It's time to flower: the genetic control of flowering time

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Summary

In plants, successful sexual reproduction and the ensuing development of seeds and fruits depend on flowering at the right time. This involves coordinating flowering with the appropriate season and with the developmental history of the plant. Genetic and molecular analysis in the small cruciform weed, *Arabidopsis*, has revealed distinct but linked pathways that are responsible for detecting the major seasonal cues of day length and cold temperature, as well as other local environmental and internal signals. The balance of signals from these pathways is integrated by a common set of genes to determine when flowering occurs. Excitingly, it has been discovered that many of these same genes regulate flowering in other plants, such as rice. This review focuses on recent advances in how three of the signalling pathways (the day-length, vernalisation and autonomous pathways) function to control flowering. *BioEssays* 26:363–373, 2004.

Introduction

The transition to flowering is controlled to coincide with conditions that enhance the production of seeds and fruits and to coordinate the flowering time of out-crossing species. Flowering at the right time requires the perception and processing of a diverse range of environmental and internal signals; these must be integrated into a single decision—to flower or not to flower.

Many organisms use environmental cues to synchronise their behaviour and development with favourable times of the year. In the experimental plant *Arabidopsis*, flowering is promoted by increasing day length, a noise-free cue that predicts the onset of spring and summer. However, *Arabidopsis* found in cooler temperate regions often do not flower until exposed to a period of cool winter temperatures. This process, called vernalisation, prevents precocious flowering during the autumn and winter when conditions for reproductive development may not be favourable. It is also important that plants can respond to changes in their local environment. For example, a strategy for dealing with overcrowded or nutrient-poor conditions is to flower rapidly. Overcrowding is detected by changes in light quality (decreases in the ratio of red to far-red light). Increased ambient temperature can also promote flowering. In addition to environmental factors, internal signals, such as plant age and stage of development, regulate flowering. Although little is known about the role that these signals play in the regulation of flowering in *Arabidopsis*, they can have a dramatic affect, preventing some trees from flowering until they are decades old.

The power of *Arabidopsis* molecular-genetics has been a key to discovering how these different signals are detected and processed (Box 1). Researchers have made use of the natural genetic variation in flowering time between *Arabidopsis* accessions collected from different parts of the world, to map and isolate quantitative trait loci (QTLs) that control flowering time. In addition, a large number of early- and late-flowering-time mutants have been identified and their corresponding genes cloned[1–7] (Table 1). Several distinct, but linked, flowering-time genetic pathways have been uncovered by this work. These regulate the flowering-time response to light and temperature signals or to internal signals (gibberellin plant hormone and autonomous pathways)[1–7] (Fig.1). The balance of signals from these pathways is integrated by a common set of genes to determine when flowering occurs. Fig. 1 shows the convergence of the different pathways at the *FT*, *SOC1* or *LFY* genes. Expression of these floral promoters leads to further upregulation of *LFY* and other floral identity genes such as *AP1*, triggering flowering.

Rapid progress is being made in the field of flowering-time research. This review will emphasise the latest discoveries in how three of the flowering-time pathways (the long-day, vernalisation and autonomous pathways) function to control flowering time. We discuss exciting experiments showing how these pathways have been modified to allow plants to adapt to different geographical regions. Finally, we review the recent findings that microRNAs regulate flowering-time.

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Abbreviations: *AP1*, *APETALA1*; *LFY*, *LEAFY*; *AP2*, *APETALA2*; Table 1 provides the full names of the *Arabidopsis* flowering-time genes; *Arabidopsis* gene names in capitals and italics; mutant gene names in lower case and italics; protein names in capitals and not italicised.

