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Crosstalk between Cold Response and Flowering in *Arabidopsis* Is Mediated through the Flowering-Time Gene *SOC1* and Its Upstream Negative Regulator *FLC*

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The appropriate timing of flowering is pivotal for reproductive success in plants; thus, it is not surprising that flowering is regulated by complex genetic networks that are fine-tuned by endogenous signals and environmental cues. The *Arabidopsis thaliana* flowering-time gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) encodes a MADS box transcription factor and is one of the key floral activators integrating multiple floral inductive pathways, namely, long-day, vernalization, autonomous, and gibberellin-dependent pathways. To elucidate the downstream targets of *SOC1*, microarray analyses were performed. The analysis revealed that the *soc1-2* knockout mutant has increased, and an *SOC1* overexpression line has decreased, expression of cold response genes such as *CBFs* (for CRT/DRE binding factors) and *COR* (for cold regulated) genes, suggesting that *SOC1* negatively regulates the expression of the cold response genes. By contrast, overexpression of cold-inducible *CBFs* caused late flowering through increased expression of *FLOWERING LOCUS C* (*FLC*), an upstream negative regulator of *SOC1*. Our results demonstrate the presence of a feedback loop between cold response and flowering-time regulation; this loop delays flowering through the increase of *FLC* when a cold spell is transient as in fall or early spring but suppresses the cold response when floral induction occurs through the repression of cold-inducible genes by *SOC1*.

INTRODUCTION

Flowering, a transition from vegetative to reproductive phase, is the most dramatic change in the plant's life cycle. To maximize reproductive success, plants have evolved an intricate mechanism determining flowering time in response to both environmental factors, such as light and temperature, and endogenous signals that reflect the plant's developmental state and age (Boss et al., 2004; Baurle and Dean, 2006; Oh and Lee, 2007). It is also known that flowering is regulated by various abiotic stresses, such as nutrient deficiency, heat, and cold (Kim et al., 2004; Balasubramanian et al., 2006; Baurle and Dean, 2006). Extensive genetic and physiological analyses of *Arabidopsis thaliana* have revealed that floral induction is regulated by at least four major genetic pathways, namely, long-day, autonomous, vernalization (a long period of cold for flowering), and gibberellin-dependent pathways. These four pathways commonly regulate so-called flowering pathway integrators *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *LEAFY* (*LFY*), and the exact flowering time is determined by the expression level of these integrators (Blazquez and Weigel, 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000; Moon et al., 2003; Moon et al., 2005). Such integrators are regulated antagonistically by two central upstream regulators: *CONSTANS* (*CO*), encoding a zinc finger protein, and *FLOWERING LOCUS C* (*FLC*), encoding a MADS box transcription factor (Michaels and Amasino, 1999; Lee et al., 2000; Samach et al., 2000). *CO*, mediating the long-day pathway, acts as a positive regulator, whereas *FLC*, mediating the autonomous/vernalization pathway, acts as a negative regulator of flowering (Lee et al., 2000; Samach et al., 2000).

In addition to these four major pathways, flowering is known to be fine-tuned by other mechanisms. For example, flowering time is adjusted by the ambient temperature such that cool temperature delays flowering, whereas warm temperature accelerates flowering (Blazquez et al., 2003; Balasubramanian et al., 2006). In *Arabidopsis*, ambient cool temperature is sensed through genes such as *FCA*, *FVE*, and *SHORT VEGETATIVE PHASE* (*SVP*): the mutants, *fca, fve*, and *svp*, exhibit insensitivity to ambient cool temperature for flowering (Blazquez et al., 2003; Halliday et al.,

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2003; Lee et al., 2007b). *FCA* and *FVE* are two autonomous pathway genes that have a function to repress *FLC* expression, and SVP is a floral repressor that makes a flowering repressor complex together with FLC (Li et al., 2008). This so-called thermosensory pathway eventually regulates *FT* expression (Blazquez et al., 2003; Lee et al., 2007b). Similarly, ambient warm temperature accelerates flowering through an increase of *FT* expression (Balasubramanian et al., 2006). However, such effect is suppressed by *FLC* and is modulated by *FLOWERING LOCUS M* (*FLM*), an *FLC* homolog (Balasubramanian et al., 2006).

Intermittent cold treatment, a short-term cold treatment during the day, also delays flowering, an effect that is mediated by *FVE* (Ausin et al., 2004; Kim et al., 2004). The *fve* mutant, showing late flowering due to increased expression of *FLC*, exhibits ectopic expression of cold-regulated (*COR*) genes without cold treatment. In addition, it shows increased freezing tolerance, and its flowering time is not delayed by intermittent cold, indicating that *FVE* is a genetic linker between flowering time and cold response (Kim et al., 2004).

Cold induces the expression of many genes encoding a diverse array of proteins that enhance the tolerance of plants to freezing temperature. Such *COR* genes share C-repeat/dehydration response elements (CRT/DRE) in their promoters, and *CRT/DRE binding factors* (*CBFs*) act as the key regulators of cold response pathway in *Arabidopsis* (Thomashow, 1999). It was reported that overexpression of *CBF1*, *CBF2*, and *CBF3* causes late flowering and dwarf phenotypes as well as phenotypes associated with freezing tolerance, such as increases of Pro and sugar concentrations and transcriptional activation of *COR* genes (Gilmour et al., 2004). *CBFs* are positively regulated by *ICE1* (for *inducer of CBF expression 1*), which encodes a MYC-like basic helix-loop-helix transcription factor (Chinnusamy et al., 2003), whereas they are negatively regulated by *HOS1* (for *high expression of osmotically responsive genes*), which encodes a RING finger protein, probably acting as a E3 ubiquitin ligase (Lee et al., 2001).

Although the regulation of flowering pathway integrator *SOC1* has been relatively well studied, the downstream factors of *SOC1* remain largely unknown. To elucidate the downstream targets of the floral integrator *SOC1* encoding a MADS box transcription factor, we performed microarray experiments using both an overexpression line and a null mutant. Here, we report that the floral activator *SOC1* functions as a negative regulator of the cold response pathway through the direct repression of *CBFs*. By contrast, overexpression of *CBFs* increases the expression level of *FLC*. In conclusion, our results suggest that *SOC1* and *FLC* are the key regulators of crosstalk between cold response and flowering time regulation. Such a feedback loop involving *SOC1*, cold response genes, and *FLC* may prevent premature flowering under cold conditions in fall or early spring and therefore may be evolutionarily advantageous.

RESULTS

SOC1 Negatively Regulates Cold-Inducible Genes

To monitor the global gene expression regulated by *SOC1*, we performed a microarray analysis using the Affymetrix ATH1 GeneChip as a preliminary screen. We used RNA extracted from an overexpression allele *soc1-101D*, a null allele *soc1-2*, and wild-type Columbia (Col) grown for 7 d under long days. Sampling was specifically done with 7-d-old seedlings because all genotypes including the early flowering *soc1-101D* are in the vegetative phase at this time, which could be determined by the absence of *APETALA1* expression (Hempel et al., 1997). Interestingly, six out of the top-ranked 20 negatively regulated genes by *SOC1* were the well-known cold-inducible (*COR*) genes.

To confirm if a loss-of-function or a gain-of-function mutation in *SOC1* affects the expression of cold-inducible genes, we analyzed the expression of a range of genes by RNA gel blot analysis in *soc1-2* and *soc1-101D* (Figure 1A). As is shown, four cold-inducible genes, *COR15a*, *COR15b*, *KIN1*, and *KIN2*, exhibited increased expression in *soc1-2* and decreased expression in *soc1-101D* under long days (16 h light/8 h dark) at 22°C.

