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Control of Flowering Time: Interacting Pathways as a Basis for Diversity

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INTRODUCTION

Flowering is controlled by environmental conditions and developmental regulation. The complexity of this regulation is created by an intricate network of signaling pathways. Arabidopsis is an excellent model system in which to approach this complexity, because it responds to many of the environmental conditions that control flowering in other species, and genetic tools are well developed. Studies in Arabidopsis have led to the identification of components within individual signaling pathways that affect flowering, and to their positioning within molecular hierarchies. Furthermore, distinct signaling pathways are known to converge on the activation of the same flowering-time genes. This convergence of pathways on a common set of genes may enable the integration of different responses, so that the plant can produce a coordinated flowering response under conditions in which multiple environmental parameters are changing simultaneously. Also, genetic analysis of Arabidopsis varieties showing natural variation in flowering time has demonstrated how the activity of these pathways can be altered in nature and how balancing the effects of different environmental stimuli on flowering time is important in plants adapting to growth in different geographical locations.

At present, the full complexity of the flowering network can only be approached in Arabidopsis where the necessary tools are available, and extensive efforts are being made to describe related pathways. For example, photoreception, circadian clock regulation, growth regulator synthesis and response, chromatin structure, and response to low temperatures play important roles in flowering-time control and are studied extensively in Arabidopsis. Nevertheless, there is a need to understand how the full diversity in flowering responses is generated. For example, Arabidopsis responds to photoperiod, but all ecotypes are long-day plants that flower earlier under long than short days, whereas many other species show the reverse response. Also, all *Arabidopsis thaliana* ecotypes are annual plants, and understanding the perennial habit will require a different model species. To understand the diversity in flowering responses, there is a need to look to other species, and here we compare detailed information from Arabidopsis with the emerging picture of the genetic control of flowering in short-day plants.

The control of flowering has been reviewed frequently over the last few years (Koornneef et al., 1998b; Simpson et al., 1999; Reeves and Coupland, 2000; Samach and Coupland, 2000; Araki, 2001). However, progress has been rapid, and in the following review we emphasize results that have emerged recently. In particular, we summarize the current understanding of the signaling pathways involved in flowering control in Arabidopsis, and stress how alterations in the balance of activity between pathways can give rise to dramatically different flowering behaviors, for example between ecotypes. We also address recent progress in describing how these pathways function in species that show different responses to environmental conditions than does Arabidopsis.

PATHWAYS CONTROLLING FLOWERING TIME IN ARABIDOPSIS

The Photoperiod Response Pathways

One of the most important factors controlling flowering time in temperate regions is the duration of the daily light period, or photoperiod. Arabidopsis is a facultative long-day plant, which flowers earlier under long days but eventually flowers under short days. Under laboratory conditions, Arabidopsis will flower in response to a single long day (Corbesier et al., 1996). Molecular-genetic approaches have identified genes required for the daylength response, and some of these encode regulatory proteins specifically involved in the regulation of flowering, while others encode components of light signal transduction pathways or are involved in circadian clock function. A representation of the relationships among

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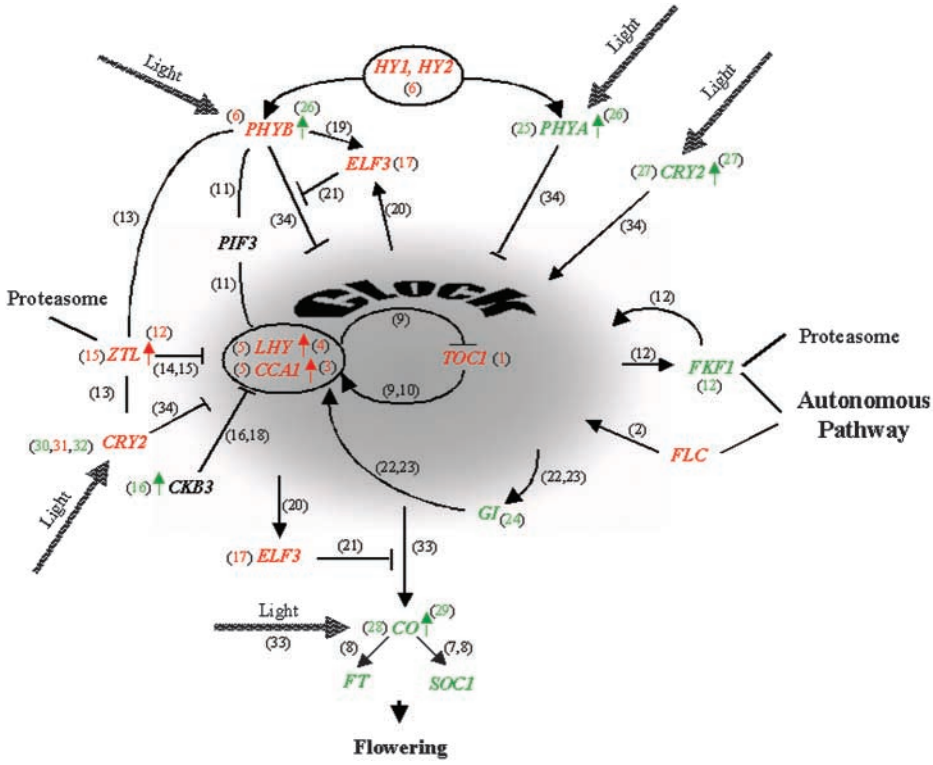

Figure 1. Signaling Pathways Involved in the Regulation of Flowering by Photoperiod in Arabidopsis.

A diagram showing the putative relationships among genes involved in the photoperiod pathway. On the basis of the phenotypes of known mutants, genes shown in red generally repress flowering, whereas those in green promote it. Small upright arrows indicate the role of the genes as determined by overexpression in transgenic plants. Arrows between genes represent a promotive effect, whereas perpendicular lines represent a repressive effect, and simple lines represent protein–protein interactions. Arrows from the clock indicate that the expression of the gene is circadian clock controlled. Arrows to the clock indicate that the gene lengthens period length, while perpendicular lines indicate that it shortens period length. Numbers between brackets refer to the following publications, which support the indications provided in the diagram. **(1)** (Somers et al., 1998b) **(2)** (Swarup et al., 1999) **(3)** (Wang and Tobin, 1998) **(4)** (Schaffer et al., 1998) **(5)** (Mizoguchi et al., 2002) **(6)** (Goto et al., 1991) **(7)** (Samach et al., 2000) **(8)** (Onouchi et al., 2000) **(9)** (Alabadi et al., 2001) **(10)** (Strayer et al., 2000) **(11)** (Martinez-Garcia et al., 2000) **(12)** (Nelson et al., 2000) **(13)** (Jarillo et al., 2001) **(14)** (Millar et al., 1995a) **(15)** (Somers et al., 2000) **(16)** (Sugano et al., 1999) **(17)** (Zagotta et al., 1992) **(18)** (Sugano et al., 1998) **(19)** (Liu et al., 2001b) **(20)** (Hicks et al., 2001) **(21)** (Covington et al., 2001) **(22)** (Fowler et al., 1999) **(23)** (Park et al., 1999) **(24)** (Fowler et al., 1999; Park et al., 1999) **(25)** (Johnson et al., 1994) **(26)** (Bagnall et al., 1995) **(27)** (Guo et al., 1998) **(28)** (Putterill et al., 1995) **(29)** (Simon et al., 1996) **(30)** (Mozley and Thomas, 1995) **(31)** (Ahmad et al., 1998) **(32)** (Koornneef et al., 1980) **(33)** (Suarez-Lopez et al., 2001) **(34)** (Somers et al., 1998a).

these processes is shown in Figure 1 and is described in the following sections.

A Pathway That Promotes Flowering of Arabidopsis in Response to Long Days

Among the flowering-time mutants of Arabidopsis, there is a group that is late flowering in long days but similar or identical to the wild type under short days. These mutants are weakly, or not at all, sensitive to vernalization, and were proposed to define a genetic pathway that promotes flowering specifically in response to long days. *CONSTANS* (*CO*), *CRYPTOCHROME2/FHA* (*CRY2*), *GIGANTEA* (*GI*), *FT* and *FWA* are part of this long day–promoting pathway (Koornneef et al., 1991). *CO* is the latest acting of the known genes that is specific to this pathway; all of the other genes also act in other pathways or have more general effects (Figure 1). *FT* and *FWA* act downstream of *CO* and in other pathways (Kardailsky et al., 1999; Kobayashi et al., 1999; Onouchi et al., 2000), whereas *GI* and *CRY2* act upstream of *CO* (Guo et al., 1998; Suarez-Lopez et al., 2001).

The *CO* gene was proposed to encode a protein with two zinc fingers loosely related to those of GATA transcription

factors (Putterill et al., 1995) and contains a carboxy-terminal domain called CCT because it is present in CO, CO-like (COL), and TIMING OF CAB 1 (TOC1) proteins (Strayer et al., 2000; Robson et al., 2001). Comparison of 16 Arabidopsis *COL* genes that code for proteins sharing the zinc fingers and CCT domains (Robson et al., 2001) demonstrated that the zinc fingers are most similar to the B-box, a type of zinc finger identified in animal proteins and believed to mediate protein–protein interactions (Borden, 1998). The CCT domain is sufficient and necessary for nuclear localisation of GFP:CO or GFP:TOC1 fusion proteins (Strayer et al., 2000; Robson et al., 2001), but may have additional roles in protein–protein interactions (Kurup et al., 2000).

