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Analysis of Flowering Pathway Integrators in *Arabidopsis*

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Flowering is regulated by an integrated network of several genetic pathways in *Arabidopsis*. The key genes integrating multiple flowering pathways are *FT*, *SOC1* and *LFY*. To elucidate the interactions among these integrators, genetic analyses were performed. *FT* and *SOC1* share the common upstream regulators CO, a key component in the long day pathway, and FLC, a flowering repressor integrating autonomous and vernalization pathways. However, the *soc1* mutation further delayed the flowering time of long day pathway mutants including *ft*, demonstrating that *SOC1* acts partially independently of *FT*. Although *soc1* did not show an obvious defect in flower meristem determination on its own, it dramatically increased the number of coflorescences in a *lfy* mutant, which is indicative of a defect in floral initiation. Therefore, double mutant analysis shows that the three integrators have both overlapping and independent functions in the determination of flowering time and floral initiation. The expression analysis showed that *FT* regulates *SOC1* expression, and *SOC1* regulates *LFY* expression, but not vice versa, which is consistent with the fact that *FT* and *LFY* have the least overlapping functions among the three integrators. The triple mutation *ft soc1 lfy* did not block flowering completely under long days, indicating the presence of other integrators. Finally, vernalization accelerated flowering of *flc ft soc1* and *ft soc1 lfy* triple mutants, which shows that the vernalization pathway also has targets other than *FLC, FT*, *SOC1* and *LFY*. Our genetic analysis reveals the intricate nature of genetic networks for flowering.

Key words: Flowering — Flowering pathway integrators — FT — LFY — SOC1.

Abbreviations: GA, gibberellic acid.

Introduction

The transition to flowering is controlled by complex genetic networks in *Arabidopsis*. The genetic and molecular analyses of *Arabidopsis* have revealed several interdependent genetic pathways for flowering, which enable plants to monitor both environmental and endogenous signals (reviewed in Mouradov et al. 2002, Simpson and Dean 2002). The long day and vernalization pathways respond to environmental signals such as light and temperature, whereas the autonomous and gibberellic acid (GA)-dependent pathways monitor the endogenous developmental state of the plant.

The autonomous pathway promotes flowering independently of environmental conditions by repressing the *FLOWERING LOCUS C* (*FLC*) gene that acts as a repressor of flowering (Michaels and Amasino 1999, Michaels and Amasino 2001, Sheldon et al. 1999). Vernalization, a long exposure to low temperature, also promotes flowering by repressing *FLC*. In contrast, *FRIGIDA* (*FRI*), a gene conferring a vernalization response on winter-annual late flowering accessions of *Arabidopsis*, acts to increase *FLC* expression level (Michaels and Amasino 1999, Sheldon et al. 1999, Johanson et al. 2000). Therefore, *FLC* is a convergence point for autonomous and vernalization pathways. The genes *FCA*, *FLOWERING LOCUS D*, *FPA*, *FVE*, *FY* and *LUMINIDEPENDENS* (*LD*) are involved in the autonomous pathway, whereas the genes *VERNALIZATION 1* and *2* (*VRN1* and *VRN2*) and *VERNALIZATION INSENSITIVE 3* (*VIN3*) are involved in the vernalization pathway (Chandler et al. 1996, Koornneef et al. 1998a, Gendall et al. 2001, Levy et al. 2002, He et al. 2003, Sung and Amasino 2004).

The genes *CONSTANS* (*CO*), *GIGANTEA* (*GI*) and *FT* are involved in the long day pathway, and mutations in these genes delay flowering under long days but not under short days (Koornneef et al. 1991, Koornneef et al. 1998b). In contrast, mutations in GA biosynthesis delay flowering only slightly under long days but cause extremely late flowering under short days, suggesting that the GA-dependent pathway has a crucial role for flowering in non-inductive photoperiods (Wilson et al. 1992, Blázquez et al. 1998).

Although the long day, GA and autonomous/vernalization pathways can act independently to promote flowering, they converge on common downstream target genes, the so-called flowering pathway integrators that regulate floral initiation genes (Simpson and Dean 2002). Thus far, three genes, *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1/AGL20*) and *LEAFY* (*LFY*), have been identified to function at this level. *FT* and *SOC1* are not only the immediate targets of transcription factor CO, a central regulator in the long day pathway, but also are negatively regulated by FLC which integrates the autonomous and vernalization pathways (Lee et al. 2000, Onouchi et al. 2000, Samach et al. 2000). Therefore, *FT* and *SOC1* integrate long day and autonomous/vernalization