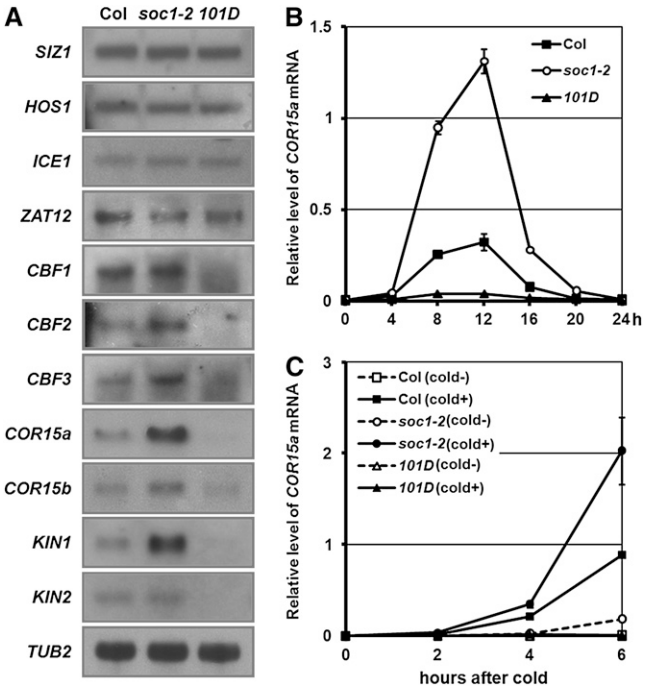

Figure 1. *SOC1* Negatively Regulates Cold-Inducible Genes.

(A) Expressions of cold-responsive genes in wild-type (Col), *soc1-2*, and *soc1-101D* (*101D*) was detected by RNA gel blot analysis. *TUB2* was used as a quantitative control. Plants grown at 22°C for 10 d under 16-h-light/8-h-dark long-day conditions were harvested at 8 h after dawn for RNA isolation.

(B) Daily rhythm of *COR15a* expression in wild-type, *soc1-2*, and *soc1-101D* grown under long days was detected by quantitative RT-PCR. The values and error bars represent mean value and SD, respectively, from three technical replicates. The 10-d-old seedlings grown 16-h-light/8-h-dark conditions were harvested every 4 h for RNA isolation. The zero time corresponds to right after dawn.

(C) Cold response of *COR15a* in wild-type, *soc1-2*, and *soc1-101D*. Expression level of *COR15a* was detected by quantitative RT-PCR. Plants were grown at 22°C for 10 d under long days and then transferred to 4°C (cold+) or maintained at 22°C (cold-) for 0, 2, 4, and 6 h in the light. The zero time corresponds to right after dawn.

Because cold response genes are known to be under circadian control (Harmer et al., 2000; Fowler et al., 2005), we checked the daily rhythm of *COR15a* expression as a representative cold-inducible gene (Figure 1B). It showed a peak at 12 h after dawn and became minimal during the night period. During the circadian cycle, *soc1-2* showed higher expression, whereas *soc1-101D* showed lower expression than the wild type, although the biggest difference was observed at the 12-h peak. This result suggests that the negative regulation of cold-inducible genes by *SOC1* is not affected by the circadian rhythm, although the amplitude is changed. Next, we addressed whether *SOC1* affects the induction kinetics of cold-inducible genes. For this, we treat with 4°C cold immediately after dawn because the daily temperature is usually the lowest at dawn in nature. As is shown in Figure 1C, *soc1-2* exhibited much stronger, and *soc1-101D* showed much weaker induction of *COR15a* expression compared with the wild type. This result strongly suggests that *SOC1* attenuates the induction of *COR* genes in response to cold.

SOC1 Directly Represses the Expression of *CBF* Genes

The majority of *COR* genes have cold- and dehydration-responsive DNA regulatory elements designated CRT/DRE in their promoter (Yamaguchi-Shinozaki and Shinozaki, 1994), and the expression of *COR* genes is mediated by CRT/DRE that is regulated by the *CBF* gene family (Stockinger et al., 1997). To determine whether the negative regulation of *COR* genes by *SOC1* is mediated through *CBFs*, we compared the expression of *CBF1*, *CBF2*, and *CBF3* in the wild type, *soc1-2*, and *soc1-101D*. The expression level of *CBFs* increased in *soc1-2* and decreased in *soc1-101D* (Figure 1A). We also compared the daily rhythm of *CBF3* expression in the three genotypes (Figure 2A). *CBF3* expression exhibited a peak at 8 h after dawn, which is 4 h before the *COR15a* peak. Similar to *COR15a*, *CBF3* was increased in *soc1-2* and decreased in *soc1-101D* during the daily cycle. In addition, the expression of *CBF3* in response to cold treatment was higher in *soc1-2* but lower in *soc1-101D* especially at 2 h after cold treatment (Figure 2B). Thus, the increased expression of various *COR* genes in *soc1-2* is most likely due to the enhanced expression of *CBFs*.

In the cold response pathway, *ICE1* and *HOS1* are positive and negative upstream regulators of the *CBF* family, respectively (Chinnusamy et al., 2007). To determine whether *SOC1* regulates the transcriptional level of *ICE1* and *HOS1*, the expression of *ICE1* and *HOS1* was also checked by RNA gel blot analysis (Figure 1A). In comparison to the wild type, the expressions of *ICE1* and *HOS1* were not changed in *soc1-2* or *soc1-101D*. In addition, expression of *ZAT12*, a negative upstream regulator of *CBF1* and *CBF2* that is also induced by cold treatment (Rizhsky et al., 2004; Vogel et al., 2005), was not affected by *soc1-101D* or *soc1-2* (Figure 1A). Thus, these results indicate that *SOC1* suppresses the cold response pathway through the repression of *CBF* genes.

It is reported that SOC1, a MADS box transcription factor, binds to variant forms of the CArG box in the promoter of *LFY* (Lee et al., 2008; Liu et al., 2008). Promoter analysis revealed that all three *CBF* genes have two variant forms of CArG boxes at the distal and proximal regions (Figure 2C). Thus, we wondered if SOC1 binds to the promoters of *CBF* genes directly. The two regions of *LFY* promoter were used as negative and positive controls for chromatin immunoprecipitation (ChIP) based on a previous report (Lee et al., 2008). ProLFY-1, a distal region of *LFY* promoter, was highly enriched in *soc1-101D* compared with *soc1-2*, whereas ProLFY-4, a proximal region of *LFY* promoter, was not enriched in *soc1-101D* as reported (Figure 2D). Interestingly, the ChIP analysis revealed that all CArG-box regions in the *CBF* promoters are enriched by *SOC1* overexpression (Figures 2D and 2E). Such results strongly suggest that SOC1 negatively regulates cold response through direct repression of the transcription of *CBFs*.

CBF Genes Activate *FLC* Expression

It has been reported that overexpression of *CBF* genes causes late flowering (Figure 3A; Liu et al., 2002; Gilmour et al., 2004), but it is not elucidated why. Because *FLC* is a central repressor of flowering in *Arabidopsis*, we checked if *FLC* expression is increased by overexpression of *CBFs*. Indeed, the *FLC* expression was increased more than twofold in *35S-CBF1*, *35S-CBF2*, and *35S-CBF3* (Figure 3B). We also checked the expression of *SVP*, a flowering repressor encoding another MADS box transcription factor (Hartmann et al., 2000) because SVP is known to mediate the ambient cool temperature delay of flowering and interacts with FLC to make a flowering repressor complex (Lee et al., 2007b; Li et al., 2008). In contrast with *FLC*, *CBF* overexpression did not affect on the expression of *SVP* (Figure 3B). *FLM*, a gene included in *FLC* clade genes, represses flowering and modulates flowering at warm temperature (Balasubramanian et al., 2006). The expression of *FLM* is not affected by *CBF* overexpression, similar to *SVP* (Figure 3B).

To address if late flowering in *35S-CBF1*, *35S-CBF2*, and *35S-CBF3* is caused by increased expression of *FLC*, these lines were vernalized to suppress *FLC* expression. After 40 d of vernalization, the *FLC* expression was strongly suppressed (Figure 3C). Correlated with this, the late flowering phenotype of *35S-CBF1*, *35S-CBF2*, and *35S-CBF3* was also suppressed (Figure 3D), indicating that the late flowering phenotype of *CBF* overexpression line is caused by increase of *FLC* expression.

