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The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*

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Summary

The floral transition in *Arabidopsis* is regulated by at least four flowering pathways: the long-day, autonomous, vernalization, and gibberellin (GA)-dependent pathways. Previously, we reported that the MADS-box transcription factor SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) integrates the long-day and vernalization/autonomous pathways. Here, we present evidences that SOC1 also integrates signaling from the GA-dependent pathway, a major flowering pathway under non-inductive short days. Under short days, the flowering time of GA-biosynthetic and -signaling mutants was well correlated with the level of *SOC1* expression; overexpression of *SOC1* rescued the non-flowering phenotype of *ga1-3*, and the *soc1* null mutant showed reduced sensitivity to GA for flowering. In addition, we show that vernalization-induced repression of *FLOWERING LOCUS C* (*FLC*), an upstream negative regulator of *SOC1*, is not sufficient to activate *SOC1*; positive factors are also required. Under short days, the GA pathway provides a positive factor for *SOC1* activation. In contrast to *SOC1*, the GA pathway does not regulate expression of other flowering integrators *FLC* and *FT*. Our results explain why the GA pathway has a strong effect on flowering under short days and how vernalization and GA interact at the molecular level.

Keywords: flowering, MADS-box gene, SOC1, gibberellin, vernalization.

Introduction

Flowering, a transition from vegetative to reproductive growth, is regulated by both environmental and endogenous cues. Extensive genetic analyses to elucidate the molecular mechanism of flowering in *Arabidopsis*, a quantitative long-day plant, have revealed at least three flowering pathways as the long-day, autonomous, and vernalization pathways (reviewed in Koornneef *et al.*, 1998a; Levy and Dean, 1998; Mouradov *et al.*, 2002; Simpson and Dean, 2002). Mutations in the genes such as *CONSTANS* (*CO*), *GIGANTEA* (*GI*), and *FT* that are involved in the long-day pathway cause late flowering under long days but do not delay flowering under short days compared to the wild type. On the contrary, mutations in genes such as *FCA*, *LUMINIDEPENDENS* (*LD*), *FVE*, and *FPA* that are involved in the autonomous pathway cause late flowering under both long days and short days compared to the wild type. In addition, mutants of the genes involved in autonomous pathway show a strong response to vernalization, a prolonged cold treatment that accelerates flowering, suggesting that the vernalization and autonomous pathways merge at some point (Koornneef *et al.*, 1991, 1998b).

The genetic analysis of natural variation of flowering among different geographical *Arabidopsis* ecotypes revealed two major genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), that confer the winter annual flowering habit (Burn *et al.*, 1993; Clarke and Dean, 1994; Koornneef *et al.*, 1994; Lee *et al.*, 1993, 1994; Michaels and Amasino, 1999a; Napp-Zinn, 1985). *FRI-FLC*-containing plants show a very late flowering phenotype and a very strong response to vernalization. Four weeks of vernalization treatment offsets the effect of *FRI FLC* and thus fully suppresses the late-flowering phenotype. *FRI* encodes a novel protein with two coiled-coil domains, whereas *FLC* encodes a MADS-box transcription factor (Johanson *et al.*, 2000; Michaels and

Amasino, 1999a; Sheldon *et al.*, 1999). *FRI* causes an increase of *FLC* expression level and thus acts as a positive regulator of *FLC. FLC* acts as a strong repressor of floral transition (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999, 2000). On the other hand, vernalization reduces *FLC* expression, thus causing early flowering (Michaels and Amasino, 1999a; Sheldon *et al.*, 2000). Interestingly, the genes involved in the autonomous pathway act as negative regulators of *FLC*; if the genes are mutated, *FLC* expression is increased and the level of FLC is well correlated with the lateness in flowering (Michaels and Amasino, 2001; Sheldon *et al.*, 2000). Therefore, the merging point of the autonomous and vernalization pathways is the *FLC* gene.

From activation-tagging mutagenesis to screening for *FRI FLC* suppressor mutants, we isolated an early flowering mutant that overexpresses *AGAMOUS-LIKE 20* (*AGL20*), another MADS-box gene (Lee *et al.*, 2000). Molecular and genetic analyses showed that *AGL20* is negatively regulated by *FLC* and positively regulated by the genes involved in autonomous pathway through *FLC*. In addition, *AGL20* is positively regulated by the long-day pathway. *AGL20* was also isolated in a screen for suppressors of *35S::CO* and was shown to be a direct target of CO (Onouchi *et al.*, 2000; Samach *et al.*, 2000). Thus, it is a synonym of *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). Hereafter, we refer to *AGL20* as *SOC1* because the function of the gene was first reported with the designation of *SOC1*. Also, we suggest that the activation-tagged mutant *agl20-101D* be named *soc1-101D* and the T-DNA-tagged *agl20* null mutant be named *soc1-2* (Lee *et al.*, 2000). As the expression of *SOC1* is regulated by both *FLC* and *CO*, two central genes regulating the autonomous/vernalization pathways and the long-day pathway, respectively, it is proposed to act as an integrator of flowering pathways (Araki, 2001; Hepworth *et al.*, 2002; Lee *et al.*, 2000; Mouradov *et al.*, 2002; Simpson and Dean, 2002).

Gibberellin (GA) has been known to induce flowering in many plant species (Bernier, 1988). Flowering of *Arabidopsis* is also promoted by GA. For example, exogenous treatment of GA accelerates flowering of *Arabidopsis* particularly under short days (Chandler and Dean, 1994; Langridge, 1957). In addition, the mutations that disrupt either GA biosynthesis or signaling show alterations in flowering time (Jacobsen and Olszewski, 1993; Wilson *et al.*, 1992). The *Arabidopsis* mutant *ga1-3* has a deletion in the gene encoding ent-kaurene synthetase A, which catalyzes the first committed step in GA biosynthesis (Sun and Kamiya, 1994). The mutant *ga1-3* fails to flower under short days and shows a slight delay in flowering under long days (Wilson *et al.*, 1992). Thus, GA is absolutely required for flowering under short days in *Arabidopsis*. The dominant *gibberellic acid insensitive-1* (*gai-1*) mutant flowers extremely late under short days, and the flowering phenotype is not rescued by the exogenous treatment of GA (Wilson *et al.*, 1992). On the other hand, the mutant *spindly* (*spy*), which causes constitutively active GA signaling, flowers early under both long days and short days (Jacobsen and Olszewski, 1993). Therefore, a GA-dependent flowering pathway has been proposed.

