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Antagonistic regulation of flowering-time gene *SOC1* by CONSTANS and FLC via separate promoter motifs

Shelley R.Hepworth[1,2], Federico Valverde[1,3], Dean Ravenscroft[1,3], Aidyn Mouradov[3] and George Coupland[1,3,4]

[1]John Innes Centre, Colney Lane, Norwich NR4 7UH, UK and [3]Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

[2]Present address: Department of Botany, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

[4]Corresponding author
e-mail: coupland@mpiz-koeln.mpg.de

Flowering in *Arabidopsis* is controlled by endogenous and environmental signals relayed by distinct genetic pathways. The MADS-box flowering-time gene *SOC1* is regulated by several pathways and is proposed to co-ordinate responses to environmental signals. *SOC1* is directly activated by CONSTANS (CO) in long photoperiods and is repressed by FLC, a component of the vernalization (low-temperature) pathway. We show that in transgenic plants overexpressing CO and FLC, these proteins regulate flowering time antagonistically and FLC blocks transcriptional activation of *SOC1* by CO. A series of *SOC1::GUS* reporter genes identified a 351 bp promoter sequence that mediates activation by CO and repression by FLC. A CArG box (MADS-domain protein binding element) within this sequence was recognized specifically by FLC *in vitro* and mediated repression by FLC *in vivo*, suggesting that FLC binds directly to the *SOC1* promoter. We propose that CO is recruited to a separate promoter element by a DNA-binding factor and that activation by CO is impaired when FLC is bound to an adjacent CArG motif.

Keywords: *Arabidopis*/*CONSTANS*/*FLC*/flowering/*SOC1*

Introduction

In plants, the transition from vegetative growth to flowering occurs in response to both environmental stimuli and endogenous signals. Genetic analyses of the control of flowering in *Arabidopsis thaliana* identified four major floral promotion pathways (reviewed in Simpson *et al*., 1999; Reeves and Coupland, 2000; Araki, 2001). The photoperiod and vernalization pathways mediate the response to environmental signals, whereas the autonomous and gibberellin (GA) pathways appear to act independently of these signals (Koornneef *et al*., 1991).

The photoperiod pathway mediates the promotion of flowering by daylength. *Arabidopsis* is a facultative long-day plant, flowering more rapidly under long-day (LD) conditions of 16 h of light than in short days (SDs) of 10 h light. *CONSTANS* (*CO*) and *FT* were placed in this pathway because mutations in these genes delay flowering in LDs, but not in SDs, and thereby modulate the response to photoperiod (reviewed in Reeves and Coupland, 2000; Araki, 2001). *CO* encodes a putative transcription factor (Putterill *et al*., 1995; Robson *et al*., 2001), whilst *FT* encodes a protein with similarity to RKIP proteins (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999; Pnueli *et al*., 2001).

The vernalization pathway promotes flowering in response to extended exposures to low temperature. This pathway acts redundantly with the autonomous pathway. Both of these pathways promote flowering by preventing accumulation of the *FLOWERING LOCUS C* (*FLC*) mRNA (Michaels and Amasino, 1999, 2001; Sheldon *et al*., 1999, 2000). *FLC* acts synergistically with *FRI* to repress flowering in late-flowering accessions (Koornneef *et al*., 1994; Sanda and Amasino, 1996; Johanson *et al*., 2000; Sheldon *et al*., 2000), and encodes a MADS-domain transcription factor that represses flowering when over-expressed in transgenic plants (Michaels and Amasino, 1999; Sheldon *et al*., 1999). Mutation of *FLC* accelerates flowering in LDs and SDs, and is epistatic to mutations in the autonomous pathway and to dominant alleles of *FRI* (Michaels and Amasino, 2001). The abundance of *FLC* mRNA and protein is elevated by mutations in the autonomous pathway and is reduced by vernalization, suggesting that modulation of *FLC* expression is central to the control of flowering time (Michaels and Amasino, 1999; 2001; Sheldon *et al*., 2000), but it is not essential for a vernalization response (Michaels and Amasino, 2001).

The floral promotion pathways ultimately converge to regulate the expression and function of the floral meristem identity genes that control flower development (Blázquez and Weigel, 2000; Borner *et al*., 2000; Lee *et al*., 2000; Samach *et al*., 2000; Rouse *et al*., 2002). For example, the floral meristem identity gene *LEAFY* (*LFY*) is regulated both by *CO*, a component of the photoperiod pathway, and GA (Blázquez and Weigel, 2000). These act through different motifs within the *LFY* promoter, although CO probably does not directly activate *LFY*, and the transcription factor that regulates *LFY* in response to GA is not yet known (Blázquez and Weigel, 2000; Samach *et al*., 2000).

The flowering-time genes *FT* and *SOC1* (or *AGL20*) are also common targets of distinct pathways and are proposed to function upstream of the floral meristem identity genes. *SOC1* and *FT* were shown to be direct targets of CO by using plants that overexpressed a translational fusion of CO to the ligand-binding domain of the glucocorticoid receptor (*35S::CO:GR*) (Samach *et al*., 2000). In agreement with this, *FT* expression is reduced in *co* mutants (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999; Samach *et al*., 2000; Suárez-López *et al*., 2001), whilst *SOC1* expression responds to photoperiod and is slightly reduced in *co* mutants (Borner *et al*., 2000; Lee *et al*., 2000;

Table I. Effect of overexpression of *CO* and *FLC* on flowering time in LDs

Genotype	No. of rosette leaves	No. of cauline leaves	Total No. of leaves
L*er*	5.5 ± 0.5	3.1 ± 0.3	8.6 ± 0.5
35S::CO	3.0 ± 0.0	1.9 ± 0.5	4.9 ± 0.5
35S::CO 35S::FLC	4.4 ± 0.5	2.5 ± 0.5	6.9 ± 0.6
35S::FLC	33.3 ± 3.4	6.9 ± 0.7	40.2 ± 3.9

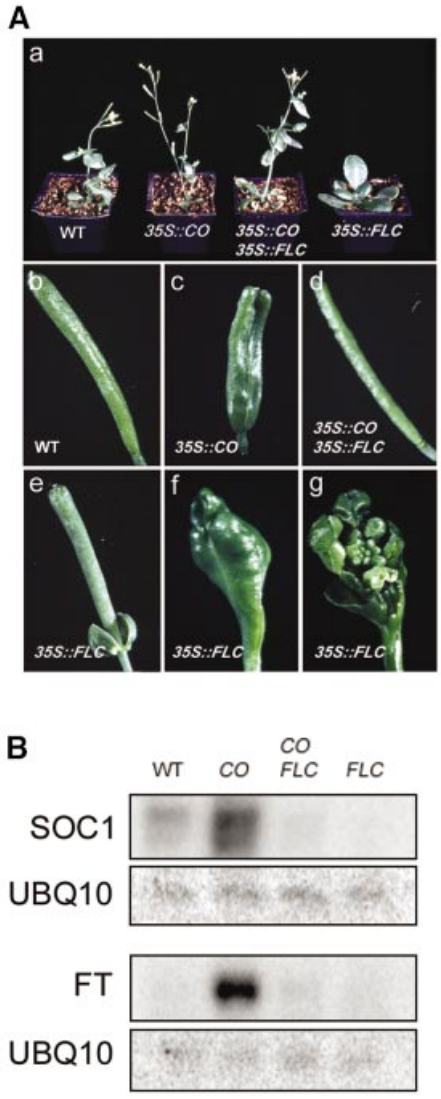

Fig. 1. Antagonistic effect of *35S::CO* and *35S::FLC* on flowering time, floral morphology, and expression of *SOC1* and *FT*. (**A**) Phenotype of wild-type (WT), *35S::CO*, *35S::CO 35S::FLC*, and *35S::FLC* plants. (a) Thirty-two-day-old plants grown in LDs; (b–f) morphology of siliques; (g) dissection of silique in (f) to show an ectopic inflorescence. (**B**) Northern analysis of *SOC1* and *FT* mRNA in WT, *35S::CO*, *35S::CO 35S::FLC*, and *35S::FLC* plants. One filter made with RNA harvested 8 h after dawn was hybridized with probes for *SOC1* and *UBQ10* (upper rows). A second filter made with RNA harvested 16 h after dawn was sequentially hybridized with probes for *FT* and *UBQ10* (lower rows).

