## Distinct Roles of CONSTANS Target Genes in Reproductive Development of *Arabidopsis*

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In plants, flowering is triggered by endogenous and environmental signals. CONSTANS (CO) promotes flowering of *Arabidopsis* in response to day length. Four early target genes of CO were identified using a steroid-inducible version of the protein. Two of these genes, *SUPPRESSOR OF OVEREXPRESSION OF CO* 1 (SOC1) and *FLOWERING LOCUS T* (*FT*), are required for CO to promote flowering; the others are involved in proline or ethylene biosynthesis. The SOC1 and *FT* genes are also regulated by a second flowering-time pathway that acts independently of CO. Thus, early target genes of CO define common components of distinct flowering-time pathways.

The transition from vegetative to reproductive development in plants involves a change in the identity of the apical meristem so that it gives rise to flowers rather than leaves. The timing of this transition is affected by the age of the plant and by environmental conditions (1-3). Genes that control flowering time in Arabidopsis were assigned to different genetic pathways on the basis of mutant phenotypes and epistasis relationships (4, 5). CO acts in a pathway that accelerates flowering in response to long photoperiods (4, 6, 7). Here, we describe the identification of genes that are activated by CO and examine their role in floral induction.

Transgenic Arabidopsis plants in which CO is overexpressed (35S::CO plants) flower early and are insensitive to day length (8, 9). Furthermore, CO function can be regulated in transgenic plants overexpressing a protein fusion of CO to the ligand-binding domain of the rat glucocorticoid receptor (35S::CO:GR plants) (9). Dexamethasone (DEX) activates proteins containing the GR domain by allowing the fusion protein to be imported into the nucleus. Application of DEX to 35S::CO:GR plants rapidly promotes flowering (9). GR fusions to transcription factors that control floral organ identity were previously used to identify their immediate targets (10). When the translational inhibitor cycloheximide (CYC) is applied together with DEX, the fusion protein affects transcript levels of CO's immediate targets, but not those of genes that act further downstream (10). This approach was used to identify early targets of CO.

FT, but not LEAFY, is an early target gene of CO. Previously, DEX application to 35S::CO:GR plants was shown to increase abundance of the mRNAs of the floral meristem identity gene LEAFY (LFY) and the flowering-time gene FT within 24 and 12 hours, respectively (9, 11). These experiments were repeated using shorter response times and including CYC (Fig. 1A). Two hours after DEX application in the presence of CYC, FT was induced from levels that were undetectable on Northern blots to clearly detectable levels, whereas the abundance of LFY mRNA was not affected (Fig. 1A). Therefore, the interaction between CO and LFY is probably indirect, whereas FT is an early target gene of CO.

FT mRNA is also present at higher abundance in 35S::CO plants than in the wild type (Fig. 2). The requirement for FT and LFY in the early-flowering phenotype of 35S::CO was tested genetically. A severe lfy mutant allele (lfy-6) did not delay reproductive development of 35S::COplants, whereas ft mutations caused a significant delay (Table 1) (8). FT is therefore required for the extreme early flowering of 35S::CO plants, but strong ft mutant alleles only partially suppress this phenotype (Table 1) (8). These findings suggest that other genes must also be activated by CO.

A gene encoding a MADS-box transcription factor is an early target of CO. In an effort to discover previously unidentified genes regulated by CO, we used suppression subtraction hybridization (12, 13)to prepare a library enriched with cDNAs of genes that are up-regulated 4 hours after application of DEX and CYC. A procedure was developed to identify cDNA clones within the library that were truly differentially expressed, and the CYC treatments were shown to influence gene expression in a manner consistent with previous reports (10, 13).

A gene present in the library encoded a MADS-box transcription factor first designated AGAMOUS-LIKE 20 (AGL20) and later named SOC1 (see below). The abundance of its mRNA in 35S::CO:GR plants increased by a factor of about 3 after the application of DEX, either alone or in combination with CYC (Fig. 1B). AGL20 mRNA was unaffected by application of CYC alone to 35S::CO:GR plants (Fig. 1B) or by application of DEX and CYC to co plants (13). AGL20 mRNA was also about 10 times as abundant in 35S::CO plants as in wild-type plants (Fig. 2). Nevertheless, no consistent difference in AGL20 mRNA abundance was detected in comparisons of co mutant and wild-type seedlings (Fig. 2) (14). The position of the AGL20 gene is close to that of a mutation causing late flowering that was recently described (8) and called suppressor of overexpression of CO 1 (soc1). The socl mutation partially suppresses the early-flowering phenotype caused by 35S::CO (Table 1) (8). We have now demonstrated that socl is a mutant allele of AGL20. This conclusion is based on the following data. First, the soc1 mutation cosegregated genetically with marker 40E1T7 in 58 F<sub>2</sub> chromosomes, and this marker is only about 100 kb from AGL20 (8, 15). Second, the AGL20 gene in the soc1 mutant contains a mutation that is predicted to truncate the AGL20 protein from 215 to 75 amino acids (13). Third, a transgene containing the AGL20 genomic region accelerated flowering of the socl mutant, but had no effect on wild-type plants (Table 1) (13). In the following sections AGL20 will be renamed SOC1. Overexpression of SOC1 from the 35S promoter caused early flowering under both long-day and short-day conditions (Table 1). The earliest transformants were also sterile. A less extreme, fertile plant was crossed to the co-2 mutant and partially suppressed the late-flowering phenotype of co-2 (Table 1).