Genetic interactions between the GA pathway and other flowering pathways have been studied. The GA pathway was proposed to act independently of the long-day pathway because the flowering defect of *ga1-3* is relatively minor under long days but a double mutant of *ga1-3* with *co* often fails to flower under long days (Putterill *et al.*, 1995; Reeves and Coupland, 2001). In addition, the comparison of double and triple mutants using *co, ga1*, and *fca* (a mutation in an autonomous pathway) showed that the GA pathway has the strongest effect on flowering under short days (Reeves and Coupland, 2001). It was proposed that GA may have a role in vernalization because a non-flowering phenotype of *ga1-3* under short days could not be overcome by vernalization (Wilson *et al.*, 1992). However, when a *ga1-3* mutation was introduced into vernalization-sensitive late-flowering backgrounds, such as *ga1-3 FRI FLC* and *ga1-3 fca-1*, it showed a complete vernalization response under long days, suggesting that GA is not required for a vernalization response under long days (Chandler *et al.*, 2000; Michaels and Amasino, 1999b). So far, it is not known why the GA pathway for flowering acts primarily under short days and how vernalization and the GA pathways interact at the molecular level.

Recently, models for the integration of the genetic networks for flowering have been proposed (reviewed in Araki, 2001; Muradov *et al.*, 2002; Simpson and Dean, 2002). In addition to *SOC1*, *FT* and *LEAFY* (*LFY*) act as flowering pathway integrators. *FT* is an immediate target of CO, and the expression of *FT* is negatively regulated by *FLC*, suggesting that *FT* integrates the long-day and autonomous pathways (Samach *et al.*, 2000). *LFY* is a gene regulating floral meristem identity, which, when overexpressed, causes premature flowering (Blázquez and Weigel, 2000; Blázquez *et al.*, 1998; Weigel and Nilsson, 1995). *LFY* expression is decreased by mutations in genes of both the long-day and the autonomous pathways (Nilsson *et al.*, 1998). *LFY* is regulated by the long-day and GA pathways through separate *cis* elements on the *LFY* promoter, suggesting that multiple flowering pathways are integrated at the *LFY* promoter (Blázquez and Weigel, 2000).

We further characterized the integrative role of *SOC1*, in particular, the integration of the GA-dependent flowering pathway. Our results showed that *SOC1* integrates the GA pathway, and that such integration is necessary for flowering under short days. In contrast, the expression of other flowering integrators, *FLC* and *FT*, is not regulated by GA. Furthermore, we show that the repression of *FLC* is not sufficient to activate *SOC1*, and that positive factors are also required. Under long days, CO acts as a major positive

factor, whereas under short days, the GA pathway provides a positive factor. Our results explain why the *ga1-3* mutant is insensitive to vernalization for flowering under short days.

Results

GA positively regulates SOC1 *expression under short days*

To address the role of *SOC1* in the GA-dependent flowering pathway, the *SOC1* expression level was analyzed in the wild type Landsberg *erecta* (L*er*) and GA-biosynthetic or -signaling mutants grown under short days with or without exogenous GA treatment (Figures 1 and 2). The L*er* wild type showed acceleration of flowering by GA treatment, and this phenotype was correlated with an increase in *SOC1* expression (Figure 2). In the *ga1-3* mutant, which fails to flower under short days, *SOC1* expression remained at a basal level. However, exogenous GA treatment led *ga1-3* to produce flowers, and this rescue of the non-flowering phenotype was accompanied by an increase in *SOC1* expression (Figure 2). The GA-insensitive mutant, *gai-1*, showed extremely late flowering under short days. In contrast to *ga1-3*, this mutant phenotype could not be rescued by exogenous GA treatment and the expression of *SOC1* remained at a basal level irrespective of GA treatment (Figure 2). The mutant, *spy-5*, showing a constitutive GA response, flowered earlier than the wild type. Exogenous GA treatment slightly enhanced the early-flowering phenotype of this mutant. In correlation with the early-flowering

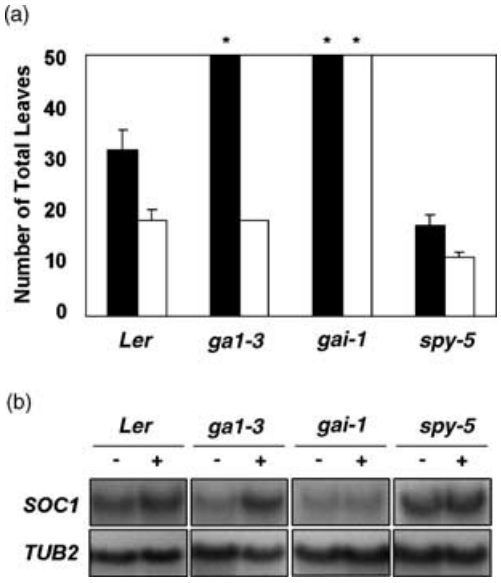

Figure 2. Effect of GA on *SOC1* expression and flowering time under short days.
(a) Flowering time of L*er*, *ga1-3, gai-1*, and *spy-5* plants grown under short days with (white bar) or without (black bar) 100 μM GA_3 treatment. Flowering time was measured as the number of total leaves produced before flowering. Values represent the mean ± SD of at least 12 plants. (*) indicates that plants did not flower during our experimental period.
(b) RNA gel blot analysis of *SOC1* transcript in L*er*, *ga1-3, gai-1*, and *spy-5* plants grown with (+) or without (–) 100 μM GA_3 treatment. Total RNA was isolated from 6-week-old plants grown under short days. A *TUBULIN 2* (*TUB2*) probe was used as a loading control.