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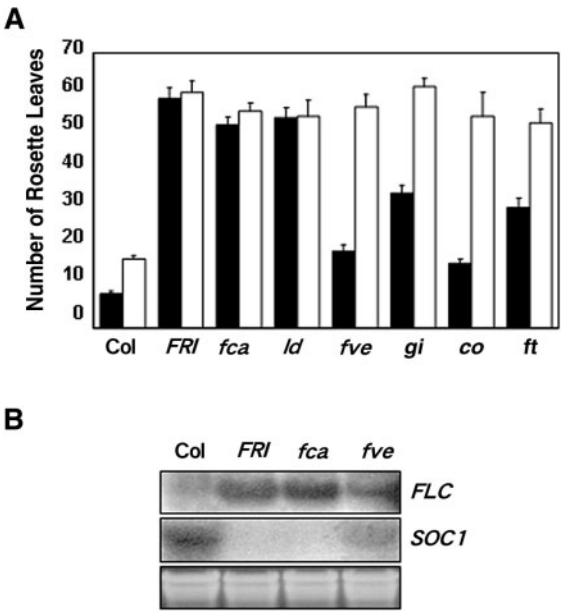

Fig. 1 Effects of *soc1* mutation on the flowering of late-flowering mutants and an *FRI*-containing line. (A) Flowering times of the single and double mutants were compared in long days. Black bars represent plants without *soc1-2* and white bars represent plants with the *soc1-2* mutation. (B) Expression of *SOC1* and *FLC* in *FRI*, *fca*, *fve* determined by RNA blot analysis.

pathways. In addition, recent studies showed that *SOC1* expression is also regulated by the GA pathway whereas *FT* expression is not, suggesting that *FT* and *SOC1* act differently in the integration of flowering pathways (Borner et al. 2000, Moon et al. 2003, Achard et al. 2004). Another integrator, *LFY*, is regulated by both the long day and GA pathways through separate *cis*-elements on the *LFY* promoter (Blázquez and Weigel 2000). In contrast to *FT* and *SOC1*, *LFY* is not an immediate target of CO, suggesting the existence of mediators between the two genes (Samach et al. 2000). It has been reported that *FT* functions in parallel to *LFY* for floral initiation and is necessary for *LFY* function (Ruiz-García et al. 1997, Nilsson et al. 1998, Kardailsky et al. 1999, Kobayashi et al. 1999). However, the relationships among the three integrators are largely unknown, especially the relationship of *SOC1* to the other integrators *FT* and *LFY*.

In this study, we further investigated the role of a flowering pathway integrator *SOC1* in the context of the genetic network. For this, we generated double mutants between *soc1-2*, a null allele and late flowering mutants that affect the long day or autonomous pathways. In addition, we generated double and triple mutants of the three flowering pathway integrators, *FT*, *SOC1* and *LFY*. The genetic and molecular analyses of the three flowering pathway integrators allowed us to scrutinize the complex genetic networks for flowering.

Results

SOC1 acts partially independently of the long day pathway for flowering

Previous studies have shown that *SOC1* integrates multiple flowering pathways (Lee et al. 2000, Samach et al. 2000, Hepworth et al. 2002, Moon et al. 2003). To determine the dependence of *SOC1* regulation on the autonomous or long day pathway, double mutants between *soc1* null and autonomous pathway mutants *fca*, *ld* and *fve*, or long day pathway mutants *gi*, *co* and *ft*, were generated. All of the mutants we used were in the Columbia (Col) background which has a functional *FLC* allele. The analysis of double mutants between *soc1* and *gi*, *co*, *ft* showed that *soc1* mutation further delayed the flowering time of the long day pathway mutants (Fig. 1A). This additive effect suggests that *SOC1* regulates flowering at least partially independently of the long day pathway. In contrast, the double mutants between *soc1* and autonomous pathway mutants *fca* and *ld* showed that the effect of *soc1* mutation on flowering was masked by these mutants (Fig. 1A). An *FRI*-containing line, which has strong expression of *FLC* like autonomous pathway mutants, also showed no further delay in flowering in combination with the *soc1* mutation (Fig. 1A, B). Such epistatic interactions suggest that *SOC1* acts in the same genetic pathway as *FCA*, *LD* and *FRI*.

Compared with *fca soc1* or *ld soc1*, elimination of *SOC1* in *fve*, another autonomous pathway mutant, led to a significant delay in flowering (Fig. 1A). To address the cause of this phenotype, *FLC* and *SOC1* transcript levels were compared among *fca*-, *fve*- and *FRI*-containing lines. In *fve*, *FLC* expression was relatively lower but *SOC1* expression higher than that in *fca* or *FRI*, which may explain why *fve* flowers earlier than *fca* or *FRI* (Fig. 1B). Thus, elimination of the remaining *SOC1* transcript in *fve* caused the additive late flowering phenotype. Considering that the *fve* mutant used is a strong allele (*fve-3*) with a premature stop codon in the first WD-40 domain (Ausin et al. 2004), a weak effect of *fve* on flowering time and *FLC* derepression might result from redundancy of FVE function in the *Arabidopsis* genome, as suggested before (He et al. 2003). Indeed, the *Arabidopsis* genome has a FVE homolog which has 75% amino acid sequence identity (Ausin et al. 2004). Taken together, our double loss-of-function mutant analysis suggests that *SOC1* acts partially independently of the long day pathway but is regulated mainly by an autonomous pathway through FLC.

ft and soc1 mutations limit vernalization response

Both of the two flowering pathway integrators *FT* and *SOC1* are negatively regulated by *FLC*, the expression of which is suppressed by vernalization (Lee et al. 2000, Samach et al. 2000). Therefore, vernalization accelerates flowering of the autonomous pathway mutants by down-regulating *FLC* expression, which subsequently increases *SOC1* and *FT* levels (Michaels and Amasino 1999, Lee et al. 2000, Samach et al.