Box 1

Arabidopsis is a small plant producing thousands of seeds after a rapid cycle. This, together with the fact that it has a compact and fully sequenced genome, makes *Arabidopsis* ideal for research into the molecular-genetic control of flowering time. Extensive mutagenesis experiments performed to discover genes regulating flowering time have led to the identification of many *Arabidopsis* mutants. These mutations can be pinpointed within the genome by mapping, utilizing the many thousands of polymorphisms that exist between the commonly used laboratory strains, and the corresponding genes identified by map-based cloning. To discover the function of each of the ~25,500 *Arabidopsis* genes, well over 200,000 insertion lines (containing foreign DNA tags, usually T-DNA or transposons, randomly inserted into the genome) have been generated by many research groups. The flanking regions of each insertion site have been sequenced from many of these lines. To analyse the function of a putative flowering-time gene, an insertion line in which the gene has been disrupted can be selected and ordered online (http://atidb.org). The role of genes in flowering can also be tested in transgenic plants as *Arabidopsis* is easily transformed using the natural genetic engineer *Agrobacterium tumefaciens*. For *Arabidopsis* resources and information, see www.arabidopsis.org. Finally, since it has been predicted that 85% of *Arabidopsis* genes are present in the distantly related plant, rice, once a flowering-time gene has been discovered in *Arabidopsis*, it can usually be found easily in any plant of interest.

Day-length control of flowering in *Arabidopsis*

Day length (photoperiod) can control several plant processes including flowering time, the onset of bud dormancy and the production of storage organs such as tubers and bulbs. *Arabidopsis* is a facultative long-day plant as its flowering is promoted by the long days of spring and summer (~16 hours light; 8 hours dark), and delayed, but not abolished, in short-day conditions (8 hours light, 16 hours dark).

How does *Arabidopsis* discriminate between long and short days, and then respond to long days by flowering rapidly? Over 60 years ago, the external coincidence model was proposed to explain how photoperiodic organisms perceived the day-length signal.[8] In this model, there is a light-inducible phase that is internally regulated and kept at the same time each day by the circadian clock. Coincidence of the light-inducible phase with light (the external signal) would then trigger flowering in long-day plants, and delay it in short-day responsive plants.

By studying *Arabidopsis* mutants with altered photoperiodic flowering, many players have been identified that function in the long-day pathway to regulate flowering in response to the long-day signal (Fig. 2A). As first predicted by the external coincidence model, they include light (photo) receptors, circadian clock components, and clock- and light-regulated genes, such as *CO,* that are key to day-length detection and floral promotion in long days.

Light signalling and photoperiodic flowering

Several different *Arabidopsis* photoreceptors perceive the light signals that regulate photoperiodic flowering.[9] The phytochromes are red and far-red light photoreceptors, existing in two photoreversible forms that detect light using a covalently bound tetrapyrrole chromophore. The cryptochromes perceive blue/UV-A light using linked flavin and pterin chromophores, and an additional family of putative blue-light photoreceptors, including ZTL and FKF1 are predicted to use similar chromophores.[10]

Light and photoreceptors play a dual role in the long-day pathway. Photoreceptors, such as FKF1, PHYA and CRY2, are proposed to promote flowering in long days by directly affecting the activity of the key long-day flowering-time gene *CO* (see below). Light is also the major signal that synchronises the circadian clock to the seasonal changes in day length.[9] This clock resetting is known as entrainment and at least four of the five phytochromes (PHYA, PHYB, PHYD and PHYE) and the two cryptochromes (CRY1 and CRY2) are involved in entraining the clock. *ZTL*, a gene related to *FKF1*, also has a role in light signalling to the clock (Fig. 2A).

How is the clock mechanism reset once light has been perceived by the photoreceptors? Of the multiple signalling events downstream of PHYA and PHYB, the one that involves direct interaction with the light-signalling component PIF3 is best understood.[9] Once PHYB perceives red light, it is activated and relocates to the nucleus where it binds to the PIF3 transcription factor. This complex is able to bind the promoter of genes that have upstream G box elements. Target genes include *CCA1* and *LHY*, which are integral components of the major circadian oscillator in *Arabidopsis* (see below).

