basic domain required for DNA binding and is highly conserved (Ramain et al., 1993). In the CO protein, this region is also positively charged: there is a net positive charge of 6 in the region adjacent to the amino finger and of 3 in the one next to the carboxyl finger. Use of the FASTA program to compare the protein sequence of the CO zinc fingers with 116 amino acids that contain the zinc fingers of hGATA1 and are conserved among members of the GATA1 family (see Ramain et al., 1993) identified one 81 amino acid region of homology that spans both zinc fingers of CO and aligns the cysteines of the zinc fingers of hGATA1 and those of CO. However, between these regions of CO and hGATA1, only 21% of the amino acids are identical, and 65% are similar or identical (Figure 2C). A further indication that these regions are important for *CO* activity is that both the *co-1* and *co-2* mutations affect residues that are conserved between the proposed finger regions (Figure 2A).

CO mRNA Is Present in Shoots and Leaves, and Its Abundance Is Reduced in SD-Grown Plants

The onset of flowering requires substances produced in the leaves and genes that act on the flanks of the shoot meristem in the cells that will form floral primordia (Bernier et al., 1993; Weigel et al., 1992). The location of the *CO* mRNA was examined to determine whether its position suggested a role for *CO* in one of these stages of the flowering process. The *CO* mRNA is extremely rare; no cDNAs were found in the libraries screened (Experimental Procedures), and it was not detected on Northern blots of poly(A) RNA (data not shown). RT–PCR was therefore used to detect the transcript.

Total RNA was extracted from leaves of LD-grown seedlings at the 3–4 leaf stage and from the stems of the seedlings after the leaves were removed. RT–PCR was then performed on these RNA preparations. The PCR was terminated after 30 cycles, because at this stage, amplification was still occurring exponentially (Experimental Procedures). The products of the reaction were separated on an agarose gel, transferred to a filter, and hybridized to a *CO*-specific probe. A hybridizing fragment of similar intensity was detected in samples derived from both RNA samples (Figure 3), indicating that *CO* mRNA is present in both leaves and shoots.

co mutations do not delay flowering under SDs, suggesting that *CO* is not required to promote flowering under these conditions (Table 1). To determine whether the absence of *CO* function under SDs is reflected in the abundance of *CO* mRNA, total RNA was extracted from LD- and SD-grown seedlings at the 2–3 leaf stage, and RT–PCR was performed. The *CO* mRNA was found to be reproducibly more abundant in the samples derived from LD-grown seedlings (Figure 3).

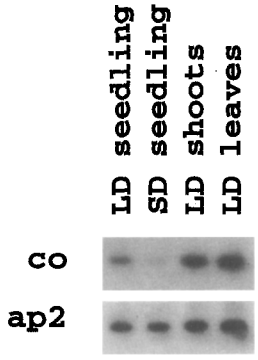

Figure 3. Detection of the *CO* mRNA by RT–PCR

RNA preparations were made from total seedlings, shoots, or leaves and used to synthesize cDNA. This was amplified by PCR using primers specific for either *CO* or *AP2*. *AP2* is expressed in all tissues tested (Jofuku et al., 1994). The amplified fragments were separated on an agarose gel, transferred to a filter, and hybridized to either a *CO* or an *AP2* probe. The abundance of *CO* mRNA between LD and SD conditions was measured in four independent experiments, and the reduction in expression was detected in each case.

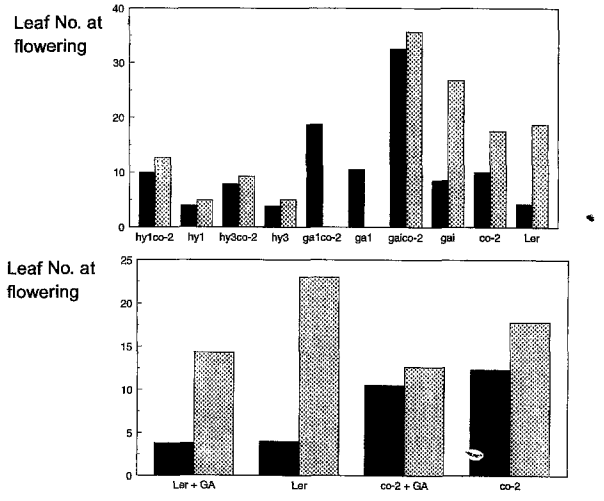

Figure 4. Flowering Time of Wild-Type and Mutant Plants under LD and SD Conditions

(Top) Flowering time of mutant and wild-type plants under LD and SD conditions. The genotype tested is shown along the horizontal axis, and the mean leaf number at flowering is plotted on the vertical axis. Dark columns show flowering time under LD conditions; light columns are those under SD conditions. Only those plants that flowered were included in the data: 13% of the *co-2 gai* plants and 35% of the *co-2 ga1* plants under LD conditions and 8% of the *co-2 gai* plants under SD conditions did not flower. *ga1* was only grown under LD conditions, as it had been previously shown not to flower under SD conditions. (Bottom) Flowering time of wild-type and *co-2* plants under LD and SD conditions. Plants were grown with or without the addition of GA. The histogram is plotted in the same way as the upper one.

ga1-3 and *gai* Enhance the Effect of *co-2* on Flowering under LD Conditions

Mutations that affect synthesis or sensitivity to the plant hormone GA were previously shown to delay flowering mainly under SD conditions (Wilson et al., 1992), while *co* mutations delay flowering only under LD conditions. This suggested that mutations affecting GA metabolism might have a more severe effect under SDs, because *CO* function is absent under these conditions, and that under LDs, the function of *CO* might partially overlap with that of genes involved in the GA system. To test this, double mutants were constructed containing *co-2* and a mutation affecting GA synthesis (*ga1-3*) or one reducing sensitivity to GA (gai).