Intermittent Cold Delays Flowering through *FLC* Activation

It is reported that intermittent cold treatment delayed flowering time through upregulation of *FLC* (Kim et al., 2004). We further analyzed the regulation of flowering by intermittent cold: a treatment of 6 h cold (4°C) beginning at dawn every day. First, we checked the effect of intermittent cold on the daily rhythm of *COR15a* and *CBF3* (Figures 4A and 4B). Without cold, *COR15a* expression peaked at 12 h after dawn and greatly decreased at dusk. However, with the intermittent cold treatment, higher peak expression of *COR15a* was observed at 6 h after dawn when the cold treatment was over and remained higher until 8 h after dawn. Subsequently, it was dramatically reduced at 12 h after dawn. *CBF3* expression was also increased by cold treatment: the expression peaked at 6 h and then abruptly decreased to a minimal level at 8 h after dawn. Our results show that intermittent cold causes highly increased expression of *COR* and *CBF* genes in the morning.

Next, we addressed how many days of intermittent cold are required to delay flowering (Figures 4C to 4E). The result showed

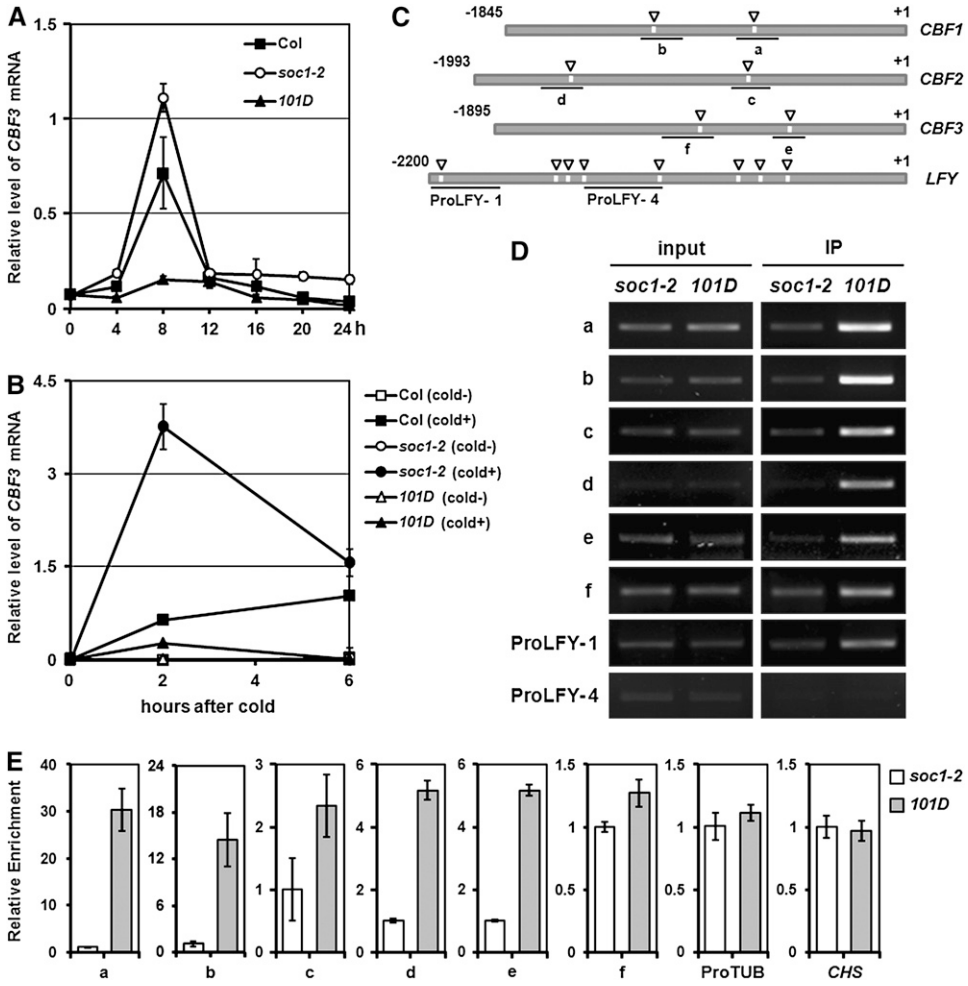

Figure 2. SOC1 Directly Represses *CBF* Expression.

(A) Daily rhythm of *CBF3* in wild-type (Col), *soc1-2*, and *soc1-101D* (*101D*) under long days. Expression level of *CBF3* was detected by quantitative RT-PCR. The 10-d-old seedlings were harvested every 4 h for RNA isolation.

(B) Cold response of *CBF3* in wild-type, *soc1-2*, and *soc1-101D*. Expression level of *CBF3* was detected by quantitative RT-PCR. Plants grown at 22°C for 10 d under long days were transferred to 4°C for 0, 2, and 6 h in the light. The quantitative RT-PCR analysis was biologically repeated three times, and each time point consisted of three technical replicates in both **(A)** and **(B)**. The error bars represent SD for three technical replicates.

(C) Four graphic bars represent the promoters of *CBF1*, *CBF2*, *CBF3*, and *LFY*. The arrowheads denote putative CArG box, and black lines (a-f, ProLFY-1, ProLFY-4) indicate the regions used for ChIP.

(D) ChIP assay with SOC1 antibody. Enrichment of *CBFs* promoters (a to f) was confirmed by ChIP-PCR. ProLFY-1 was used as a positive control, and ProLFY-4 was used as a negative control.

(E) Quantitative real-time PCR analysis using the same ChIP-PCR products in **(D)**. Values are normalized against *soc1-2* and are means of triplicate experiments with error bars representing SD. Negative controls, *pTUB* and *CHS*, are shown at right.

that 10 d of cold slightly delays flowering and 20 d of cold delays it further, indicating that the flowering is delayed in proportion to the days of the cold treatment. Consistent with this, *FLC* expression was increased according to the days of cold. By contrast, *SVP* expression was not changed by intermittent cold, which is correlated well with the fact that *SVP* expression is not affected in the *35S-CBF* lines.

If the delay of flowering that is induced by intermittent cold is caused by the increase of *FLC*, it is expected that the flowering of *flc*, a null mutant, would not be delayed by intermittent cold. Indeed, *flc* showed the same flowering time with and without intermittent cold (Figure 4F). Interestingly, *svp* mutants also showed no response to intermittent cold, although *SVP* expression was not affected by cold treatment (Figure 4F). This may be because SVP produces a flowering repressor complex together with FLC as reported (Li et al., 2008). The *soc1-101D* mutants, in which strong suppression of *CBFs* is observed, also showed insensitivity to the intermittent cold (Figure 4F). Together, our results suggest that intermittent cold delays flowering through FLC activity, which is induced by *CBFs*.

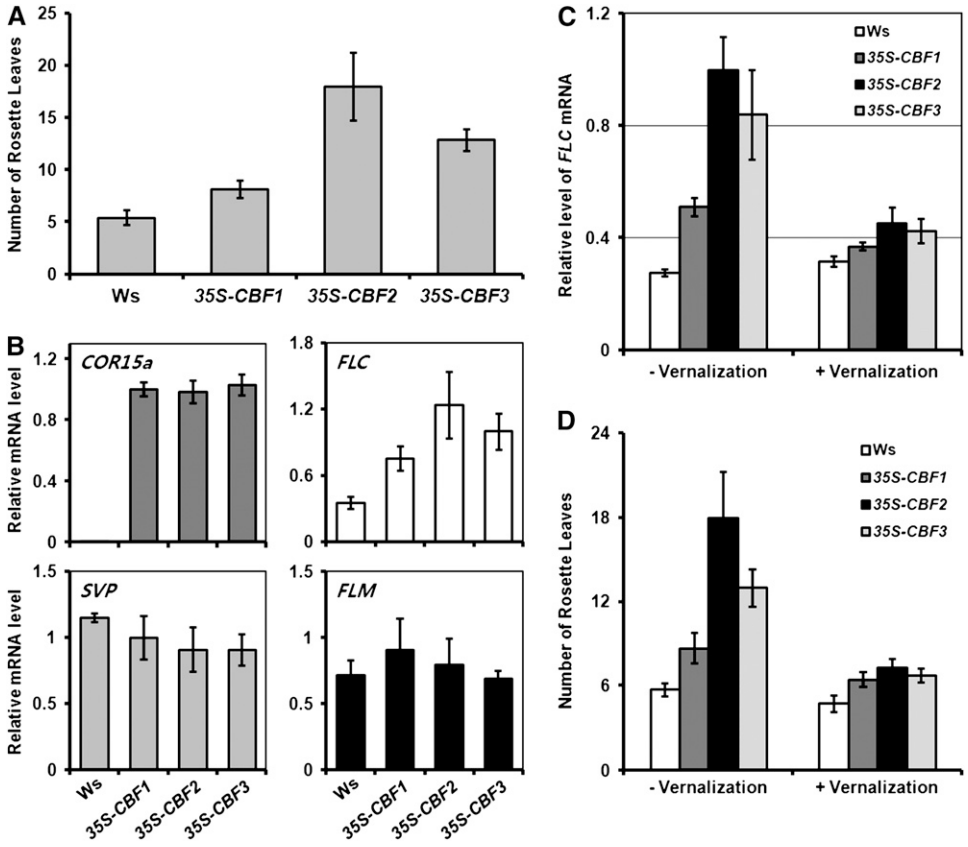

Figure 3. *CBFs* Positively Regulate *FLC* Expression.