Samach *et al*., 2000). In addition, *ft* or *soc1* mutations partially suppress the early flowering of *35S::CO* plants (Onouchi *et al*., 2000). Thus, *FT* and *SOC1* act downstream of *CO* in the photoperiod pathway and *CO* promotes their expression. However, *FT* and *SOC1* also act downstream of the floral inhibitor *FLC*, which is a component of the autonomous/vernalization pathway and does not affect *CO* expression. For example, *SOC1* mRNA abundance is reduced in genotypes with high levels of *FLC* and is increased in *flc* mutants (Borner *et al*., 2000; Lee *et al*., 2000; Samach *et al*., 2000; Michaels and Amasino, 2001). These observations are consistent with the proposal that *FLC* represses *SOC1*.

Thus, the antagonistic effect of transcription factors CO and FLC on the expression of downstream genes *FT* and *SOC1* may represent a direct convergence of signalling pathways and provide a means of co-ordinating the control of flowering by daylength and temperature. Here we further characterize how *CO* and *FLC* interact to generate antagonistic effects on *SOC1* expression.

Results

Phenotypes associated with overexpression of FLC are suppressed by overexpression of CONSTANS

To examine the antagonistic effect of *CO* and *FLC* on flowering, the phenotypes of plants overexpressing both genes from the strong CaMV 35S promoter were examined. A *35S::CO 35S::FLC* line was generated by crossing plants carrying *35S::CO* (Onouchi *et al*., 2000) with those containing *35S::FLC* (Michaels and Amasino, 1999). The flowering times of wild-type (L*er*), *35S::CO*, *35S::CO 35S::FLC*, and *35S::FLC* plants were scored in LDs. *35S::CO* plants flowered earlier than wild type, whilst *35S::FLC* plants flowered much later (Table I; Figure 1A). The *35S::CO 35S::FLC* plants flowered much earlier than *35S::FLC* and at a time between that of *35S::CO* and wild-type plants (Table I). *CO* and *FLC*, therefore, have antagonistic effects on flowering time and overexpression of *CO* can largely overcome the delay in flowering caused by overexpression of *FLC*.

Overexpression of *FLC* also caused defects in floral morphogenesis. A proportion of flowers produced anthers with little or no pollen (data not shown), the petioles were often retained at the base of mature siliques (Figure 1A) and floral reversion caused development of an inflorescence inside some siliques (Figure 1A). These defects were absent in *35S::CO 35S::FLC* lines (Figure 1A), although the mRNA expressed from *35S::FLC* was still present in these plants. Similarly, defects associated with overexpression of *CO*, such as the presence of extra carpels and the short club-like appearance of siliques, were absent in *35S::CO 35S::FLC* lines (see Onouchi *et al*., 2000; Figure 1), although *35S::CO* mRNA was present.

This genetic analysis supports the notion that CO and FLC interact antagonistically to regulate flowering time,

and indicates that this antagonism persists throughout development when these genes are overexpressed.

Antagonistic effect of CONSTANS and FLC on expression of target genes

Previously, *SOC1* and *FT* were shown to be immediate targets of *CO*, and their mRNA levels correlate with the level of *CO* expression (Samach *et al*., 2000). *SOC1* mRNA levels also correlate with the level of *FLC* expression (Lee *et al*., 2000; Michaels and Amasino, 2001). We compared the level of *SOC1* and *FT* mRNAs in *35S::CO*, *35S::CO 35S::FLC*, *35S::FLC* and wild-type plants to determine whether they correlate with flowering time. RNA was extracted from 10-day-old seedlings and subjected to northern analysis. As expected, *SOC1* and *FT* mRNAs accumulated to a higher level in *35S::CO* plants than in wild type and were not detected in *35S::FLC* plants (Figure 1B). In *35S::CO 35S::FLC* plants, the levels of *SOC1* and *FT* mRNAs were dramatically reduced compared with those in *35S::CO* plants. Thus, 8.3-fold less *SOC1* mRNA and 11.3-fold less *FT* mRNA was detected in *35S::CO 35S::FLC* plants compared with *35S::CO* plants (Figure 1B). Nevertheless, *35S::CO 35S::FLC* plants flowered only slightly later than *35S::CO* plants (Figure 1A; Table I). Furthermore, *SOC1* mRNA in *35S::CO 35S::FLC* plants was 3-fold less abundant in comparison to wild-type plants, although *35S::CO 35S::FLC* plants flowered earlier than wild type. Despite the lack of correlation between *SOC1* mRNA levels and flowering time, our analysis confirmed that *CO* and *FLC* have antagonistic effects on *SOC1* and *FT* expression.

Expression of SOC1 in cauline leaves and flowers and accumulation of transcript with age require promoter sequences between nucleotides –4105 and –1911

Previously, *SOC1* mRNA was shown to accumulate early in development and to be present in most tissues of mature plants (Borner *et al*., 2000; Lee *et al*., 2000; Samach *et al*., 2000). To identify *SOC1* promoter sequences, expression of a *SOC1::GUS* reporter gene containing ~4 kb of sequence upstream of the *SOC1* transcriptional start site was monitored (Materials and methods). This was assumed to contain the full-length promoter since a genomic DNA fragment containing 1.4 kb of upstream sequence complemented the *soc1* mutation (Samach *et al*., 2000). Twenty primary transformants carrying *SOC1::GUS* were analysed by β-glucuronidase (GUS) staining. Staining was first visible in the germinating seed after 1 day of growth in LDs, and was subsequently detected in the roots, apex and cotyledons of seedlings. GUS expression was observed in mature plant tissues such as rosette leaves, cauline leaves, inflorescences and flowers, but not in mature siliques or in seeds (Figure 2B). The pattern of expression of the 4 kb *SOC1::GUS* reporter gene was, therefore, similar to that of the endogenous *SOC1* gene as monitored by RT–PCR (Borner *et al*., 2000; Lee *et al*., 2000).

To identify a minimal promoter sequence that would still mediate activation by CO and repression by FLC, the effects of sequential 5′ deletions of the 4 kb promoter on expression of *SOC1::GUS* were monitored in wild-type plants. *SOC1::GUS* reporter genes with deletion endpoints at nucleotide (nt) –1911 (2 kb *SOC1::GUS*), nt –966 (1 kb *SOC1::GUS*) and nt –89 (0 kb *SOC1::GUS*) were introduced into wild-type plants (Figure 2A; Materials and methods). Twenty primary transformants were obtained for each construct and analysed for activity by GUS staining. The patterns of expression for the 1 and 2 kb *SOC1::GUS* reporter genes were similar (Figure 2B). Staining was detected in seedlings and rosette leaves, but was reduced or absent in cauline leaves and was not detected in inflorescences or flowers. GUS staining was not detected for plants transformed with a 0 kb *SOC1::GUS* reporter gene (Figure 2B). Therefore, the 5′ boundary of promoter sequences required for *SOC1* expression in cauline leaves and in flowers was located upstream of nt –1911, but sequences between nt –966 and –89 were sufficient for *SOC1* expression in seedlings.

Previously, *SOC1* mRNA was detected at a low level early in development and slowly accumulated over a 12 day period (Lee *et al*., 2000). To determine whether the 4 kb *SOC1::GUS* reporter gene was similarly expressed in developing seedlings and to monitor activity of the truncated 2 and 1 kb promoters, seedlings with 4, 2 or 1 kb *SOC1::GUS* reporter genes were monitored for GUS activity over 21 days (Figure 2C). Expression of 4 kb *SOC1::GUS* was detected early in development (day 3) and gradually increased until about day 12. For the 1 and 2 kb *SOC1::GUS* reporter genes, activity was also detected early in development (day 3), but at ~10-fold lower levels than for 4 kb *SOC1::GUS*. Expression reached a maximum at about day 5 and remained constant until day 21. Therefore, truncation of the *SOC1* promoter resulted in an overall decrease in the level of reporter gene expression and abolished the age-dependent increase in expression that is observed for the 4 kb promoter. The 5′ boundary of sequences required for maximal accumulation of *SOC1* mRNA in seedlings must be located upstream of nt –1911.

The 1 kb SOC1::GUS reporter is activated by CO and repressed by FLC

To test whether the 1 kb *SOC1::GUS* reporter retained the ability to be activated by CO and/or repressed by FLC, 1 kb *SOC1::GUS* was introduced into *35S::CO*, *35S::CO 35S::FLC*, and *35S::FLC* plants by crossing. Expression of 1 kb *SOC1::GUS* in these lines was then monitored by GUS staining and northern blotting (Figure 3).

GUS activity was higher in all tissues of *35S::CO* seedlings in comparison to wild type (Figure 3A). An increase in GUS activity was also detected in cauline leaves and in flowers relative to that of wild-type plants (Figures 2B and 3A). In contrast, GUS activity was dramatically reduced in *35S::FLC* seedlings in comparison with wild-type seedlings. Repression was strongest in the leaves; a residual amount of expression was retained in the roots, at the apex and in the veins of the leaves (Figure 3A). GUS expression in *35S::CO 35S::FLC* seedlings was similar to that in *35S::FLC* seedlings (data not shown).