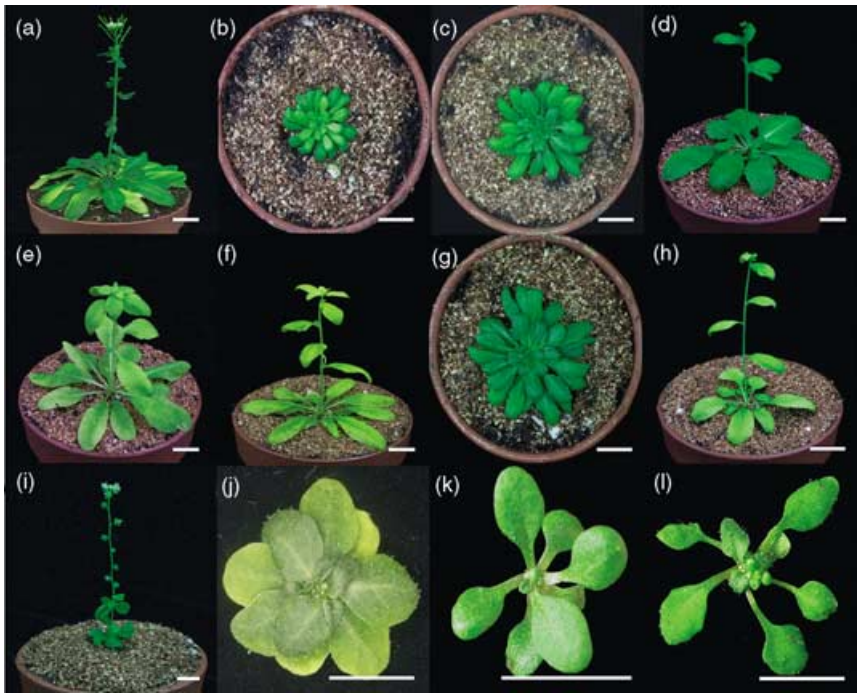

Figure 1. Flowering phenotype of lines described in this study.
All plants were grown under short days (8-h light/16-h dark). The plants (e) to (h) were treated with 100 μM GA_3 once a week, and all other plants were not treated with GA_3. Scale bars are 1 cm.
(a) and (e) L*er* wild type.
(b) and (f) *ga1-3*.
(c) and (g) *gai-1*.
(d) and (h) *spy-5*.
(i) *soc1-101D*.
(j) *ga1-3 soc1-101D*.
(k) *ga1-3 35S::CO*.
(l) *ga1-3 35S::FT*.

phenotype, *SOC1* was highly expressed even without GA treatment, and the expression level was similar to that of the GA-treated wild type (Figure 2). This correlation between the flowering time and the *SOC1* expression level in GA mutants strongly suggests that *SOC1* is a target of the GA signals for flowering.

Expressions of FLC *and* FT *are not affected by GA*

As *SOC1* functions together with other genes in the flowering pathways, we compared the temporal expression pattern of *SOC1* with those of two other flowering time genes, *FLC* and *FT*, in short-day-grown *ga1-3* mutants with and without GA treatment (Figure 3). *SOC1* expression remained at a basal level throughout the development of *ga1-3* grown under short days if GA was not treated. However, GA-treated *ga1-3* mutants showed a significantly increased *SOC1* transcript level after 6 weeks. Consistently GA-treated *ga1-3* started to express *AP1* after 6 weeks, which indicates that flowering had occurred (Hempel *et al.*, 1997, 1998; Kardailsky *et al.*, 1999). In contrast to *SOC1*, the expression patterns of *FLC* and *FT* were not changed by GA treatment. *FLC* showed uniform expression, and *FT* showed a gradual increase of expression during the time course irrespective of GA treatment (Figure 3).

Overexpression of SOC1 *rescues the block to flowering in* ga1-3

The non-flowering phenotype of *ga1-3* under short days was correlated with the lack of increase in *SOC1* expression (Figures 2 and 3). If the minimal *SOC1* expression level is the main cause of the block to flowering in *ga1-3*, overexpression of *SOC1* would be expected to induce flowering irrespective of the endogenous GA level. To test this hypothesis, we treated *soc1-101D*, a mutant constitutively overexpressing *SOC1*, with GA and paclobutrazol (PAC), an inhibitor of GA biosynthesis, and checked their flowering times (Figure 4). The wild type L*er* showed an acceleration of flowering and a concomitant increase in *SOC1* levels in response to exogenous GA treatments. When treated with PAC, L*er* failed to produce flowers that phenocopied the *ga1-3* mutant (Figure 4a). *SOC1* expression was also decreased to a basal level in these plants (Figure 4b). In contrast to the counteracting effects of GA and PAC on L*er*, *soc1-101D* did not show significant changes in flowering time in response to GA and PAC (Figure 4a). Such insensitivity of *soc1-101D* supports our hypothesis that saturation

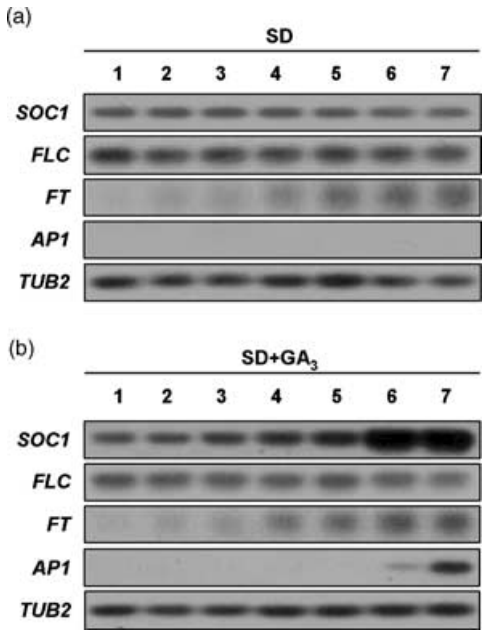

Figure 3. Effect of GA on expression of *SOC1, FLC*, and *FT* in *ga1-3* mutant. Semiquantitative RT-PCR analysis was carried out for each gene. RNA was isolated from the plants harvested every week up to 7 weeks. Plants were harvested at 6 h after dawn all times. Numbers indicate weeks after germination. *AP1* was used as a marker for floral transition, and *TUB2* was used as a quantitative control.
(a) Expression of *SOC1, FLC*, and *FT* in *ga1-3* grown under short days. *AP1* transcript was not detected until 7 weeks, indicating that floral transition did not occur.
(b) Expression of *SOC1, FLC*, and *FT* in *ga1-3* grown under short days with 100 μM GA_3 treatment. *AP1* transcript was detected from 6 weeks, indicating that floral transition occurred after 6 weeks.