(A) Flowering time of wild-type (Wassilewskija) and *CBF* overexpression lines. Thirty plants were used to measure the flowering time, and the error bars represent SD.

(B) Expression levels of *COR15a*, *FLC*, *SVP*, and *FLM* were determined by quantitative RT-PCR.

(C) Suppression of *FLC* expression in *CBF* overexpression lines by vernalization. Expression level of *FLC* was detected by quantitative RT-PCR.

(D) Flowering time of *CBF* overexpression lines without or with 40 d of vernalization. Plants with −Vernalization were grown at 22°C for 9 d under long days, whereas plants with +Vernalization were grown at 22°C for 5 d under long days and then transferred to 4°C for 40 d.

Vernalization Overrides the Effect of Cold Stress on Delaying Flowering

Vernalization, an exposure to prolonged cold temperature, has the opposite effect on flowering compared with intermittent cold treatment. To understand the molecular basis of this difference, we examined the effects of vernalization and intermittent cold on *COR15a* and *CBFs* expression in Col:*FRI*^{SF2}, a line showing high expression of *FLC* and a dramatic acceleration of flowering by vernalization (Michaels and Amasino, 1999; Choi et al., 2005). As expected, intermittent cold treatment caused strong increase in *CBF3* and *COR15a* and slight increase in *FLC* expression level, which causes a slight delay in flowering time (Figures 5A and 5B). By contrast, when the tissues were harvested immediately after 40 d of vernalization treatment, *FLC* expression was strongly suppressed in Col:*FRI*^{SF2}, although the plants showed strong induction of *CBF1* and *CBF3* and much stronger induction of *COR15a* (Figure 5A). Taken together, our results show that vernalization overrides the effect of cold stress on flowering and suggest that vernalization and cold stress affect *FLC* expression and flowering via distinct mechanisms.

Effect of *SOC1* Mutation on Freezing Tolerance

Because *soc1-2* increases and *soc1-101D* decreases the induction of *CBFs* and *COR* genes, it was of interest to determine whether these mutants exhibit differences in freezing tolerance. To address this question, plants were exposed to −5°C for 6 h and transferred to room temperature to check the survival rate (Figure 6). As expected, more *soc1-2* mutants survived than the wild type, whereas few *soc1-101D* mutants survived after the freezing treatment. Our result demonstrates that *SOC1* regulates not only flowering but also freezing tolerance.

COR Gene Expression Is Regulated by Some Other Flowering-Time Genes

Because *COR15a* expression has known to be increased in *fve* mutants as well as in *soc1-2* (Kim et al., 2004), we tested whether

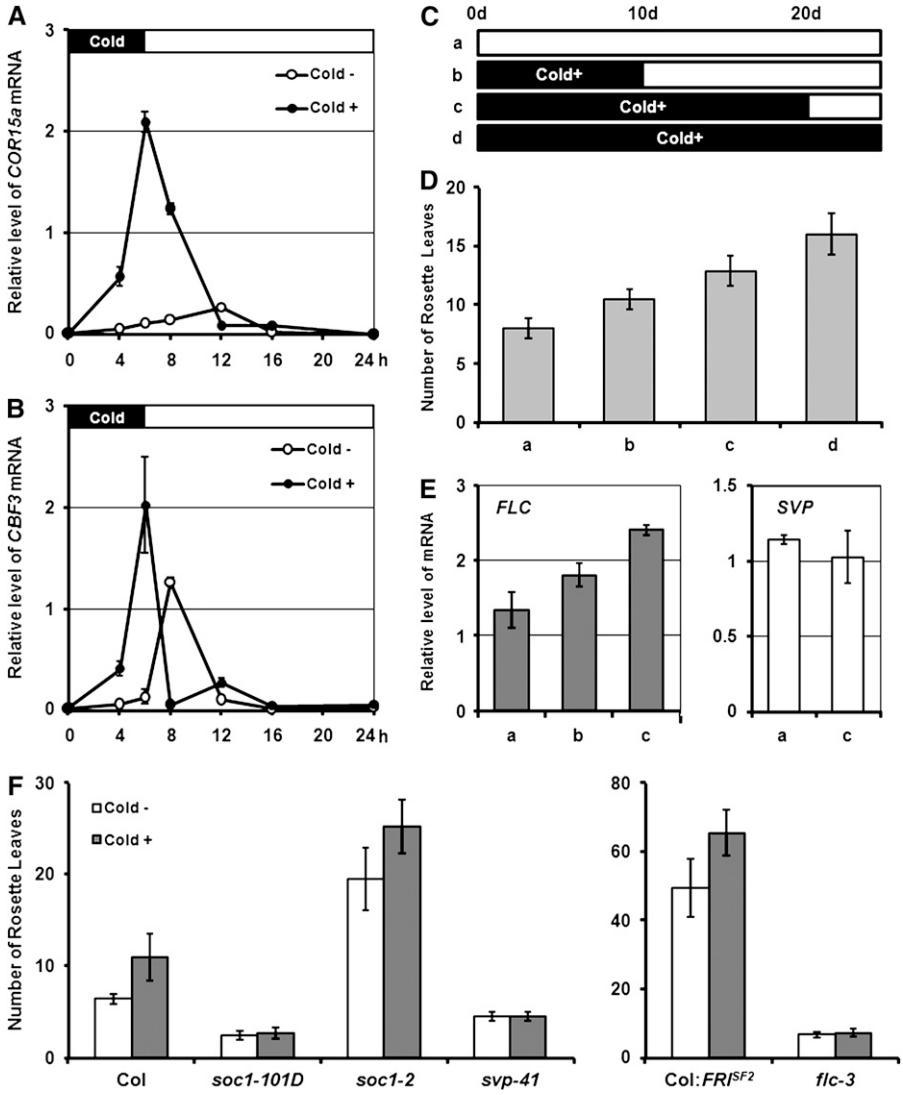

Figure 4. Effect of Intermittent Cold on Flowering.

(A) Comparison of *COR15a* expressions between plants grown with (Cold +) and without (Cold −) intermittent cold (4°C). Expression level of *COR15a* was detected by quantitative RT-PCR. Intermittent cold treatments were for 6 h from dawn every day. For RNA isolation, the 10-d-old seedlings were harvested at 0, 4, 6, 8, 12, 16, and 24 h after dawn.

(B) Expression level of *CBF3* detected by quantitative RT-PCR. The quantitative RT-PCR analysis was biologically repeated three times, and each time point consisted of three technical replicates in both **(A)** and **(B)**. The error bars represent SD for three technical replicates.

(C) The schematics of intermittent cold treatment. The white bars represent normal growth conditions, and the black bars represent intermittent cold treatment.

(D) Effect of intermittent cold treatment length on the flowering time.

(E) Effect of intermittent cold treatment length on the expression of *FLC* and *SVP.* Expression level of *FLC* and *SVP* was detected by quantitative RT-PCR. Col plants grown 20 d in each condition were harvested at 6 h after dawn for RNA isolation.

