Analysis of Flowering Pathway Integrators in *Arabidopsis*

Jihyun Moon¹, Horim Lee¹, Minsoo Kim¹ and Ilha Lee¹,²,³

¹ Department of Biological Sciences, Seoul National University, Seoul, 151-742, Korea
² Plant Metabolism Research Center, Kyung Hee University, Suwon, 449-701 Korea

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 regulator 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 coleterices in an *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, Gandall 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/AGL1*) 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 pathways.
genes (Samach et al. 2000). It has been reported that CO, suggesting the existence of mediators between the two relationships among the three integrators are largely unknown, (Kardailsky et al. 1999, Kobayashi et al. 1999). However, the LFY function (Ruiz-García et al. 1997, Nilsson et al. 1998, Kardailsky et al. 1999, Kobayashi et al. 1999). In contrast to 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 (Ruíz-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, la and fve, or long day pathway mutants gi, co and fl, 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, fl showed that soc1 mutation further delayed the flowering time of the long day pathway 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 (Austin 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 (Austin 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.
2). As was reported previously, mutants between 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 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.

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-
increased only slightly flowering. Vern, vernalization treatment; RLs, rosette leaves produced when compared with that of coflorescences. When the flowering time of bolting time) but had a significant effect on the number of number of coflorescences that produce secondary shoots, whereas like structures and failed to produce lfy flower-like structures, as previously reported (Table 1, Ruiz-García et al. 1997). Such

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of RLs</th>
<th>No. of coflorescences</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>8.6 ± 0.7</td>
<td>2.3 ± 0.3</td>
<td>15</td>
</tr>
<tr>
<td>soc1-2</td>
<td>23.2 ± 3.0</td>
<td>3.4 ± 1.1</td>
<td>15</td>
</tr>
<tr>
<td>ft-1</td>
<td>30.7 ± 2.3</td>
<td>8.1 ± 0.6</td>
<td>15</td>
</tr>
<tr>
<td>lfy-12</td>
<td>8.9 ± 0.3</td>
<td>6.8 ± 0.4</td>
<td>15</td>
</tr>
<tr>
<td>ft-1 soc1-2</td>
<td>52.3 ± 3.5</td>
<td>10.3 ± 0.5</td>
<td>15</td>
</tr>
<tr>
<td>ft-1 lfy-12</td>
<td>33.8 ± 2.1</td>
<td>&gt;70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>soc1-2 lfy-12</td>
<td>27.2 ± 1.3</td>
<td>30.6 ± 2.3</td>
<td>15</td>
</tr>
<tr>
<td>ft-1 soc1-lfy-12</td>
<td>61.1 ± 3.6</td>
<td>&gt;70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>Vern ft soc1 lfy</td>
<td>41.3 ± 2.2</td>
<td>&gt;70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> More than 70 coflorescences were counted before growth arrest occurred. Vern, vernalization treatment; RLs, rosette leaves produced when flowering.

### Table 2

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

RLs, rosette leaves; CLs, cauline leaves.

FT, soc1, and lfy expression further confirms 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 the 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.

### 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.

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-
to cauline leaf-like structures. A similar phenotype is also
found in a strong allele of embryonic flower (emf) (Sung et al.

Molecular interactions among flowering pathway integrators

To elucidate the molecular relationship of SOC1 to the
other integrators, expression patterns of these genes were ana-
alyzed 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 regu-
late SOC1.