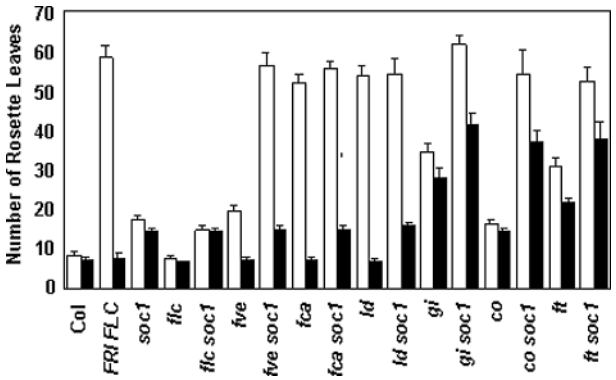

Fig. 2 Effect of vernalization on the flowering times of the double loss-of-function mutants. Plants were subjected to 6 weeks of vernalization treatment before being grown in long days. White bars represent plants without vernalization treatment, and black bars represent plants with 6 weeks of vernalization treatment.

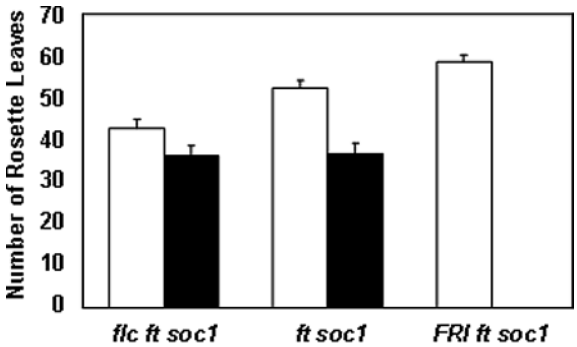

Fig. 3 Effect of endogenous *FLC* levels on the flowering time of *ft soc1*. Plants without (white) and with (black) 6 weeks of vernalization treatment were grown in long days.

2000, Sheldon et al. 2000). Consistently, *soc1* and *ft* as well as *flc* mutants show a partial loss in sensitivity to the vernalization response (Koornneef et al. 1991, Michaels and Amasino 2001, Michaels et al. 2003). We determined the extent to which the vernalization response is dampened by *soc1* or *ft* in double mutants between *soc1* and other late flowering mutations (Fig. 2). As was reported previously, *soc1*, *ft* and *flc* single mutants showed a weak response to 6 weeks of vernalization under long days (Fig. 2). When the double mutants between *soc1* and autonomous pathway mutants *fca*, *fve* and *ld* were subjected to vernalization, flowering was accelerated but only to the vernalized *soc1* level, indicating that up-regulation of *SOC1* is required for full promotion of flowering by vernalization.

The *ft soc1* double mutant also showed a response to vernalization, although the response was relatively weak as vernalized *ft soc1* flowered later than unvernalized *ft* or *soc1* (Fig. 2). Interestingly, vernalization of the double mutants between *soc1* and long day pathway mutants *gi* and *co* accelerated flowering to a level similar to the vernalized *ft soc1* double mutant (Fig. 2). This presumably is due to the lack of *FT* expression in *gi* and *co* mutants because the two genes *CO* and *GI* are upstream regulators of *FT* (Kardailsky et al. 1999, Kobayashi et al. 1999). This result shows that *ft* and *soc1* limit the vernalization response, but *FT* and *SOC1* are not the only targets of vernalization.

FLC-dependent and -independent additional factors for vernalization response

The vernalization response of *ft soc1* can be explained by two mechanisms. One is that there are additional factor(s) regulated by *FLC* and the other is that vernalization promotes flowering in *FLC*-independent ways. The flowering time of the *ft soc1* double mutant was close to that of autonomous pathway mutants *fca* and *ld* and *FRI*-containing lines that have high *FLC* expression (Fig. 1). Thus, it is not clear if *FLC* regulates additional downstream target(s) other than *FT* and *SOC1*. To address this question, we compared the flowering time of *ft soc1* with *flc ft soc1* and *FRI ft soc1* that have a null allele and strong expression of *FLC*, respectively (Fig. 3). Although *ft soc1* in the Col background expresses almost undetectable levels of *FLC* by RNA blot analysis (Michaels and Amasino 1999, Sheldon et al. 1999, data not shown), it still flowered ~10 leaves later than the triple mutant *flc ft soc1* under long days. When *FRI* was introduced into *ft soc1*, flowering was delayed ~10 leaves further. This result shows that flowering time depends on the levels of *FLC* expression even in the absence of *FT* and *SOC1*, demonstrating the existence of additional targets downstream of *FLC*.