The *ga1-3* allele affects GA biosynthesis at an early stage in the pathway and is caused by a deletion that removes most of the gene (Sun et al., 1992). Plants homozygous for *ga1-3* do not germinate unless supplied with GA, have short internodes and poorly developed petals and stamens, and do not flower under SD conditions (Koornneef and van der Veen, 1980; Wilson et al., 1992). A double mutant carrying *co-2* and *ga1-3* was constructed and its flowering time compared with that of *ga1-3* homozygotes under LD conditions. The double mutant flowered later than either parental line, and around 35% of the double mutants never flowered (Figure 4). The *ga1-3* mutation therefore increases the severity of the late-flowering phenotype associated with *co-2* under LD conditions.

gai mutants resemble plants homozygous for mutations that have a relatively weak effect on GA biosynthesis: plants germinate normally but are dwarfed, dark green, and late flowering, particularly under SD conditions (Peng and Harberd, 1993; Wilson et al., 1992). However, the gai phenotype cannot be corrected by supplying GA, and although the function of the product of *GAI* is unknown, the gene has been postulated to encode a protein involved in GA signal transduction (discussed by Peng and Harberd, 1993). *gai* delayed flowering under both LD and SD conditions (Figure 4). In addition, the phenotype of *gai* mutants was more severe under SD conditions: the internodes of the bolting stem did not elongate, the flowers often did not open, the anther filaments and petals did not extend to their normal length, the plants were usually infertile, and the leaves developed a wrinkled morphology (Figure 5; Wilson et al., 1992).

The flowering time of *co-2 gai* double mutants was tested under LD conditions. Of these plants, 13% did not flower. The remaining *co-2 gai* double mutants flowered much later than either the *co-2* or *gai* parental lines (see Figure 4).

co-2 enhanced phenotypes, other than late flowering, that are associated with *gai*. For example, the bolting stems of *co-2 gai* plants often did not elongate and were shorter than those of *gai* mutants, the petals and stamens did not develop, and the flowers were sterile, although in some individuals the floral phenotype was restored to that of wild type in the later flowers (Figure 5). *co-2 gai* double mutants therefore resembled *gai* plants growing under SD conditions and mutants that are more severely affected in the GA system, such as *ga1* (Wilson et al., 1992). The phenotype of the *co-2 gai* double mutant suggests that in a *gai* background, *CO* influences characteristics such as internode elongation that apparently do not require *CO* activity in wild-type plants and suggests that *CO* and *GAI* might have overlapping functions.

Under SD conditions, the *co-2* mutation has little effect on the gai phenotype (Figures 4 and 5).

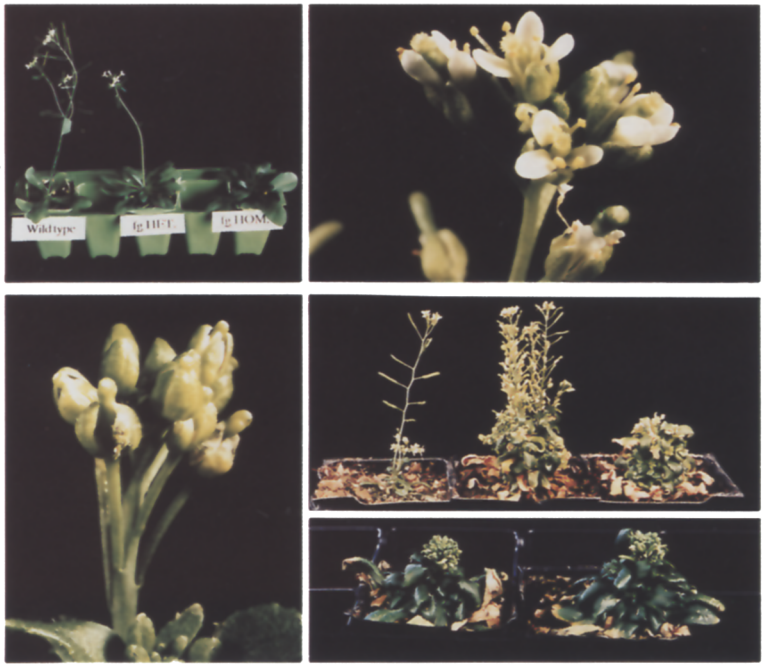

Figure 5. Phenotype of *co* and *gai* Mutant Plants

(Top left) Landsberg erecta, a *co-2* heterozygote, and a *co-2* homozygote. All plants are growing under LD conditions and are the same age.

(Top right) Flowers of a *gai* homozygote. Petals and anthers have developed normally, and the flowers are fertile.

(Bottom left) Flowers of a *co-2 gai* double mutant. The petals and stamens have not extended, and the flowers are infertile.

(Middle right) A *gai* homozygote on the left, and two *co gai* mutants that are approximately 2 months older than the *gai* plant. Both double mutants produced a mass of rosette leaves prior to flowering; the one on the right never bolted, and the internodes remained short, while the plant in the center eventually bolted and produced a few fertile flowers late in development.

(Bottom right) Two *gai* plants growing under SD conditions. These plants never bolted and were not fertile.