When constitutively expressed from a cauliflower mosaic virus 35S promoter, *CO* induces early flowering and loss of photoperiod sensitivity (Onouchi et al., 2000). Mutations in the *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*, also called *AGAMOUS-LIKE 20* [*AGL20*]) and *FWA* genes partially suppress *35S*::*CO* (Onouchi et al., 2000). In addition, *FT* and *SOC1* expression levels are increased in *35S*::*CO* plants or *35S*::*CO*:*GR* plants in which *CO* activity is induced by dexamethasone (Samach et al., 2000), indicating that these genes act downstream of *CO*. In agreement with this, *35S*::*FT* can suppress the effect of *co* mutations (Kardailsky et al., 1999; Kobayashi et al., 1999). However, no mutations were recovered that totally suppress the *35S*::*CO* early-flowering phenotype, suggesting that parallel pathways are activated by *CO* (Onouchi et al., 2000).

The *CO* transcript level shows a diurnal rhythm in long days, with a broad biphasic peak between 12 and 24 h after dawn and maximum levels 16 and 24 h after dawn. This peak is narrower in short days, ending 4 h earlier (Suarez-Lopez et al., 2001). Plants entrained in long days show a circadian rhythm in *CO* transcript level when transferred to continuous light (LL), indicating that this rhythm is controlled by the circadian clock (Suarez-Lopez et al., 2001).

FT is an early target of *CO* (Samach et al., 2000), and its transcript level follows a circadian rhythm that peaks 20 h after dawn in long days (Suarez-Lopez et al., 2001). This peak is absent in the *co* mutant. In addition, the circadian clock–related mutants, *LATE ELONGATED HYPOCOTYL* (*LHY*), *GI*, and *EARLY FLOWERING3* (*ELF3*), show an altered rhythm in *CO* transcript level that correlates with their flowering phenotype. Overall, *CO* appears to mediate between the circadian clock and the flowering-time gene *FT* (Suarez-Lopez et al., 2001). This model suggests that the circadian clock acts within the long-day pathway to regulate the expression of downstream genes such as *CO* and *FT*.

Two general models have been proposed for how flowering may be regulated by daylength (Thomas and Vince-Prue, 1997; Samach and Coupland, 2000; Samach and Gover, 2001). The external coincidence model proposes that light acts as an external signal that interacts with a light-sensitive rhythm at certain times of day. In long-day plants, such as Arabidopsis, if the plant is exposed to light at the crucial phase of the rhythm, flowering is promoted. Another model, the internal coincidence model, suggests that in photoperiods that are inductive, two rhythms are brought into the same phase and interact to promote flowering, whereas in noninductive photoperiods, these rhythms are out of phase. Observing the activation of *FT* by *CO*, Suarez-Lopez et al. (2001) proposed that the expression pattern of *CO* may represent a light-sensitive rhythm that is exposed to light only under long-day conditions that promote flowering, and that post-transcriptional activation of *CO* by light may lead to the activation of *FT* transcription and ultimately to flowering. This proposal represents a version of the external coincidence model.

Candidates for photoreceptors that might be involved in the post-transcriptional activation of CO protein under long days are CRY2 and PHYTOCHROME A (PHYA). On the basis of the observation that *phyA* mutants are slightly late flowering in long days (Johnson et al., 1994) and transgenic plants overexpressing PHYA flower earlier than the wild type in short days (Bagnall et al., 1995), PHYA is a photoreceptor that promotes flowering. Similarly, the *cry2* mutant is late flowering in long days and transgenic plants overexpressing *CRY2* are early flowering in short days, indicating that *CRY2* is involved in sensing photoperiod (Guo et al., 1999). Furthermore, a novel allele of *CRY2* was recently isolated by positional cloning of a quantitative trait locus for early flowering identified between two Arabidopsis ecotypes (El-Assal et al., 2001). This new allele causes early flowering under short days because of a single amino acid substitution that impairs the light-induced downregulation of CRY2 under short days. Mutation in *CO* suppresses the early flowering caused by the *CRY2* allele, suggesting that *CRY2* does regulate flowering through *CO*. *PHYA* and *CRY2* are therefore good candidates for photoreceptors that perceive the photoperiod in the external coincidence model.

The Circadian Clock and the Central Oscillator

Circadian rhythms have a period length (the duration of one cycle) of ~24 h. These rhythms do not require daily transitions from light to dark, but continue under constant conditions. Circadian rhythms have been observed at different levels of organization, from leaf movement to stomatal aperture, CO_2 assimilation, or gene transcription. The mechanism that generates these rhythms is often described in three interrelated sections. These are input pathways that synchronise the clock mechanism to daily cycles of light and dark, a central oscillator that generates the 24-h time-keeping mechanism, and output pathways that regulate particular processes (Roenneberg and Merrow, 2000; McClung, 2001). The control of flowering via *CO* and *FT* represents one such output pathway (Suarez-Lopez et al., 2001), whereas many others have been described in detail using global gene expression assays (Harmer et al., 2000; Schaffer et al., 2001).

One output pathway, represented by the *CHLOROPHYLL A/B BINDING PROTEIN2* (*CAB2*) gene, has enabled detailed analysis of clock regulation. The *timing of CAB* (*toc*) mutants were isolated from transgenic plants carrying the *LUCIFERASE* (*LUC*) reporter gene under the control of the *CAB2* promoter (Millar et al., 1995a). The *toc1* mutant showed a 2- to 3-h shorter period length for *CAB* transcription and other gene expression rhythms, leaf movement (Millar et al., 1995a), and stomatal conductance (Kreps and Simon, 1997; Somers et al., 1998b). The *toc1* mutant flowered early in short days (Kreps and Simon, 1997; Somers et al., 1998b), and this phenotype could be rescued by growing the plants in daily cycles of 21 h rather than 24 h, a reduction of 3 h corresponding to the shorter period length observed in *toc1* (Strayer et al., 2000). This supports the proposal that shortened circadian period length is the cause of the early-flowering phenotype of *toc1* mutants.

The TOC1 protein shows two characteristic domains. The first of these, at the N terminus, is similar to the receiver domain of response regulators from two-component signal transduction systems. However, invariant residues of this motif, including the Asp residue that is normally phosphorylated in response regulators, are substituted in TOC1, suggesting that TOC1 does not function as a classic response regulator. Indeed, TOC1, also known as APRR1, and other pseudo–response regulators, are not phosphorylated in an in vitro assay (Makino et al., 2000). The second motif, at the C terminus, is the CCT domain, shared with the CO family of transcriptional regulators. *TOC1* transcript level cycles in light/dark cycles with a peak in the evening and shows a circadian rhythm in LL (continuous light; Strayer et al., 2000). In the *toc1* mutant, the period of the circadian rhythm of *TOC1* expression in LL is shorter, indicating that *TOC1* controls its own rhythm of expression through a feedback loop. The rhythm in *TOC1* transcript level, the period-shortening effect of the *toc1* mutation with the identical influence of the mutation on multiple outputs, and the participation of *TOC1* in a feedback loop make it a candidate component of the oscillator.

Other likely components of the oscillator are the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) genes, which are also involved in the photoperiodic induction of flowering. These genes encode highly similar single Myb domain DNA binding proteins and are each regulated by the circadian clock, with a peak in their expression soon after dawn (Schaffer et al., 1998; Wang and Tobin, 1998). The overexpression of *LHY* or *CCA1* disrupts all circadian rhythms tested, such as those in leaf movement and gene expression rhythms in different phases, including those in *LHY* or *CCA1* expression (Schaffer et al., 1998; Wang and Tobin, 1998). Recent reports, nevertheless, suggest that *LHY* overexpression may not disrupt rhythms in expression of the circadian clock–regulated gene *EARLY FLOWERING 3* (*ELF3*), which would be an unexpected exception to the effect of *LHY* (Hicks et al., 2001). Loss-of-function alleles of *LHY* or *CCA1* cause circadian period length to be shortened by ~3 h, and inactivation of both genes prevents the maintenance of circadian rhythms under constant conditions (Green and Tobin, 1999; Mizoguchi et al., 2002). The observations that *CCA1* and *LHY* present a circadian rhythm of expression, that they regulate their own transcripts through a feedback loop, and that they both suppress all observed rhythms when constitutively expressed suggest that they could be components of the oscillator. There is also a close relationship between the effect of *LHY* and *CCA1* on clock regulation, and flowering time. Overexpression of either gene causes late flowering under long-day conditions, whereas loss-of-function alleles of *CCA1* and *LHY*, which, like the *toc1* mutant, show a shortening of the period length in LL, also resulted in early flowering in short days (Mizoguchi et al., 2002).

The proposal that *TOC1* and *LHY/CCA1* may be part of a central oscillator was recently strengthened by the demonstration that they participate in an autoregulatory loop (Alabadi et al., 2001). LHY and CCA1 bind to the *TOC1* promoter in vitro and reduce *TOC1* expression when overexpressed, whereas in *toc1* mutants, the period length of *LHY* and *CCA1* expression is shorter and their level of expression is reduced. Therefore, TOC1, LHY and CCA1 may act in a negative feedback loop, in which *LHY* expression and *CCA1* expression rise in the morning and repress the expression of *TOC1*. According to this model, LHY and CCA1 feed back to repress their own expression, and as their protein levels fall, the expression of *TOC1* rises. TOC1, in turn, promotes the expression of *LHY* and *CCA1*, so initiating another cycle (Alabadi et al., 2001). This feedback loop may regulate flowering time by determining the time of day that flowering-time genes in the long-day pathway, such as *CO* and *GI*, are expressed (Figure 1).

Entraining the Clock

Circadian rhythms must be synchronized with the daily rhythm of light and dark and of temperature. The phase of circadian rhythms can therefore be reset by environmental conditions such as light and temperature that fluctuate during day/night cycles. Mutations that affect entrainment of circadian rhythms by light signals have been described, and some of them directly affect photoreceptors, whereas others affect light signal transduction. Many of these mutations also have severe effects on flowering time, but for many of them, it is not clear whether their effect on flowering time is due to an impairment of circadian clock entrainment or to another aspect of light signal transduction. The intensity and quality of light will also affect flowering independently of the effects of light on circadian clock regulation, and the role of the blue light–receptor cryptochromes is reviewed in this volume (Lin, 2002).