Previous results showed that vernalization promotes flowering of an *flc* null mutant, showing the presence of an *FLC*-independent mechanism mediating the vernalization response (Michaels and Amasino 2001). In addition, vernalization up-regulates *FT* and *SOC1* expression in the *flc* null, suggesting that an *FLC*-dependent and *FLC*-independent mechanism share the common downstream targets *FT* and *SOC1* (Moon et al. 2003). To see if an *FLC*-independent mechanism has its own targets for promotion of flowering, the vernalization response was determined in the *flc ft soc1* triple mutant (Fig. 3). The triple mutant showed a response to vernalization, although weak, indicating that additional target(s) regulated by an *FLC*-independent mechanism exist. Therefore, the acceleration of flowering in the *ft soc1* double mutant by vernalization is due to the additive effects of *FLC*-dependent and *FLC*-independent mechanisms (note the same flowering time of vernalized *ft soc1* and *flc ft soc1*).

Genetic interactions among flowering pathway integrators

Studies on *FT*, *SOC1* and *LFY* suggested that the three genes function to integrate the flowering pathways (Araki 2001, Simpson and Dean 2002, Mouradov et al. 2002). However, not much is known about the interaction among the integrators. To determine the genetic interactions among the three flowering pathway integrators, double and triple loss-of-func-

Table 1 Flowering times of double and triple mutants of *ft, soc1* and *lfy*

Genotype	No. of RLs	No. of coflorescences	*n*
Col	8.6 ± 0.7	2.3 ± 0.3	15
soc1-2	23.2 ± 3.0	3.4 ± 1.1	15
ft-1	30.7 ± 2.3	8.1 ± 0.6	15
lfy-12	8.9 ± 0.3	6.8 ± 0.4	15
ft-1 soc1-2	52.3 ± 3.5	10.3 ± 0.5	15
ft-1 lfy-12	33.8 ± 2.1	>70 [a]	5
soc1-2 lfy-12	27.2 ± 1.3	30.6 ± 2.3	15
ft-1 soc1-2 lfy-12	61.1± 3.6	>70 [a]	15
Vern *ft soc1 lfy*	41.3± 2.2	>70 [a]	23

[a] More than 70 coflorescences were counted before growth arrest occurred.

Vern, vernalization treatment; RLs, rosette leaves produced when flowering.

Table 2 Flowering times with double or triple overexpression of *FT*, *SOC1* and *LFY*

Genotype	No. of RLs	No. of CLs	*n*
Col	8.6 ± 0.7	2.3 ± 0.3	15
35S::FT/+	5.2 ± 0.3	2.1 ± 0.2	10
soc1-101D/+	3.9 ± 0.2	2.3 ± 0.3	10
35S::LFY/+	7.4 ± 0.5	1.2 ± 0.4	10
35S::FT/+ soc1-101D/+	2.1 ± 0.2	1.9 ± 0.3	10
35S::FT/+ 35S::LFY/+	2.0 ± 0.0	None	10
soc1-101D/+ 35S::LFY/+	2.0 ± 0.2	2.0 ± 0.0	10
35S::FT/+ soc1-101D/+ 35S::LFY/+	None	2.0 ± 0.0	10

RLs, rosette leaves; CLs, cauline leaves.

tion mutants among them were analyzed (Table 1). The *soc1* single mutant delayed flowering but had little effect on the number of coflorescences that produce secondary shoots, whereas *lfy* showed little effect on the flowering time (based on bolting time) but had a significant effect on the number of coflorescences. When the flowering time of *soc1 lfy* was compared with that of *soc1*, the rosette leaf number at bolting increased only slightly. However, the total number of leaves before flowering showed a synergistic effect as the number of coflorescences subtended by cauline leaves in the double mutant increased dramatically (Table 1). This result suggests that *SOC1* has a functional redundancy with *LFY* in regulating floral initiation. As described, the double mutant *ft soc1* showed an additive late flowering phenotype, suggesting that *FT* and *SOC1* regulate flowering time in parallel (Fig. 1, Table 1). Compared with *soc1 lfy*, *ft lfy* had a more severe coflorescence phenotype, as it continuously produced secondary shoot-like structures and failed to produce *lfy* flower-like structures, as previously reported (Table 1, Ruiz-García et al. 1997). Such