Consistent with the role CO plays in the promotion of flowering, SOC1 expression responds to long photoperiods in wild-type plants. SOC1 mRNA was not detected in sections of the shoot apex of plants grown under short-day conditions, but was present in the shoot apical meristem and

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Fig. 1. Effect of 35S::CO:GR transgene on gene expression. Total RNA samples on Northern blots were hybridized with gene-specific probes (13). Ubiquitin mRNA (UBQ10) or ribosomal 18S RNA were loading controls. Similar results were obtained in independent experiments. (A) Effect of 35S::CO:GR transgene





on *LFY* and *FT* expression. Plants were treated with 1  $\mu$ M DEX and/or 10  $\mu$ M CYC as indicated. Start time is the time of day at which the treatment was applied and is represented as the number of hours after dawn. After treatment for 2 hours, plants were immediately harvested. If both DEX and CYC were applied, they were added simultaneously, although pretreatment with CYC gave similar results (*13*). (**B**) An experiment similar to that described in (A), analyzing expression of the *ACS10*,

**Table 1.** Effects of mutations and transgenes on flowering time. Flowering time is presented as the number of leaves (rosette, cauline, and total) formed on the main shoot; plants that flower later form more leaves. At least five plants of each genotype were examined, except 35S::SOC1 #3, which did not

AtPC5B2, and SOC1 genes that were obtained from the library [for methods, see Science Online (13)]. Labeling is as described for (A). Numbers below each signal enable comparison of expression levels for the same gene between different treatments. They were calculated by first normalizing each signal against that for UBQ10 (13) and then against the treatment giving the lowest level of expression for that particular gene.

form seeds. The flowering time of *lfy-6* is measured using rosette leaves only, because this mutation causes the growth of extra cauline leaves. AGL20 T2 plants were selected with the herbicide Challenge (active ingredient glufosinate ammonium) before scoring to identify those that contained the transgene.

Genotype	Long days				Short days			
	Rosette	Cauline	Total	SD	Rosette	Cauline	Total	SD
Landsberg erecta	5.7	2.7	8.4	0.8	31.4	12.2	43.6	2.0
co-2	13.8	6.9	20.7	3.3				
35S::AGL20 #1	5.0	2.3	7.3	0.5	12.3	2.7	15.0	1.6
35S::AGL20 #2	4.0	2.3	6.3	1.0	9.8	2.2	12.0	3.3
35S::AGL20 #3	0.0	2.0	2.0	_	ND	ND	ND	ND
35S::AGL20 #2/co-2	7.0	4.5	11.5					
35S::CO	2.5	2.0	4.5	0.8	2.4	2.0	4.5	0.5
35S::CO/ft-7	5.7	3.1	8.8	0.8				
35S::CO/soc1	4.4	2.5	6.9	0.6				
35S::CO/ft-7/soc1	11.4	3.6	15.0	1.4				
35S::CO/lfy-6	3.0	9.8	12.8	1.3				
lfy-6	5.5	25.3	30.8	4.0				
Col	7.6	2.8	10.3	0.9				
35S:CO in col+	4.4	1.3	5.7	0.5	4.8	1.4	6.1	0.5
35S:CO/ein2-1	5.0	1.9	6.9	0.8				
ein2-1	17.0	6.0	23.0	7.2				
Landsberg erecta	5.5	3.0	8.5	0.7				
soc1	10.6	2.5	13.2	1.0				
AGL20 soc1 #1 T2 <sup>R</sup>	7.8	3.0	10.8	1.2				
AGL20 soc1 #22 T2 <sup>R</sup>	7.9	2.9	10.8	1.4				
AGL20 soc1 #25 T2 <sup>R</sup>	8.0	3.0	10.9	1.0				
AGL20 L.er #1 T2 <sup>R</sup>	5.3	2.8	8.0	1.2				
AGL20 L.er #4 T2 <sup>R</sup>	5.7	2.6	8.3	0.9				
AGL20 L.er #8 T2 <sup>R</sup>	5.5	2.6	8.0	0.6				

leaf primordia 16 hours after the shift to continuous light (Fig. 3A). This response is similar to that of the mustard MADSA gene, whose product shows strong similarity to that of SOC1 (13, 16). These results, taken together with the findings of induction of SOC1 by 35S::CO:GR and partial suppression of the 35S::CO phenotype by the soc1 mutation, demonstrate that CO promotes flowering in part through activation of SOC1.