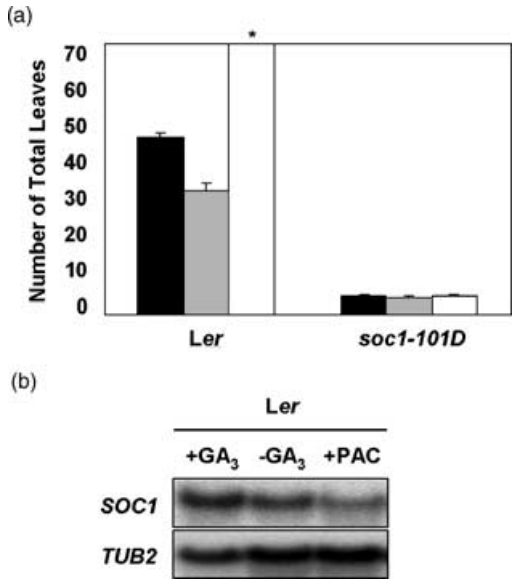

Figure 4. Effects of GA and PAC on flowering time and *SOC1* expression in the wild type and *soc1-101D* grown under short days.
(a) Effect of GA and PAC on flowering time. Non-treated control (black bar), GA treatment (gray bar), and PAC treatment (white bar). Flowering time was measured as the number of total leaves produced before flowering. Values represent the mean ± SD of at least 20 plants.
(b) RNA gel blot analysis of *SOC1* transcript in L*er* grown with (+GA_3) or without (–GA_3) 100 μM GA_3 treatment or with 37 mg l^{-1} concentrated PAC treatment (+PAC).

Table 1 Flowering times of transgenic and mutant plants grown under short days

Genotype	Number of total leaves[a]	*n*
L*er*	43.2 ± 2.8	14
soc1-101D	4.3 ± 0.5	12
35S::CO	2.9 ± 0.7	12
35S::FT	3.0 ± 0.8	12
ga1-3 soc1-101D	13.9 ± 0.8	12
ga1-3 35S::CO	4.8 ± 0.8	12
ga1-3 35S::FT	4.6 ± 0.5	12

[a]Flowering time was measured as total leaves produced before flowering.

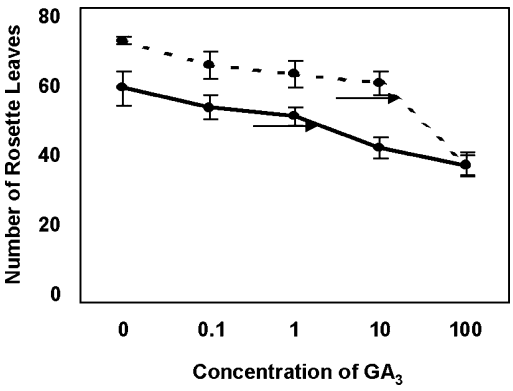

Figure 5. Dose–response curve of GA effect on flowering time of *soc1-2*. The wild type Col (solid line) and *soc1-2* (dashed line) were sprayed with different concentrations of GA_3 once a week under short days. Arrows designate half-maximal response to GA_3.

of *SOC1* expression can sufficiently overcome the effects of changes in endogenous GA signaling.

To further confirm this result, we introduced *soc1-101D* into the *ga1-3* mutant background by a genetic cross. The *ga1-3 soc1-101D* double mutant successfully produced flowers under short days, although the double mutant produced more leaves than the *soc1-101D* single mutant (Figure 1i,j and Table 1). Therefore, this result suggests that the failure of *ga1-3* to flower under short days is at least in part caused by reduced SOC1 activity. We compared the flowering phenotype of *ga1-3 soc1-101D* with *ga1-3 35S::CO* and *ga1-3 35S::FT*. In a *soc1-101D* background, *ga1-3* still had a relatively strong effect in delaying flowering, whereas in *35S::CO* and *35S::FT* backgrounds, *ga1-3* had little effect on flowering under short days (Table 1 and Figure 1j,k,l). As *ga1-3* has little effect on flowering under long days, such suppression of the defect in *ga1-3* by the overexpression of long-day-pathway genes was expected. On the contrary, the flowering phenotype of *ga1-3 soc1-101D* suggests that GA regulates additional factor(s) as well as *SOC1* under short days.

Together, our results suggest that GA signaling for flowering is targeted to *SOC1* and sufficient levels of *SOC1* are able to suppress the defects of *ga1-3* mutants in flowering under short days.

The soc1 *null mutant is less sensitive to GA*

If *SOC1* mediates the GA pathway for flowering, it is expected that the *soc1* null mutant is insensitive to GA for a flowering response. To test this possibility, we checked the sensitivity of the *soc1-2* null mutant to various concentrations of GA (Figure 5). The mutant *soc1-2* showed an acceleration of flowering with increasing amounts of GA. However, it showed a weak response to GA, compared to the wild type. The half-maximal concentration of GA for flowering was 4 nM for the wild type and 30 nM for *soc1-2*. Such a partial sensitivity to GA in a *soc1-2* null supports the hypothesis that *SOC1* integrates the GA pathway for flowering. In addition, the result suggests the presence of additional factors regulated by a GA-dependent flowering pathway. Such an interpretation is also consistent with the fact that *soc1-1* mutation has a little effect on flowering under short days compared to the *ga1-3* or *gai-1* (Onouchi *et al.*, 2000). Another floral pathway integrator, *LFY*, is most likely one of the additional factors because the *LFY* promoter has a *cis* element mediating the GA pathway for flowering (Blázquez and Weigel, 2000).