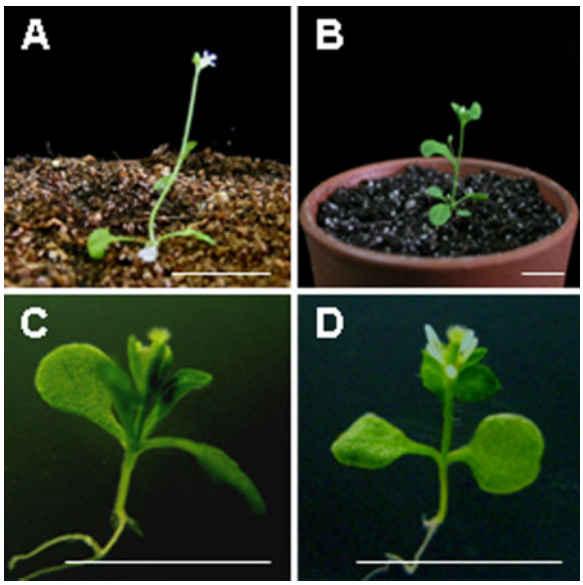

Fig. 4 Flowering phenotypes of plant double and triple mutants overexpressing *FT*, *SOC1* and *LFY*. (A) *35S::FT/+ soc1-101D/+*, (B) *soc1-101D/+ 35S::LFY/+*, (C) *35S::FT/+ 35S::LFY/+* and (D) *35S::FT/+ soc1-101D/+ 35S::LFY/+*.

genetic analysis shows that the three flowering pathway integrators *FT*, *SOC1* and *LFY* have both overlapping and independent functions on flowering time determination and floral initiation. It also suggests that *FT* and *LFY* have the least overlapping function among three integrators.

We generated *ft soc1 lfy* triple mutants to see if flowering is completely blocked by the removal of the three integrators. Compared with each double mutant, the *ft soc1 lfy* triple mutant had a slightly later flowering time than *ft soc1* and a severe coflorescence phenotype similar to *ft lfy* (Table 1). The triple mutant showed a vernalization response similar to *ft soc1*, although vernalization could not ameliorate the coflorescence phenotype of the triple mutant (Table 1). Thus, triple mutant phenotype strongly suggests that there are other factors regulating flowering at the integrative level.

The double and triple combinations of *FT*, *SOC1* and *LFY* overexpression were also generated. In general, double overexpression showed additive effects on flowering (Table 2). For example, the double overexpression line *35S::FT/+ soc1-101D/+* flowered earlier than the single overexpression lines *35S::FT/+* or *soc1-101D/+* (an *SOC1* overexpression line) (Table 2, Fig. 4A). The double line *soc1-101D/+ 35S::LFY/+* also flowered earlier than *soc1-101D/+* or *35S::LFY/+* (Table 2, Fig. 4B). Among the double lines, *35S::FT/+ 35S::LFY/+* showed the earliest flowering as it produced only two leaves before producing a terminal flower, which is consistent with previous reports (Table 2, Fig. 4C; Kardailsky et al. 1999, Kobayashi et al. 1999). Such an additive effect of double overexpression further confirms that the three flowering pathway integrators have partially independent functions. Also, the ear-

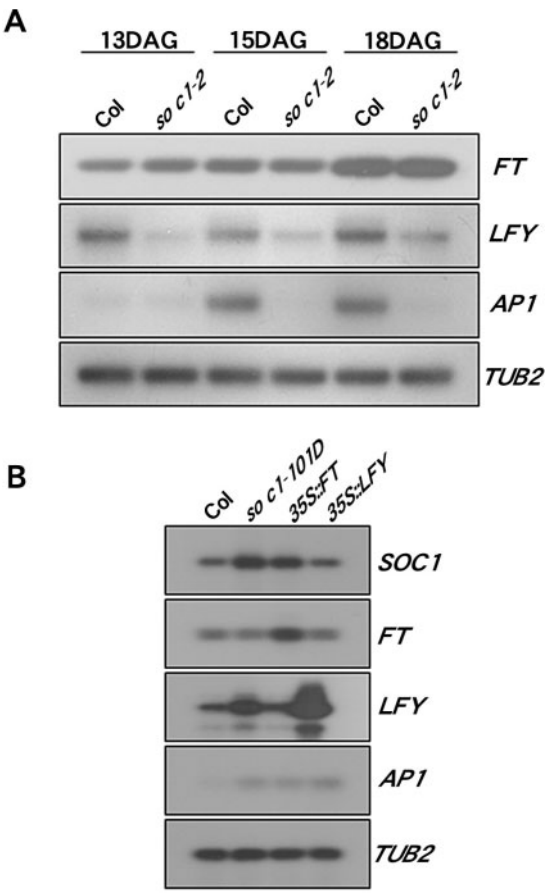

Fig. 5 Expression of *FT*, *SOC1* and *LFY* in the mutants or transgenics of the three integrators. (A) The expression of *FT* and *LFY* in the *soc1* null mutant was determined by RT–PCR. Tissues were harvested 13, 15 and 18 days after germination at 12 h after dawn. (B) The expression of *FT*, *SOC1* and *LFY* in the three overexpression lines, *35S::FT*, *soc1-101D* and*35S::LFY*, was determined by RT–PCR. Tissues were harvested 4 days after germination at 12 h after dawn. *AP1* was used as a marker for the floral transition, and *TUB2* was used as a quantitative control.

liest phenotype of *35S::FT/+ 35S::LFY/+* among double overexpression lines suggests that such independence is stronger between *FT* and *LFY* than between *FT* and *SOC1* or between *SOC1* and *LFY*.