For example, a general effect of phytochromes on flowering can be deduced from the *hy1* and *hy2* mutants, which are impaired in the synthesis of the chromophore of all phytochromes. Both mutations cause severe early flowering in

short days and in long days (Goto et al., 1991), indicating that phytochromes generally are repressors of flowering. However, only a part of this effect is likely to be due to the long circadian period also caused by these mutations (Millar et al., 1995b).

The photoreceptors PHYA, PHYB, CRY1, and CRY2 all influence clock entrainment under specific light conditions. However, the quadruple mutant *phyA phyB cry1 cry2* still shows robust circadian rhythms that can be entrained, indicating that either another phytochrome or an unknown photoreceptor is also part of the clock input machinery (Yanovsky et al., 2000). The influence of *PHYA* and *CRY2* on clock entrainment occurs only at low fluence rates (Somers et al., 1998a), suggesting that the late-flowering phenotype associated with mutations in these two photoreceptors probably is not caused by a direct effect on circadian rhythms (see also section describing the long-day pathway).

Mutations in a PHYB-interacting protein, ZEITLUPE (ZTL), also have dramatic effects on clock function and flowering time. A semidominant mutation in *ZTL* caused late flowering in long days and a 3-h increase in the period length of gene expression rhythms (Millar et al., 1995a; Somers et al., 2000), whereas the period of leaf movements was lengthened by 5 to 6 h. The lengthening of the period in *CAB* transcription, as followed using a *CAB*::*LUC* reporter, was shown to be strongly dependent on light intensity, but no effect of light quality was observed (Somers et al., 2000), whereas leaf movements were arrhythmic under red light (Jarillo et al., 2001). Constitutive overexpression of *ZTL* also gave a late-flowering phenotype in long days (Nelson et al., 2000). The ZTL protein contains a PAS domain, an F-box, and six repeated kelch motifs that are predicted to form a β-propeller. The β-propellers are believed to be implicated in protein–protein interactions (Adams et al., 2000). The F-box interacts with SKP1 within the Skp1p-Cdc53p-F-box protein (SCF) complex that acts as a ubiquitin ligase implicated in the ubiquitination of target proteins (Patton et al., 1998). The PAS domain mediates protein–protein interactions and has been found in a group of blue light photoreceptors (Briggs and Huala, 1999). The combination of domains within ZTL suggested that it might recruit proteins for degradation by the proteosome in a way that is influenced by light. Protein–protein interactions have been described between ZTL and PHYB and between ZTL and CRY1 (Jarillo et al., 2001). Because the transcript level of *ZTL* is not regulated by the clock and its effect is dependent on light intensity (Somers et al., 2000), *ZTL* may affect circadian rhythms by the impairment of a light input pathway.

ZTL is a member of a family of three genes that also includes *FKF1* and *LKP2* (Somers et al., 2000). The *fkf1* mutant shares the late-flowering phenotype of *ztl*. However, its transcript level shows a circadian rhythm, and an *fkf1* null allele has no effect on the period length of *CAB* and *CCA1* expression; only a change in the form of the peak was observed (Nelson et al., 2000). The late-flowering phenotype of *fkf1* can be rescued by vernalization and gibberellin (GA) application, suggesting that *fkf1* may be associated with the autonomous flowering pathway (Nelson et al., 2000). The association of F-box proteins with circadian clock function is consistent with the importance of protein instability in circadian clock regulation.

Target protein recognition by F-box proteins is strictly dependent on their phosphorylation (del Pozo and Estelle, 2000). CCA1 and LHY can be bound by CKB3, a regulatory subunit of the protein kinase CK2 (Sugano et al., 1998, 1999). Transgenic plants overexpressing CKB3 show a period length of *CCA1* and *LHY* expression under LL that is shorter by ~4 h, and are early flowering under long or short days. The period shortening was also detected in the rhythms in expression of several other genes, which peak at different times of the day, indicating the importance of CK2 in the regulation of the circadian clock. CK2 activity is increased in the transgenic plants, and this activity has been shown to phosphorylate CCA1 and LHY in vitro. Thus, in plants overexpressing CKB3, CCA1 may be phosphorylated more rapidly, perhaps causing a shorter period length due to an increased rate of degradation of the protein. This may involve F-box proteins and the ubiquitin pathway.

The *elf3* mutant was isolated as an early-flowering mutant insensitive to photoperiod (Zagotta et al., 1992), and this phenotype is likely to be due, at least in part, to increased expression of CO (Suarez-Lopez et al., 2001). *elf3* shows similarities to *phyB* mutants, including elongated hypocotyls and petioles, early flowering, and defects in the red light response (Reed et al., 2000). *ELF3* encodes a nuclear protein proposed to act as a transcriptional activator (Hicks et al., 2001; Liu et al., 2001b). The ELF3 protein interacts with PHYB and requires *PHYB* for the regulation of hypocotyl elongation but not for the control of flowering time, indicating that although *ELF3* is part of the *PHYB* signaling pathway, they control flowering time independently (Liu et al., 2001b). *ELF3* transcript level is circadian clock regulated, and peaks at the beginning of the subjective night (Hicks et al., 2001). The *elf3* mutant is arrhythmic for leaf movements and gene expression rhythms when grown in LL, but not in continuous darkness (DD). It also shows increased sensitivity to red light, as measured by light-induced expression of *CAB2* in etiolated seedlings, which is independent of circadian clock control, or induction of *CAB2* during the night, a process regulated by the circadian clock. *ELF3*-overexpressing plants show a reduced sensitivity to acute light induction and an increased period length of circadian rhythms. Taken together, these data suggest that ELF3 is a repressor of light signaling that represses light input to the clock during the night. It also inhibits phytochrome-induced responses such as the acute induction of *CAB* (McWatters et al., 2000; Covington et al., 2001). *ELF3* illustrates the complexity of the function of genes that simultaneously control circadian rhythms and light regulation while they are themselves influenced by the clock.

The *gi* mutant is late flowering in long days, and *gi* seedlings show a loss of sensitivity to red light but not to far-red

light, typical of a disruption of the *PHYB* signal transduction pathway (Huq et al., 2000). However, how this role for *GI* in *PHYB* signal transduction relates to its late-flowering phenotype is unclear because in contrast to *gi* mutants, *phyB* mutants are early flowering. Mutant alleles of *GI* also cause alterations in period length of gene expression rhythms. The GI protein contains six putative transmembrane domains (Fowler et al., 1999; Park et al., 1999), suggesting a membrane protein, but GUS:GI and GFP:GI fusion proteins were targeted to the nucleus (Park et al., 1999). The *GI* transcript level is circadian clock regulated. In the *elf3* mutant, the *GI* transcript level is increased in long days and short days, and the rhythm is disrupted in LL, suggesting that *GI* acts after *ELF3* (Fowler et al., 1999). The late flowering of *gi* mutants is at least in part due to reduced amplitude of *CO* expression, but how this relates to the roles of *GI* in light signal transduction or in regulating circadian clock period length is unknown.

The Vernalization Response Pathway

Exposure to low temperatures for several weeks will often accelerate flowering. Susceptibility to this treatment can differ markedly between varieties of a species. For example, many naturally occurring Arabidopsis varieties will flower very late if they are not exposed to a vernalization treatment but flower early if exposed to low temperatures for 4 to 8 weeks (Michaels and Amasino, 2000). Such a requirement for vernalization is associated with a winter annual growth habit. In nature, these varieties do not flower until they have been exposed to winter conditions. They typically germinate in the summer, grow vegetatively through the winter until the following spring, and then flower, often in response to appropriate daylengths during the spring or summer. This is in contrast to summer annuals, which germinate and flower in the same summer without a requirement for vernalization. The genetic control of vernalization was addressed by crossing winter annual varieties that require vernalization with summer annual varieties that do not. These varieties differed at two loci, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), and dominant alleles at these loci in the winter annual are required to confer a vernalization requirement (Burn et al., 1993a; Lee and Amasino, 1993; Clarke and Dean, 1994).

FLC encodes a MADS box transcription factor (Michaels and Amasino, 1999b; Sheldon et al., 1999). The mRNA and protein of *FLC* are expressed at much higher levels in vernalization-requiring winter annual varieties of Arabidopsis than in early-flowering summer annual varieties. In addition, expression of *FLC* in summer annual varieties from the 35S promoter causes a dramatic late-flowering phenotype. Therefore, *FLC* encodes a repressor of flowering, and high-level expression of *FLC* correlates with the vernalization requirement of winter annual varieties (Michaels and Amasino, 1999b; Sheldon et al., 1999, 2000). The association between *FLC* and vernalization was strengthened by demonstration that the abundance of *FLC* mRNA falls when the plants are exposed to cold, and that this reduction occurs progressively in a way that is consistent with the progressive effect on flowering time. For example, treatment of plants at 4°C for 14 days causes a partial reduction in *FLC* expression, and incomplete vernalization as measured by flowering time (Sheldon et al., 2000). Flowering-time genes whose expression is repressed by FLC have been isolated (see below, Integration of Flowering Pathways).