Several other genes are involved in light signalling to the clock including *GI* and *ELF3*.[9] The role of *ELF3* is best characterised; it is needed for entrainment of the clock to long days and is proposed to increase the sensitivity of the plant to the photoperiodic dawn signal by blocking (or ''gating'') light input at dusk to the clock. This may be achieved by ELF3 binding PHYB and inhibiting the activity of this photoreceptor (Fig. 2A).

The circadian clock and photoperiodic flowering

In all organisms studied, the daily rhythms with a hallmark ~24 hours periodicity, are generated by autoregulatory feedback loops, in which clock proteins cyclically regulate their own

Table 1. *Arabidopsis* flowering time genes[1]

Abbreviation	Gene name	Predicted gene product	Pathway/Function
AGL24	*AGAMOUS-LIKE 24*	MADS transcription factor	Floral promoter
CCA1	*CIRCADIAN CLOCK ASSOCIATED 1*	MYB domain transcription factor	Circadian clock
CK2	*CASEIN KINASE 2*	Protein kinase	Circadian clock
CO	*CONSTANS*	Zinc finger transcription factor	Long day pathway
CRY1 and *2*	*CRYPTOCHROME 1* and *2*	Blue/UV light photoreceptors	Light perception
EAT	*EARLY ACTIVATION TAGGED*	Transcript contains *MiR172*	Represses *AP2-LIKE* genes
ELF3	*EARLY FLOWERING 3*	Novel nuclear protein	Circadian clock
ELF4	*EARLY FLOWERING 4*	Novel protein	Circadian clock
FCA	*FCA*	RNA binding protein	Autonomous pathway
FKF1	*FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*	Putative blue light photoreceptor	Long day pathway
FLC	*FLOWERING LOCUS C*	MADS transcription factor	Floral repressor
FLD	*FLOWERING LOCUS D*	Component of histone deacetylase complex	Autonomous pathway
FPA	*FPA*	RNA binding protein	Autonomous pathway
FRI	*FRIGIDA*	Novel protein	Floral repressor
FT	*FLOWERING LOCUS T*	Phosphatidylethanoleamine binding protein	Floral promoter
FVE	*FVE*	WD40 repeat protein	Autonomous pathway
FY	*FY*	Polyadenylation factor	Autonomous pathway
GI	*GIGANTEA*	Novel nuclear protein	Long day pathway
HOS1	*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1*	RING finger protein	Cold signalling
LD	*LUMINIDEPENDENS*	Nuclear-localised homeobox protein	Autonomous pathway
LHY	*LATE ELONGATED HYPOCOTYL*	MYB domain transcription factor	Circadian clock
MAF1-4	*MADS AFFECTING FLOWERING 1–4*	MADS transcription factors	Floral repressors
MAF5	*MADS AFFECTING FLOWERING 5*	MADS transcription factor	Putative floral promoter
PHY A-E	*PHYTOCHROME A-E*	Red/far-red light photoreceptors	Light perception
PIE1	*PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1*	Chromatin remodeling protein	Floral repressor
PIF3	*PHYTOCHROME INTERACTING FACTOR 3*	Basic/helix-loop-helix transcription factor	Light signaling
SMZ & SNZ	*SCHLAFMÜTZE and SCHNARCHZAPFEN*	AP2- like transcription factor	Putative floral repressors
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	MADS transcription factor	Floral promoter
TFL2	*TERMINAL FLOWER 2*	Heterochromatin 1 like chromatin repressor	Floral repressor
TOC1	*TIMING OF CAB 1*	Nuclear-localised putative transcription factor with pseudo-response regulator domain	Circadian clock
TOE1-2	*TARGET OF EAT 1* and *2*	AP2-like transcription factors	Putative floral repressor
VIP1-7	*VERNALISATION INDEPENDENCE 1–7*	VIP3 has multiple WD repeats; VIP4 is a novel protein	Floral repressors
VRN1	*VERNALISATION 1*	B3 DNA binding protein	Vernalisation pathway
VRN2	*VERNALISATION 2*	Polycomb group protein	Vernalisation pathway
ZTL	*ZEITLUPE*	Putative blue light photoreceptor	Circadian clock