Supplying GA Has Little Effect on the Phenotype of *co-2* Mutants

Previous reports that supplying exogenous GA to Arabidopsis can promote flowering (Langridge, 1957) suggested that *CO* might simply be required to activate the transcription of genes encoding enzymes required for GA biosynthesis. If this were the case, then the late-flowering phenotype of the mutant might be corrected by supplying the hormone. Wild-type plants and individuals homozygous for *co-2* were grown under LD and SD conditions and sprayed with 10^{-4} M GA3 once a week from soon after germination until appearance of the flower bud. This treatment was previously shown to correct the severe dwarf phenotype of *ga1* mutants (Koornneef and van der Veen, 1980). Under LD conditions, both genotypes flowered slightly earlier when supplied with GA3 (see Figure 4). Nevertheless, even after the addition of GA3, *co-2* mutants flowered later than wild-type. Under SD conditions, the addition of GA3 reduced the time to flowering of wild type and *co-2* mutants, suggesting that GA levels are limiting on flowering time under these conditions (see Figure 4).

Early Flowering of Mutants Affected in Phytochrome Activity Requires *CO* Activity

The *phyB* mutation is within the gene encoding the photoreceptor phytochrome B, and the *hy1* mutation affects the synthesis of the chromophore thought to be used by all forms of phytochrome (Reed et al., 1993; Parks and Quail, 1991). These mutations cause early flowering, particularly under SD conditions (Goto et al., 1991), suggesting that phytochrome B is involved in repressing flowering. We constructed double mutants containing *co-2* and *hy1* or *phyB* to determine whether *CO* is required for the early flowering of the phytochrome mutants under SD conditions.

The double mutants flowered earlier than wild-type and *co-2* mutant plants under SD conditions, but considerably later than either *phyB* or *hy1* (see Figure 4). Therefore, in contrast with its effect in wild-type plants, *CO* activity is required under SD conditions for the early-flowering phenotype of *hy1* and *phyB*. This suggests that phytochrome B might normally be involved in reducing *CO* activity under SD conditions.

Under LD conditions, *co-2 hy1* and *co-2 phyB* plants flowered slightly earlier than *co-2* mutants, but later than either wild-type or *phyB* mutants (see Figure 4), indicating that *CO* is also required for the early flowering of the *hy1* and *phyB* mutants under LD conditions.

The Phenotype of *co lfy* Double Mutants

Mutations in the *LFY* gene affect the transition from the inflorescence meristem to the floral meristem, with the result that mutant plants produce a higher number of secondary inflorescences subtended by cauline leaves than wild-type plants (Huala and Sussex, 1992; Weigel et al., 1992). *LFY* is also required for floral organ development (Weigel and Meyerowitz, 1993). The *LFY* mRNA is first detectable at an early stage in floral primordium development, suggesting that the gene might be activated by gene products controlling floral induction (Weigel et al., 1992). We reasoned that if activity of the *CO* gene is required directly or indirectly to activate *LFY*, then the phenotype of plants homozygous for a weak *lfy* allele might be enhanced in individuals also homozygous for *co*.

Under LD conditions, plants homozygous for *lfy-5* or *lfy-6*, weak and strong alleles, respectively, produced a higher number of secondary inflorescences than either wild-type plants or *co-2* homozygotes (Table 2). However, the *co-2 lfy-5* double mutants produced three times more secondary inflorescences than either parent (Table 2). The

Table 2. Effect of *lfy-5* on Number of Secondary Inflorescences of *co-2* Mutants, and of *lfy-5* and *lfy-6* on Number of Secondary Inflorescences of Wild-Type Plants under LD and SD Conditions

Genotype	Flowering Time under LD Conditions	Number of Secondary Inflorescences under LD Conditions[a]	Flowering Time under SD Conditions	Number of Secondary Inflorescences under SD Conditions[b]
Landsberg erecta	4.9 ± 0.34; n = 18	2.6 ± 0.86; n = 18	18.7 ± 2.2; n = 19	5.8 ± 1.2; n = 18
co-2	9.9 ± 0.55; n = 46	3.7 ± 0.41; n = 45	24.3 ± 2.8; n = 3	9.8 ± 1.3; n = 4
lfy-5	5.6 ± 0.55; n = 20	6.2 ± 0.77; n = 18	31.6 ± 2.1; n = 20	37.8 ± 3.8; n = 18
lfy-6	5.5 ± 0.55; n = 6	8.8 ± 2.6; n = 6	Not scored[c]	64.3 ± 18.1; n = 3
co-2 lfy-5[b]	9.9 ± 0.68; n = 20	21.4 ± 3.4; n = 20	—[d]	—
co-2 lfy-5[e]	11.4 ± 1.1; n = 9	18.3 ± 7.6; n = 9	—	—

[a] Only secondary inflorescences subtended by cauline leaves are included. Some *lfy* and *co lfy* plants also included a small number of secondary inflorescences that were not subtended by cauline leaves.
[b] Progeny of *co-2/co-2 lfy-5/+* plants.
[c] The high number of secondary inflorescences made it difficult to assess leaf number at flowering.
[d] These genotypes were not grown under these conditions.
[e] Progeny of *co-2/+ lfy-5/lfy-5* plants.

co-2 mutation therefore greatly enhances this aspect of the lfy-5 phenotype. The effect of the *lfy-5* mutation on floral development is also greatly enhanced by the *co-2* mutation. *lfy-5* plants give rise to floral structures similar to those of wild-type plants, except that fewer petals and stamens are formed, and fertility is reduced (Figure 6). However, the main stem and the secondary shoots of *co-2 lfy-5* double mutants terminated in an inflorescence of leaf-like organs (Figure 6). On some secondary inflorescences of the double mutants, tertiary inflorescences bearing four or five flowers occasionally emerged late in development, and some of these flowers were fertile (Figure 6). The phenotype of the *co-2 lfy-5* double mutant was more extreme than that caused by the severe *lfy-6* allele (Table 2; Figure 6), suggesting that the *co* mutation was not simply enhancing the phenotype of *lfy-5* by reducing residual *LFY* activity.