The accumulation of *GUS* mRNA in whole seedlings was also measured by northern blotting (Figure 3B). The 1 kb *SOC1::GUS* transcript was upregulated 3.1-fold in *35S::CO* seedlings and repressed 5.1-fold in *35S::FLC* seedlings in comparison with wild type (Figure 3B). In addition, a transient assay was developed to determine

whether the 1 kb fragment of the *SOC1* promoter responded to FLC in wild-type plants and in *fca* mutants, which contain elevated levels of FLC (Michaels and Amasino, 1999, 2001; Sheldon *et al*., 2000). A 1 kb *SOC1::LUCIFERASE* fusion was introduced into wild-type plants, *flc* loss-of-function mutants and *fca* mutants by microprojectile bombardment. Levels of luciferase expression were compared with those of co-bombarded 35S::GFP (Materials and methods). Luciferase expression was ~1.75-fold higher in *flc* loss-of-function mutants than in wild-type plants, and ~0.6-fold wild-type levels in *fca* mutants (Figure 3C). The differences between the mutants and wild-type plants were confirmed as significantly different ($P < 0.001$) using the Mann–Whitney rank sum test. The 1 kb *SOC1* promoter fragment therefore also confers responses to FLC at levels of expression found in wild-type and *fca* mutant plants.

In *35S::CO 35S::FLC* seedlings carrying 1 kb *SOC1::GUS*, *GUS* mRNA was 4.1-fold less abundant than in wild type, and was most similar to that in *35S::FLC* (Figure 4B). Analysis of *SOC1* mRNA demonstrated that the endogenous gene was upregulated in *35S::CO* plants and repressed in *35S::FLC* plants to a similar extent to 1 kb *SOC1::GUS* (Figure 3B).

We also tested whether upregulation of 1 kb *SOC1::GUS* expression in *35S::CO:GR* lines was likely to be mediated directly by CO (Samach *et al*., 2000). Expression of 1 kb *SOC1::GUS* was monitored in *35S::CO:GR* plants by northern blotting (Figure 3B). A 3.5-fold increase in *GUS* mRNA abundance was detected after 4 h of dexamethasone (Dex) treatment in comparison to untreated control plants (Figure 3B), whereas no *GUS* mRNA was detected for the minimal 0 kb *SOC1::GUS* reporter gene in *35S::CO:GR* plants (Figure 3B). The endogenous *SOC1* gene was also analysed, demonstrating that treatment with Dex was sufficient for upregulation of endogenous *SOC1* mRNA.

These experiments indicated that sequences between nt –966 and –89 in the *SOC1* promoter mediate activation by CO and repression by FLC, and that 1 kb *SOC1::GUS* is regulated by CO and FLC in a similar manner to the endogenous gene.

Overlapping 300 bp fragments in the 1 kb promoter of SOC1 mediate activation by CO and repression by FLC

To further define the sequences that mediate activation by CO and repression by FLC, the 1 kb promoter was subdivided into four overlapping fragments of ~300 bp each. These fragments were cloned upstream of the minimal 0 kb *SOC1::GUS* reporter gene to generate the 300 bp A, B, C and D *SOC1::GUS* reporter genes

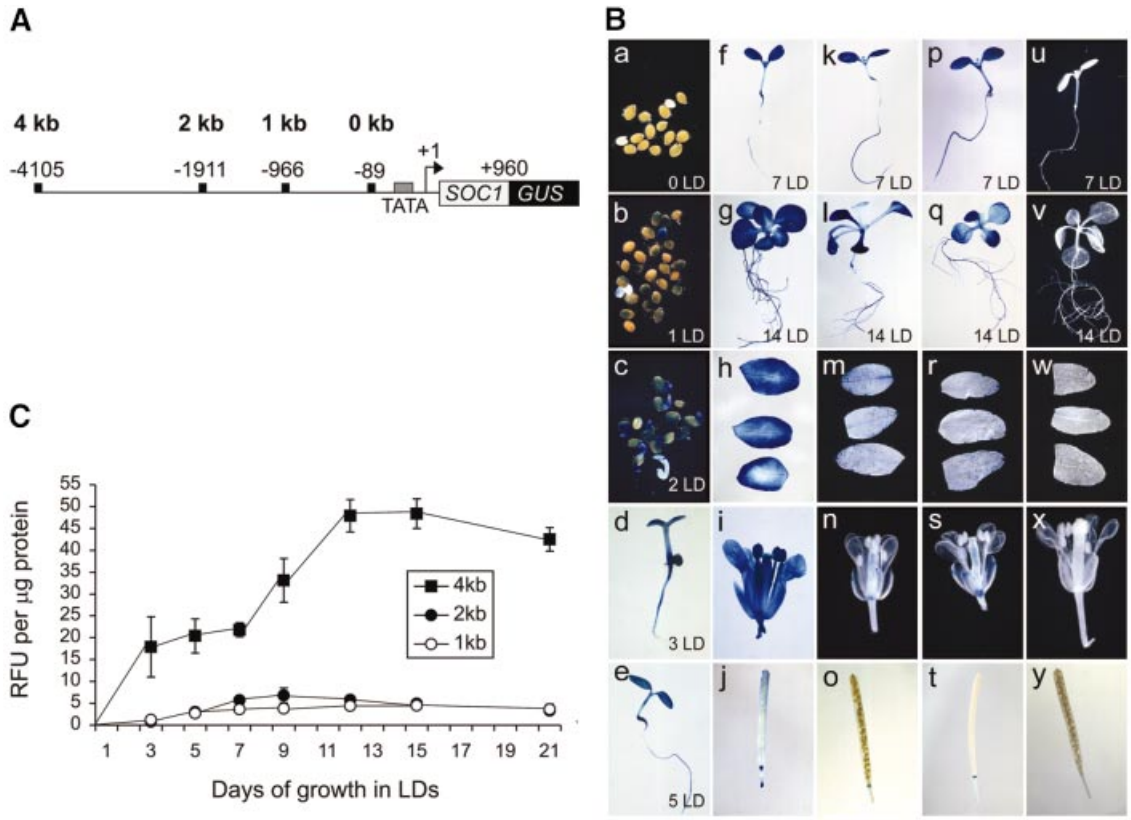

Fig. 2. Analysis of expression of *SOC1::GUS* reporter genes in wild-type plants. (**A**) Diagram of *SOC1::GUS* reporter genes with full-length (4 kb) or truncated promoters (2, 1 and 0 kb). The 5′ endpoints of constructs are numbered relative to the transcription start site (+1). (**B**) Expression of *SOC1::GUS* reporter genes in seedlings, cauline leaves, flowers and siliques as monitored by GUS staining. Days of growth are in the lower right of panels. Four kilobase *SOC1::GUS* reporter gene expression in (a–g) seedlings, (h) cauline leaves, (i) flowers and (j) siliques; 2 kb *SOC1::GUS* reporter gene expression in (k and l) seedlings, (m) cauline leaves, (n) flowers and (o) siliques; 1 kb *SOC1::GUS* expression in (p and q) seedlings, (r) cauline leaves, (s) flowers and (t) siliques; 0 kb *SOC1::GUS* expression in (u and v) seedlings, (w) cauline leaves, (x) flowers and (y) siliques. (**C**) Time course of 4, 2 and 1 kb *SOC1::GUS* reporter gene expression in wild-type seedlings as determined by GUS activity assays (see Materials and methods). RFU, relative fluorescence units.