In floral primordia, SOC1 shows a complex and specific pattern of expression (Fig. 3, B and C). SOC1 mRNA is present in the inflorescence meristem but is excluded from young floral primordia. It reappears in stage 2 to stage 3 flowers (17) in a domain overlapping whorls 3 and 4 that is similar to the pattern of expression of AGAMOUS (18). This pattern suggests additional roles for SOC1 in the regulation of organ identity, although these may be redundant because soc1 has no effect on floral morphology.

The early-flowering phenotype of 35S::CO was only partially suppressed by the *soc1* and *ft* mutations (Table 1) ( $\delta$ ). However, the delay in flowering of

35S::CO plants carrying both mutations was much stronger than in plants carrying only one of them. These *ft soc1* 35S::COplants flowered only slightly earlier than *co-2* mutants (Table 1). The molecular and genetic experiments therefore demonstrate that CO activates *FT* and *SOC1*, and that these then promote flowering in partially redundant pathways.

Early target genes of CO involved in proline or ethylene biosynthesis. Two other genes that were differentially expressed in 35S::CO:GR plants in response to DEX were AtP5CS2 (19), which en-





codes an enzyme controlling the ratelimiting step of glutamate-derived proline biosynthesis, and ACS10, which encodes a putative 1-aminocyclopropane-1carboxylic acid (ACC) synthase involved in ethylene biosynthesis (13). Treatment of 35S::CO:GR plants with DEX and CYC or with DEX alone increased the abundance of ACS10 mRNA by a factor of 3 to 4, and of AtP5CS2 mRNA by a factor of 2 to 3 (Fig. 1B). Transcript levels were not affected by control treatments (Fig. 1B) (13). The rapid induction of the mRNAs in CO:GR plants treated with DEX and CYC shows that these genes are also early targets of CO.

These genes may also have roles in reproductive development (Fig. 4). No mutation affecting ACS10 has been described, but mutations that reduce ethylene signal transduction and cause insensitivity to ethylene, such as ein2-1, also cause late flowering (Table 1) (20, 21). Furthermore, ein2-1 slightly delays flowering of 35S::CO plants (Table 1) (21), and ethylene was proposed to play a role in flower initiation in other plant species (22). AtP5CS2 encodes an enzyme that catalyzes synthesis of a precursor of the amino acid proline (19). Reduction in AtP5CS2 gene expression in transgenic Arabidopsis plants did not delay flowering but prevented elongation of the stem (bolting) that occurs after the appearance of the first floral buds, and this was corrected by supplying the plants with proline (23).

The FT and SOC1 genes are common components of two flowering-time pathways. In addition to the long-day pathway in which CO acts, flowering of Arabidopsis is regulated by a day length-insensitive pathway (the autonomous pathway) (1-3). The autonomous and long-day pathways both regulate the expression of the floral meristem identity gene LFY (24), but they may also converge earlier.





Fig. 3. Temporal and spatial expression of SOC1, as revealed by RNA in situ hybridization probed with SOC1-specific antisense probe (13). (A) Shoot apex sections of 28-day-old Landsberg erecta plants grown under short-day conditions (8 hours light/16 hours dark) and shifted to continuous light at dawn (32). Eight hours after the shift, these plants were first exposed to a change in photoperiod, and this was designated as the zero time point. Left, bright field; middle, dark field; right, bright/dark field double exposure with a red filter to make the hybridization signal appear red. A low level of SOC1 expression was detected at 0 hours. In contrast, at 16 hours SOCT RNA was easily detected in the shoot apex and in the emerging leaf primordia. A lower but significant level of expression was also observed at 8 hours (not shown). (B) Shoot apex sections of seedlings grown under continuous light with bright/dark field double exposure as in (A); d, days after sowing. SOC1 RNA shows a pronounced localized increase in expression at the apex between days 7 and 10, around the time of the transition from vegetative to reproductive development. Once initiated, high levels of SOC1 RNA in the shoot apex are maintained (day 13) but the transcript is excluded from emerging floral meristems (fm). (C) Longitudinal section of à mature inflorescence of a plant grown under continuous light (im, inflorescence meristem). SOC1 RNA was not detected in stage 1 floral primordia [stages defined in (17)] but reappeared in late stage 2 flowers in the central dome, the cells that will later give rise to stamens and carpels. Expression persists in stamens (s) and carpels (g) throughout flower development, although at reduced levels. Flowers at stage 3 and 4 are also indicated.