Although *FLC* plays a central role in vernalization, and much of this response involves reductions in *FLC* mRNA, this probably does not explain the whole response to vernalization. Mutants carrying null alleles of *FLC* still show a response to vernalization (Michaels and Amasino, 2001). There is a clade of MADS box transcription factors closely related to FLC, and partial redundancy between these might explain the ability of *flc* mutants to respond to vernalization (Ratcliffe et al., 2001; Scortecci et al., 2001). Inactivation of at least one of these transcription factors, FLM, causes early flowering, and its overexpression delays flowering (Scortecci et al., 2001). However, none of these genes are regulated by vernalization in a way similar to *FLC*. There is also suggestive physiological and genetic evidence that vernalization does not simply act to reduce the expression of an inhibitor of flowering such as FLC, but that it more actively promotes flowering. For example, vernalization of *flc* mutants in a Columbia background causes early flowering in short days, suggesting that promotion of flowering by vernalization can overcome the requirement for long-day induction of flowering (Michaels and Amasino, 2001). Similarly, vernalization causes a *co ga1 fca* triple mutant to flower surprisingly early, suggesting that, even in this background, in which the three major pathways are impaired, vernalization will promote early flowering (Reeves and Coupland, 2001). However, the molecular nature of a pathway that promotes flowering via vernalization but independently of *FLC* is not known (Figure 2).

The other locus at which dominant alleles confer a vernalization requirement is *FRI*. The product of this gene somehow increases *FLC* mRNA abundance (Michaels and Amasino, 1999b; Sheldon et al., 1999). That this effect on *FLC* expression is required for *FRI* to delay flowering is supported by the observation that loss-of-function *flc* mutations suppress the effect of *FRI* on flowering time. The biochemical function of FRI protein is unknown, but it contains coiled-coil domains that may be involved in protein–protein interactions (Johanson et al., 2000). Most early-flowering varieties of Arabidopsis carry one of two deletions that disrupt the open reading frame of *FRI*, suggesting that these early-flowering varieties are derived from late-flowering ones by inactivation of the *FRI* gene (Johanson et al., 2000).

The molecular-genetic analysis of *FRI*/*FLC* suggests that vernalization acts by reducing *FLC* expression in response to extended exposure to cold. The effect of vernalization was previously shown to be stable through mitosis

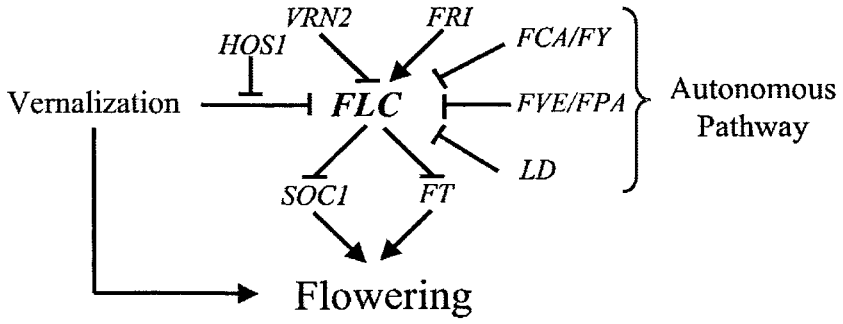

Figure 2. The Effects of Vernalization and the Autonomous Pathway on Flowering Time, Emphasizing the Central Role of FLC.

The autonomous pathway genes and vernalization promote flowering by repressing *FLC* expression. Once *FLC* expression is reduced by vernalization, it is stably repressed by the *VRN2* gene product. Vernalization promotes flowering independently of *FLC* as well as by *FLC* repression. HOS1 seems to act as a repressor of the vernalization pathway. *FLC* expression is promoted by *FRI*. FLC represses flowering and, at least in part, this occurs through repressing the flowering time genes *SOC1* and *FT*. The data used to derive this model are described in detail in the text.

(Michaels and Amasino, 2000), and once *FLC* expression is reduced, it remains low even in plant organs that are formed subsequent to the vernalization treatment (Michaels and Amasino, 1999b; Sheldon et al., 1999). Similarly, the effect of vernalization is reset during meiosis, and high-level expression of *FLC* is also restored in the progeny of vernalized plants. *FLC* expression therefore shows features in common with the vernalized state. These features of vernalization show similarities to epigenetic control of gene expression, and led to the proposal that regulation of gene expression by methylation may be the basis of vernalization (Burn et al., 1993b). Consistent with this proposal, demethylating agents caused early flowering of late-flowering mutants of Arabidopsis, transient reductions in methylation occurred in vernalized plants, and reductions in methylation in transgenic plants carrying an antisense methyltransferase construct caused early flowering of late-flowering plants that respond to vernalization (Finnegan et al., 1998). *FLC* expression was also reduced in a transgenic line carrying the antisense methylase transgene (Sheldon et al., 1999).

A genetic approach was also taken to identifying genes required for the vernalized state. In this case, vernalization-requiring plants (*fca* mutants; see below, The Autonomous Pathway) were mutagenised to identify mutations that prevented early flowering after vernalization (Chandler et al., 1996). The resulting *vernalization* (*vrn*) mutants appeared to prevent downregulation of *FLC* expression by exposure to cold, because when *fca vrn* double mutant plants were vernalized and then grown at normal temperatures for 2 to 3 weeks, *FLC* mRNA was expressed strongly in the double mutant but reduced to low levels in the *fca* mutant (Sheldon et al., 1999). However, recently the role of *VRN2* was described in more detail (Gendall et al., 2001). These experiments demonstrated that *FLC* mRNA levels in an *fca vrn2* double mutant were as low as in an *fca* mutant immediately after vernalization, but that as plants were grown at normal growth temperatures, the abundance of *FLC* mRNA rose in the double mutant but not in *fca*. This suggests that the role of *VRN2* is not the reduction of *FLC* expression in response to low temperatures, but the maintenance of the vernalized state (Gendall et al., 2001). Cloning of *VRN2* showed that its product is related in sequence to a Drosophila Polycomb group protein, Su(z)12, that regulates gene expression by modifying chromatin structure (Birve et al., 2001; Gendall et al., 2001). A similar role for *VRN2* was indicated by the demonstration of altered chromatin structure around *FLC* in *vrn2* mutants. The epigenetic regulation of vernalization may, then, be mediated by alterations in chromatin structure; how this relates to the effect of methylation is not clear.

The analysis of *VRN2* gene function demonstrated that this protein is required for the maintenance of the vernalized state but not for the initial response to cold. A gene that appears to regulate the response to cold is *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1* (*HOS1*) (Lee et al., 2001). Mutations in this gene were identified by isolating mutants that showed enhanced expression of the cold-inducible gene *RD29* in response to cold stress. The *hos1* mutants were also early flowering, and showed reduced expression of *FLC* (Ishitani et al., 1998; Lee et al., 2001). These observations are consistent with the idea that *HOS1* encodes a negative regulator of cold signaling, and that in the absence of *HOS1*, enhanced activity of this pathway leads to increased expression of cold-induced genes such as *RD29* and reduced expression of *FLC*, which is normally repressed by cold (Lee et al., 2001). The *hos1* mutant provides a genetic link between vernalization and cold stress. The *HOS1* gene encodes a RING finger protein that may serve as a ubiquitin ligase; these have been implicated as negative regulators of a number of other plant signal transduction pathways, but a role in cold signal transduction

has not been demonstrated (Bachmair et al., 2001). Further genes involved in vernalization and required for cold perception and/or response to the cold will surely emerge from further genetic screens, and the indication, based on the analysis of *HOS1*, that there may be similarities to the mechanism of cold stress is a tantalizing one.

The Autonomous Pathway

The autonomous pathway was identified via a group of mutants that are late flowering under all photoperiods, and are highly responsive to vernalization (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991). These mutants include *fca*, *fy*, *fpa*, *luminidependens* (*ld*), and *fve*. The similarity between these mutants was further emphasized by the demonstration that they all contain much higher levels of *FLC* mRNA than do wild-type plants or late-flowering mutants affected in the long-day or GA pathways (Michaels and Amasino, 1999b; Sheldon et al., 1999). Thus, autonomous pathway mutations appear to delay flowering by causing increased expression of *FLC*, and therefore in wild-type plants this pathway can be considered to negatively regulate *FLC* expression (Figure 2). Such a role for autonomous pathway mutants was also demonstrated genetically, because loss-of-function *flc* alleles suppress the late-flowering phenotype of autonomous pathway mutants (Michaels and Amasino, 2001).

Nevertheless, the interaction of autonomous pathway mutants with naturally occurring alleles of *FLC* differs. The *fca*, *fy* and *fpa* mutations cause late flowering in a Landsberg *erecta* background, and the *FLC* allele present in this ecotype is therefore sensitive to loss-of-function alleles in these autonomous pathway genes. However, the *ld* mutation does not cause late flowering in Landsberg *erecta*, but does in other ecotypes such as Columbia, suggesting that the Columbia but not the Landsberg allele of *FLC* responds to loss-of-function of *LD* (Koornneef et al., 1994; Lee et al., 1994a). These allelic differences in the regulation of *FLC* remain to be explained.

Although all of the autonomous pathway mutations act by increasing *FLC* expression, genetic evidence suggests that they may not all act in a simple linear pathway (Figure 2). For example, *fca fpa* double mutants are much later flowering than would be expected as a result of a simple additive effect of these mutations, and combining *fpa* and *fy* mutations appears to be lethal, indicating a much wider role for these genes in plant development than simply regulating flowering time (Koornneef et al., 1998a).

Several of the genes within the autonomous pathway have been cloned. The predicted FCA protein contains two copies of an RNA binding domain, the RNP (also referred to as RNA recognition motif or consensus sequence RNA-binding domain), and a WW protein–protein interaction domain (Macknight et al., 1997). FCA was shown to bind RNA in vitro. Strikingly, *FPA* also encodes an RNA binding protein containing RNP motifs, suggesting that post-transcriptional regulation may play a general role in the pathway (Schomburg et al., 2001). The LD protein contains a homeobox and putative nuclear localization sequences, and may encode a transcription factor (Lee et al., 1994b). All three genes are expressed in a similar pattern, with maximal expression at the apex of the plant and inflorescences, and low-level expression in mature leaves and roots. Overexpression of *FPA* causes severe early flowering under short days, whereas increased expression of *FCA* had relatively minor effects on flowering time, but in this experiment, post-transcriptional regulation of *FCA* RNA splicing prevented large increases in the abundance of the fully processed *FCA* mRNA (Macknight et al., 1997; Schomburg et al., 2001).