[1] *Arabidopsis* flowering time genes described in the text. Molecular identification of the genes is described in reviews and references therein[1,9,63] or in recent papers.[17,36,38–40,46,48,50,51,55,57]

expression. Substantial progress has been made on identifying molecular components of the major *Arabidopsis* circadian feedback loop[8,9,11] (Fig. 3).

The proposed *Arabidopsis* clock loop begins in the morning with *LHY* and *CCA1* expression peaking, and the translation of LHY protein being enhanced by the light.[12] LHY and CCA1 then repress the expression of *TOC1*. This repression is probably direct as the LHY and CCA1 proteins bind the *TOC1* promoter in vitro via promoter sequences called evening elements. Since TOC1 is required for the expression of *LHY* and *CCA1*, the reduction in TOC1 is followed by a decrease in *LHY* and *CCA1* mRNA levels. By the evening, LHY and CCA1 protein levels have dropped, allowing the mRNA levels of *TOC1*, and other evening expressed genes including *ELF4*[13] and *GI*, to peak (Fig. 3). The subsequent increase in these evening-expressed genes is proposed to upregulate *LHY* and *CCA1* mRNA to peak levels in the morning, and the cycle begins again.

This circadian feedback loop generates a series of rhythmic outputs including circadian-regulated flowering-time genes *GI*, *FKF* and *CO*. How these genes function to promote flowering in long days is discussed below.

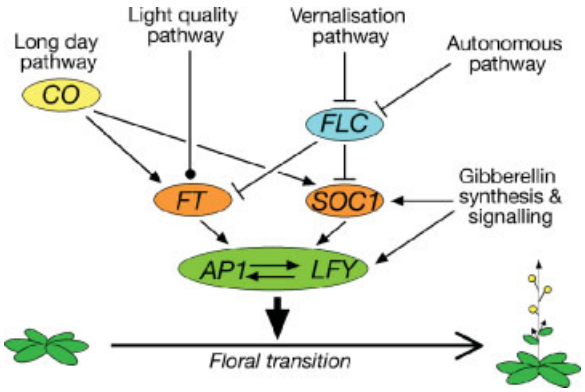

Figure 1. Cross talk between flowering-time pathways in *Arabidopsis*. Distinct but linked genetic pathways are responsible for detecting the major seasonal cues of day length and cold temperature, as well as other local environmental, and internal signals. The signals from these pathways are integrated through upregulation of the expression of one or more common target genes; *FT*, *SOC1* and *LFY*. Expression of the floral promoters leads to further upregulation of *LFY* and other floral identity genes, such as *AP1*, triggering flowering. The floral repressor, *FLC*, is the major target of the vernalisation and autonomous pathways, and within the long-day pathway, the *CO* gene integrates circadian clock and light signals. Lines with arrows indicate upregulation of gene expression, lines with bars indicate gene repression. The light quality pathway can function to activate or repress flowering depending on light quality (low ratios of red to far red light promote flowering, while high ratios delay it).

Communication of the long-day signal and promotion of flowering

Analysis of mutant and transgenic plants strongly suggests that the regulation of *CO* expression and activity is important for photoperiodic flowering.[8] *CO* promotes flowering in *Arabidopsis* in long-day conditions (as *co* mutants are late flowering in long days, but flower at a similar time to wild type in short days). However, constitutive overexpression of *CO* in plants causes rapid flowering even in short days, demonstrating that *CO* misexpression alone can trigger flowering in non-inductive conditions.