We also examined the phenotype of overexpression of all three integrators. The total leaf numbers in the triple overexpression plants were the same as in *35S::FT/+ 35S::LFY/+* (Table 2, Fig. 4C, D). However, in contrast to *35S::FT/+ 35S::LFY/+*, in which the true leaves were in the form of rosette leaves, the triple overexpression line had two cauline leaves on the stem without rosette leaves. This phenotype may be reminiscent of the transition from vegetative leaf primordia to cauline leaves by strong floral induction (Hempel and Feldman 1994). By adding the *SOC1* overexpression to *35S::FT/+ 35S::LFY/+*, the only two leaf primordia present were changed to cauline leaf-like structures. A similar phenotype is also found in a strong allele of *embryonic flower* (*emf*) (Sung et al. 1992).

Molecular interactions among flowering pathway integrators

To elucidate the molecular relationship of *SOC1* to the other integrators, expression patterns of these genes were analyzed in loss-of-function and gain-of-function mutants of *FT*, *SOC1* and *LFY*. Because the *SOC1* transcript level was lower in *ft* mutants, *SOC1* was proposed to function in one of the downstream pathways of *FT* (Lee et al. 2000). To confirm such a relationship further, *SOC1* expression was determined in *FT* overexpression plants. As shown in Fig. 5B, *SOC1* expression was increased in *35S::FT.* In contrast, *FT* expression was not affected in either the *soc1-101D* (Lee et al. 2000) or *soc1* null mutant. This result confirms the previous suggestion that *FT* regulates *SOC1*, but not vice versa. In the same context, the relationship between *SOC1* and *LFY* was also determined. *LFY* expression was reduced in the *soc1* null mutant even at 13 days after germination when *AP1*, a marker for floral initiation, was not induced (Fig. 5A). Consistently, *soc1-101D* showed higher expression of *LFY* than wild-type Col (Fig. 5B), indicating that *SOC1* functions upstream of *LFY*. However, *SOC1* expression was not changed in *35S::LFY*, showing that *LFY* does not regulate *SOC1*.

Discussion

Studies of flowering time control in *Arabidopsis* have identified an integrated network of genetic pathways (Araki 2001, Simpson and Dean 2002, Mouradov et al. 2002, Boss et al. 2004). The multiple 'input' pathways such as the long day, vernalization and autonomous pathways regulate an overlapping set of common targets, called flowering pathway integrators, that function in the transition to flowering. The key integrators are *FT*, *SOC1* and *LFY*. In this study, the complete genetic analyses of double and triple mutants of the three integrators were performed to elucidate the functional significance of the interactions among the integrators.

Our results, which are consistent with previous reports, show that the three flowering pathway integrators have both overlapping and distinct functions. Among the integrators, *FT* and *SOC1* have more direct function in determining flowering time, but the main function of *LFY* is in the initiation of flower meristem formation. Thus mutations in *FT* or *SOC1* cause late flowering, whereas *lfy* mutation causes only a slight delay in flowering (defined as bolting time) but a strong delay in floral initiation (Koornneef et al. 1991, Weigel et al. 1992, Blázquez et al. 1997, Lee et al. 2000, Samach et al. 2000). Although *FT* and *SOC1* have similar functions and even share the common upstream regulators CO and FLC (Samach et al. 2000), they also have independent functions. *FT* is more strongly dependent on the long day pathway such that the effect of an *ft* mutation is masked by the mutations in the long day pathway but *ft* further delays flowering of the autonomous pathway mutants

(Koornneef et al. 1998b). In contrast, *SOC1* is more strongly dependent on the autonomous pathway, as shown in this study. Such a result is also consistent with the report showing that *soc1* is epistatic to *flc*, a mutant that eliminates the effects of *FRI* or autonomous pathway mutations on flowering, which indicates that *SOC1* is regulated by an autonomous pathway through FLC (Michaels and Amasino 2001). The additive phenotype of the double gain-of-function mutant *35S::FT soc1-101D* further supports the independent function of the two integrators (Fig. 1, Table 2).

The overlapping and distinct functions of the three integrators were also found in the initiation of flower meristem formation. Although the *soc1* single mutant does not show any defect in floral initiation, evaluated by the number of coflorescences, the *soc1 lfy* double mutant shows a dramatic increase in coflorescences compared with the *lfy* single mutant, suggesting that *SOC1* regulates floral initiation in parallel with *LFY* (Table 1). Similarly, the *ft lfy* double mutant shows strong suppression in floral initiation such that *ft lfy* failed to produce flower-like structures although it produced a similar number of rosette leaves to the *ft* single mutant when bolting (Ruiz-García et al. 1997). Consistently, it was reported that *FT* and *LFY* have overlapping function in the expression of *APETALA1*, another gene regulating floral initiation (Ruiz-García et al. 1997). Altogether, it shows that *FT* also functions in parallel with *LFY* for floral initiation.