(F) The effect of intermittent cold on the flowering time of each mutant. The mutants of *soc1-101D*, *soc1-2*, and *svp-41* in the left graph are in the Col background, whereas the *flc-3* mutants in the right graph are in the Col:FRI^{SF2} background. Plants were treated with (gray bars, Cold +) or without (white bars, Cold −) intermittent cold for 6 h from the dawn every day until they flowered.

any other late-flowering mutants show a similar increase in *COR15a* expression (Figure 7). Increased levels of *COR15a* transcript were observed in *gi*, a long-day pathway mutant, and in *fpa*, an autonomous pathway mutant. By contrast, *co* and *ft*, two long-day pathway mutants, as well as *fca* and *fld*, two autonomous pathway mutants, did not show any difference in the expression of *COR15a* in comparison to that in the wild type. Interestingly, the *ld* mutant consistently showed reduced expression of *COR15a* (Figures 7A and 7B). This result indicates that late flowering per se is not the cause of the ectopic

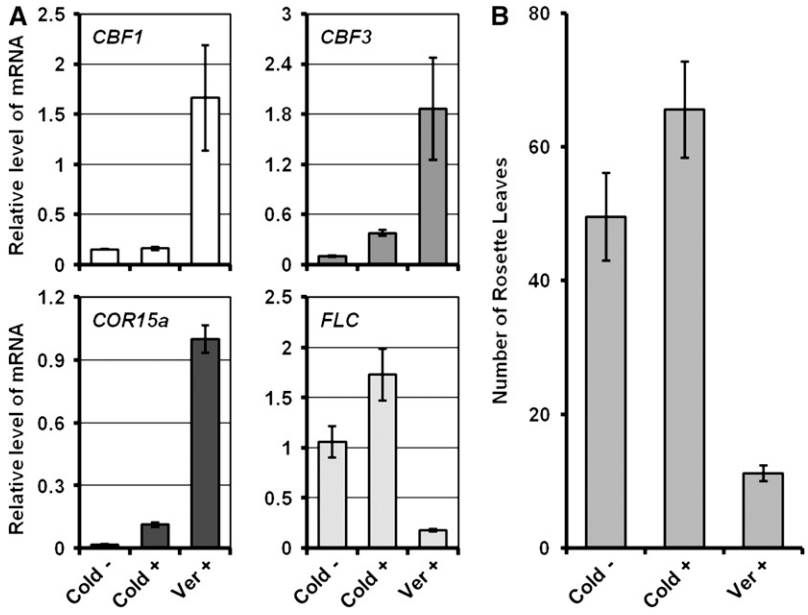

Figure 5. Comparison of Vernalization and Intermittent Cold.

(A) Expression levels of *CBF1*, *CBF3*, *COR15a*, and *FLC* in Col:*FRI*^{SF2} grown with intermittent cold (4°C) for 6 h every day (Cold +) or with 40 d of vernalization (Ver +). The quantitative RT-PCR analysis was biologically repeated three times, and each time consisted of three technical replicates. The error bars represent SD from triplicate samples.

(B) Effect of vernalization and intermittent cold on the flowering time of Col:*FRI*^{SF2}. Thirty plants were used to measure the flowering time, and the error bars represent SD.

expression of *COR15a*. It also suggests that the increased *COR15a* expression is not simply due to the decreased level of *SOC1* in the late-flowering mutants because all the late-flowering mutants used in this study have reduced levels of *SOC1* transcripts (Lee et al., 2000). For example, among the late-flowering mutants analyzed here, the *fca* mutant, which has the lowest expression of *SOC1*, did not show any difference, whereas *gi* and *fpa*, which have relatively higher *SOC1* expression, showed increased *COR15a* expression (Figures 7A and 7B; Lee et al., 2000). These findings suggest that the suppression of cold-inducible genes occurs through *SOC1*-dependent and *SOC1*-independent pathways.

To confirm the hypothesis of two independent pathways, we compared the level of *COR15a* in the single mutant *soc1-2* and the double mutants *soc1-2 fve-3* and *soc1-2 gi-2* (Figure 7D). The mutants, *soc1-2*, *fve-3*, and *gi-2*, we used in this experiment are null (Fowler et al., 1999; Borner et al., 2000; Ausin et al., 2004). As expected, the double mutants showed higher expression of *COR15a* than *soc1-2*. We also compared the level of *COR15a* between *soc1-101D* and the double mutants *soc1-101D fve-3* and *soc1-101D gi-2* (Figure 7E). The double mutants showed a similar reduced level of *COR15a* as the *soc1-101D* single mutant, indicating that overexpression of *SOC1* overcomes the derepression caused by the mutations in *GI* and *FVE*.

Similar to *soc1-2*, the *gi* and *fve* mutants showed an increase in *CBF1* expression, although the increase in *fve* was relatively less (Figure 7C). Thus, *GI* and *FVE* are also likely to suppress the cold response pathway through the repression of *CBF* genes. Taken together, our results indicate the existence of *SOC1*-dependent and *SOC1*-independent pathways for regulating flowering in response to cold signals.

DISCUSSION

In this study, we identified downstream targets of *SOC1*, which is a key integrator of flowering pathways, by microarray analysis. Unexpectedly, many of genes that are negatively regulated by *SOC1* were identified as cold-inducible genes. By contrast, the overexpression of cold response genes delays flowering through the activation of *FLC*, as does cold stress. This finding reveals the presence of a feedback loop between cold response and flowering, which is another fine-tuning mechanism for flowering time regulation. We propose to name this mechanism as an intermittent cold-sensing pathway for flowering.

Model of Intermittent Cold-Sensing Pathway for Flowering

A model of the intermittent cold-sensing pathway for flowering in *Arabidopsis* is presented in Figure 8. When the ambient temperature is cold during vegetative growth, the expression of *CBFs* is induced in response to cold (Thomashow, 1999). The increased expression of *CBFs* then causes the activation of *FLC*, which represses the two flowering pathway integrators *FT* and *SOC1*, thereby delaying the flowering. On the other hand, a decreased level of *SOC1* causes derepression of cold-inducible genes.

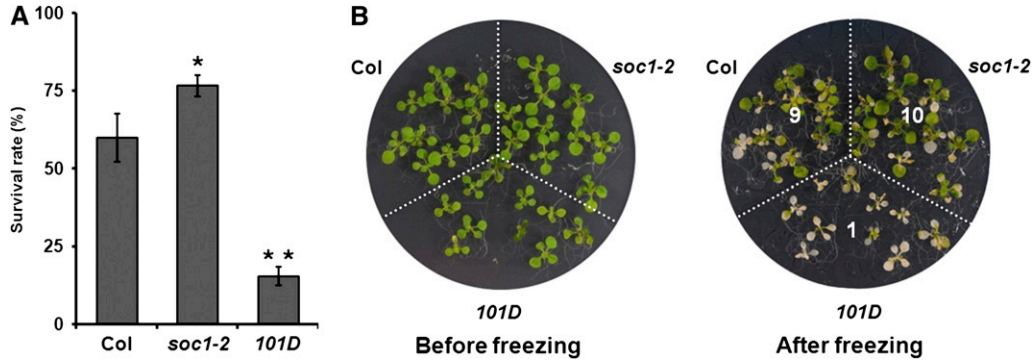

Figure 6. Effect of *soc1* Mutations on Freezing Tolerance.

(A) The freezing-tolerance of *soc1-2* and *soc1-101D* (*101D*) plants compared with wild-type plants. Experiments were performed in triplicate, and percentage of the plants survived was calculated: $n \geq 30$. Mean values and standard errors were plotted. The * and ** denote statistical significance with $P < 0.05$ and $P < 0.01$ (Student's *t* test), respectively.

(B) Sample plates showing plants subjected to freezing tolerance assays. Each plate contains 10 plants per line. The numbers on the plate to the right denote the number of plants that survived after freezing.

Such derepression appears to enable plants to respond to cold more strongly, as was seen in the *soc1-2* mutant (Figures 1C and 2B). Consistent with this, the *soc1-2* mutant showed enhanced resistance to freezing tolerance (Figure 6). Therefore, cold temperature during vegetative growth not only delays flowering but also makes the plants more sensitive to cold. However, when flowering occurs, usually in late spring, *SOC1* expression increases (Lee et al., 2000), and increased *SOC1* represses cold-inducible genes and thus suppresses the cold response. Such a suppression of cold response can be observed in *soc1-101D*, an overexpression mutant (Figures 1C and 2B). In addition to *SOC1*, other flowering time genes, such as *FPA*, *FVE*, and *GI*, are involved in the cold-sensing pathway, although the mechanism needs to be further analyzed (Figure 7).