Studies using plants expressing an inducible *CO* gene revealed that *CO* appears to promote flowering by directly upregulating the expression of the *FT* and *SOC1* genes[8] (Fig. 1). *FT* and *SOC1* transcript levels are upregulated in long-day compared to short-day conditions, resulting in rapid flowering in long days, but delayed flowering in short days. The reason why *CO* upregulates *FT* transcript levels in long days is explained by the sensitivity of *CO* to both light stimuli and internal circadian clock signals.[8,14,15] *CO* is cyclically expressed with a broad bi-phasic peak of transcript in long days, and a slightly narrower peak in short days. In long days, *CO* expression coincides with the light at dawn and in the afternoon. This correlates with upregulation of *FT* transcript levels and induces flowering. In short days, however, high levels of *CO* expression occur in the dark, *FT* expression is not upregulated and flowering is delayed (Fig. 4). The importance of the coincidence of elevated *CO* expression with light for *FT* upregulation is confirmed by experiments altering the timing of expression of *CO*, in wild-type plants or the circadian mutant *toc1*.[15,16] The upregulation of *FT* transcript levels by CO in the afternoon requires coincident light perception by two photoreceptors, PHYA and CRY2, which is thought to activate the CO protein (Fig. 2A).[15]

In addition to these floral promoters, a number of floral repressors have been discovered that repress *FT* expression and oppose the *CO*-dependent activation of *FT*. These floral repressors include *FLC* (Fig. 1; see later) and *TFL2*. The *tfl2* mutant flowers early in long and short days due to de-repression of *FT*.[17] *TFL2* weakens, but does not abolish, the *CO*-dependent activation of *FT* and, in so doing, probably helps to limit flowering in response to transient changes in *CO*

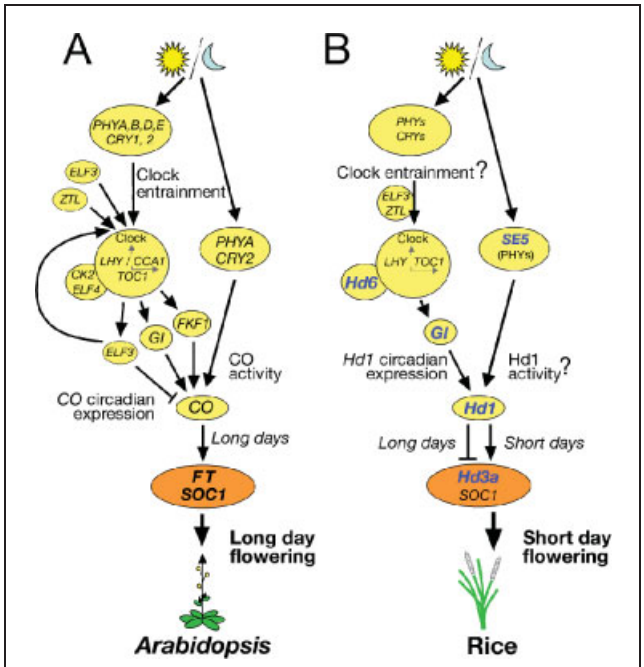

Figure 2. The *Arabidopsis* long-day pathway and how it has been modified in rice, a short-day plant. **A:** Simplified outline of the long-day pathway in *Arabidopsis*, which functions to promote flowering in long-day conditions. The genes indicated are described in the text. **B:** Model of genetic regulation of flowering in rice in response to day length. Rice flowering is promoted by short days, but repressed by long days. The rice genes in blue have been shown to be involved in flowering and the other rice genes are homologues of *Arabidopsis* genes mentioned in the text.[26]