Inflorescence development of *lfy-5* and *lfy-6* mutants under SD conditions was affected similarly to that of *co-2 lfy-5* double mutants under LD conditions: the number of secondary inflorescences was increased, and the stem terminated in an inflorescence of leaf-like organs (Figure 6; Huala and Sussex, 1992; Schultz and Haughn, 1993). This result is consistent with low levels of *CO* function under SD contributing to the extreme lfy phenotype seen under these conditions. However, under SD conditions, *lfy-5* plants produced more fertile flowers on tertiary inflorescences than were observed on the *co-2 lfy-5* plants (Figure 6).

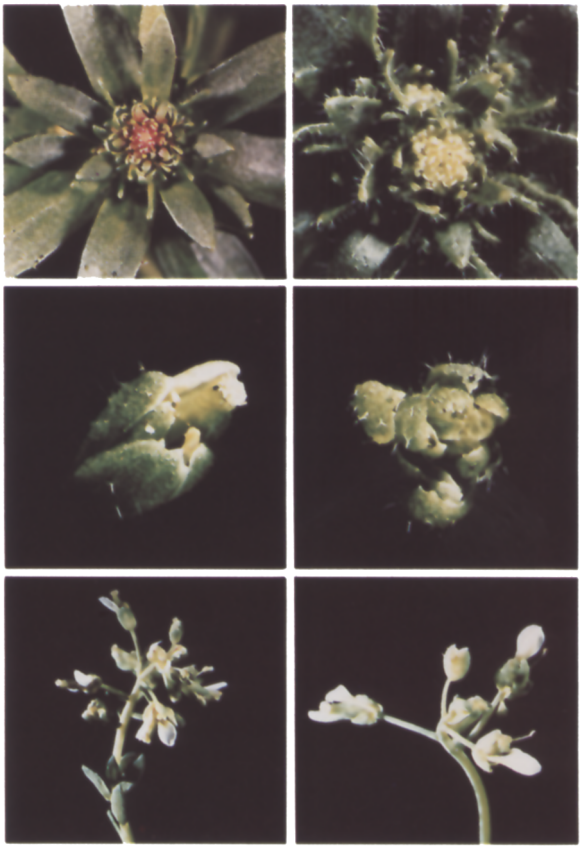

Figure 6. The Phenotype of *lfy* and *co-2 lfy-5* Plants

(Top left) The terminal inflorescence of a *lfy-5* plant growing under SD conditions. Only a disorganized mass of small leaf-like structures are visible; no floral organs were detected.
(Top right) The terminal inflorescence of a *co-2 lfy-5* plant growing under LD conditions, showing the similarity to the *lfy-5* plant growing under SD conditions.
(Middle left) A typical flower of a plant homozygous for the weak *lfy-5* allele growing under LD conditions.
(Middle right) A typical flower of a plant homozygous for the strong (*lfy-6*) allele growing under LD conditions.
(Bottom left) Fertile flowers on a tertiary inflorescence of a *lfy-5* homozygote growing under SD conditions.
(Bottom right) Floral structures on a tertiary inflorescence of a *co-2 lfy-5* double mutant growing under LD conditions.

Discussion

The Protein Encoded by the *CO* Gene Contains Putative Zinc Finger Motifs

The *CO* sequence shows features in common with zinc finger transcription factors (Figure 2). The spacing of the cysteines within the putative CO zinc fingers is most similar to those found in members of the GATA1 family (Sánchez-García and Rabbitts, 1994). GATA1 was first identified as being required to promote the expression of globin genes in humans (Pevny et al., 1991) and contains two zinc fingers of the $C\text{-}X_2\text{–}C\text{-}X_{17}\text{–}C\text{-}X_2\text{–}C$ type adjacent to each other. A family of proteins related to GATA1 and

showing around 85% homology over 116 amino acids across the zinc fingers was identified in human, mouse, chick, Drosophila melanogaster and Caenorhabditis elegans (discussed by Ramain et al., 1993; Sánchez-García and Rabbitts, 1994), and related proteins containing only one zinc finger were identified in fungi and tobacco (Kudla et al., 1990; Daniel-Vedele and Caboche, 1993). CO does not show the degree of identity to GATA1 that is characteristic of members of the family, so it could be considered a divergent member of the GATA1 family or as representing a novel family of zinc finger–containing proteins (Figure 2; Sánchez-García and Rabbitts, 1994).

CO Promotes Flowering of Wild-Type Plants under LD Conditions in a Gene Dosage–Dependent Manner

The *co* mutant flowered later than wild type under LD but at the same time as wild type under SD conditions. *co* therefore showed a reduced response to photoperiod, flowering only slightly earlier under LD conditions than SD.

Plants that are heterozygous for a wild-type allele and either *co-1* or *co-2* flower at a time intermediate between *co* homozygotes and Landsberg erecta under LD conditions (Koornneef et al., 1991; F. R., unpublished data; Figure 5). The sequence of the *co-1* and *co-2* alleles indicates that they are caused by an in-frame deletion of three amino acids and an alteration to a single amino acid, respectively (Figure 2). This suggests two models for the partial dominance of *co*. First, the mutant alleles give rise to altered products that interfere with floral induction, or, second, the mutations cause loss of function, and the 2-fold reduction in the level of the CO protein in a heterozygote leads to a delay in flowering time (haploinsufficiency). We prefer the haploinsufficiency argument for the following reasons. In the complementation experiments, transgenic plants containing two copies of cosmids B or C and homozygous for the *co-2* allele often flowered at the same time as wild-type plants under LD conditions. If the mutant allele encoded a product that interfered with the activity of the wild-type protein, then this would not be expected to occur. Moreover, the need to use RT–PCR to detect the *CO* transcript suggests that it is present at very low levels, which is consistent with the possibility that further reductions in transcript level cause late flowering.