It was proposed that the multiple input pathways quantitatively regulate the pathway integrators responding to environmental and endogenous signals (Simpson and Dean 2002, Boss et al. 2004). The results of our genetic analysis also reflect the differential regulation of the integrators by each of the input pathways. Although *SOC1* is a direct transcriptional target of CO, the *SOC1* expression remained at ~70% of the wild-type level in the *co* mutation (Lee et al. 2000, Hepworth et al. 2002). Thus, genetic removal of *SOC1* in long day pathway mutations causes a further delay in flowering, which is similar to the additive effect of *soc1* on flowering of the *fve* mutant that has a residual amount of *SOC1* expressed (Fig. 1). On the contrary, *SOC1* expression is regulated predominantly by FLC. The high expression of *FLC* due to the presence of *FRI* or mutations in the autonomous pathway causes a strong block in *SOC1* expression (Lee et al. 2000). Thus, the *soc1* effect on the autonomous pathway mutations is largely masked. Such a predominance of the effect of *FLC* on the regulation of *SOC1* expression was also observed in the transgenic lines overexpressing both *FLC* and *CO* where *SOC1* activation by *CO* was completely blocked by *FLC* (Hepworth et al. 2002). Similarly, it is likely that FT activity is regulated differentially by long day and autonomous pathways because the *ft* mutant shows epistatic interaction with the long day pathway mutations but an additive effect on the autonomous pathway mutations. However, the expression of *FT*, similarly to that of *SOC1*, is strongly blocked by *FLC* (Samach et al. 2000, Hepworth et al. 2002). Thus, the molecular mechanism of how FT activity is differentially regulated by the two input pathways needs to be addressed.

The genetic interaction of the *ft soc1* double mutant with *FLC* revealed the presence of additional targets of *FLC* repression. In addition, it was shown that the activity of these additional targets is sensitive to the level of *FLC*; the flowering time of the *ft soc1* mutant is 20% accelerated by the *flc* null and 10% delayed by the presence of *FRI* (Fig. 3). Considering that there is a quantitative correlation between the expression level of *FLC* and those of *FT* and *SOC1* (Lee et al. 2000, Samach et al. 2000, Hepworth et al. 2002), the additional factors downstream of *FLC* may have functional similarity to *FT* and *SOC1*. A possible candidate for the additional target is *LFY* because higher expression of *FLC* in *FRI soc1-101D* caused the suppression of *LFY* expression (Lee et al. 2000). It was also proposed that *LFY* is regulated by *LD*, and thus by *FLC* (Aukerman et al. 1999). However, it is likely that additional targets are also present because the triple mutant of *ft soc1 lfy* responds to vernalization that suppresses *FLC* expression.

The expression analysis of three integrators among loss-of-function mutants and overexpression lines showed that *FT* regulates *SOC1* expression whereas *SOC1* regulates *LFY* expression, but not vice versa (Fig. 4). Such regulatory hierarchy among the three integrators suggests that the functional overlap among the integrators may be smallest between *FT* and *LFY*. Indeed, the double mutant *ft lfy* has the strongest effect on the floral initiation among double loss-of-function mutants, and the double overexpression line *35S::FT/+ 35S::LFY/+* showed the earliest flowering phenotype among double overexpression lines. Thus, the genetic interactions among the three integrators are well correlated with the molecular regulatory hierarchy. The regulatory hierarchy among the integrators seems not to be conserved in plant species. In rice, the T-DNA-inserted mutation in *OsMADS50*, a *SOC1* ortholog, showed reduced expression of *Hd3a*, an *FT* ortholog, indicating that the *SOC1* ortholog activates the expression of the *FT* ortholog (Lee et al. 2004). It is probable that *OsMADS50* and *Hd3a* cross-regulate each other's expression, which was observed between *AGL24*, another gene promoting flowering, and *SOC1* in *Arabidopsis* (Michaels et al. 2003). Therefore, more complicated networks of flowering pathway integration may have evolved in rice.

Previously, it was shown that vernalization promotes flowering via *FLC*-dependent and *FLC*-independent mechanisms, and both mechanisms activate the expression of *FT* and *SOC1* (Michaels and Amasino 2001, Moon et al. 2003). Our analysis of the *flc ft soc1* triple mutant shows that the *FLC*-independent vernalization pathway regulates additional factor(s) as well as *FT* and *SOC1* (Fig. 3). *AGL24* was proposed as a target of the *FLC*-independent vernalization pathway because the expression level of *AGL24* was not affected by the increase of *FLC* expression but was increased by vernalization treatment (Michaels et al. 2003). Furthermore, *AGL24* was proposed to act upstream of another integrator, *LFY* (Yu et al. 2002, Yu et al. 2004). Thus, it is possible that the *FLC*-inde-

pendent vernalization pathway is also integrated into the three pathway integrators. However, the triple mutant *ft soc1 lfy* still showed a vernalization response, which strongly supports the presence of other targets of vernalization.