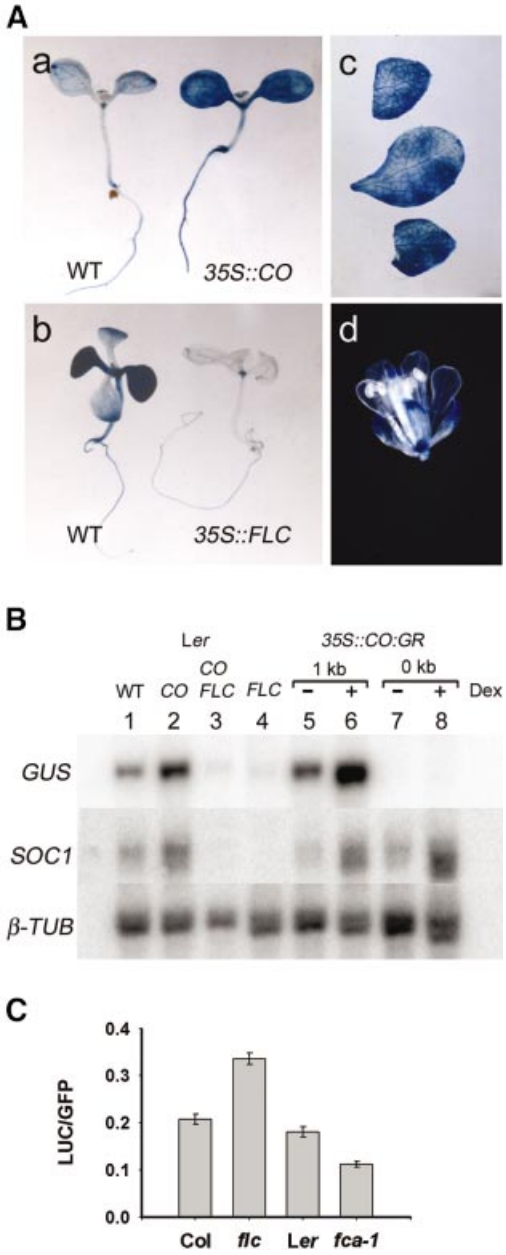

Fig. 3. One kilobase *SOC1::GUS* reporter gene expression is activated in *35S::CO* lines and repressed by *FLC*. (**A**) Analysis of 1 kb *SOC1::GUS* expression in wild-type (WT), *35S::CO* and *35S::FLC* plants by GUS staining: (a) 10-day-old seedlings, 1 kb *SOC1::GUS* WT (left) and 1 kb *SOC1::GUS 35S::CO* (right); (b) 14-day-old seedlings, 1 kb *SOC1::GUS* WT (left) and 1 kb *SOC1::GUS 35S::FLC* (right); 1 kb *SOC1::GUS 35S::CO* in (c) cauline leaves and (d) flower. (**B**) Northern analysis of 1 and 0 kb *SOC1::GUS* expression. RNA was purified from 1 kb *SOC1::GUS* or 0 kb *SOC1::GUS* seedlings: wild type (WT), *35S::CO* (CO), *35S::CO 35S::FLC* (CO FLC), *35S::FLC* (FLC) and *35S::CO:GR*. Dexamethasone (Dex) treatment, (+) or (–). The filter was sequentially hybridized with probes for *GUS*, *SOC1* and *β-TUB* (loading control). (**C**) Transient expression assays of 1 kb *SOC1::LUC*. Leaves of Columbia (Col), *flc-1* (in Col background), Landsberg *erecta* (L*er*) and *fca-1* (in L*er*) plants were bombarded with beads coated with DNA of plasmids carrying 1 kb *SOC1::LUC* and *35S::GFP* (Materials and methods). The ratio of luciferase to GFP expression is shown for each genotype (Materials and methods). In each case, the column represents the mean value, with the standard error.

(Figure 4A; Materials and methods). Each of these constructs was used to transform wild-type plants. Twenty primary transformants for each construct were obtained. Reporter genes present in these lines were introduced into *35S::CO* and *35S::FLC* lines by crossing.

The expression pattern of each construct was first analysed qualitatively by staining seedlings for GUS activity in the T_2 generation (Figure 4A). In wild-type plants, constructs containing fragments B and C supported expression of the minimal 0 kb *SOC1::GUS* gene. In general, constructs containing fragment B supported higher levels of GUS activity than those containing fragment C. However, the GUS activity mediated by fragment C was strongly increased in *35S::CO* lines, whereas that mediated by fragment B was not. The GUS expression caused both by fragment B and C was significantly repressed by *35S::FLC*, indicating that the

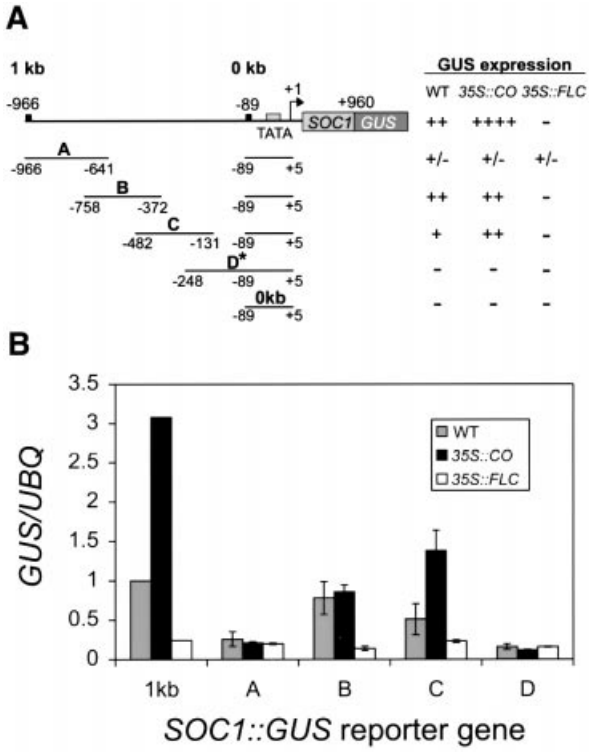

Fig. 4. Identification of a 234 bp region of the *SOC1* promoter that mediates activation by CO and repression by FLC. (**A**) Summary of expression of *SOC1::GUS* reporter genes in wild-type (WT), *35S::CO* and *35S::FLC* lines. Relative activities were determined by GUS staining. Top line, 1 kb *SOC1::GUS* reporter gene; second to fifth lines, 300 bp *SOC1::GUS* reporter genes containing overlapping fragments A, B, C or D from the *SOC1* promoter. Fragments of 300 bp were each inserted upstream of the minimal 0 kb reporter gene at a unique *Bam*HI site (see Materials and methods). Asterisk denotes that fragment D contains two copies of the minimal promoter region between –89 and +5. Bottom line, minimal 0 kb *SOC1::GUS* reporter gene that contains the TATA box and transcription start site (+1) for *SOC1*. (**B**) Promoter fragment C mediates activation in *35S::CO* plants and repression in *35S::FLC* plants. Expression of 1 kb *SOC1::GUS* (1 kb) and 300 bp *SOC1::GUS* (A, B, C, D) reporter genes was monitored in WT, *35S::CO* and *35S::FLC* seedlings by northern blotting using probes for *GUS* and *UBQ10* (loading control). These data were quantified and are presented in histogram format. The amount of *GUS/UBQ* transcript in WT plants containing the 1 kb *SOC1::GUS* reporter gene was given an arbitrary value of 1. The relative level of transcript for each reporter gene construct in each background is presented as an average.