Assuming that these mutant alleles simply encode products with no or reduced activity, then the flowering time data are consistent with the conclusion that the product of the *CO* gene is required to promote flowering under LD but not under SD conditions. The eventual flowering of *co* mutants under LD conditions could be due to genes with a redundant function partially compensating for loss of *CO* activity, or because the *co* alleles that are available are not complete nulls.

That CO protein promotes flowering is further supported by the observation that increases in the dosage of *CO* can lead to slightly earlier flowering under LD conditions. This was concluded from the observation that some of the transgenic lines carrying extra copies of the *CO* gene flowered slightly earlier than wild-type plants (Table 1). This observation, together with the haploinsufficiency phenotype discussed above, suggests that the level of expression of *CO* is a critical determinant of flowering time of Arabidopsis under LD conditions.

Substances required to promote flowering have previously been reported to be formed in the leaves (reviewed by Bernier et al., 1993), while genes that act at the shoot apex to carry out the early stages of flower development have been isolated and their expression analyzed. *CO* mRNA was detected in both leaves and shoots, and therefore its function in promoting flowering cannot readily be assigned to one or other of these organs. It is possible that *CO* function is required in both organs, or that the *CO* gene is expressed in cells in which *CO* gene function is not required. The identification of those cells in which *CO* function is required to promote flowering will require the construction of mosaic plants in which the gene is only expressed in defined sectors.

The Level of *CO* Activity Is a Limiting Factor on Flowering Time under SD Conditions

Some of the lines carrying extra copies of the *CO* gene flowered much earlier than wild-type plants under SDs (Table 1). The level of *CO* activity is therefore limiting on the flowering time of wild-type plants under SDs, and potentially, therefore, regulation of *CO* function contributes to the facultative LD phenotype of Arabidopsis. This is supported by the observation that the abundance of *CO* mRNA is lower in SD- than in LD-grown seedlings. As discussed above, the haploinsufficiency phenotype of LD-grown *co/CO* heterozygotes indicates that flowering time is sensitive to reductions in the level of the product of *CO*, and therefore it is very likely that the reduction in the level of *CO* mRNA in SD-grown plants would lead to a delay in flowering.

The observation that increasing the dosage of *CO* under SD conditions caused early flowering suggested that mutations that cause early flowering under SDs could do so by increasing *CO* activity. This is supported by our observation that early flowering of *phyB* and *hy1* mutants under SDs requires *CO* activity, while under these conditions the flowering time of wild-type plants is unaffected by *co-2* mutations. Phytochrome B might therefore delay flowering of wild-type plants under SDs by reducing *CO* activity directly, or by indirectly antagonizing its effect on flowering time.

co Has an Effect Similar to That of SD Conditions in Enhancing the Phenotype of Mutations Affecting GA Metabolism or Meristem Identity

The *ga1-3* mutation, a deletion in a GA-biosynthetic gene, enhanced the *co* mutant phenotype. The first step of GA biosynthesis is promoted by LD conditions in spinach plants (Zeevaart and Gage, 1993); therefore, one interpretation of the co ga1 phenotype is that *CO* is required for elevated rates of GA biosynthesis in Arabidopsis. However, the co phenotype could not be corrected by the addition of exogenous GA3, although the amounts applied do correct the effect of GA-biosynthetic mutations on stem elongation (Koornneef and van der Veen, 1980), and GA3

did promote flowering of wild-type plants and *co* mutants under SD conditions.

The phenotype of the *co gai* double mutant under LD conditions is more extreme, both in flowering time and GA responses, than would be expected from simple addition of the parental genotypes. Thus, *gai* has an extreme effect on flowering under LD conditions that is only uncovered in a *co* background. In addition, the effect of *co* on GA-related processes such as stem elongation is only revealed in a genetic background such as *gai* that is compromised for GA responses. *CO* may therefore have a role in GA signal transduction similar to that affected by *gai*, so that mutation of either one of these genes is partially compensated by the activity of the other one. That there is a relationship between *CO* and *GAI* is further implied by the observation that the co gai phenotype under LD conditions is similar to the phenotype of *gai* mutants under SD conditions. Since genetic and molecular analyses suggest that *CO* is more active under LD than SD conditions, the extreme phenotype of *gai* mutants under SDs could be a consequence of a reduction in *CO* activity.