The manner in which these proteins regulate *FLC* expression is not known. However, plants containing meristem sectors of *fca* homozygous mutant cells within the L2 and L3 layers of otherwise wild-type plants flowered at the same time as did the wild type. This suggests that FCA wild-type cells, either in adjacent L1, L2, or L3 cells within the meristem or in other tissues of the plant, were able to correct the phenotype of the *fca* mutant cells (Furner et al., 1996). Thus non–cell autonomous factors may act downstream of *FCA*.

The GA Pathway

The growth regulator GA promotes flowering of Arabidopsis (Wilson et al., 1992; Putterill, et al., 1995; Blazquez et al., 1998). This was initially demonstrated by applications of exogenous GA (Langridge, 1957), and has been more recently studied using mutations that disrupt either GA biosynthesis or signaling (Wilson et al., 1992). These mutations also have effects on many other aspects of plant growth and development, including stem elongation, germination, and floral development. In this section, we summarize the impact of GA on flowering (Figure 3), and GA signaling is thoroughly reviewed by Olszewski et al. (2002).

Several mutations affecting GA biosynthesis have been identified in Arabidopsis, and the mutant genes cloned. Roles for the *GA1*, *GA4* and *GA5* genes in regulating flowering time have been described. *GA1* encodes copalyl diphosphate synthase, an enzyme that catalyzes the first committed step in GA biosynthesis (Sun and Kamiya, 1994). The corresponding mutant, *ga1-3*, is unable to flower in short days, and is later flowering than is the wild type under long days (Wilson et al., 1992). These plants are also severely dwarfed, do not germinate in the absence of exogenous GA, and exhibit reduced apical dominance.

In contrast to *ga1*, the *ga4* and *ga5* mutations have less-severe effects, giving rise to semidwarf plants that produce fertile flowers with normal siliques (Koornneef and van der Veen, 1980). The *ga4* and *ga5* mutants are defective in GA 3β-hydroxylase and GA 20-oxidase activity, respectively (Talon et al., 1990). GA 20-oxidase is regulated by environmental or physiological changes, suggesting that it may be

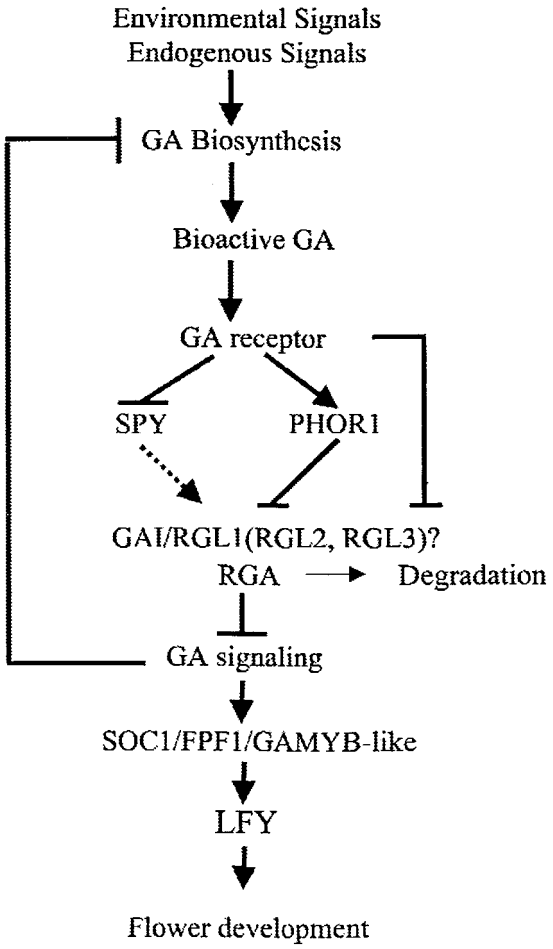

Figure 3. GA-Signaling Pathway That Regulates Flowering in Arabidopsis.

Activation of a hypothetical transmembrane receptor by GA inhibits repressors of GA signaling. These repressors are encoded by the *RGA*, *GAI*, and *RGL* genes. The *SPY* gene also represses GA signaling and genetically acts upstream of *RGA* and *GAI*. It may act to promote the activity of GAI/RGA/RGL by GlcNAc modification, in which case GA signaling may inhibit GAI/RGA/RGAL by repressing SPY function. *PHOR1* has not been described in Arabidopsis, but has been shown to be involved in GA signaling in potato. Its possible involvement in ubiquitination and protein degradation leads to the tentative proposal that it is involved in the demonstrated degradation of the repressing protein RGA in response to GA. The floral meristem identity gene *LFY* is upregulated at the transcriptional level by GA. The flowering-time gene *SOC1* is also upregulated by GA, whereas FPF1 and GA-MYB were proposed to mediate between GAs and the regulation of flowering time. These three genes may therefore act downstream of GAI/RGA/RGL but upstream of LFY. The data underlying this scheme are described in detail in the text.

involved in a key regulatory step in GA biosynthesis (Xu et al., 1995). Expression of this gene increases when plants are transferred from short days to long days, and therefore high-level expression correlates with conditions that induce early flowering (Xu et al., 1997). Furthermore, transgenic plants containing elevated levels of GA 20-oxidase also contained more GA4 and flowered earlier than did wild-type control plants under both long-day and short-day conditions (Huang et al., 1998; Coles et al., 1999). This suggests that GA levels are limiting on flowering time, and is consistent with previous observations that application of exogenous GA causes early flowering of wild-type plants.

A role for GA in the control of flowering is also suggested by studying genes involved in GA signaling. Three related genes with key roles in GA signaling, *GIBBERELLIC ACID INSENSITIVE* (*GAI*), *REPRESSOR OF GA1-3* (*RGA*) and *RGA-LIKE 1*(*RGL1*) have overlapping functions. GAI and RGA proteins share 71% sequence identity with each other (Peng et al., 1997; Silverstone et al., 1998), and 61% identity to RGL1 (Sanchez-Fernandez et al., 1998; Wen and Chang, 2002). These proteins belong to the plant-specific GRAS (for GAI, RGA, SCARECROW) family of regulatory proteins (Pysh et al., 1999; Bolle et al., 2000). All GRAS family members contain highly conserved central (VHIID) and carboxy-terminal (RVER) regions (Silverstone et al., 1998). RGA, GAI and RGL1 also contain a conserved sequence near their N termini that was called DELLA after the amino acids making up the region, although in RGL1 an alanine-to-valine substitution changes the sequence to DELLV (Wen and Chang, 2002). This sequence is not present in most GRAS family members (Peng et al., 1997; Silverstone et al., 1998). GAI, RGA and RGL1 are believed to inhibit GA responses in the absence of active GA, and GA relieves this inhibition.

A prediction of this model is that GA responses would occur in the absence of GA if *GAI*, *RGA*, and *RGL1* genes were inactivated. This prediction was recently confirmed by constructing *ga1* mutant plants, which contain very low amounts of active GA, in which *RGA* and *GAI* were also inactivated. The introduction of *rga gai* loss-of-function alleles completely rescued the stem growth defects of *ga1-3* and suppressed the nonflowering phenotype of *ga1* mutants so that the triple mutant flowered slightly earlier than did the wild type under short days (Dill and Sun, 2001; King et al., 2001). This, together with the observation that *rga gai* double loss-of-function mutants are slightly earlier flowering than wild-type plants, indicates that the late-flowering phenotype shown by GA biosynthetic mutants is due to active repression of flowering by *GAI* and *RGA*. This is an important observation, because the *rga gai* mutations do not rescue all aspects of the *ga1* phenotype. For example, the *rga gai ga1* triple mutant retains the germination and impaired floral development phenotype of *ga1*. These aspects of the *ga1* phenotype may be negatively regulated by *RGL1* and other related genes, such as *RGL2* and *RGL3* (Wen and Chang, 2002). Indeed, flower development of transgenic plants in which *RGL1* expression was reduced by co-suppression

was resistant to the effects of inhibitors of GA biosynthesis (Wen and Chang, 2002). Unlike *GAI* and *RGA*, expression of *RGL1* is almost restricted to the inflorescence. In flowers, *RGL1* expression was localized to ovules and developing anthers. However, *RGL1* may also be involved in sepal and petal development. In transgenic plants overexpressing a modified RGL1 protein lacking the DELLA domain, sepals, petals, and stamens were underdeveloped and the flowers were male sterile. RGL1 may therefore play a role in repressing GA responses in the inflorescence, where apparently GAI and RGA are less important.

The DELLA sequence appears to play an important role in the mechanism by which GA inhibits the function of GAI, RGA, and RGL1. This was suggested by the analysis of a dominant *gai* allele carrying a deletion within the DELLA domain, because this mutant form inhibited shoot growth and delayed flowering even in the presence of GA (Peng et al., 1997). Deletion of the DELLA domain may therefore cause GAI to become insensitive to GA, so that it continues to repress shoot growth and flowering even in the presence of GA (Peng et al., 1997). Further evidence for this model comes from recent analysis of RGA. The RGA protein accumulates in the nucleus in the absence of GA, but in the presence of GA is rapidly degraded (Silverstone et al., 2001). However, this effect is prevented by removal of the DELLA domain (Dill et al., 2001). Therefore, regulation of *RGA* by GA may be caused by RGA degradation through a mechanism that requires the DELLA domain of RGA. Transgenic plants expressing a modified RGL1 protein lacking the DELLA domain also exhibited repression of GA responses and were phenotypically similar to GA-deficient plants (Wen and Chang, 2002). However, unlike GFP:RGA, GFP:RGL1 was not degraded upon GA treatment, suggesting this might not be a universal mechanism for the regulation of these DELLA proteins.