Such a feedback loop between the cold response and flowering could be evolutionarily advantageous. When cold conditions prevail in fall, the intermittent cold-sensing pathway would delay flowering time, providing protection against premature flowering. In addition, for annual plants that start growing from early spring, such a mechanism would delay flowering until full-blown spring has come. By contrast, if flowering sets in, plants suppress the cold response because the expression of *CBFs* is not desirable for reproductive development, as seen in overexpression lines of *CBFs*, which show growth retardation (Gilmour et al., 2004).

Cold Response and Vernalization

Both vernalization and intermittent cold-sensing pathways recognize cold temperature; however, their effects on flowering are opposite: whereas vernalization accelerates flowering, intermittent cold sensing delays it. Interestingly, the target of both vernalization and intermittent cold sensing is *FLC*. It is well known that vernalization suppresses the expression of *FLC* through histone modification (Sung and Amasino, 2004a). Here, we provide evidence demonstrating that *FLC* is also a target of the intermittent cold-sensing pathway. First, intermittent cold stress increases the transcript level of *FLC* and delays the flowering. Second, the *flc* null mutant does not exhibit delayed flowering in response to cold stress. Third, overexpression of *CBFs* delays flowering through the activation of *FLC*. Finally, vernalization, which suppresses the expression of *FLC*, offsets the effect of *CBFs* overexpression (Figures 3C and 3D). It is noteworthy that Liu et al. (2002) did not find that the *CBF1* overexpression line increases *FLC* expression in the Col:FRI^{SF2} background. However, this difference may be because Col:FRI^{SF2} line has such a high basal level of *FLC* expression.

Although vernalization has an opposite effect on *FLC*, its effect on *CBFs* and *COR* genes is the same as that of cold stress; that is, vernalization causes a strong induction of *CBF1*, *CBF3*, and *COR15a* (Figure 5A). Thus, cold stress and vernalization cannot be distinguished at the *CBF* and *COR15a* expression level. On the other hand, this distinction can be made at the *VIN3* gene expression level because *VIN3* is induced not by a short period of cold but by a long period of cold that is sufficient to trigger vernalization (Sung and Amasino, 2004b). Vernalization-induced *VIN3* expression initiates inactivation of *FLC* by histone modification of *FLC* chromatin. Thereafter, *VRN1*, *VRN2*, and *LHP1* permanently inactivate *FLC* chromatin structure via heterochromatin formation (Bastow et al., 2004; Sung and Amasino, 2004b; Mylne et al., 2006; Sung et al., 2006). Thus, vernalization suppresses *FLC* expression epigenetically despite the presence of a positive regulator, such as the FRI complex (Kim et al., 2006). Currently, it is not known how *CBFs* regulate *FLC* expression, but it is very likely that they cannot resolve the heterochromatic state of *FLC* caused by vernalization. There are two CRT/DRE cold response elements at the proximal region of the *FLC* promoter; thus, it would be worthwhile to determine whether CBFs bind the *FLC* promoter in vivo.

SOC1-Dependent and *SOC1*-Independent Mechanisms for the Intermittent Cold-Sensing Pathway

SOC1 is not the only genetic factor that affects the intermittent cold-sensing pathway with respect to flowering. This and a

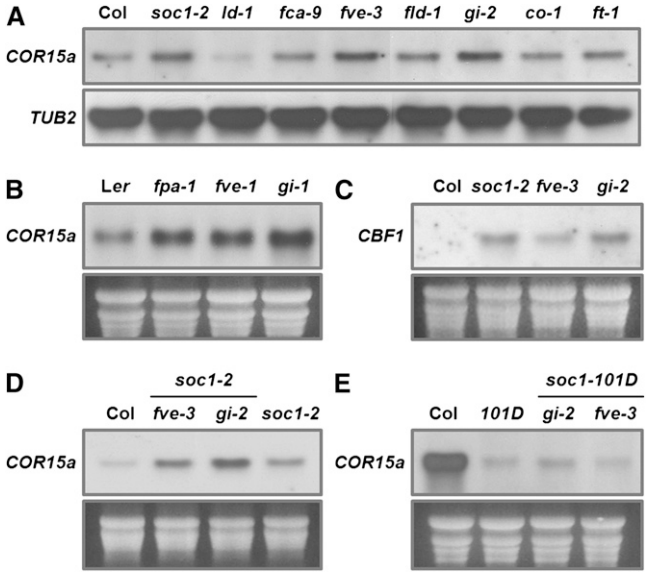

Figure 7. Expression of *COR15a* Is Regulated by Other Flowering Time Genes.

(A) RNA gel blot analysis of *COR15a* in various late-flowering mutants in Col background. *TUB2* probe was used as a loading control.
(B) RNA gel blot analysis of *COR15a* in *fpa-1*, *fve-1*, and *gi-1* mutants in Landsberg *erecta* background.
(C) RNA gel blot analysis of *CBF1* in Col, *soc1-2*, *fve-3*, and *gi-2*.
(D) RNA gel blot analysis of *COR15a* in double mutants with *soc1-2.*
(E) RNA gel blot analysis of *COR15a* in double mutants with *soc1-101D.*
Total RNAs were presented as quantitative control for RNA gel blot analysis in **(B)** to **(E)**.

previous study showed that three other flowering genes, namely, *FPA*, *FVE*, and *GI*, act in this pathway (Kim et al., 2004). In the current model of flowering time regulation, all three genes regulate *SOC1* through the long-day pathway or the autonomous pathway (Parcy, 2005). Thus, *SOC1* appeared to integrate the cold-sensing signal from the upstream factors *FPA*, *FVE*, *GI*, and *FLC*. However, our results indicate a more complex pathway. First, other flowering time mutants with strongly reduced expression of *SOC1*, such as *fca* and *ld*, did not exhibit increased expression of cold-inducible genes (Figure 7A). Second, *CO* and *FT* do not participate in the intermittent cold-sensing pathway, although *GI* regulates *SOC1* through the activation of *CO* and *FT* (Figure 7A). Third, the double mutants *soc1 gi* and *soc1 fve* showed an additive effect with regard to the increase in *COR15a* expression (Figure 7D). This strongly indicates that the cold-sensing pathway is distinct from other well-defined genetic pathways for flowering. It also suggests that *FPA*, *FVE*, and *GI* act on the intermittent cold-sensing pathway independent of *SOC1*. One caveat is that the decreased level of *SOC1* in the *fca*, *fld*, *ld*, *co*, and *ft* mutants does not cause increased *COR15a* expression, although the *soc1* null mutation does. The low level of *SOC1* remaining in such mutants probably is sufficient to repress *COR15a* since it has been reported that ~30 to 70% of the wild-type *SOC1* level is detected in these mutants (Lee et al., 2000).

FVE and *GI* are classified in different flowering pathways (Parcy, 2005; Oh and Lee, 2007). Consistently, no differences in *GI* expression between *fve-1* mutants and wild-type plants were detected, indicating they do not affect the transcription of the other (Fowler et al., 1999). Thus, it is likely that these two genes affect the intermittent cold-sensing pathway via separate mechanisms. However, both *fve* and *gi* showed epistatic interaction with *fpa* in a double mutant analysis (Koornneef et al., 1998; Veley and Michaels, 2008). Thus, there is still an open possibility that these three genes participate in the same intermittent cold-sensing pathway for flowering, which is independent of *SOC1*. It is noteworthy that *GI* was identified as a gene that is highly induced in response to cold from a microarray analysis (Fowler and Thomashow, 2002). In addition, the *gi* mutant shows increased resistance against paraquat-induced oxidative stress (Kurepa et al., 1998). Therefore, *GI* may have a function in stress responses as well as in flowering.