Vernalization activates SOC1 *and* FT *expression irrespective of* FLC

Vernalization promotes flowering of late-flowering ecotypes and autonomous pathway mutants by the repression of *FLC* expression (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). Subsequently, *SOC1*, which acts downstream of *FLC*, is upregulated by this repression (Lee *et al.*, 2000). Recently, the *flc* null mutant (*flc-3*) was reported to respond to vernalization under short days, suggesting that vernalization is able to promote flowering via *FLC*-dependent and *FLC*-independent mechanisms (Michaels and Amasino, 2001). This result prompted us to check if vernalization can upregulate *SOC1* expression in an *flc* null background. As previously reported, the *flc-3* mutant showed an acceleration of flowering by intensive vernalization under short days (Figure 6a). The vernalization effect was saturated by approximately 9 weeks of cold treatment. Consistently, *SOC1* expression in *flc-3* was increased by prolonged vernalization under short days and the maximal expression of *SOC1* was reached after around 9 weeks of vernalization (Figure 6b). This result clearly showed that vernalization increases *SOC1* expression even in the absence of *FLC*.

As another flowering pathway integrator *FT* is also repressed by FLC (Samach *et al.*, 2000), we checked if vernalization upregulates *FT* in the *flc* null background (Figure 6b). Similar to *SOC1*, *FT* also showed an increase

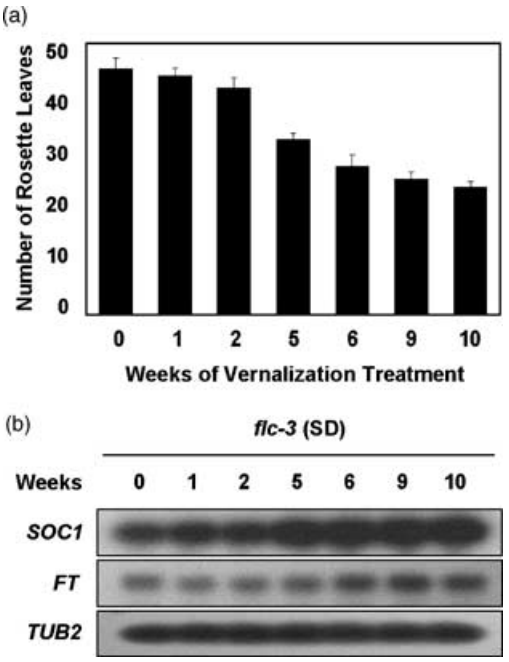

Figure 6. Effects of vernalization on flowering time and expression of *SOC1* and *FT* in *flc* null mutant.
(a) Effect of vernalization on flowering time of *flc* null. Flowering time was measured as the number of rosette leaves produced before flowering. Values represent the mean ± SD of at least 20 plants. Plants were grown under short days after vernalization treatment.
(b) Semiquantitative RT-PCR analysis of *SOC1* and *FT* in *flc* null. Tissues were harvested for RNA extraction when plants produced 10 rosette leaves. *TUB2* was used as a quantitative control.

in expression in *flc-3* by more than 9 weeks of vernalization. Thus, both *FLC*-dependent and *FLC*-independent mechanisms of vernalization promote flowering through the regulation of flowering pathway integrators, *FT* and *SOC1*. This result may indicate the presence of additional common upstream repressors of *FT* and *SOC1* for which expression is repressed by vernalization.

Interaction of GA and vernalization for the activation of SOC1 *under short days*

The role of GA in the vernalization response remains controversial because *ga1-3* mutants grown under short days are insensitive to vernalization but *ga1-3 FRI FLC* and *ga1-3 fca* grown under long days show a strong acceleration of flowering by vernalization (Chandler *et al.*, 2000; Michaels and Amasino, 1999b; Wilson *et al.*, 1992). It is possible that GA mediates the vernalization response only under short days. Alternatively, vernalization may cause only the derepression of flower-promoting genes like *SOC1,* but for the activation of such genes, GA may be absolutely required under short days. As our results showed that both vernalization and GA activate the same target gene, *SOC1*, we addressed this issue by determining *SOC1* expression in *FRI FLC* and *ga1-3 FRI FLC* grown under different environmental conditions.

FRI FLC and *ga1-3 FRI FLC* were subjected to 0, 2, 4, and 6 weeks of vernalization and divided into two groups that were grown under long days and short days until plants produced four rosette leaves under long days and 10 rosette leaves under short days, and then the *FLC* and *SOC1* expression levels were determined by RNA gel blot analysis (Figure 7). As previously reported, *ga1-3 FRI FLC* responded normally to vernalization under long days (Michaels and Amasino, 1999b). The flowering time of these plants was accelerated as the *FRI FLC* wild types after 6 weeks of vernalization if grown under long days (Figure 7c). In correlation, the expression of *FLC* was decreased to an undetectable level after 6 weeks of cold treatment, while the *SOC1* level reached the maximum (Figure 7a). Therefore, under long days, *ga1-3 FRI FLC* showed a response to vernalization very similar to that of the *FRI FLC* wild type, both physiologically as an acceleration of flowering time and molecularly as a decrease in *FLC* and an increase in *SOC1* expression.

However, under short-day conditions, *ga1-3 FRI FLC* lacked their response to vernalization and failed to produce flowers while the *FRI FLC* wild type showed normal acceleration of flowering by vernalization (Figure 7c). Consistently, the *FRI FLC* wild type showed normal repression of *FLC* expression and a concomitant increase in *SOC1* expression by vernalization (Figure 7b). In contrast, vernalization of *ga1-3 FRI FLC* failed to activate *SOC1* expression, although vernalization reduced *FLC* transcript levels sufficiently (Figure 7b). After 6 weeks of vernalization, *SOC1* expression remained at a basal level in *ga1-3 FRI FLC* even though vernalization completely repressed *FLC* expression. This result clearly shows that vernalization represses *FLC* expression irrespective of GA under both long days and short days. However, it shows that *SOC1* activation is dependent on the presence of GA under short days.

The *ga1-3* mutant, which has low *FLC* expression and fails to respond to vernalization under short days, did not show any increase in *SOC1* expression even after 9 weeks of vernalization, although the wild type L*er* showed a strong increase in *SOC1* expression after 6 weeks of vernalization under short days (Figure 7d,e). Together, our results suggest that the repression of *FLC* expression is not sufficient for activation of *SOC1*, but that positive factors are also required for the activation of *SOC1* expression. The GA pathway provides such a positive factor under short days as the long-day pathway does under long days. The possibility that GA mediates the vernalization response under short days was excluded because vernalization activates both *FT* and *SOC1* but GA activates only *SOC1* under short days (Figure 6). This suggests that the vernalization and GA pathways are independent.