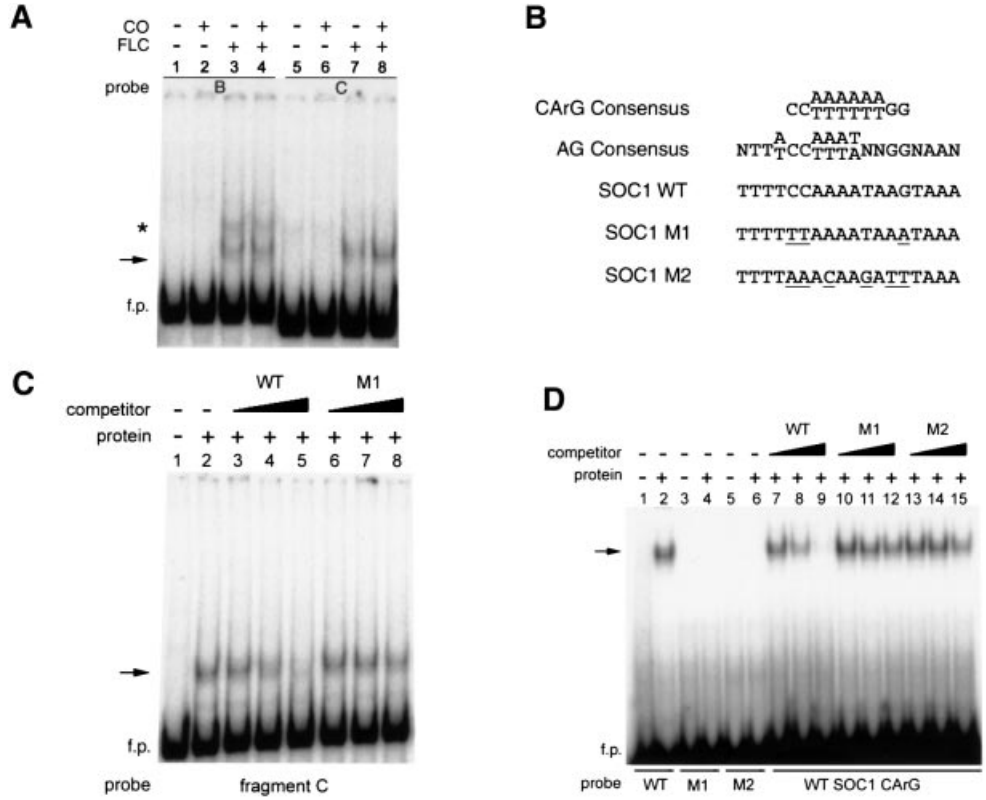

Fig. 5. Specific binding of FLC protein to a CArG box at nt –400 of the *SOC1* promoter. (**A**) FLC protein forms a gel retardation complex (arrow) with 300 bp fragments B and C. FLC and/or CO protein were incubated with promoter fragments B or C as probes. The protein contained in each reaction is indicated above the panel. f.p., free probe. The asterisk indicates an apparent non-specific protein–DNA complex that was not consistently observed and was competed with non-specific competitor. (**B**) Comparison of wild-type (WT) and mutant MADS-box protein binding sites (CArG boxes): includes CArG box at nt –400 of the *SOC1* promoter (CCAAAATAAG), as well as mutant versions of *SOC1* CArG box. (**C**) Specific binding of FLC protein to fragment C probe. The presence of FLC protein is indicated above the panels and competitor DNAs are described in the text. Lane 1, no protein and no competitor DNA; lanes 3–5, SOC1 WT fragment as competitor DNA; lanes 6–8, SOC1 M1 fragment as competitor DNA. Non-labelled DNA in molar excess was used as competitor in lanes 3 and 6 (10-fold), lanes 4 and 7 (100-fold), and lanes 5 and 8 (1000-fold). (**D**) Specific binding of FLC protein to a 30 bp fragment containing the CArG sequence at nt –400. FLC protein was incubated with 30 bp fragment probes as indicated below the panel. The presence of FLC protein is indicated above the panels and competitor DNAs are described in the text. Lanes 1, 3, 5, no protein and no competitor DNA; lanes 2, 4, 6, FLC and no competitor DNA; lanes 7–9, SOC1 WT fragment as competitor DNA; lanes 10–12, SOC1 M1 fragment as competitor DNA; lanes 13–15, SOC1 M2 fragment as competitor DNA. Non-labelled DNA in molar excess was used as competitor in lanes 7, 10 and 13 (10-fold), lanes 8, 11 and 14 (100-fold), and lanes 9, 12 and 15 (1000-fold).

overlapping region between these two sequences was likely to contain sequences that mediate repression by FLC. Little or no GUS activity for constructs containing fragments A and D was detected in wild-type, *35S::CO* or *35S::FLC* lines.

The accumulation of *GUS* transcript in homozygous T_3 lines was also monitored in whole seedlings by northern blotting. Figure 4B shows that for constructs containing fragment C, *SOC1::GUS* mRNA abundance increased by an average of 2.7-fold in *35S::CO* seedlings. This increase is similar to that observed for 1 kb *SOC1::GUS* mRNA, which was increased 3.1-fold in *35S::CO* seedlings in comparison to wild type. *SOC1::GUS* mRNA expressed from fragments A, B or D was not significantly increased in abundance in *35S::CO* seedlings compared with wild type. Figure 4B also shows that constructs containing fragment B or C were repressed by an average of 5.6- or 2.1-fold, respectively, in *35S::FLC* seedlings compared with wild type. The level of repression of fragment B is similar to that observed for the 1 kb *SOC1::GUS* reporter gene in *35S::FLC* seedlings (Figure 4). The mRNA of *SOC1::GUS* constructs containing fragments A and D was not significantly reduced in abundance in *35S::FLC* seedlings in comparison to wild type.

This analysis showed that overlapping fragments B and C support activity of a minimal 0 kb *SOC1::GUS* reporter gene in wild-type plants, and that this activity is repressed by *35S::FLC*. Therefore, sequences between nt –482 and –372 of the *SOC1* promoter mediate repression by FLC. Furthermore, only fragment C could support efficient activation of the minimal 0 kb *SOC1::GUS* reporter in *35S::CO* lines, suggesting that the sequences required for this response are located between nt –372 and –248.

Specific in vitro binding of FLC to DNA containing the CArG box at nt –400 of the SOC1 promoter

To test whether CO and/or FLC bind directly to the *SOC1* promoter, gel retardation experiments were performed using fragments B and C as probe. A protein–DNA complex was detected by gel retardation after incubation of recombinant FLC protein with labelled fragments B and C (Figure 5A). Formation of a similar complex was observed when FLC and CO proteins were present together in the reaction mixture, and no complex was

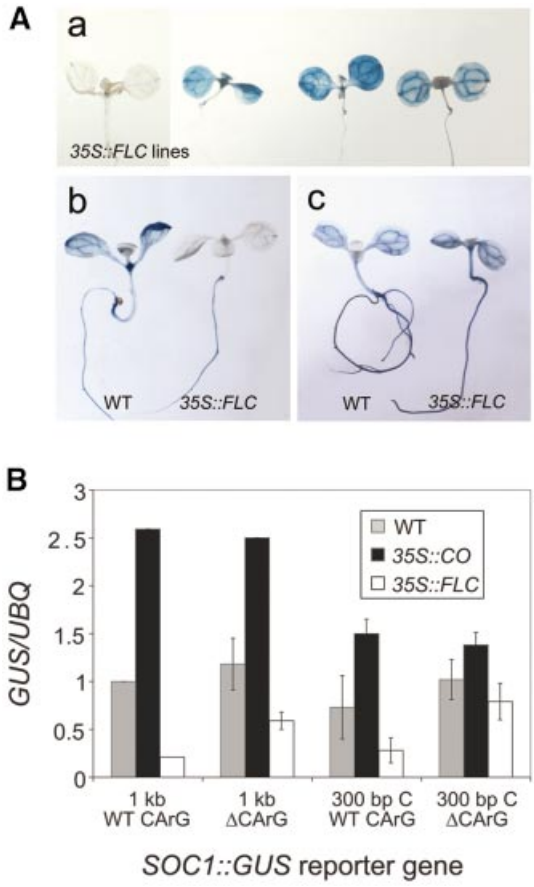

Fig. 6. The CArG box at nt –400 of the *SOC1* promoter mediates repression of *SOC1::GUS* expression in *35S::FLC* lines. (**A**) Mutation of the CArG box at nt –400 leads to derepression of *SOC1::GUS* expression in *35S::FLC* lines. Ten-day-old seedlings were stained for GUS activity. (a) One kilobase *SOC1::GUS* expression in a *35S::FLC* control seedling (left); three independent lines showing 1 kb ΔCArG *SOC1::GUS* expression in *35S::FLC* seedlings (right); (b) 300 bp C *SOC1::GUS* expression in a wild-type (WT) seedling and in a *35S::FLC* seedling; (c) mutated 300 bp C ΔCArG *SOC1::GUS* expression in a WT seedling and in a *35S::FLC* seedling. (**B**) Analysis of the effect of the CArG box mutation on *SOC1::GUS* activation in *35S::CO* lines and repression in *35S::FLC* lines. Expression of WT and mutated ΔCArG reporter genes was monitored in WT, *35S::CO* and *35S::FLC* seedlings by northern blotting using probes for *GUS* and *UBQ10* (loading control). The data were quantified and are presented in histogram format.

observed when recombinant CO protein alone was incubated with the probes (Figure 5A).

Inspection of the overlapping region between fragments B and C revealed a sequence motif centred at nt –400 that resembled a CArG box, the site to which MADS-domain proteins bind (Figure 5B; reviewed in Shore and Sharrocks, 1995). This CArG sequence in the *SOC1* promoter is most similar to the consensus binding site for AGAMOUS (AG) (Figure 5B; Huang *et al*., 1993; Shiraishi *et al*., 1993).

The specificity of the interaction of FLC protein with fragment C was tested (Figure 5C). For this, a 30 bp fragment (SOC1 WT) that spanned the CArG box at nt –400 was used as specific competitor (Figure 5B, third line). As non-specific competitor, a similar fragment with three base-pair changes in the CArG site was used (SOC1 M1; Figure 5B). Formation of a complex was observed when FLC protein was incubated with radioactively labelled fragment C (Figure 5C, lane 2). Formation of this complex was inhibited by the presence of excess non-labelled WT fragment (Figure 5C, lanes 3–5), but not by fragment M1. Therefore, this protein–DNA complex reflects a specific interaction of FLC with the CArG box within fragment C.