Recently, it was reported that low red/far-red light ratio at a low ambient temperature (16°C) induces the expression of *COR15a* and *COR15b* through CBF activity (Franklin and Whitelam, 2007). In such low ambient temperature, phytochrome B (phyB) and phyD suppress the expression of *COR* genes; therefore, the mutations in *phyB* and *phyD* or low red/far-red increase the expression of *COR* genes. Although the mechanism is not well understood, such results with ours here can explain why *phyB* mutant at 16°C flowers later than the wild type, while it flowers earlier than the wild type at normal temperature, 22°C (Halliday et al., 2003). The increased activity of CBF in the *phyB* mutant at 16°C is likely to delay flowering. However, *phyB* at 16°C did not show an increase of *FLC* (Halliday et al., 2003), which is inconsistent with our results. Thus, it adds another layer of complexity in the crosstalk between the regulation of *COR* genes and flowering time control.

Crosstalk with the Thermosensory Pathway for Flowering

The key components of intermittent cold-sensing pathway are *SOC1*, *CBFs*, and *FLC* (Figure 8). SOC1 directly binds to the *CBF*

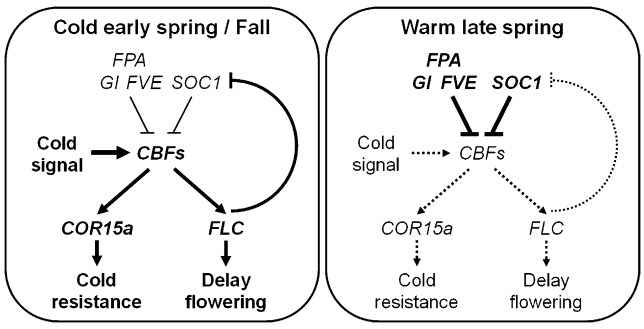

Figure 8. Model of Crosstalk between Cold Response and Flowering Time Regulation.

Arrows indicate promotion, and T bars indicate repression. In cold early spring or fall, the expression of *CBF* genes is activated by the cold signal, and the increased *CBFs* activate *FLC* expression, which eventually delays flowering time. By contrast, in warm late spring, floral induction occurs and the increased *SOC1*, *GI*, *FVE*, and *FPA* suppress the *CBF*-dependent cold response pathway.

promoters, which have modified CArG boxes, for the transcriptional repression (Figure 2). *CBFs* positively regulate *FLC* expression, then FLC represses flowering pathway integrators to delay flowering. In this report, we showed that SOC1 acts as transcriptional repressor of *CBF* genes. It is well known that SOC1 acts as transcriptional activator for the expression of *LFY* (Lee et al., 2008; Liu et al., 2008). However, it is reported that SOC1 also acts as transcriptional repressor by directly binding the *SEP3* promoter (Liu et al., 2009). Therefore, it is likely that SOC1 can act as both an activator and a repressor depending on the cofactors.

Additional components in this pathway are *FPA*, *FVE*, and *GI* that negatively regulate *CBF* expression. In the thermosensory pathway, it is suggested that *FT* and *SVP* are major players, and *FCA* and *FVE* are involved in this pathway (Blazquez et al., 2003; Lee et al., 2007b). In addition, it is proposed that *FLC* is not involved in this pathway because the *flc* null mutant shows delayed flowering time in response to low temperature, although *FLC* expression is increased in this condition (Blazquez et al., 2003; Lee et al., 2007b). Thus, it appears that the two pathways are independent. However, our results here show that the two pathways are intertwined. First, the two pathways share the same component, *FVE*, one of the autonomous pathway genes that regulate *FLC* expression. Second, *SVP* is also involved in the intermittent cold-sensing pathway in genetic terms. Although the expression of *SVP* is not affected by either intermittent cold or *CBFs*, the *svp* mutant shows insensitivity to intermittent cold for flowering as does the *flc* mutant (Figures 3B, 4E, and 4F). Because SVP makes a flowering repressor complex with FLC (Li et al., 2008), it indicates that SVP-FLC complex is involved in intermittent cold-sensing pathway. Therefore, there is a cross-talk between the thermosensory pathway and the intermittent cold-sensing pathway, although they are partially independent as well. In conclusion, we elucidated a fine-tuning mechanism of flowering in response to cold, which must confer adaptability to an ever-changing environment.

METHODS

Plant Materials and Growth Conditions

Sterilized seeds were incubated on 0.85% plant agar (Duchefa) containing 1% sucrose and half-strength Murashige and Skoog (Duchefa) medium for 3 d at 4°C to break seed dormancy. The normal condition in long days was followed as previously described (Lee et al., 2008). To test the induction kinetics by cold, plants were grown at 22°C for 10 d under long days and then transferred to 4°C (cold+) or maintained at 22°C (cold-) for 0, 2, 4, or 6 h in the light. The zero time corresponds to right after dawn. For intermittent cold treatment, plants were placed at 4°C for 6 h from right after dawn every day. For vernalization treatment, 5-d-old seedlings were incubated at 4°C for 40 d under short-day conditions. The *soc1-2* and *soc1-101D* mutants were described previously as *agl20* and *agl20-101D*, respectively (Lee et al., 2000). The mutants of *svp-41*, *ft-1*, *co-1*, *gi-2*, *ld-1*, *fve-3*, *fca-9*, and *fld-1* are in the Col background, and *fpa-1*, *fve-1*, and *gi-1*, are in the Landsberg *erecta* background (Koornneef et al., 1991; Lee et al., 1994; Putterill et al., 1995; Fowler et al., 1999; Kardailsky et al., 1999; Kobayashi et al., 1999; Page et al., 1999; Hartmann et al., 2000; He et al., 2003; Ausin et al., 2004; Kim et al., 2004). The *flc-3* mutants are in the Col:FRI^{SF2} background (Lee et al., 2000). At least 16 plants were used to measure the flowering time. The flowering time was measured as the number of rosette leaves produced when flowering occurs. The overexpression lines of *CBF1*, *CBF2*, and *CBF3* in the Wassilewskija background were previously described (Gilmour et al., 2004).

Analysis of Gene Expression

Total RNA was isolated from plant tissues by the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. For RNA gel blot analysis, 20 μg of RNA was separated on 1.5% denaturing formaldehyde agarose gels and transferred to Hybond N^+ nylon membranes (Amersham Biosciences). All RNA probes were prepared from plasmid vectors containing the cDNA fragments of each gene amplified by RT-PCR with primers as follows: *SIZ1*, 5′-GCAGGAACTTGTCGACCGG-3′ and 5′-GCTTGCACCATCATTTGGGATAG-3′; *HOS1*, 5′-ATGGATACGAGAGAAATCAACGG-3′ and 5′-ATACAGACATTGGTGATATAATG-3′; *ICE1*, 5′-ATGGGTCTTGACGGAAACAATGG-3′ and 5′-ACAGAACTCAAATCCCTGTTCCC-3′; *ZAT12*, 5′-ATGGTTGCGATATCGGAGATC-3′ and 5′-TCAATAAACTGTTCTTCCAAGCTC-3′; *CBF1*, 5′-ATGAACTCATTTTCAGCTTT-3′ and 5′-TTAGTAACTCCAAAGCGACA-3′; *CBF2*, 5′-CTTCTACTTACTCTACTCTCATAAAC-3′ and 5′-ATTTGCATTTGACAACAACTTTTACC-3′; *CBF3*, 5′-GACGACGGATCATGGCTTC-3′ and 5′-TAATAACTCCATAACGATACGTCG-3′; *COR15a*, 5′-ATGCTCTCGAGGCTTCAGATTTCGTGACGG-3′ and 5′-ATGCTGGTACCTGAAGAGAGAGGATATGG-3′; *COR15b*, 5′-ATGGCGATGTCTTTATCAGGAG-3′ and 5′-TCAGGACTTTGTGGCATTCTTAG-3′; *KIN1*, 5′-AAGCCCACATCTCTTCTCATC-3′ and 5′-TTATTTGAATATAAGTTTGGCTCGTC-3′; *KIN2*, 5′-CATAATTGATTCTCGTACTCATCG-3′ and 5′-GGTAAAACAAAGTTCTTAGAACTTAAAC-3′; *TUB2*, 5′-CTCAAGAGGTTCTCAGCAGTA-3′ and 5′-TCACCTTCTTCATCCGCAGTT-3′. Each fragment was inserted at single 3′-T overhangs of pGEM-T Easy Vector (Promega) between T7 and SP6 RNA polymerase promoters. These plasmids were linearized with *Nco*I restriction enzyme that leaves a 5′ overhang. One microgram of the purified, linearized plasmid was used as template of in vitro transcription by SP6 RNA polymerase. RNA probes were made by the digoxigenin RNA labeling kit (Roche) according to the manufacturer's instructions. Prehybridization, hybridization, wash, and detection were performed as described in the digoxigenin application manual (Roche).