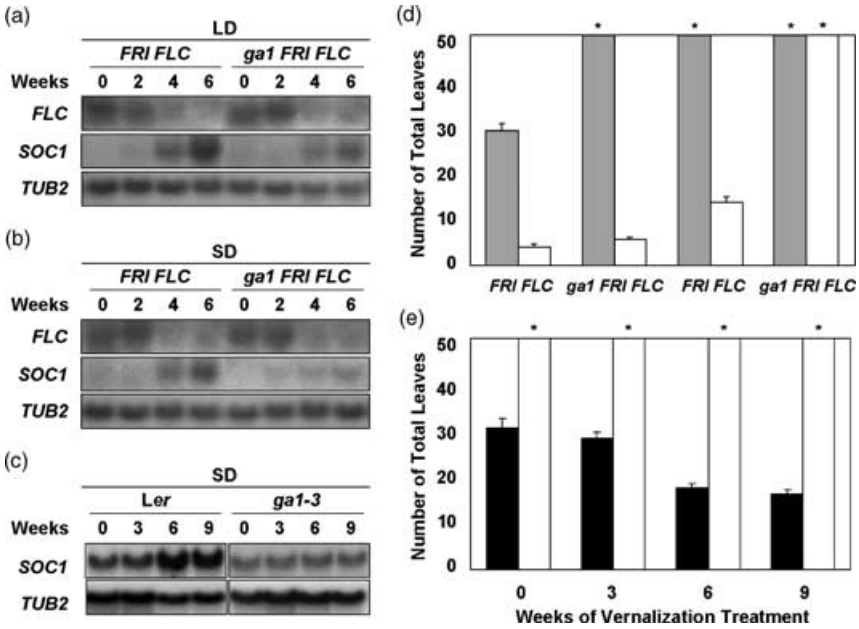

Figure 7. Effect of vernalization on expression of *FLC* and *SOC1* in plants containing *ga1-3*.
(a) RNA gel blot analysis of *FLC* and *SOC1* transcript in *FRI FLC* and *ga1-3 FRI FLC* grown under long days after 0, 2, 4, and 6 weeks of vernalization treatment. Plants were harvested for total RNA extraction when four rosette leaves were produced.
(b) RNA gel blot analysis of *FLC* and *SOC1* transcripts in *FRI FLC* and *ga1-3 FRI FLC* grown under short days after 0, 2, 4, and 6 weeks of vernalization treatment. Plants were harvested for total RNA extraction when 10 rosette leaves were produced. *TUB2* probe was used as a loading control.
(c) RNA gel blot analysis of *SOC1* transcript in L*er* and *ga1-3* plants grown under short days after 0, 3, 6, and 9 weeks of vernalization treatment. Total RNA was isolated when 10 rosette leaves were produced.
(d) Effect of vernalization on the flowering time of *FRI FLC* and *ga1-3 FRI FLC* grown under long (left) and short (right) days. Gray bars represent the plants without vernalization treatment, and white bars represent the plants with vernalization. Flowering time was measured as the number of total leaves produced before flowering. Values represent the mean ± SD of at least 12 plants. (*) indicates that plants were unable to flower.
(e) Flowering time of L*er* (black bar) and *ga1-3* (white bar) plants grown under short days after 0, 3, 6, and 9 weeks of vernalization treatment.

Discussion

Previous studies on flowering time in *Arabidopsis* have revealed many genes that can be placed in four genetic pathways: the autonomous, vernalization, long-day, and gibberellin pathways (Koornneef *et al.*, 1998a; Simpson *et al.*, 1999). Recently, three genes, *FT, SOC1*, and *LFY*, were shown to be regulated by multiple flowering pathways and were termed floral integrators (Araki, 2001; Blázquez and Weigel, 2000; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Lee *et al.*, 2000; Onouchi *et al.*, 2000; Samach *et al.*, 2000; Simpson and Dean, 2002). Particularly, *SOC1* is antagonistically regulated by a B-box zinc finger protein CO, encoded by a gene involved in the long-day pathway, and a MADS-box protein FLC, which itself is an integrator of the autonomous/vernalization pathway (Hepworth *et al.*, 2002; Lee *et al.*, 2000; Samach *et al.*, 2000).

In this study, we focused on the role of *SOC1* in relation to the GA-dependent flowering pathway. Our studies showed that the flowering time of GA-biosynthetic and -signaling mutants are well correlated with the *SOC1* expression level, and that the introduction of *SOC1* overexpression into *ga1-3* rescues the non-flowering phenotype under short days, demonstrating that *SOC1* also integrates a GA-dependent flowering pathway. In addition, we showed that the repression of *FLC* by vernalization is not sufficient for *SOC1* activation, but the GA pathway is also required under short days.

SOC1 *integrates a GA-dependent flowering pathway*

Previously, Borner *et al.* (2000) showed that exogenous GA treatment increased the *SOC1* expression level in short-day-grown *Arabidopsis* plants, which were already at the reproductive stage. It was also shown that GA treatment activated the expression of the *SOC1* ortholog *SaMADSA* in the shoot apex of *Sinapsis alba* plants grown under non-inductive photoperiods (Bonhomme *et al.*, 2000). However, the correlation between flowering time and the increase in *SOC1* expression by GA has not been firmly addressed yet. Here, we intensively studied how the *SOC1* expression level correlated with flowering time by using short-day-grown GA-biosynthetic and -signaling mutants with or without GA treatment. The GA-biosynthetic mutant *ga1-3* failed to flower under short days, and it was correlated with the minimal expression of *SOC1* (Figure 2). On the contrary, exogenous GA treatment caused *ga1-3* to flower at a time similar to that for a GA-treated wild type, and caused the *SOC1* expression in *ga1-3* to increase to a level similar to