Fig. 4. Convergence of flower-promoting pathways. CO promotes flowering of Arabidopsis in response to long photoperiods. The processes associated specifically with flowering in response to photoperiod are shown in blue. CO can directly increase expression of the FT, SOC1, ACS10, and AtP5CS2 genes. FT and SOC1 promote the transition to flowering. Floral meristem identity genes confer floral identity on primordia (33). Possible roles for ACS10, which encodes an enzyme involved in ethylene biosynthesis, and AtP5CS2, which encodes an enzyme that catalyzes proline biosynthesis, are described in the text.



The processes associated with these genes also occur in short days where CO does not promote flowering, which suggests that they are also regulated by other floral-promoting pathways. FT and SOC1 expression is also reduced by fca mutations that affect the autonomous flowering pathway. FCA negatively regulates *FLC*, which encodes a floral repressor. *FLC* expression is also reduced by vernalization and promoted by FRI (29, 30). FLC and CO are proposed to have antagonistic effects on the expression of *SOC1* and *FT*. The abundance of *FT*, *SOC1*, and *AtP5CS2* mRNAs (boxed) increases with age independently of CO (Fig. 2) (13).

To determine whether SOC1 and FT are regulated by the autonomous pathway, we tested whether the fca-1 mutation, which affects the autonomous pathway (4, 5, 25), reduces their expression. The abundance of SOC1 mRNA was reduced in 6- and 13-day-old *fca-1* mutants relative to the wild type, and it was reduced to a similar extent in fca-1 35S::CO plants relative to 35S:: CO plants (Fig. 2) (13). FT expression was also reduced by fca-1 in a 35S::CO background. Although the effect on FT was less extreme than for SOC1 (Fig. 2) (13), it is consistent with a reduction in FT expression observed in fca mutants relative to the wild type (26). Therefore, SOC1 and FT are regulated by the day length-dependent pathway that includes CO and the autonomous pathway that involves FCA (Fig. 4).

In addition, SOC1 expression rises in co and fca mutants between days 6 and 13 (Fig. 2), demonstrating a response of SOC1 to the age of the plant that is independent of CO or FCA (Fig. 4).

**Conclusions.** CO plays a pivotal role in promoting flowering by activating at least four early target genes with diverse biochemical functions that act in combination to promote flowering and reproductive development (Fig. 4). Their activation by CO is probably dependent on other factors that differ for each of the target genes, because they are not all constantly overexpressed in 35S::COplants. In *co* mutants, the target genes

retain responses to time of day and the age of the plant (Fig. 2) (13), suggesting that part of their regulation occurs independently of CO. Two of the target genes, FT and SOC1, have major roles in promoting flowering time, and their functions are genetically redundant. The molecular mechanism of this redundancy is unclear, because SOC1 encodes a putative transcription factor, whereas FT encodes a protein with similarity to Raf kinase inhibitor protein (11, 27). SOC1 may be part of a transcriptional cascade within the shoot apex that results in activation of floral meristem identity genes such as LFY. In contrast, FT was proposed to activate floral meristem identity genes other than LFY (28). Strikingly, the MADS-box transcription factor encoded by the Arabidopsis FLC gene (29, 30) acts as a floral repressor. Mutations in the autonomous pathway cause elevated levels of FLC, and this increase is required for the late-flowering phenotype of fca-1 mutants (31). Thus, the reduction in SOC1 and FT expression by the fca-1 mutation may be due to an increase in the floral repressor FLC. Indeed, levels of FLC mRNA were much higher in fca-1 35S::CO plants than in 35S::CO plants (Fig. 2). The interactions of CO with SOC1 and FT are probably direct, but those of FLC may be indirect. The levels of SOC1 and FT expression may be determined by a balance of CO and FLC activity (Fig. 4). Because FLC plays

a role in vernalization (29-31), SOC1 and FT may be common components of the pathways by which flowering time is regulated by different environmental conditions (Fig. 4).

## **References and Notes**

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- 34. We thank P. Suarez-Lopez for advice, S. Melzer for sharing his data on AGL20 before publication, and G. Simpson, Y. Levy, C. Dean, and R. Sablowski for comments on the manuscript. Supported by grants from the European Community and the Human Frontier Science Program (G.C.).

15 February 2000; accepted 4 May 2000