We also tested whether FLC protein could bind to 30 bp fragments containing either WT or mutant CArG sites: WT and M1 fragments are described above; fragment M2 contains six base-pair changes in the CArG site (Figure 5B). Formation of a complex was observed when FLC protein was incubated with WT 30 bp fragment, but not with the M1 or M2 fragments (Figure 5D). Formation of this complex was inhibited by the presence of excess non-labelled WT fragment, but not by M1 or M2 fragments (Figure 5D, compare lanes 7–9 with 10–15). These data indicate that the interaction of FLC protein with the CArG box at nt –400 in the *SOC1* promoter is specific.

The CArG box at nt –400 is required for repression in vivo

To test whether the CArG box at nt –400 is important for FLC-mediated repression of SOC1 *in vivo*, site-directed mutagenesis was used to create 1 kb and 300 bp C *SOC1* promoter fragments containing the changes present in M2 that prevented FLC binding (Figure 5B; Materials and methods). *SOC1::GUS* reporter genes with these mutant promoters, denoted 1 kb ΔCArG *SOC1::GUS* and 300 bp C ΔCArG *SOC1::GUS*, were introduced into wild-type plants. Reporter genes present in these lines were introduced into *35S::CO* and *35S::FLC* lines by crossing. The activity of the 1 kb ΔCArG and 300 bp C ΔCArG *SOC1::GUS* reporter genes was monitored by GUS staining and northern blotting (Figure 6).

In wild-type plants, both the 1 kb ΔCArG and 300 bp C ΔCArG mutant promoters supported strong expression of *SOC1::GUS*, which was efficiently upregulated in the corresponding *35S::CO* line. In contrast, both the 1 kb ΔCArG and 300 bp C ΔCArG promoters failed to mediate efficient repression of *SOC1::GUS* in *35S::FLC* lines (Figure 6A). Loss of repression was most evident in leaves, which did not stain for GUS activity in *35S::FLC* plants carrying constructs containing wild-type promoters (Figure 6A). The accumulation of *GUS* transcript was also tested in whole seedlings by northern blotting. For constructs containing 300 bp ΔCArG fragment C, *SOC1::GUS* transcript was on average 1.4-fold more abundant in *35S::CO* seedlings in comparison to wild type (Figure 6B). This ratio of activation appears lower than that observed for the WT 300 bp C *SOC1::GUS* reporter gene in *35S::CO* seedlings in comparison to wild type because there is a higher level of GUS activity in WT ΔCArG 300 bp lines. GUS mRNA produced from constructs containing 300 bp ΔCArG fragment C was on average 1.3-fold lower in abundance in *35S::FLC* seedlings compared with wild-type seedlings (Figure 6B). Repression of this construct by FLC is, therefore, much lower than that observed for the WT 300 bp fragment C *SOC1::GUS* reporter gene, in which *GUS* mRNA abundance was on average 2.1-fold less in *35S::FLC* seedlings (see also Figure 4). Similarly, in plants carrying 1 kb

ΔCArG *SOC1::GUS*, the level of GUS mRNA was reduced 2.2-fold compared with 5.1-fold in plants carrying 1 kb *SOC1::GUS* and *35S::FLC* (Figure 6B).

In summary, this analysis indicates that mutation of the CArG box reduced the repression of *SOC1* expression by *35S::FLC*, whereas the level of activation of reporter gene expression in *35S::CO* lines was not significantly affected.

Discussion

FT and *SOC1* were recently shown to be common targets of multiple flowering-time pathways. In particular, they are positively regulated by the photoperiod pathway through the B-box transcription factor CO and negatively regulated by the MADS-domain transcription factor FLC, a component of the autonomous/vernalization pathway (reviewed in Araki, 2001). The antagonistic effect of these transcription factors on the expression of downstream genes *FT* and *SOC1* may provide a means of co-ordinating the control of flowering time by daylength and temperature.

We explored the specific relationship between *CO* and *FLC* using plants overexpressing each of these transcription factors. This demonstrated the antagonistic activity of these transcription factors on flowering time and expression of *SOC1* and *FT*, and identified a 351 bp region of the *SOC1* promoter that mediates activation by CO and repression by FLC. A CArG box within this sequence was bound specifically by FLC *in vitro* and mediated repression by FLC *in vivo*, but was not required for activation by CO.

Flowering time is not completely correlated with SOC1 and FT mRNA level

Although the expression of *SOC1* and *FT* was antagonistically regulated by CO and FLC, the flowering time of plants specifically overexpressing these transcription factors did not correlate with *SOC1* and *FT* mRNA levels. For example, *35S::CO 35S::FLC* plants flowered 1.6 leaves earlier than wild-type plants despite containing 3-fold less *SOC1* mRNA and only 1.4-fold more *FT* mRNA in comparison to wild-type plants (Table I; Figure 1B). Similarly, *35S::CO* plants flowered just two leaves earlier than *35S::CO 35S::FLC* plants, despite having 11.3-fold more *FT* mRNA and 8.3-fold more *SOC1* mRNA than *35S::CO 35S::FLC* plants (Table I; Figure 1B). This suggests that the determination of flowering time by CO and FLC is not mediated solely by *SOC1* and *FT*. Alternatively, a small increase in *FT* mRNA abundance may be responsible for the early flowering of *35S::CO 35S::FLC* compared with wild type, and the effect of FT may reach saturation so that the higher levels present in *35S::CO* compared with *35S::CO 35S::FLC* do not promote greatly earlier flowering.

Ubiquitous expression of SOC1 requires a complex promoter with multiple elements

Analysis of *SOC1* mRNA previously demonstrated that the gene has a broad pattern of expression and is regulated by multiple flowering-time pathways, suggesting that the regulatory sequences may be complex. Our analysis of 4, 2 and 1 kb *SOC1::GUS* expression patterns shows that the correct regulation of *SOC1* requires up to 4 kb of promoter sequence. Specifically, promoter sequences between nt –1955 and –4105 are needed for the expression of *SOC1* in cauline leaves and in flowers, for an age-dependent increase of expression in developing seedlings, and for full levels of expression in seedlings. A similar pattern of age-dependent regulation was reported for both *LFY* and *FT* (Blázquez *et al*., 1997; Kardailsky *et al*., 1999; Kobayashi *et al*., 1999).

A CArG box mediates repression by FLC in vivo

A CArG box motif at nt –400 in the *SOC1* promoter is bound specifically by FLC *in vitro* and mediates repression by FLC *in vivo*. *SOC1* may, therefore, be an immediate target of FLC, although it is also possible that expression of another MADS-domain protein is activated by FLC and this binds to the CArG box in the SOC1 promoter. Consistent with this notion, these two genes share a ubiquitous pattern of expression prior to the transition to flowering (Michaels and Amasino, 1999; Borner *et al*., 2000; Lee *et al*., 2000). However, mutation of the CArG box that is recognized *in vitro* by FLC in the promoter of *SOC1* did not lead to full derepression of *SOC1::GUS* expression *in vivo*. No other CArG box motifs were apparent in the 300 bp fragment C. This may indicate that FLC retains some ability to interact with the mutated CArG site *in vivo* that was not detected *in vitro*. Alternatively, FLC may also repress *SOC1* expression indirectly.

The gel shift data suggest that FLC can interact *in vitro* with the CArG box motif in the SOC1 promoter as a homodimer. FLC may also form complexes with other MADS-domain partners to interact with this motif as a heterodimer *in vivo*, as has been described for other MADS-domain proteins (reviewed in Riechmann and Meyerowitz, 1997). Phylogenetic analysis places *FLC* in a subfamily with two other MADS-box genes: *FLM*/*MAF1*/*AGL27* and *AGL31* (Alvarez-Buylla *et al*., 2000; Ratcliffe *et al*., 2001). *FLM* was recently demonstrated to function as a floral repressor, to have a pattern of expression similar to *FLC* and to be down-regulated by vernalization in some ecotypes (Ratcliffe *et al*., 2001; Scortecci *et al*., 2001). Another candidate for heterodimerization with FLC is the MADS-box protein encoded by *SVP*, which is also a negative regulator of the floral transition and has a pattern of expression that is similar to that of *FLC* (Hartmann *et al*., 2000). The effect of overexpression of these genes on *FT* and *SOC1* expression is untested.

A separate motif is required for activation by CO

Analysis of the overlapping 300 bp fragments A, B, C and D from the 1 kb *SOC1* promoter indicated that only fragment C (nt –482 to –131) could efficiently mediate upregulation of *SOC1::GUS* expression in *35S::CO* lines. This suggests that the motif that mediates activation by CO lies downstream of the CArG box and is located between the 3′ endpoint of fragment B at nt –372 and the 5′ endpoint of fragment D nt –248. Consistent with the idea that a separate motif is required for activation by CO, mutation of the CArG box at nt –400 did not impair the ability of fragment C to mediate activation in *35S::CO* lines.