For cDNA production, 4 μg of total RNA was reverse transcribed with oligo$(dT)_{18}$ primer (Fermentas) in a 20-μL reaction mixture using RevertAid M-MuLV reverse transcriptase (Fermentas). After heat inactivation, total volume of the reaction mixture was diluted in 580 μL of sterilized water, and 4 μL was used for the real-time quantitative RT-PCR. All quantitative RT-PCR analyses were performed by iQ5 multicolor real-time PCR detection system (Bio-Rad) using iQ SYBR Green supermix (Bio-Rad). We adopted the guidelines for the experimental design and statistical analysis of quantitative RT-PCR data (Rieu and Powers, 2009). The PCR condition was as follows: 40 cycles of PCR (95°C for 30 s, 60°C for 30 s, and 72°C for 20 s) after the initial denaturation step of 5 min at 95°C. Data was collected at 72°C in each cycle, and the expression levels of genes were calculated by iQ5 optical system software version 2.0 using *TUB2* as the reference gene. The quantitative RT-PCR analysis was biologically repeated three times, and each time consisted of three technical replicates. The primers used for quantitative RT-PCR are as follows: *COR15a*, 5′-CTTACCTAATCAGTTAATTTCAAGCA-3′ and 5′-TTAAACATGAAGAGAGAGGATATGG-3′; *CBF1*, 5′-CTTGAAAAAGAAATCTACCTG-3′ and 5′-AGTACGTAGTTACTAGAGTTCTC-3′; *CBF3*, 5′-CGACGTATCGTTATGGAGTTATTA-3′ and 5′-CTAAAAATAATAATAAAATAAAAAGTATCGTAC-3′; *FLC*, 5′-GAGAATAATCATCATGTGGGAGC-3′ and 5′-CAACCGCCGATTTAAGGTGG-3′; *SVP*, 5′-CCGGAAAACTGTTCGAGTTC-3′ and 5′-TGACTGCAAGTTATGCCTCTCT-3′; *FLM*, 5′-TGAAGAACCAAATGTCGATAATGT-3′ and 5′-ATCAGTTCTGCCTTCCTAGC-3′; and *TUB2*, 5′-ATCGATTCCGTTCTCGATGT-3′ and 5′-ATCCAGTTCCTCCTCCCAAC-3′.

ChIP Assay

ChIP with SOC1 antibody was performed by following the method described previously (Lee et al., 2007a, 2008). Briefly, 600 mg of *soc1-2* and *soc1-101D* seedlings grown under long days for 8 d was used for ChIP. After cross-linking with 1% formaldehyde, extracted cells were lysed and the DNA is broken into pieces of 0.3 to 1.0 kb length by sonication. Then, immunoprecipitation using anti-SOC1 serum, raised in rabbits by repeated injection of $SOC1_{IKC}$-GST fusion proteins, was performed. The purified protein-DNA complexes were heated to reverse cross-linking, allowing the DNA to be separated from the proteins. One-twentieth of the purified DNA was used for PCR analysis, and 1/100 was used for real-time quantitative PCR. Fifteen microliters of the ChIP products resuspended in 400 μL of TE was used for PCR, and 4 μL of them was used for real-time quantitative PCR. In PCR analysis, sonicated input DNA (0.5%) was used as a quantitative control. In quantitative PCR analysis, expression levels were normalized against the expression in *soc1-2.* The primers for the *CBFs* promoter regions containing CArG box are as follows: a, 5′-CAGGACAGGACTAAGCGAAG-3′ and 5′-GCGA-GAGGTAACGAGAGAGA-3′; b, 5′-CGTACGGACGTTCGTTTTTGAA-3′ and 5′-CCTCAATTATCTTCTTATCTCGC-3′; c, 5′-GAATATGCTAG-AGTAATTTCCTAAGA-3′ and 5′-CCCTGCCACTTGTTAATTCTC-3′; d, 5′-GCCAAGGATTAGACCGATATAG-3′ and 5′-CATTCCTTGTCGA-TATATTTCTCC-3′; e, 5′-GAATTGGGAGAGTAGATATTTGTG-3′ and 5′-AAAATGTTACATTTGATCATTCACCC-3′; f, 5′-AGATCAATTAGAAG-CATGCAGTTG-3′ and 5′-GAGGGCGTTGAGATTGTGATC-3′; ProLFY-1, 5′-CCGGATCCATCCATTTTTCGCAAAGG-3′ and 5′-CCGGATCCATCT-GTTCTAAAGCCTCC-3′; ProLFY-4, 5′-CCGGATCCCCCATATGTCCAA-TCCCA-3′ and 5′-CCGGATCCATCTATCTGCGTTTTAGG-; ProTUB, 5′-ACAAACACAGAGAGGAGTGAGCA-3′ and 5′-ACGCATCTTCGGTT-GGATGAGTGA-3′; and *CHS*, 5′-CCACCATTCCAATCTTGGTAAGTA-3′ and 5′-AGAAGCACCAGCCATCACCAT-3′.

Freezing-Tolerance Assays

Eleven-day-old plants were placed at −5°C for 6 h, and then they were incubated at 23°C for 2 d for recovery. The percentage of plants that survived after this freezing and recovery was calculated. Experiments were performed in triplicate, and each experiment was accomplished in a plate (diameter 150 × 20 mm) containing 10 plants per each control or mutant.

Microarray Analysis

Total RNA was prepared using Trizol reagent (Sigma-Aldrich). Double-stranded cDNA was synthesized using 10 μg of total RNA mixed with T7-$(dT)_{24}$ primer using SuperScript Choice System (Invitrogen). Next, the cDNA was used to synthesize biotinylated cRNA using the Enzo BioArray High Yield RNA transcript labeling kit (Affymetrix). Twenty micrograms of cRNA was fragmented in a fragmentation buffer (40 mM Tris acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc in DEPC water) at 94°C for 35 min before undergoing chip hybridization. We used *Arabidopsis* ATH1 Genome Array (Affymetrix). Hybridization, washing, and scanning steps were performed at the Affymetrix Service Center (Seoulin Bioscience Institute). Affymetrix GCOS software was used for scanning and basic analysis. More detailed analysis was performed using Affymetrix DMT software. The microarray data have been submitted to the Gene Expression Omnibus of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). Series accession number is GSE3279, which contains the whole experimental samples. Each sample accession number is GSM73643, GSM73646, GSM73647, GSM73648, GSM73649, GSM73650, and GSM73651.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *SOC1* (AT2G45660), *FLC* (AT5G10140), *CBF1* (AT4G25490), *CBF2* (AT4G25470), *CBF3* (AT4G25480), *KIN1* (AT5G15960), *KIN2* (AT5G15970), *COR15a* (AT2G42540), *COR15b* (AT2G42530), *ICE1* (AT3G26744), *HOS1* (AT2G39810), *SIZ1* (AT5G60410), *ZAT12* (AT5G59820), *SVP* (AT2G22540), *FLM* (AT1G77080), *FVE* (AT2G19520), *FPA* (AT2G43410), *GI* (AT1G22770), *FCA* (AT4G16280), *LD* (AT4G02560), *FLD* (AT3G10390), *FT* (AT1G65480), *LFY* (AT5G61850), *CO* (AT5G15840), *AP1* (AT1G69120), *VIN3* (AT5G57380), *VRN1* (AT3G18990), *VRN2* (AT4G1684), *LHP1* (AT5G17690), *FRI* (AT4G00650), *phyB* (AT2G18790), *phyD* (AT4G16250), and *TUB2* (AT5G62690).

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Crosstalk between Cold Response and Flowering in Arabidopsis Is Mediated through the Flowering-Time Gene SOC1 and Its Upstream Negative Regulator FLC
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