that in a GA-treated wild type. Therefore, similar levels of *SOC1* expression seem to reflect similar flowering times. Such an activation of *SOC1* expression by GA is most likely to be mediated by the GA-signaling pathway because the GA-signaling mutants *gai* and *spy* also showed this correlation between flowering time and *SOC1* expression level. *GAI* encodes a GRAS (for GAI, RGA, SCARECROW) family regulatory protein with a DELLA domain at the N-terminus (Peng *et al*., 1997). The function of GAI is to inhibit GA responses in the absence of active GA, and such inhibition is suppressed by GA. The suppression of GAI activity is likely to be mediated through the DELLA domain because the DELLA-domain-deleted mutant *gai-1* shows the dominant gain-of-function phenotype, thus showing the GA-signaling defect even with exogenous GA treatment (Peng *et al*., 1997). The flowering of *gai-1* is extremely delayed irrespective of GA treatment, and the plant shows a minimal level of *SOC1* expression, showing the correlation between flowering time and a low *SOC1* expression level as a result of the GA-signaling defect (Figure 2). The *SPY* gene, encoding a tetratricopeptide repeat protein, is another negative regulator of GA response, and *SPY* has been proposed to act upstream of *GAI* (Jacobsen *et al*., 1996; Silverstone *et al*., 1998). The recessive mutant, *spy-5*, shows a constitutive GA response and earlier flowering than the wild type. The earlier flowering phenotype in *spy-5* was again correlated with higher expression of *SOC1* than that in the wild type (Figure 2). In addition to the correlation between flowering time and *SOC1* expression level, the introduction of *SOC1* overexpression into a *ga1-3* mutant rescued the non-flowering phenotype under short days. Similarly, PAC treatment could not block the flowering of *35S::SOC1* under short days (Blázquez *et al*., 2002). These results suggest that the failure of flowering by GA deficiency under short days is caused by the lack of *SOC1* activation. Therefore, all our results strongly suggest that *SOC1* integrates the GA-dependent flowering pathway.

GA pathway activates additional factors

Although the expression level of *SOC1* is regulated by GA and is correlated with the flowering time, *SOC1* is not the only flowering time determinant regulated by GA. The double mutant *ga1-3 soc1-101D*, which overexpresses *SOC1*, flowered later than *soc1-101D* single mutant (Table 1). Such a delaying effect of *ga1-3* on the flowering time of *soc1-101D* suggests the presence of additional factor(s) regulated by GA. Consistent with this, the *soc1* null mutant showed partial sensitivity to various concentrations of applied GA for a flowering response (Figure 5). Compared to the *ga1-3* or *gai-1*, the *soc1-1* mutant shows only a slight delay in flowering under short days (Onouchi *et al*., 2000). It also supports the presence of partially redundant factor(s) regulated by GA. A flower meristem identity gene, *LFY,* may be one of the additional factors regulated by GA (Blázquez *et al*., 1998). The expression of *LFY* is also regulated by GA; *LFY* expression remains at a minimal level in *ga1-3* mutants throughout development under short days, whereas stronger expression is detected in *spy-5* mutants than in the wild type. Similar to *soc1-101D*, *35S::LFY* rescued the flowering defect of *ga1-3* under short days but *ga1-3 35S::LFY* flowered later than *35S::LFY*, indicating that *LFY* is also not sufficient to mediate the GA pathway (Blázquez *et al*., 1998). *SOC1* was proposed to act partially upstream of *LFY* because overexpression of *SOC1* in the absence of *FRI* caused the production of ectopic flowers subtended by cauline leaves, which are usually observed in *35S::LFY* plants (Lee *et al*., 2000). Thus, it is possible that GA-promoted *LFY* expression is mediated through *SOC1*. However, the *cis* element on the *LFY* promoter, which mediates the GA-signaling pathway for flowering, is known to be bound to a GAMYB-like protein, AtMYB33, which shows an increase in the expression at the shoot apex during floral transition (Blázquez and Weigel, 2000; Gocal *et al*., 2001). Therefore, it is more likely that GA regulates the two flowering pathway integrators *SOC1* and *LFY* independently. In addition, the presence of additional factor(s), other than *SOC1* and *LFY*, that regulate flowering in response to GA cannot be excluded.

GA pathway is targeted downstream of FLC

The *SOC1* promoter contains a MADS-domain protein binding element (CArG) box that mediates the repression by FLC (Hepworth *et al*., 2002). Although the GA pathway activates the expression of *SOC1*, GA does not influence the expression of *FLC*. *FLC* showed uniform expression throughout development in the *ga1-3* mutants grown under short days, and the expression level was not changed by GA treatment (Figure 3). On the contrary, *SOC1* expression was gradually increased after GA treatment, suggesting that the GA pathway targets downstream of *FLC*. Consistent with our result, Sheldon *et al*. (1999) showed that the flowering of plants overexpressing *FLC* was accelerated by GA treatment, and that the *FLC* level in wild-type plants was not changed by the GA application. Similarly, the expression of another flowering pathway integrator, *FT*, which is also regulated by FLC, is not affected by the GA pathway (Figure 3). Therefore, we propose that the GA pathway is directly integrated via *SOC1*.

It is noteworthy that there is some specificity in the integration of flowering pathways among pathway integrators. *FT* integrates the long-day and autonomous/vernalization pathways, but not the GA pathway. *SOC1* integrates all pathways: the long-day, autonomous/vernalization, and the GA pathways. Finally, *LFY* integrates the autonomous/vernalization and the GA pathways but is not an immediate target of CO, a central regulator of the

long-day pathway. Such integration specificity may further elaborate the fine tuning of environmental and endogenous cues for flowering.

Interaction of GA, photoperiod, and vernalization

Previously, it was proposed that *SOC1* is directly repressed by FLC through a CArG box on the *SOC1* promoter and is activated by CO under long days through a separate *cis* element (Hepworth *et al.*, 2002). In this study, we showed that *SOC1* is also positively regulated by the GA pathway mainly under short days. We propose that the GA pathway is the only flower-promoting pathway under short days, and that removal of FLC repression is a prerequisite but is not sufficient for flowering. Six weeks of vernalization reduced the expression of *FLC* in *ga1-3 FRI FLC* to an undetectable level, but *SOC1* expression remained at a basal level under short days (Figure 7). This result shows that the repression of *FLC* expression is not sufficient to activate *SOC1* expression but positive factors are also required. Our results also show not only that CO acts as a positive factor but also that the GA pathway provides a positive factor for *SOC1* activation and concomitant flowering. Under long days, CO and GA redundantly activate *SOC1* expression. Although a *ga1-3* or *co* single mutation causes only a slight decrease in *SOC1* expression, blocking the GA pathway in a *co* mutant causes strong reduction in *SOC1* expression under long days (data not shown). On the contrary, under short days, only the GA pathway activates *SOC1* expression. The mutant *ga1-3* fails to activate *SOC1* expression under short days even with prolonged vernalization treatment (Figure 7). Our results suggest that the GA pathway is a constitutive flower-promoting pathway regardless of the photoperiod, but the long-day pathway is a physiologically conditional flower-promoting pathway. In contrast, vernalization causes a de-repressed state of flower-promoting genes such as *SOC1*. Such a model explains how vernalization interacts with the GA pathway at a molecular level. The vernalization and GA pathways eventually affect the same target molecules, but vernalization acts through de-repression while the GA pathway acts through activation of flower-promoting genes.