CO probably does not bind directly to DNA to mediate activation of *SOC1*. We did not detect CO binding to the

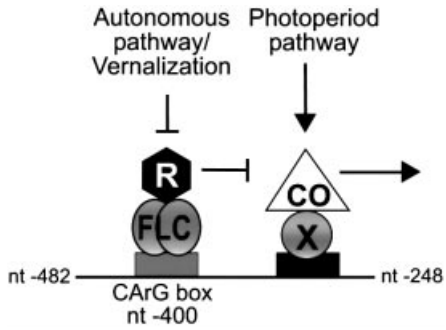

Fig. 7. Model for the antagonistic regulation of *SOC1* expression by CO and FLC. The horizontal line represents a 234 bp region of the *SOC1* promoter with the indicated nucleotide endpoints. Arrows indicate promotion and T-bars indicate repression of *SOC1* expression. We propose that FLC binds directly to a CArG box motif at nt –400 of the *SOC1* promoter and may recruit a global repression complex (R) to DNA to repress transcription. CO is proposed to be recruited by a sequence-specific DNA-binding factor (X) to an adjacent motif located between nt –372 and –248 in the *SOC1* promoter to mediate activation. Deletion of the CArG box does not abolish FLC-mediated repression, suggesting that FLC also represses *SOC1* expression by other more indirect mechanisms.

SOC1 promoter using yeast one-hybrid selection or gel retardation assays with either purified recombinant CO protein and/or nuclear extracts prepared from *35S::CO* or *35S::CO:GR* plants (Figure 5; S.R.Hepworth and G.Coupland, unpublished results). Also, sequence analysis shows that the zinc fingers of CO present at the N-terminus of the protein are most similar to those of B-box proteins (Robson *et al*., 2001). In animal proteins, the B-box is usually accompanied by a RING finger and closely followed (5–8 amino acids) by a predicted α-helical coiled coil. This RBCC motif is believed to mediate protein–protein interactions (Borden, 1998; Hassler and Richmond, 2001). The C-terminus region of CO, which contains a CCT domain, also appears to mediate protein–protein interactions with other transcription factors. For example, the CCT domains of CO and TOC1 will interact with the DNA-binding protein ABI3 (Kurup *et al*., 2000), suggesting that ABI3 or related proteins could recruit CO or its homologues to DNA.

Model for the regulation of SOC1 by CO and FLC

We propose that the antagonistic regulation of *SOC1* by CO and FLC requires a CArG box at nt –400 that mediates repression by FLC, and a separate motif located between nt –372 and –248 that mediates activation by CO (Figures 4A and 7). Based on previous experiments utilizing *35S::CO:GR* (Samach *et al*., 2000), we favour a model in which CO is recruited directly to the *SOC1* promoter by a DNA-binding factor to activate the expression of *SOC1* in LD photoperiods, and FLC binds directly to DNA to mediate signals from the autonomous and vernalization pathways. FLC may mediate repression through recruitment of a global repression complex to the promoter, although it remains possible that another MADS-domain protein mediates between FLC and the repression of *SOC1* expression. The finding that CO and FLC act through separate motifs implies that other flowering-time genes may be regulated by only CO or FLC, but not both. Furthermore, deletion of the FLC binding site from the *SOC1* promoter did not abolish repression of SOC1 by FLC, suggesting that in addition to the direct mechanism of repression described here, FLC can also repress *SOC1* expression indirectly. Determining how widespread is the antagonism between CO and FLC, and whether direct repression of CO targets by FLC is a general mechanism, will require analysing the response of other CO target genes to FLC.

Materials and methods

Plant material and growth conditions

Wild type was the Landsberg *erecta* ecotype of *A.thaliana*. *35S::FLC* plants and *flc* mutants were provided by R.Amasino (Michaels and Amasino, 1999, 2001). The *fca-1* mutant was provided by M.Koornneef (University of Wageningen, Wageningen, The Netherlands). The *35S::CO* and *35S::CO:GR* plants were described previously and contain the *co-2 tt4* mutations (Simon *et al*., 1996; Onouchi *et al*., 2000). We introduced *SOC1::GUS* reporter genes into *35S::CO*, *35S::CO 35S::FLC*, *35S::FLC* and *35S::CO:GR* plants by crossing. For most lines, homozygous plants were selected in the F_3 generation. Flowering time was measured as described by Putterill *et al*. (1995). The numbers of rosette and cauline leaves on the main stem were scored and data are expressed as means ± SEM.

RNA isolation and northern analysis

RNA was extracted from whole seedlings grown on GM agar plates as described by Putterill *et al*. (1995). Tissue was harvested 5 h after dawn except where noted otherwise. Northern hybridization techniques were as in Suárez-López *et al*. (2001). The *UBQ10*-, *FT*- and *β-TUB*-specific probes have been described (Snustad *et al*., 1992; Wang *et al*., 1997; Samach *et al*., 2000). A *SOC1*-specific probe (nt +181 to 640 of the cDNA) was amplified by PCR from template pSOC1P (P.Reeves and G.Coupland, unpublished) using M13-20 and M13 Reverse primers (Stratagene). A *GUS*-specific probe was amplified by PCR from template pSLJ4K1 (Jones *et al*., 1992) using as primers GUS-2 and GUS-3. Images were visualized using a PhosphorImager (Molecular Dynamics) and band intensities quantified using ImageQuant software (Molecular Dynamics). Information on all primers cited in the text is provided in the Supplementary data available at *The EMBO Journal* Online.

Gel retardation assay

Gel retardations were performed as described by Hepworth *et al*. (1995) using as probe fragments of the *SOC1* promoter whose 5′ overhangs were labelled by filling in with M-MLV reverse transcriptase (Life Technologies) in the presence of [α-^{32}P]dCTP. A 20 µl gel retardation mixture was as described in Hepworth *et al*. (1995) plus 10 000 c.p.m. of radioactively labelled probe, the indicated competitor DNA and ~120 ng of recombinant protein. After a 40 min incubation at room temperature, the samples were applied to an 8 or 12% polyacrylamide gel and run at 120 V. Images were visualized as above. Probes corresponding to all 300 bp fragments were amplified by PCR, digested with *Bam*HI to give 5′-GATC overhangs, gel purified and labelled as above. The 30 bp fragment probes (SOC1 WT, SOC1 M1, SOC1 M2) were formed by annealing complementary oligonucleotides, which also generated 5′-GATC overhangs. Oligonucleotide sequences are provided in the Supplementary data.

Synthesis of His-tag CO and His-tag FLC proteins

Plasmids pET19b-CO and pET19b-FLC, which expressed His-tag CO and His-tag FLC proteins, respectively, were generated as described in the Supplementary data. Recombinant proteins were expressed in *Escherichia coli* BL21(DE3)pLysS and purified to apparent electrophoretic homogeneity over a cobalt resin column (Talon®; Clontech) according to the manufacturer's instructions. The purified proteins were dialysed twice against PBS buffer at 4°C and stored at –20°C in 50% (v/v) glycerol.

Construction of SOC1::GUS reporter gene plasmids

SOC1::GUS reporter plasmids were derivatives of pGreen 0229, and the details of their construction are described in the Supplementary data.

Construction of mutant SOC1::GUS reporter gene plasmids

Site-directed mutagenesis was performed by PCR using the splice overlap extension procedure as described by Rowland and Segall (1998). To generate plasmid 1 kb ΔCArG SOC1::GUS, complementary reverse and forward oligonucleotides containing the desired mutations were used in separate PCRs with pSK208 as the template. Oligonucleotide sequences used to generate the mutations are provided in the Supplementary data.

GUS staining and activity measurement

For histochemical analysis of GUS expression, T_2 or T_3 seedlings were grown on GM agar. Homozygous T_3 seedlings were used in most cases, but preliminary analyses were often performed in the T_2, and the extreme late-flowering phenotype of *35S::FLC* plants delayed the availability of T_3 seedlings, making it necessary to use T_2 material. Tissue was incubated in heptane for 5 min to remove the cuticle, air dried and then incubated in X-Gluc staining solution (Jefferson *et al*., 1987). Quantitative measurements of GUS activity in seedlings were determined based on the method of Jefferson *et al*. (1987). The protein concentration of samples was determined using a Bradford assay (Bio-Rad) according to the manufacturer's instructions. GUS activity for each time point was determined in triplicate and expressed as relative fluorescence units (RFU) per microgram of protein. Data represent the means ± SEM.