The *SPY* locus encodes another negative regulator of GA responses that influences flowering time. Mutations in *SPY* cause partial suppression of the effects of reduced GA levels, whether these are due to mutations in GA biosynthetic genes or the presence of GA biosynthesis inhibitors (Jacobsen and Olszewski, 1993). The *spy* mutant is early flowering, probably due to the effects of increased activity of the GA-signaling pathway. Cloning of *SPY* (Jacobsen et al., 1996) and its homolog in barley, HvSPY (Robertson et al., 1998), revealed that SPY is highly similar to Ser/Thr O-linked N-acetylglucosamine transferases in rat and humans (Kreppel et al., 1997). The manner in which this enzyme acts to regulate GA responses is not known, but genetically it acts upstream of *GAI*, and may be required for GAI/RGA activity.

Other proteins implicated in GA signal transduction and flowering time regulation are PHOR1 (PHOTOPERIOD RESPONSIVE 1), FPF1, and SHI. *PHOR1* was initially identified in potato, and has not been incorporated into the signal transduction pathway defined in Arabidopsis (Amador et al., 2001). However, PHOR1 is transported into the nucleus in response to GA. This protein belongs to a family of armadillo-related helical repeat proteins, which serve as scaffolding proteins on which other proteins and/or nucleotides can assemble, and contains a protein domain related to that present in components of the ubiquitination system. The GA-responsive nuclear import of PHOR1 and its relationship to the ubiquitination system suggest that it could act upstream of RGA and could be involved in its degradation in the nucleus in response to GA, although there is no direct demonstration of this (Figure 3). *FPF1* is upregulated in the shoot meristem at the transition to flowering, and when overexpressed causes early flowering (Kania et al., 1997; Melzer et al., 1999). On the basis of genetic tests performed by combining transgenes that overexpress *FPF1* with mutations that delay flowering, FPF1 was proposed to promote flowering via the GA pathway. *FPF1* encodes a small protein, whose biochemical function is unknown. Finally, the dominant *shi* mutation causes a phenotype similar to a GA-deficient mutant, including late flowering, and is caused by overexpression of a zinc finger protein (Fridborg et al., 1999, 2001).

Several studies have described genetic interactions between the GA pathway and other flowering-time pathways. For example, genetic analysis suggests that the GA pathway probably acts in parallel to the long-day pathway because there is redundancy between mutations affecting the two pathways. The effect of mutations that impair the GA pathway is strongest under short days, and combining *ga1* with mutations that impair the long-day pathway, such as *co*, produced double mutants that often did not flower under long days (Putterill et al., 1995; Reeves and Coupland, 2001). This suggests that in short days, where the long-day pathway is not active, GA is the major flowering pathway and loss of function of this pathway can prevent flowering. Under long days, the effect of inactivation of the GA pathway is less severe because of the activity of the long-day pathway.

There is also recent evidence for a connection between *FPA*, a gene encoding an RNA binding protein that acts in the autonomous pathway, and GA (Meier et al., 2001). Two late-flowering mutants, *fpa1-3* and *fpa1-4*, markedly increased activity of a *GA5*::*LUC* transgene. These plants showed elevated levels of GA_{19} and GA_4, which is consistent with overexpression of the *GA5* gene (Coles et al., 1999). *fpa1-3* and *fpa1-4* plants also show reduced sensitivity to GA levels for floral promotion and germination (Meier et al., 2001). Finally, the early flowering caused by vernalization was proposed to be due to the GA pathway (Sheldon et al., 1999). This model indicated that FLC acts to repress GA activity at the apex and thereby to repress flowering, and vernalization overcomes this repression by reducing FLC levels. However, *ga1-3 FRI FLC* plants, which never flower under long days without vernalization, flower as early as do *ga1-3* single mutants after cold induction, suggesting the ability to respond to vernalization under long days does not require GA (Michaels and Amasino, 1999a). Although this suggested that the GA pathway is not the basis of vernalization, the recent demonstration that expression of the flower-

ing-time gene *SOC1* is repressed by FLC and promoted by GA or the long-day pathway indicates that the GA pathway does regulate the same flowering-time genes as the other flowering-time pathways (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000) (see also below, section on pathway integration).

One way in which GAs promote flowering is by increasing the transcriptional activity of the floral meristem identity gene LEAFY (*LFY*). Expression of *LFY*::*GUS* is reduced in mutants defective in GA biosynthesis, such as *ga1-3*, and increased in mutants with constitutive GA signaling, such as *spy* and *35S*::*FPF1* (Blazquez et al., 1998; Melzer et al., 1999). Overexpression of *LFY* also restores flowering of *ga1-3* in short days (Blazquez et al., 1998). The effect of GA on *LFY* transcription appears to act through a promoter motif that is similar to the consensus binding site for MYB transcription factors (Blazquez and Weigel, 2000). A particular MYB protein, AtMYB33, which resembles a MYB transcription factor of barley that mediates activation of amylase gene expression by GA, and is upregulated at the apex during floral initiation was proposed to interact with this *LFY* promoter motif in vivo (Gocal et al., 2001). Although *LFY* transcription is activated by GA, *35S*::*LFY ga1-3* plants still produced more leaves than did *35S*::*LFY* plants, suggesting that GA plays an additional role in the regulation of flowering time (Blazquez et al., 1998).

Chromatin Structure and Floral Repression in Arabidopsis

Arabidopsis mutants have been described that flower without forming any adult leaves, but progress directly from embryonic development to flowering (Sung et al., 1992; Yang et al., 1995; Kinoshita et al., 2001). The original representative of this class of mutants was *embryonic flower 1* (*emf 1*), which formed a reduced inflorescence and abnormal flowers that lacked petals without first forming any rosette (Sung et al., 1992). A second mutant with a similar phenotype, *emf2*, was identified subsequently (Yang et al., 1995). The *embryonic flower* mutations are recessive, and were interpreted as identifying genes that are required either to promote vegetative growth or to repress flowering during embryo and seedling development. The floral meristem identity gene *APETALA1* (*AP1*) and the *AGAMOUS* (*AG*) gene, which specifies carpel and stamen identity in the flower, are ectopically expressed in germinating seedlings of *emf* mutants (Chen et al., 1997). However, the mutant phenotype of *emf2* was not reduced in severity when combined in double mutants with the *ap1*, *ap2*, or *lfy* mutations, suggesting that ectopic expression of each of these genes is not essential for the *emf* phenotype.

A mechanism of action of the *EMF* genes was suggested by their cloning. The predicted EMF2 protein contains a zinc finger, an N-terminal basic domain and a C-terminal acidic domain (Yoshida et al., 2001). This protein shows similarity to two Arabidopsis proteins, VRN2 and FERTILISATION INDEPENDENT SEED 2 (FIS2), which were previously identified by mutations, and to a Drosophila protein, Su(z)12. The *Su(z)12* gene encodes a Polycomb group (PcG) protein involved in the repression of homeobox gene expression during development of Drosophila (Birve et al., 2001). The *VRN2* gene is required for maintenance of the vernalized state and continued repression of *FLC* expression after vernalization (Gendall et al., 2001), and mutations in *FIS2* allow partial development of the seed without fertilization (Chaudhury et al., 1997).

The similarity of EMF2, VRN2 and FIS2 to Su(z)12 suggests that these three plant proteins may act in a way similar to PcG proteins of animals. These proteins act in large protein complexes to repress transcription by altering chromatin structure. The EMF2 protein may then act as part of a protein complex that during embryo and seedling development represses the expression of genes that promote reproductive development.

The importance of PcG genes in repressing reproductive development was also emphasized by the demonstration that another Arabidopsis PcG gene, *FERTILISATION INDEPENDENT ENDOSPERM* (*FIE*), which is related to the PcG protein EXTRA SEX COMBS OF Drosophila, represses reproductive development in the seedling (Kinoshita et al., 2001). Loss-of-function *fie* alleles were originally described because they allow partial endosperm formation prior to fertilization. However, the effect of the mutation in the seedling or adult was not described, because maternal *FIE* alleles are essential for embryo development (Ohad et al., 1999). By expressing *FIE* from a defective *FIE* promoter that allows expression during seed development but not in the germinated seedling, it could be demonstrated that *FIE* is required for the repression of flowering in the seedling (Kinoshita et al., 2001). Plants homozygous for *fie* and expressing *FIE* from such a defective promoter initiate reproductive development as seedlings and resemble *emf* mutants.

The effects of *fie* mutations on embryo and seed development are similar to those of the *mea* and *fis2* mutations. *MEDEA* (*MEA*) encodes a SET-domain PcG group protein while *FIS2* encodes a Su(z)12 homolog (see above), and MEA/FIS2/FIE probably form a protein complex that regulates seed development (Luo et al., 1999; Spillane et al., 2000; Yadegari et al., 2000). However, *mea* and *fis2* mutant seedlings do not initiate reproductive development in the seedling. Therefore, FIE may interact in the seedling with other proteins related to MEA and FIS2 to generate a protein complex that represses reproductive development. One candidate for such a protein is CURLY LEAF (CLF), a SET-domain protein implicated in the repression of homeotic gene expression during vegetative growth, and *clf* mutants show an early-flowering phenotype (Goodrich et al., 1997). The analysis of mutants that initiate flowering prematurely in the seedling therefore illustrates a requirement for active transcriptional repression to prevent reproductive development

occurring so early that no vegetative development can take place. The activation of flowering by environmental conditions such as photoperiod and vernalization must be able to overcome these repression mechanisms and ensure that flower development is initiated at appropriate times.