Our hypothesis explains well the phenotype of *ga1-3 FRI FLC* plants. Without vernalization, the repression of *SOC1* by FLC is too strong to be overcome by CO activation under long days; thus, *ga1-3 FRI FLC* shows a minimal expression of *SOC1* and a failure to flower (Michaels and Amasino, 1999b). However, if vernalized, the repression by FLC is relieved and the positive factor CO activates *SOC1* expression under long days. On the other hand, under short days, vernalization of *ga1-3 FRI FLC* fails to activate *SOC1* because the positive factor provided by GA as well as CO is absent in short-day-grown *ga1-3 FRI FLC*, although *FLC* expression is reduced completely as under long days by vernalization (Figure 7). Consistent with this hypothesis, overexpression of *CO* in *ga1-3* almost completely rescues the flowering defect of *ga1-3* under short days (Table 1). Alternative to this hypothesis, vernalization may have promotive effect on flowering and GA-dependent activation of *SOC1* is enhanced by vernalization.

Michaels and Amasino (1999b) proposed that vernalization affects meristem competency for flowering whereas GA affects a flowering signal under short days. Our data provide some of the molecular details of the relationship between the vernalization and GA pathways. It is tempting to propose that the meristem competency is a reduced state of floral repressor genes like *FLC*, and that GA acts as a flowering signal at least under short days. In addition, our results may have an implication about the nature of qualitative photoperiod response. Although *Arabidopsis* is a quantitative long-day plant, the mutation in *GA1* converts it into qualitative long-day plant as *ga1-3* never flowers under short days. It is because the only flowering pathway under short days is blocked in *ga1-3*. This may be the evolutionary mechanism through which plants acquire a qualitative photoperiod response. If a constitutive pathway, the GA pathway in the case of *Arabidopsis*, is blocked by mutation, flowering would be absolutely dependent upon inductive photoperiods.

Although the promoter analysis of *SOC1* gene revealed the presence of *cis* elements that mediate activation by CO and repression by FLC (Hepworth *et al.*, 2002), there must be many more *cis* elements on the promoter that mediate integration of other flowering pathways. More detailed analysis of *SOC1* promoter and comparison with the promoters of *FT* and *LFY* will lead to further understanding of the molecular mechanism of flowering pathway integration.

Experimental procedures

Plant materials

The GA mutants *ga1-3, gai-1,* and *spy-5* were all in the *Arabidopsis thaliana* L*er* ecotype. The seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH 43210, USA). The mutant *soc1-2* is re-named from T-DNA-tagged null allele of *agl20*, and *soc1-101D* is re-named from activation-tagged allele of *agl20-101D* (Lee *et al.*, 2000). The mutant *flc-3* in Col background has been previously described by Michaels and Amasino (1999a).

To generate double mutants between *ga1-3* and *soc1-101D, soc1-101D* in L*er* was generated by backcrossing the *soc1-101D* in Col background to L*er* three times (Lee *et al.*, 2000). The double mutants of *ga1-3 35S::CO* were obtained by crossing *ga1-3* and *35S::CO* in a L*er* background. The *35S::CO* seeds were kindly provided by Dr George Coupland (Max-Planck-Institut für Züchtungsforschung), and the *ga1-3 35S::FT* seeds by Dr Miguel Blázquez (CSIC-UPV). The *ga1-3 FRI FLC* line provided by Dr Rick Amasino (University of Wisconsin-Madison) has been previously described by Michaels and Amasino (1999b).

Growth conditions

To break seed dormancy, seeds were stratified on 0.65% phytoagar containing 1.5% sucrose and half-strength MS (Gibco-BRL, Gaitherburg, MD, USA) plates for 2–3 days at 4°C. Afterwards, plants were transferred and grown at 23°C under long (16-h light/8-h dark) or short (8-h light/16-h dark) photoperiod conditions in cool white fluorescent lights (100 μmol m^{-2} sec^{-1}). At least 20 plants were used to measure the flowering time of each genotype. The flowering time was measured as a mean of the total leaf number including rosette and cauline leaves.

To germinate *ga1-3* mutants, seeds were soaked in 100 μM GA_3 (Duchefa, Biochemie, the Netherlands) for 5 days under 4°C dark conditions, and then rinsed thoroughly with water before sowing on MS plates, which were then moved to the chambers. For vernalization treatment, the MS plates were incubated for several weeks at 4°C under short-day conditions. Exogenous application of GA_3 was carried out by spraying the plants with 100 μM GA_3 every week. L*er* and *soc1-101D* were treated with PAC by watering the plants with 37 mg l^{-1} concentrated solution twice a week.

RNA analysis

Total RNA was extracted as described before by Puissant and Houdebine (1990). For RNA gel blot analysis, 20 μg of RNA was separated on 1% denaturing formaldehyde agarose gels and transferred to NYTRAN-PLUS membranes (Schleicher and Schuell, Keene, NH, USA). The *SOC1* and *FLC* probe were cDNA fragments lacking MADS-domain sequences. Blots were probed with *TUBULIN 2* (*TUB2*)-coding regions as a control for the quantity of RNA loaded.

The RT-PCR procedure and primers used for *SOC1, FLC, AP1*, and *TUB2* were described previously by Lee *et al.* (2000). For *FT*, 5′-ATG TCT ATA AAT ATA AGA GAC C-3′ and 5′-CTA AAG TCT TCT TCC TCC GCA G-3′ were used as primers.

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