Luciferase transient assay

The 1 kb SOC1 promoter was inserted upstream of the luciferase (LUC+; Promega) open reading frame and the *nos* poly(A) sequence. Details of the construct are provided in the Supplementary data. Thirty milligrams of 1 μm gold microcarriers were coated with both 1 kb SOC1::LUC and 35S::GFP according to the manufacturer's instructions. A Biolistic PD-100/He particle delivery Gun (Bio-Rad) with Hepta adapter allowing the use of seven macrocarrier discs was used to deliver the microcarriers to the leaves of 15-day-old plants. Luciferase expression was detected after 24 h by spraying leaves with 1 mM luciferin, and detection of luminescence with a Hamamatsu Argus-50(20)/CA Imaging and Analysis system. Luminescence intensity was quantified using Hamamatsu HPD:CP software, and integrated density of the GFP images was analysed using Adobe PhotoShop and Scion Image software.

Transformation of plants and selection of homozygous lines

Wild-type plants were transformed with *SOC1::GUS* reporter gene constructs by floral dipping (Clough and Bent, 1998). The *Agrobacterium* strain used was C58C1 pGV101 pMP90. Basta®-resistant transformants were selected on soil by treatment of seedlings with the herbicide Challenge containing glufosinate-ammonium as the active ingredient (AgrEvo). Homozygous lines were selected in the T_3 generation on GM agar plates that contained 12 mg/l phosphinothricin (Duchefa).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Alvarez-Buylla,E.R., Liljegren,S.J., Pelaz,S., Gold,S.E., Burgeff,C., Ditta,G.S., Vergara-Silva,F. and Yanofsky,M.F. (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J.*, **24**, 457–466.

Araki,T. (2001) Transition from vegetative to reproductive phase. *Curr. Opin. Plant Biol.*, **4**, 63–68.

Blázquez,M.A. and Weigel,D. (2000) Integration of floral inductive signals in *Arabidopsis*. *Nature*, **404**, 889–892.

Blázquez,M.A., Soowal,L.N., Lee,I. and Weigel,D. (1997) *LEAFY* expression and flower initiation in *Arabidopsis*. *Development*, **124**, 3835–3844.

Borden,K.L.B. (1998) RING fingers and B-boxes: zinc-binding protein–protein interaction domains. *Biochem. Cell Biol.*, **76**, 351–358.

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

Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.

Hartmann,U., Höhmann,S., Nettesheim,K., Wisman,E., Saedler,H. and Huijser,P. (2000) Molecular cloning of *SVP*: a negative regulator of the floral transition in *Arabidopsis*. *Plant J.*, **21**, 351–360.

Hassler,M. and Richmond,T.J. (2001) The B-box dominates SAP-1–SRF interactions in the structure of the ternary complex. *EMBO J.*, **20**, 3018–3028.

Hepworth,S.R., Ebisuzaki,L.K. and Segall,J. (1995) A 15-base-pair element activates the *SPS4* gene midway through sporulation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **15**, 3934–3944.

Huang,H., Mizukami,Y., Hu,Y. and Ma,H. (1993) Isolation and characterization of the binding sequences for the product of the *Arabidopsis* floral homeotic gene *AGAMOUS*. *Nucleic Acids Res.*, **21**, 4769–4776.

Jefferson,R.A., Kavanagh,T.A. and Bevan,M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, **6**, 3901–3907.

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

Jones,J.D.J., Shlumukov,L., Carland,F., English,J., Scofield,S.R., Bishop,G.J. and Harrison,K. (1992) Effective vectors for transformation, expression of heterologous genes and assaying transposon excision in transgenic plants. *Transgenic Res.*, **1**, 285–297.

Kardailsky,I., Shukla,V.K., Ahn,J.H., Dagenais,N., Christensen,S.K., Nguyen,J.T., Chory,J., Harrison,M.J. and Weigel,D. (1999) Activation tagging of the floral inducer *FT*. *Science*, **286**, 1962–1965.

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

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.

Koornneef,M., Blankestijn-de Vries,H., Hanhart,C., Soppe,W. and Peeters,T. (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. *Plant J.*, **6**, 911–919.

Kurup,S., Jones,H.D. and Holdsworth,M.J. (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seeds. *Plant J.*, **21**, 143–155.

Lee,H., Suh,S.-S., Park,E., Cho,E., Ahn,J.H., Kim,S.-G., Lee,J.S., Kwon,Y.M. and Lee,I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes Dev.*, **14**, 2366–2376.

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

Michaels,S.D. and Amasino,R.M. (2001) Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell*, **13**, 935–941.

Onouchi,H., Igeño,M.I., Périlleux,C., Graves,K. and Coupland,G. (2000) Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among *Arabidopsis* flowering-time genes. *Plant Cell*, **12**, 885–900.

Pnueli,L., Gutfinger,T., Hareven,D., Ben-Naim,O., Ron,N., Adir,N. and Lifschitz,E. (2001) Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. *Plant Cell*, **13**, 2687–2702.

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

Ratcliffe,O.J., Nadzan,G.C., Reuber,T.L. and Riechmann,J.L. (2001) Regulation of flowering in *Arabidopsis* by an *FLC* homologue. *Plant Physiol.*, **126**, 122–132.

Reeves,P.H. and Coupland,G. (2000) Response of plant development to environment: control of flowering by daylength and temperature. *Curr. Opin. Plant Biol.*, **3**, 37–42.

Riechmann,J.L. and Meyerowitz,E.M. (1997) MADS domain proteins in plant development. *Biol. Chem.*, **378**, 1079–1101.

Robson,F., Costa,M.M.R., Hepworth,S., Vizir,I., Piñeiro,M., Reeves, P.H., Putterill,J. and Coupland,G. (2001) Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. *Plant J.*, **28**, 619–631.

Rouse,D.T., Sheldon,C.C., Bagnall,D.J., Peacock,W.J. and Dennis,E.S. (2002) FLC, a repressor of flowering, is regulated by genes in different inductive pathways. *Plant J.*, **29**, 183–191.

Rowland,O. and Segall,J. (1998) A hydrophobic segment within the 81-amino-acid domain of TFIIIA from *Saccharomyces cerevisiae* is essential for its transcription factor activity. *Mol. Cell. Biol.*, **18**, 420–432.

Samach,A., Onouchi,H., Gold,S.E., Ditta,G.S., Schwarz-Sommer,Z., Yanofsky,M.F. and Coupland,G. (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis. Science*, **288**, 1613–1616.

Sanda,S.L. and Amasino,R. (1996) Interaction of *FLC* and late-flowering mutations in *Arabidopsis thaliana*. *Mol. Gen. Genet.*, **251**, 69–74.

Scortecci,K.C., Michaels,S.D. and Amasino,R.M. (2001) Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. *Plant J.*, **26**, 229–236.

Sheldon,C.C., Burn,J.E., Perez,P.P., Metzger,J., Edwards,J.A., Peacock,W.J. and Dennis,E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell*, **11**, 445–458.

Sheldon,C.C., Rouse,D.T., Finnegan,E.J., Peacock,W.J. and Dennis,E.S. (2000) The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC) *Proc. Natl Acad. Sci. USA*, **97**, 3753–3758.

Shiraishi,H., Okada,K. and Shimura,Y. (1993) Nucleotide sequences recognized by the AGAMOUS MADS domain of *Arabidopsis thaliana in vitro*. *Plant J.*, **4**, 385–398.

Shore,P. and Sharrocks,A.D. (1995) The MADS-box family of transcription factors. *Eur. J. Biochem.*, **229**, 1–13.

Simon,R., Igeño,I.M. and Coupland,G. (1996) Activation of floral meristem identify genes in *Arabidopsis*. *Nature*, **384**, 59–62.

Simpson,G.G., Gendall,A.R. and Dean,C. (1999) When to switch to flowering. *Annu. Rev. Cell Dev. Biol.*, **15**, 519–550.

Snustad,D.P., Haas,N.A., Kopczak,S.D. and Silflow,C.D. (1992) The small genome of *Arabidopsis* contains at least nine expressed β-tubulin genes. *Plant Cell*, **4**, 549–556.

Suárez-López,P., Wheatley,K., Robson,F., Onouchi,H., Valverde,F. and Coupland,G. (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature*, **410**, 1116–1119.

Wang,Z.Y., Kenigsbuch,D., Sun,L., Harel,E., Ong,M.S. and Tobin,E.M. (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis Lhcb* gene. *Plant Cell*, **9**, 491–507.

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