There is also evidence that chromatin modeling can affect flowering by changing methylation patterns. The *decreased DNA methylation* (*ddm1*) mutation affects a gene encoding a SWI2/SNF2–like protein that is most closely related to genes of the SNF2 family implicated in chromatin remodeling (Jeddeloh et al., 1999). Repeated self fertilization of *ddm1* mutants creates epigenetic alleles, some of which affect flowering time (Kakutani et al., 1996). One of the loci affected by these alleles is *FWA* (Kakutani, 1997). Epigenetic alleles of FWA result in late flowering due to reduced methylation in the vicinity of the gene, which causes increased and ectopic expression of *FWA* (Soppe et al., 2000). *FWA* encodes a homeodomain containing transcription factor (Soppe et al., 2000), whose ectopic expression delays flowering. Analysis of *FWA*, together with the other loci causing early and late flowering in the *ddm* background, suggests that methylation levels regulate the expression of a number of genes involved in flowering-time regulation.

Integration of Arabidopsis Flowering Pathways

Separate genetic pathways regulate flowering in response to different environmental signals, but these pathways eventually converge to regulate the expression of the same downstream genes. For example, all of the flowering-time pathways ultimately lead to the transcriptional activation of the same set of floral identity genes that act within the floral primordia (Pineiro and Coupland, 1998). *LFY* is the earliest of the known floral identity genes to be expressed, and directly activates at least one of the later genes, *AP1* (Wagner et al., 1999). Plants carrying fusions of the *LFY* promoter to the *GUS* marker gene were used to demonstrate that *LFY* expression responds both to the long-day flowering pathway and to GA. Furthermore, deletion of a putative MYB transcription factor binding site within the *LFY* promoter prevented activation by GA, but not by the long-day pathway (Blazquez and Weigel, 2000). Plants homozygous for the *lfy* mutation and carrying a *LFY* transgene in which this GA-responsive element was deleted appeared wild type under long days but exhibited the *lfy* mutant phenotype under short days, where *LFY* expression requires the GA pathway. This conditional activation of the mutant *LFY* transgene demonstrated that activation of *LFY* by each of these two flowering pathways is separable. Thus the GA and long-day pathways converge on the *LFY* promoter, rather than both pathways activating an earlier acting gene that in turn increases the expression of *LFY* (Blazquez and Weigel, 2000).

Convergence of the long-day and autonomous pathways has also been studied. These pathways are clearly separate until the *FLC* and *CO* genes (Figure 4). For example, expression of the floral repressor *FLC*, a component of the autonomous pathway, delays flowering but does not reduce expression of *CO*, a component of the long-day pathway (Suarez-Lopez et al., 2001). Similarly, mutations in the *CO* gene do not affect expression of *FLC* (Sheldon et al., 1999). Nevertheless, flowering-time genes have been identified that act downstream of both *CO* and *FLC*. The *SOC1* (or *AGL20*) gene that encodes a MADS box protein is both activated by CO and repressed by FLC, suggesting that the pathways represented by these genes converge on *SOC1* (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000; Michaels and Amasino, 2001). *SOC1* is also regulated by GA, and therefore is a common target of all three flowering pathways (Borner et al., 2000). Mutations that inactivate *SOC1* delay flowering, while *SOC1* overexpression causes early flowering and suppresses the effect of mutations in the long-day and autonomous pathways. Expression of the *Sinapis alba* ortholog of *SOC1* is increased by daylength, as well as by applications of GA and cytokinin (Bonhomme et al., 2000). This is consistent with the results from Arabidopsis, and in addition indicates that *SOC1* responds to cytokinins. Expression of *FT*, a second flowering-time gene regulated by more than one pathway, is reduced by mutations that impair the function of the long-day and autonomous pathways and activated by overexpression of *CO*, indicating that *FT* acts downstream of both of these pathways (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000).

The repression of *SOC1* and *FT* expression by *FLC*, even in the presence of wild-type *CO* represents an important aspect of the adaptation of flowering time to changing seasons. This adaptation involves responses to several environmental stimuli, including temperature and daylength, which need to be balanced to produce a coherent response. For example, the winter annual growth habit requires repressing the induction of flowering by daylength until the plant has been exposed to winter temperatures, after which the plant must respond to lengthening daylength the following spring. The antagonism between *FLC* and *CO* in the activation of downstream genes such as *SOC1* and *FT* can explain such an adaptation because the activation of these genes by *CO* may be prevented by *FLC* during the first summer, but reduction in *FLC* expression during winter would allow *CO* to activate expression of the downstream genes during the long photoperiods of the following summer.

The analysis of *SOC1* expression demonstrates convincingly that this gene represents a point of convergence of several flowering-time pathways. Although *SOC1* expression increases rapidly within the floral meristem in response to inductive conditions, such as exposure to inductive photoperiods, the manner in which its function is related to increases in expression of the floral meristem identity genes is not understood.

The relationship between *FT* and floral meristem identity gene expression has been studied at the genetic and molecular levels. The *ft* mutation did not reduce the expression

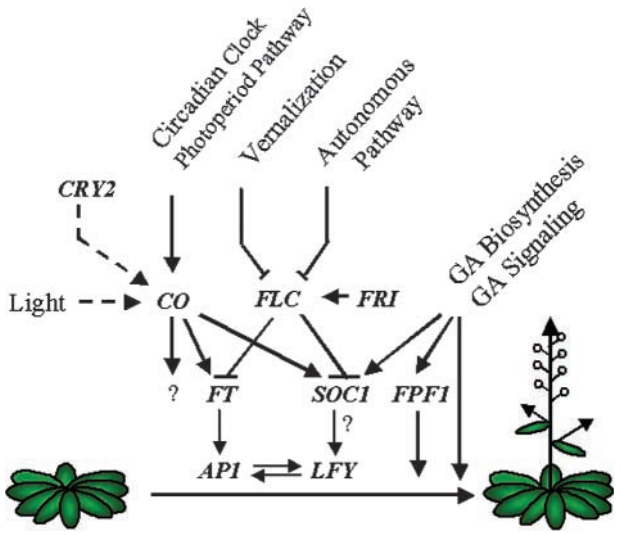

Figure 4. Overview of the Relationships among Arabidopsis Flowering Pathways.

The pathways described in detail in Figures 1 to 3 are combined to emphasize their relationships. In particular, the effects of the integration of the photoperiod, GA, and vernalization pathways on the regulation of expression of *FT* and *SOC1* is illustrated. The data underlying this model are described in detail in the text.

of *LFY*::*GUS* expression, suggesting that *FT* does not act upstream of *LFY* (Nilsson et al., 1998). Furthermore, the late flowering caused by *ft* mutations was epistatic to the early flowering of *35S*::*LFY*. Finally, *ft lfy* double mutants showed strongly synergistic effects on floral development, whereas *ft ap1* double mutants did not (Ruiz-Garcia et al., 1997). All of these results were taken as evidence that *FT* does not act upstream of *LFY* to regulate its expression, but rather acts in a parallel pathway. In particular, *FT* might activate the expression of other floral meristem identity genes such as *AP1* (Ruiz-Garcia et al., 1997). Thus the primary activation of *LFY* and *AP1* may occur by parallel pathways, and subsequent direct activation of *AP1* by *LFY* may be required to rapidly amplify floral meristem identity gene expression in young primordia. Amplification of floral meristem identity gene expression was proposed on the basis of the phenotypes of plants carrying mutations in more than one of these genes (Bowman et al., 1993).

FT is a member of a small gene family in Arabidopsis that also contains *TERMINAL FLOWER 1* (*TFL1*). The function of this family of proteins in plants is not known. However, as was shown for the Antirrhinum ortholog of *TFL1*, *CENTRORADIALIS* (*CEN*), they have homology to mammalian phosphatidylethanolamine binding proteins (PEBP), which were originally shown to bind phospholipids (Bradley et al., 1997). These proteins are also known as Raf1 kinase inhibitor proteins because of their ability to prevent Raf1 phosphorylation. The crystal structure of CEN confirmed its relationship with PEBP, and in particular demonstrated that it also contained the ligand binding site that is believed to interact with phosphate groups (Banfield and Brady, 2000). The sequence of *FT* is therefore consistent with it controlling flowering by regulating a phosphorylation cascade.

Conservation of Flowering Pathways in Plants Showing Responses Different from Those of Arabidopsis

Plants that flower in response to photoperiod are classified as short day, long day or day-neutral. Arabidopsis is a facultative long-day plant, and genetic and biochemical analyses have identified a pathway responsible for early flowering in response to inductive long photoperiods, and a GA-mediated pathway that promotes flowering under short days. However, GAs have no florigenic effect in most short-day plants in noninductive photoperiods, and whereas day-length perception appears to occur mainly under darkness in short-day plants, it seems to occur predominately during the day in long-day plants (Thomas and Vince-Prue, 1997). These observations suggest that there may be major differences in the mechanisms of flower induction in the long-day plant Arabidopsis compared with most short-day plants, and that genetic models based on Arabidopsis may not be sufficient to explain photoperiodic responses in short-day plants. However, recent molecular-genetic experiments in short-day plants have demonstrated striking parallels between these and Arabidopsis.

Molecular and genetic approaches were used to study the transition from the vegetative to the reproductive phase in the classic short-day plants, rice, *Pharbitis nil*, and tobacco. In rice, flowering time (or heading date) is critical for the adaptation to different cultivation areas and cropping seasons and may be affected by environmental conditions such us photoperiod, temperature, and light intensity. There are several major genes affecting heading date that relate to vegetative growth or photoperiod sensitivity. Five quantitative trait loci, *HEADING DATE 1* (*Hd1*) to *Hd5*, that control heading date were identified by comparing genetic differences between a *japanica* rice variety (Nipponbare) and an *indica* variety (Kasalath) (Yano et al., 1997). Genetic interactions among three loci involved in photoperiod sensitivity, *Hd1*, *Hd2*, and *Hd3* identified epistatic interactions between some combinations, suggesting that they act in the same genetic pathway (Lin et al., 2000).