Discussion

Studies of flowering time control in Arabidopsis have
identified an integrated network of genetic pathways (Araki
al. 2004). The multiple ‘input’ pathways such as the long day,
vernalization and autonomous pathways regulate an overlap-
ping set of common targets, called flowering pathway integra-
tors, 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 inte-
grators 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
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 depend-
ent on the long day pathway such that the effect of an ft muta-
tion 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, \textit{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 \textit{soc1} is epistatic to \textit{flc}, a mutant that eliminates the effects of \textit{FRI} or autonomous pathway mutations on flowering, which indicates that \textit{SOC1} is regulated by an autonomous pathway through \textit{FLC} (Michaels and Amasino 2001). The additive phenotype of the double gain-of-function mutant 3SS::FT \textit{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 \textit{soc1} single mutant does not show any defect in floral initiation, evaluated by the number of cobs, the \textit{soc1 lfy} double mutant shows a dramatic increase in cobs compared with the \textit{lfy} single mutant, suggesting that \textit{SOC1} regulates floral initiation in parallel with \textit{LFY} (Table 1). Similarly, the \textit{fl lfy} double mutant shows strong suppression in floral initiation such that \textit{fl lfy} failed to produce flower-like structures although it produced a similar number of rosette leaves to the \textit{ft} single mutant when bolting (Ruiz-García et al. 1997). Consistently, it was reported that \textit{FT} and \textit{LFY} have overlapping function in the expression of \textit{APETALA1}, another gene regulating floral initiation (Ruiz-García et al. 1997). Altogether, it shows that \textit{FT} also functions in parallel with \textit{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 \textit{SOC1} is a direct transcriptional target of CO, the \textit{SOC1} expression remained at \textasciitilde70% of the wild-type level in the \textit{co} mutation (Lee et al. 2000, Hepworth et al. 2002). Thus, genetic removal of \textit{SOC1} in long day pathway mutations causes a further delay in flowering, which is similar to the additive effect of \textit{soc1} on flowering of the \textit{fte} mutant that has a residual amount of \textit{SOC1} expressed (Fig. 1). On the contrary, \textit{SOC1} expression is regulated predominantly by \textit{FLC}. The high expression of \textit{FLC} due to the presence of \textit{FRI} or mutations in the autonomous pathway causes a strong block in \textit{SOC1} expression (Lee et al. 2000). Thus, the \textit{soc1} effect on the autonomous pathway mutations is largely masked. Such a predominance of the effect of \textit{FLC} on the regulation of \textit{SOC1} expression was also observed in the transgenic lines overexpressing both \textit{FLC} and \textit{CO} where \textit{SOC1} activation by \textit{CO} was completely blocked by \textit{FLC} (Hepworth et al. 2002). Similarly, it is likely that \textit{FT} activity is regulated differentially by long day and autonomous pathways because the \textit{ft} mutant shows epistatic interaction with the long day pathway mutations but an additive effect on the autonomous pathway mutations. However, the expression of \textit{FT}, similarly to that of \textit{SOC1}, is strongly blocked by \textit{FLC} (Samach et al. 2000, Hepworth et al. 2002). Thus, the molecular mechanism of how \textit{FT} activity is differentially regulated by the two input pathways needs to be addressed.

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

The expression analysis of three integrators among loss-of-function mutants and overexpression lines showed that \textit{FT} regulates \textit{SOC1} expression whereas \textit{SOC1} regulates \textit{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 \textit{FT} and \textit{LFY}. Indeed, the double mutant \textit{ft lfy} has the strongest effect on the floral initiation among double loss-of-function mutants, and the double overexpression line 3SS::FT/+/ 3SS::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 \textit{OsMADS50}, a \textit{SOC1} ortholog, showed reduced expression of \textit{Hd3a}, an \textit{FT} ortholog, indicating that the \textit{SOC1} ortholog activates the expression of the \textit{FT} ortholog (Lee et al. 2004). It is probable that \textit{OsMADS50} and \textit{Hd3a} cross-regulate each other’s expression, which was observed between \textit{AGL24}, another gene promoting flowering, and \textit{SOC1} in \textit{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 \textit{FLC}-dependent and \textit{FLC}-independent mechanisms, and both mechanisms activate the expression of \textit{FT} and \textit{SOC1} (Michaels and Amasino 2001, Moon et al. 2003). Our analysis of the \textit{flc ft soc1} triple mutant shows that the \textit{FLC}-independent vernalization pathway regulates additional factor(s) as well as \textit{FT} and \textit{SOC1} (Fig. 3). \textit{AGL24} was proposed as a target of the \textit{FLC}-independent vernalization pathway because the expression level of \textit{AGL24} was not affected by the increase of \textit{FLC} expression but was increased by vernalization treatment (Michaels et al. 2003). Furthermore, \textit{AGL24} was proposed to act upstream of another integrator, \textit{LFY} (Yu et al. 2002, Yu et al. 2004). Thus, it is possible that the \textit{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 gal1 does not flower even under inductive photoperiods. Considering that CO, FCA and GAL1 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 enf 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 floren 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 this 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 of the gi-2 allele (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). The 35S::FT transgene was kindly provided by Dr. Ji Hoon Ahn (Korea University). The 35S::LFY strains used were DW151.2.9. 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′-TGAGGACGAGGAGCTTGAAAG-3′ and LFY-R, 5′-TTGCCACGTGCCTTCCTC-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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References