The *co-2* mutation greatly enhanced the phenotype caused by the weak *lfy-5* mutation. The phenotype of the *co lfy-5* double mutant is more severe than that of the strong *lfy-6* allele, which is very likely to be a null allele, as it contains a translational stop after 32 codons (Weigel et al., 1992). This suggests that the enhancement of the lfy-5 phenotype is not simply due to *co* reducing residual *LFY* activity of *lfy-5*. However, in addition to *LFY*, at least three other genes, *APETALA1* (*AP1*), *AP2*, and *CAULIFLOWER* (*CAL*), are required for the development of flowers on the inflorescence meristem. These gene products are redundant to different degrees: *cal* mutations are almost undetectable in an *AP1* wild-type background, but the *cal ap1* double mutant has a more severe phenotype than *ap1*, and *lfy ap1* double mutants show a more severe phenotype than either parental line (Huala and Sussex, 1992; Bowman et al., 1993). The phenotypes of double and triple mutants led several groups to suggest that the meristem identity genes *LFY*, *AP1*, and *AP2* positively regulate each other (Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). That *co-2 lfy-5* double mutants show a more severe phenotype than *lfy-6* can therefore be explained if *CO* function is required to activate more than one of these meristem identity genes. The enhancement of the phenotype of *lfy* mutants that is detected under SD conditions (Huala and Sussex, 1992; Schultz and Haughn, 1993), and the similarity between *lfy-5* SD-grown plants and *co lfy-5* LD-grown plants, would suggest that the strong lfy-5 phenotype under SDs is partly caused by a lack of *CO* function under these conditions.

Although the *co-2 lfy-5* double mutant has a strong effect on floral morphology, the flowers of *co-2* mutants are indistinguishable from those of wild-type plants. A reduction in *LFY* activity is therefore required to detect the effect of the *co-2* mutation on floral morphology. This could be because *CO* function is required to activate other floral meristem identity genes, and this function of *CO* and *LFY* is partially redundant, or because none of the *co* alleles are amorphic, and residual *CO* function is sufficient to activate the meristem identity genes in *co* mutants.

A Model for the Role of *CO* during Floral Induction

On the basis of the genetic interaction between *gai* or *ga1* and *co*, and the failure of exogenous GA3 to correct the co phenotype, we propose that *CO* has a function related to that of gene products required for GA signal transduction and that in wild-type plants its major role is to promote flowering under LD conditions. It is also likely that *CO* is involved in other GA-related processes, but that this cannot be detected in the available *co* mutants, owing to functional redundancy with other genes. This is supported by the observation that *co* enhances the reduced stem elongation phenotype of *gai*.

The presence of the zinc fingers suggests that the CO protein binds DNA. It is likely therefore that *CO* is required to affect transcription, either as a transcriptional activator or repressor. Our interpretation is that *CO* function is required to promote flowering. In previous models for *CO* action, it was proposed that *CO* is required to antagonize the activity of inhibitors of flowering encoded by genes such as *EMBRYONIC FLOWER* (*EMF*) (Martinez-Zapater et al., 1994; Sung et al., 1992). This is not disproven by our data, and it is possible that *CO* has an inhibitory role rather than our simpler proposal that it is required to activate the transcription of other genes involved in the transition from vegetative growth to flowering. The strong interaction between *lfy-5* and *co* suggests that *CO* might be required to activate directly the transcription of floral meristem identity genes, although it is also possible that this interaction is less direct.

Experimental Procedures

Growth Conditions and Measurement of Flowering Time

Flowering time was measured under defined conditions by growing plants in Gallenkamp controlled environment rooms at 20°C. SDs comprised a photoperiod of 10 hr lit with 400 W metal halide power star lamps supplemented with 100 W tungsten halide lamps. This provided a level of photosynthetically active radiation of 114 μmol of photons/m^2/s and a red:far red light ratio of 2:4. A similar cabinet was used for LD conditions, with 10 hr under the conditions described above and extended for a further 8 hr using only the tungsten lamps.

The flowering times of large populations of plants were measured in the greenhouse. In the summer, the plants were grown in sunlight. In winter, supplementary light was provided so that the minimum day length was 16 hr.

Flowering time was measured by counting the number of leaves, excluding the cotyledons, in the rosette at the time the flower bud was visible. Mean leaf numbers are shown with the standard error at 95% confidence limits. The number of days from sowing to the appearance of the flower bud was also recorded. The close correlation between leaf number and flowering time was previously demonstrated for Landsberg erecta and *co* alleles (Koornneef et al., 1991).

Plant Material

The standard wild-type genotype used was Arabidopsis thaliana Landsberg erecta. The *co-1* mutation was isolated by Redei (1962) and is in an *ERECTA* background that in our experiments showed no sequence variation from Landsberg erecta. All other mutations are in Landsberg erecta. The details of the lines used for the RFLP mapping of *co* have been described previously (Putterill et al., 1993).

All lines carrying *co-2* also carried *tt4*. The *tt4* mutation prevents anthocyanin accumulation in the seed coat but does not affect flow-

ering time. The mutation is approximately 3.3 cM from *co* (Putterill et al., 1993).

Construction of Double Mutants

Double mutants were usually made by crossing lines homozygous for each of the mutations, identifying F2 plants homozygous for one of the mutations, self-fertilizing several of these F2 plants, and identifying F3 families in which phenotypes characteristic of the second mutation were visible. Further information on the construction of particular double mutants can be obtained from the authors.

RNA Extractions

RNA was extracted by use of a method described by Stiekema et al. (1988).

Isolation of cDNA and Detection of mRNA by RT-PCR

For first-strand cDNA synthesis, 10 μg of total RNA was used, and cDNA synthesis was primed by using the standard dT_{17} adapter primer as described by Frohman et al. (1988).

The product of the first-strand synthesis reaction was then used for PCR with the primers *CO49* (5′-GCTCCCACACCATCAAACTTACTAC, located 38 bp upstream of translational start) and *CO50* (5′-CTCCTCGGCTTCGATTTCTC, located 57 bp upstream of translational termination codon). Two independently amplified cDNAs were ligated into the EcoRV site of Bluescript (Stratagene) and sequenced.