Hd1 was cloned on the basis of its map position. Sequence analysis revealed that *Hd1* encodes a B-box zinc finger protein containing a C-terminal CCT domain and exhibits a high degree of similarity to the Arabidopsis *CO* gene (Yano et al., 2000). Experiments with various rice lines showed that the presence of a functional Nipponbare *Hd1* allele was associated with a stronger photoperiod response, causing early heading under short days and later heading under long days. Lines homozygous for the recessive

Kasalath *Hd1* allele in a Nipponbare background flowered extremely early under long-day field conditions. Thus, the *CO/Hd1* gene is important in photoperiod response in long-day and short-day plants. However, unlike *CO* in Arabidopsis, *Hd1* appears to have two roles in rice, promoting heading under short days and delaying it under long days.

Detailed comparison of the sequences of wild-type and mutant forms of CO or Hd1 also suggests that there may be differences in the biochemical mechanism of CO and Hd1 function. For example, *co-3* is a strong mutant allele that converts a His residue predicted to be involved in zinc binding within the second B-box to a Tyr residue (Robson et al., 2001). Strikingly, the active Ginbouzu allele of *Hd1* has exactly the same change of His to Tyr, suggesting that in rice this does not impair protein function. Similarly, the *co-1* allele of Arabidopsis has a deletion of three residues within the second B-box, while the active Nipponbare allele has a deletion of 36 bp that removes two of these residues. Taken together, these data suggest that the second B-box is essential for CO but not for Hd1 function. Detailed comparisons of COL proteins among species may help in determining their biochemical function. Although a COL gene displaying a high level of homology was found in gymnosperms (GenBank accession number AF001136), suggesting conservation of these sequences, there is also evidence that the family evolved rapidly (Lagercrantz and Axelsson, 2000).

Among the other major genes that affect heading date and are related to vegetative growth or photoperiod sensitivity in rice are *Hd6* and *SE5*, which were identified as the α subunit of protein kinase CK2 (Takahashi et al., 2001) and a putative heme oxygenase (Izawa et al., 2000), respectively. *Hd6* is a quantitative trait locus involved in rice photoperiod sensitivity, which was also detected in backcross progeny derived from a cross between the Nipponbare and Kasalath varieties (Yamamoto et al., 2000). The Nipponbare *Hd6* allele is nonfunctional. Introduction of the functional Kasalath allele delayed heading date of Nipponbare. CK2 belongs to the family of messenger-independent serine/threonine kinases that are present in all eukaryotic cells examined to date. This also suggests a relationship with photoperiod response in Arabidopsis. The expression of CK2 antisense RNA in Arabidopsis affected the expression of some light-regulated genes (Lee et al., 1999). CK2 interacts with and phosphorylates the Arabidopsis CCA1 protein in vitro (Sugano et al., 1998; Yamamoto et al., 2000). Overexpression of CK2 shortened the period of rhythmic expression of the *CCA1* and *LHY* genes, and caused early flowering in both long- and short-day conditions (Sugano et al., 1999). Therefore, protein kinase CK2 may have a role in the photoperiod pathway of Arabidopsis and rice.

Finally, the rice *photoperiodic sensitivity 5* (*se5*) mutation prevents the delay in flowering caused by exposure to noninductive long-day conditions (Izawa et al., 2000). *SE5* encodes a putative heme oxygenase, which shows 70% identity to *HY1* from Arabidopsis within the heme oxygenase domain (Davis et al., 1999; Muramoto et al., 1999). Both *hy1* and *se5* mutants are deficient in phytochrome responses such as coleoptile responses to light pulses and seedling growth under continuous red and far-red light. The *hy1* mutant of Arabidopsis also flowers very early under noninductive conditions, emphasizing the involvement of the phytochromes in the repression of flowering under noninductive conditions in both long-day and short-day plants.

The Japanese Morning Glory, *P. nil* cv Violet, has also been used extensively as a model system for the physiology of flowering in short-day plants (Takeba and Takimoto, 1966; Vince-Prue and Gressel, 1985). A *P. nil* homolog of the *CO* gene, *PnCO* was isolated using differential display to identify genes with increased expression under short-day conditions (Liu et al., 2001a). The *PnCO* mRNA is inefficiently spliced, but the fully processed cDNA under the control of the cauliflower mosaic virus 35S promoter in Arabidopsis *co-1* complements the late-flowering phenotype of *co-1* mutants, confirming that PnCO has the same biochemical function as CO. *PnCO*, like *CO*, is under circadian clock control (Liu et al., 2001a). In continuous inductive darkness, the level of *PnCO* mRNA reaches a peak between 16 and 20 h, but it is not clear how this circadian clock control of *PnCO* regulates flowering in response to short days. Much lower accumulation of *PnCO* mRNA was detected after the noninductive 8, 10, and 12 h of darkness. Interestingly, the daily cycle in Arabidopsis *CO* expression under short days also shows a broad peak between 16 and 20 h with a strong peak at 20 h (Suarez-Lopez et al., 2001). In Arabidopsis, circadian clock control is thought to regulate exposure of CO protein to light, and post-transcriptional regulation of CO by light is proposed to promote flowering in long days. A night break (NB), 5 min of red light given 8 h into 14 h of darkness, inhibits flowering of *P. nil*. However, the level of *PnCO* RNA is only slightly reduced under these conditions, suggesting that the NB does not inhibit flowering by reducing transcription of *PnCO*. However, CO protein is very unstable in light (Suarez-Lopez et al., 2001), and *P. nil* PnCO protein could also be unstable and a target for degradation in response to NB.

The mechanism by which *CO* and its orthologs in short-day plants mediate the photoperiodic response is not clear. In Arabidopsis, *CO* promotes flowering under long-day conditions, activating at least two flowering-time genes, *SOC1* and *FT*, which have major roles in promoting flowering time (Samach et al., 2000). Light-activated post-transcriptional regulation of *CO* could be a part of this signal transduction pathway. It remains possible that differences in photoperiod-regulated activity of CO in short-day plants lie in the interaction of CO with different sets of proteins and subsequent upregulation of the target flowering-time genes. In addition, in short-day plants, CO may act in protein complexes that actively repress flowering under long days. The fact that the Hd1 protein is involved in two opposite processes—activation of heading under short-day conditions and inhibition of heading under long-day conditions—supports this suggestion.

Isolation and characterization of homologs of the key genes involved in photoperiod-mediated transition to flowering in short-day plants as well as expression of the homologous genes in transgenic long-day and short-day plants will enable comparison of the molecular mechanisms that regulate the floral transition in short-day and long-day plants. In a study of this type, the *SOC1* homolog from mustard, *MADSA*, was overexpressed in long-day (*Nicotiana sylvestris*), day-neutral (*N. tabacum*), and short-day tobacco (*N. tabacum* cv Maryland Mammoth) species (Borner et al., 2000). Analysis of the transgenic plants showed that only in the short-day cultivar could overexpression of MADSA overcome the photoperiodic barrier of floral induction. MADSA overexpression did not cause long-day and day-neutral cultivars to flower under noninductive photoperiod, suggesting a difference in the behavior of long- and short-day plants at the molecular level, even in downstream processes.

PERSPECTIVES

Genetic analysis in Arabidopsis has enabled the isolation of genes that control flowering time, and the identification of interacting pathways that promote flowering in response to different environmental conditions. However, our present understanding of these pathways represents only a skeletal framework, and future work in this area will concentrate on understanding the biochemical function of pathway components and the manner in which the signaling pathways convey information that ultimately regulates flowering time. Furthermore, the incorporation into these models of classic observations, such as the graft transmissibility of flowering signals formed in the leaf, is a major challenge.

The complexity of the data regarding the network of flowering pathways will also increase further. Some environmental effects on flowering have not been placed within the pathway structure. For example, it is not clear whether the effects of nutrient availability or light quality on flowering act through the pathways described here or through further pathways, although high sucrose levels delay flowering and reduce *FT* expression (Ohto et al., 2001). There is also physiological evidence that vernalization may act partly through an additional pathway that has so far not been described genetically (Figure 2). In addition, although there is strong genetic and molecular evidence for the separation of the photoperiod and autonomous pathways, as shown in Figure 4, there are also indications of interactions between the pathways at unexpected levels. For example, *FLC*, a major repressor within the autonomous pathway, influences circadian period length, a feature of the photoperiod pathway (Swarup et al., 1999).

Explaining the diversity in flowering-time responses also represents a great challenge. The analysis of natural variation in flowering time between ecotypes of Arabidopsis indicates that modifiying the balance of the activity of the described flowering pathways can create different reproductive strategies, such as winter annual or summer annual types (Michaels and Amasino, 2000). For example, in comparing winter annual and summer annual varieties of Arabidopsis, changing the relative activities of the vernalization and photoperiod pathways generates a vernalization requirement. It seems likely that altering the balance of the activity of distinct pathways on the activation of genes such as *FT* and *SOC1* that integrate the different pathways may be a more general mechanism of generating diversity in flowering behavior. However, there are also fundamental differences among species in the roles of pathways. The comparison of photoperiod responses in long-day and short-day plants demonstrates that for this example of diversity in flowering behavior, the pathway described in Arabidopsis is likely to be relevant for species that behave very differently. However, the function of key pathway components is likely to be altered in short-day plants to change their target genes or the activity of the protein complexes in which they act. Similar changes may explain why GA promotes flowering in Arabidopsis but represses flowering in trees and some short-day plants. There are flowering strategies, however, for which Arabidopsis may be a less-effective model. For example, although Arabidopsis genes can be used to drive very early flowering of trees and to overcome developmental delays of flowering (Weigel and Nilsson, 1995; Pena et al., 2001), studies of an annual plant such as Arabidopsis will probably not reveal the molecular mechanisms underlying many of the unique features of perennial plants (Battey, 2000).

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