Reeves and Coupland (2001) have reported previously that the triple mutant *co fca ga1* does not flower even under inductive photoperiods. Considering that *CO*, *FCA* and *GA1* represent the long day, autonomous and GA pathway, this result demonstrated that the three pathways are essential for flowering to occur. The three pathways are integrated to the flowering pathway integrators, *FT*, *SOC1* and *LFY*. However, the triple mutant *ft soc1 lfy* did not show a complete block to flowering, which demonstrates the presence of other factors regulating flowering at the integration level. In contrast, the overexpression of all of the three integrators caused flowering immediately after germination with only two cauline leaves, similar to a strong allele of the *emf* mutant (Sung et al. 1992), illustrating the importance of quantitative regulation of the three integrators for determining flowering time.

The overlapping and independent functions of flowering pathway integrators and the existence of additional integrators shown in this study may explain why mutants that never flower have not been obtained. It is also consistent with the multifactorial hypothesis proposed by Bernier (1988) instead of the single florigen concept. In conclusion, our study reveals the complicated nature of the genetic network for flowering which enables the plant to respond more flexibly to the changes in environmental and endogenous floral signals.

Materials and Methods

Plant materials

The wild type used was *Arabidopsis* strain Col. All of the mutants and transgenic lines are in the Col background which has a functional *FLC* allele. The *FRI*-containing line is a Columbia near isogenic line with *FRIGIDA* of SF2 by eighth backcross into Col, which was described before (Michaels and Amasino 1999, Lee et al. 2000). The *gi-2* has a frameshift mutation at the N-terminus, and thus is a strong allele (Fowler et al. 1999). The *co-1* mutation has a three amino acid deletion at the zinc finger region and causes a strong phenotype relative to the other alleles at these loci (Koornneef et al. 1991, Putterill et al. 1995). *ft-1* is an intermediate allele with a missense mutation in the last exon of its open reading frame (Kardailsky et al. 1999, Kobayashi et al. 1999). The *fca-9*, *fve-3* and *ld-1* mutations also cause strong phenotypes. The *fca-9* and *fve-3* mutants have nonsense mutations at the N-terminus while the *ld-1* phenotype is caused by a three amino acid deletion (Page et al. 1999, Ausin et al. 2004, Lee et al. 1994). The *lfy-12* mutant is also a strong allele with a premature stop codon (Weigel et al. 1992). The *soc1-2* and *soc1-101D* mutants were described previously as *agl20* and *agl20–101D* (Lee et al. 2000). *35S::FT* was kindly provided by Dr. Ji Hoon Ahn (Korea University). The *35S::LFY* strains used were *DW151.2.9* in the Col background (Weigel and Nilsson 1995). To generate the double mutants, the late flowering mutants were crossed with *soc1-2* or *soc1-101D*. The genotypes of the F_2 plants were checked using polymerase chain reaction (PCR)-based markers. The presence of the *soc1-2*, *soc1-101D*, *gi-2*, *co-1* and *flc-3* mutations was analyzed by SSLP markers, while that of *ft-1*, *fca-9*, *fve-3* and *lfy-12* mutations was analyzed using (d)CAPS markers that could distinguish between mutant and wild-type alleles (see Supplementary Table 1). PCR markers were not available for the *ld-1* mutant, thus the double mutants were confirmed by the backcross to *ld-1*.

Growth conditions

To break seed dormancy, seeds were stratified on 0.65% phytoagar containing 1.5% sucrose and half strength Murashige–Skoog (MS; Gibco-BRL) plates for 2–3 days at 4°C. Afterwards plants were transferred and grown at 23°C in long (16 h light/8 h dark) or short (8 h light/16 h dark) photoperiod conditions under cool white fluorescent lights (100 μmol m^{-2} s^{-1}). For vernalization treatment, the MS plates were incubated for 6 weeks at 4°C under short day conditions. At least 20 plants were used to measure the flowering time of each genotype unless stated otherwise. The flowering time was measured as the mean of the total leaf number including rosette and cauline leaves.

RNA analysis

Total RNA was extracted as described before (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 & Schuell). The *SOC1* and *FLC* probes were made from the cDNA fragments lacking MADS-domain sequences. The reverse transcription (RT)–PCR procedure and primers used for *SOC1*, *FT* and *TUB2* were described previously (Lee et al. 2000, Moon et al. 2003). For *LFY*, LFY-F, 5′-TGAAGGACGAGGAGCTTGAAGAG-3′ and LFY-R, 5′-TTGCCACGTGCCACTTC C-3′ were used.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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