The conditions described above were also used to detect the *CO* mRNA. A primer spanning the *CO* intron (5′-CATTAACCATAACGCATACATTTC, where the position of the intron is indicated by the hyphen) was used for PCR along with *CO50* (described above). The use of a primer spanning the intron prevented small amounts of contaminating Arabidopsis DNA contributing to the abundance of the amplified fragment. Amplification of *AP2* mRNA was used as a control to ensure that equal amounts of cDNA were added to each PCR. The primers used to amplify *AP2* cDNA were 5′-CTCAATGCCG-AGTCATCAGG (this spans an intron, and its position is indicated by the hyphen) and 5′-CATGAGAGGAGGTTGGAAGC. For both *CO* and *AP2* cDNA, the PCR was terminated while the amplification was still occurring exponentially. In both cases, the PCR was performed for 25, 30, and 35 cycles. For the *CO* cDNA, the amplified fragment was barely detectable after 25 cycles and was clearly visible after 30 cycles, but after 35 cycles it was only slightly more intense than after 30 cycles. After 35 cycles, the difference in *CO* cDNA abundance under LD and SD conditions was no longer as clear. A similar procedure was used for the *AP2* mRNA, but in this case the amplified fragment was readily detected after 25 cycles.

Isolation of cDNA Fragments by 3′ RACE

First strand cDNA synthesis was performed using the same conditions, RNA preparation and dT_{17} adapter as described above for RT-PCR. The PCR was then performed using the standard adapter primer (5′-gactcgagtcgacatcg; Frohman et al., 1988) and the *CO49* primer described above. A smear of fragments between 1 and 2 kb was excised from an agarose gel and subjected to a second round of PCR using the adapter primer and another *CO*-specific primer (*CO28*, 5′-tgcagattctgcctacttgtgc, located 94 bp downstream of translational start). When this PCR was monitored on an agarose gel, a fragment of 1.3 kb was detected. Four amplified fragments recovered from two independent amplifications were ligated to Bluescript (Stratagene) and sequenced.

Isolation of Clones Containing *co-1* and *co-2* Alleles

Primers *CO41* (5′-ggtcccaacgaagaagtgc, located 263 bp upstream of translational start) and *CO42* (5′-cagggaggcgtgaaagtgt, located 334 bp downstream of translational stop) were used. The DNA sequences of two independently amplified fragments from each allele were determined.

Screening Phage and Cosmid Libraries

Twenty thousand colonies of the cosmid library were screened (Olszewski and Ausubel, 1988). Three cDNA libraries were screened to try to identify a *CO* cDNA. The numbers of plaques screened were 5×10^5 from the aerial parts library (Arabidopsis Stock Center, Max Planck Institute, Cologne), 3×10^5 plaques of a library made from plants growing in sterile beakers (European Union Arabidopsis Stock Center), and 1×10^6 plaques of the CD4-71-PRL2 library (Arabidopsis Biological Resource Center, Ohio State University).

Transformation of Arabidopsis

The cosmids containing DNA from the vicinity of *CO* were mobilized into Agrobacterium tumefaciens C58C1, and the T-DNA was introduced into Arabidopsis plants as described by Valvekens et al. (1988).

Acknowledgments

Correspondence should be addressed to G. C. Our interest in *CO* was inspired by the work of George Redei, who first identified the mutant, and by Maarten Koornneef and colleagues who analyzed its behavior. We are grateful to Justin Goodrich for suggestions on the double-mutant analysis, and to Enrico Coen, Nic Harberd, and Alison Wilson for their comments on the manuscript. We thank George Murphy for providing some of the sequence data and for help in analyzing the sequence. Neil Olszewski kindly supplied the cosmid library. This work was supported by grants from the Biotechnology and Biological Sciences Research Council Plant Molecular Biology Programmes I and II to G. C.

Received October 10, 1994; revised January 20, 1995.

References

Bagnall, D. J. (1993). Light quality and vernalization interact in controlling late flowering in *Arabidopsis* ecotypes and mutants. Ann. Bot. *71*, 75–83.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., and Lejeune, P. (1993). Physiological signals that induce flowering. Plant Cell *5*, 1147–1155.

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M., and Smyth, D. R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development *119*, 721–743.

Clarke, J. H., and Dean, C. (1994). Mapping *FRI*, a locus controlling flowering time and vernalization response. Mol. Gen. Genet. *242*, 81–89.

Coen, E. S., Romero, J. M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *floricaula*: a homeotic gene required for floral development in Antirrhinum majus. Cell *63*, 1311–1322.

Daniel-Vedele, F., and Caboche, M. (1993). A tobacco cDNA clone encoding a GATA-1 zinc finger protein homologous to regulators of nitrogen metabolism in fungi. Mol. Gen. Genet. *240*, 365–373.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA *85*, 8998–9002.

Goto, N., Kumagai, T., and Koornneef, M. (1991). Flowering responses to night breaks in photomorphogenic mutants of *Arabidopsis*. Physiol. Plant. *83*, 209–215.

Gustafson-Brown, C., Savidge, B., and Yanofsky, M. F. (1994). Regulation of the floral homeotic gene *APETALA1*. Cell *76*, 131–144.

Huala, E., and Sussex, I. M. (1992). *LEAFY* interacts with floral homeotic genes to regulate *Arabidopsis* floral development. Plant Cell *4*, 901–913.

Huijser, P., Klein, J., Lönnig, W.-E., Meijer, H., Saedler, H., and Sommer, H. (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *squamosa* in *Antirrhinum majus*. EMBO J. *11*, 1239–1249.

Jofuku, K. D., den Boer, B. G. W., Van Montagu, M., and Okamuro, J. K. (1994). Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. Plant Cell *6*, 1211–1225.

Koornneef, M., and van der Veen, J. H. (1980). Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.). Heynh. Theor. Appl. Genet. *58*, 257–263.

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

Kudla, B., Caddick, M. X., Langdon, T., Martinez-Rossi, N. M., Bennett, C. F., Sibley, S., Davies, R. W., and Arst, H. N., Jr. (1990). The regulatory gene *areA* mediating nitrogen metabolic repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. *9*, 1355–1364.

Langridge, J. (1957). Effect of day-length and gibberellic acid on the flowering of *Arabidopsis*. Nature *180*, 36–37.

Lee, I., Aukerman, M. J., Gore, S. L., Lohman, K. N., Michaels, S. D., Weaver, L. M., John, M. C., Feldmann, K. A., and Amasino, R. M. (1994). Isolation of *LUMINIDEPENDENS*: a gene involved in the control of flowering time in *Arabidopsis*. Plant Cell *6*, 75–83.

Martinez-Zapater, J. M., and Somerville, C. R. (1990). Effect of light quality and vernalization of late-flowering mutants of *Arabidopsis thaliana*. Plant Physiol. *92*, 770–776.

Martinez-Zapater, J. M., Coupland, G., Dean, C., and Koornneef, M. (1994). The transition to flowering in *Arabidopsis*. In *Arabidopsis*, C. R. Somerville and E. M. Meyerowitz, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 403–434.

Napp Zinn, K. (1957). Untersuchungenzur Genetik des Kältebedürfnisses bei Arabidopsis thaliana L. Heynh. Z. Indukt. Abstamm. Vererbungsl. *88*, 253–285.

Olszewski, N., and Ausubel, F. M. (1988). Specialized binary vector for plant transformation: expression of *Arabidopsis thaliana AHAS* gene in *Nicotiana tabacum*. Nucl. Acids Res. *16*, 10765–10782.

Parks, B., and Quail, P. H. (1991). Phytochrome-deficient *hy1* and *hy2* long hypocotyl mutants of *Arabidopsis* are defective in phytochrome chromophore biosynthesis. Plant Cell *3*, 1177–1186.

Peng, J., and Harberd, N. P. (1993). Derivative alleles of the *Arabidopsis* gibberellin-insensitive (*gai*) mutation confer a wild-type phenotype. Plant Cell *5*, 351–360.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S.-F., D'Agati, V., Orkin, S. H., and Constantini, F. (1991). Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature *349*, 257–260.

Putterill, J., Robson, F., Lee, K., and Coupland, G. (1993). Chromosome walking with YAC clones in *Arabidopsis*: isolation of 1700 kb of contiguous DNA on chromosome 5, including a 300 kb region containing the flowering-time gene *CO*. Mol. Gen. Genet. *239*, 145–157.

Ramain, P., Heitzler, P., Haenlin, M., and Simpson, P. (1993). *pannier*, a negative regulator of *achaete* and *scute* in *Drosophila*, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. Development *119*, 1277–1291.

Redei, G. P. (1962). Supervital mutants of *Arabidopsis*. Genetics *47*, 443–460.

Reed, J. W., Nagpal, P., Poole, D. S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell *5*, 147–157.

Sánchez-García, I., and Rabbitts, T. H. (1994). The LIM domain: a new structural motif found in zinc-finger-like proteins. Trends Genet. *10*, 315–320.

Schultz, E. A., and Haughn, G. W. (1993). Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. Development *119*, 745–765.

Shannon, S., and Meeks-Wagner, D. R. (1993). Genetic interactions that regulate inflorescence development in *Arabidopsis*. Plant Cell *5*, 639–655.

Stiekema, W. J., Heidekamp, F., Dirkse, W. G., Vanbeckum, J., Dehaan, P., Tenbosch, C., and Louwerse, J. D. (1988). Molecular cloning and analysis of four potato tuber mRNAs. Plant Mol. Biol. *11*, 255–269.

Sun, T., Goodman, H., and Ausubel, F. M. (1992). Cloning the *Arabidopsis GA1* locus by genomic subtraction. Plant Cell *4*, 119–128.

Sung, Z. R., Belachew, A., Shunong, B., and Bertrand-Garcia, R. (1992). *EMF*, an *Arabidopsis* gene required for vegetative shoot development. Science *258*, 1645–1647.

Uberbacher, E. C., and Mural, R. J. (1991). Locating protein-coding regions in human DNA sequences by a multiple sensor–neural network approach. Proc. Natl. Acad. Sci. USA *88*, 11261–11265.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA *87*, 5536–5540.

Vetrilova, M. (1973). Genetic and physiological analysis of induced late mutants of *Arabidopsis thaliana* (L.) Heynh. Biol. Plant. *15*, 391–397.

Weigel, D., and Meyerowitz, E. M. (1993). Activation of floral homeotic genes in *Arabidopsis*. Science *261*, 1723–1726.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F., and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. Cell *69*, 843–859.

Wilson, R. N., Heckman, J. W., and Somerville, C. R. (1992). Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. Plant Physiol. *100*, 403–408.

Zagotta, M. T., Shannon, S., Jacobs, C., and Meeks-Wagner, D. R. (1992). Early flowering mutants of *Arabidopsis thaliana*. Austr. J. Plant Physiol. *19*, 411–418.

Zeevaart, J. A. D., and Gage, D. A. (1993). ent-kaurene biosynthesis is enhanced by long photoperiods in the long-day plants *Spinacia oleracea L.* and *Agrostemma githago L.* Plant Physiol. *101*, 25–29.