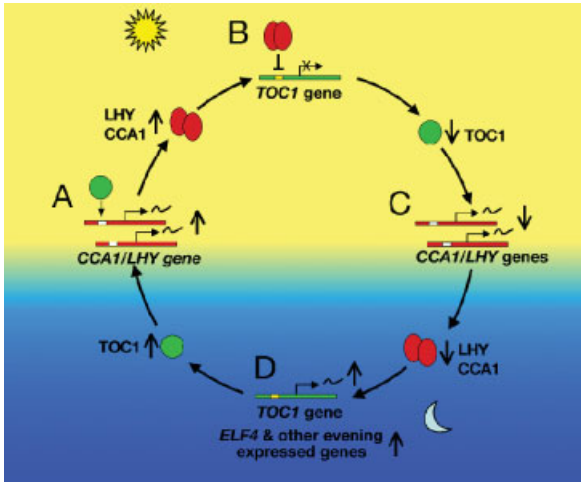

Figure 3. Model showing how the central clock genes form an autoregulatory feedback loop. **A:** *CCA1/LHY* gene expression is activated in the morning by light and directly, or indirectly, by TOC1 protein. **B:** CCA1/LHY proteins bind to the *TOC1* promoter and inhibit *TOC1* expression causing the levels of TOC1 protein to decrease. **C:** Since *TOC1* is required for the expression of *CCA1/LHY*, the decrease in TOC1 protein level, causes a decrease in their expression. **D:** This decrease in the *TOC1* gene repressors, CCA1/LHY, allows *TOC1* and other evening-expressed genes such as *ELF4* to increase. As the TOC1 levels and those of other evening-expressed genes levels rise during the night, *CCA1/LHY* expression begins to increase and the cycle begins again. It is not known if the TOC1 protein interacts directly with the *CCA1* or *LHY* promoter. The filled red ovals represent CCA1 and LHY proteins. The filled green circles represent TOC1 protein. The coloured boxes on the genes show the position of their promoters.

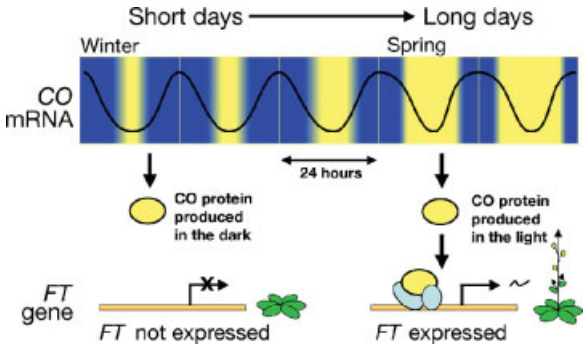

Figure 4. Model of how long days promote flowering in *Arabidopsis*. Day length during five representative days from winter to spring is shown. *CO* mRNA is expressed in a circadian rhythm with a peak of expression in the second half of the day. During the short-day length of winter, this peak in *CO* expression occurs in the dark. CO protein produced in the dark is proposed not to promote *FT* gene expression and this results in delayed flowering. In spring, once the day length has increased sufficiently for *CO* expression to coincide with the light, active CO protein is produced, which induces *FT* gene expression and flowering. The molecular mechanism by which CO protein is activated by light and induces *FT* gene expression is not known. It is represented here by CO protein in the light interacting with DNA-binding nuclear factors (filled blue circles) and switching on transcription of *FT*.

activity prior to perception of the long-day signal.[18] Even more intriguing is that *CO* and *TFL2* appear to regulate *FT* expression in the vascular tissue of leaves,[18] long thought to be the initial site of perception of the day-length signal.

At least three other clock output genes lie upstream of *CO* in the long-day pathway and are important for regulating expression of the *CO* transcript[8,10] (Fig. 2A). The *GI* gene promotes expression of CO (plants with mutations in the *GI* gene are late flowering largely due to reduced expression of *CO*). In contrast, in the early-flowering *elf3* mutant, *CO* levels are high at all time points suggesting that *ELF3* functions to repress *CO* expression.

A recent exciting discovery was that a third upstream gene, *FKF1*, is needed for the peak of *CO* expression that occurs in the afternoon in long days.[10] The *fkf1* mutant is late flowering in long days, lacks this peak of *CO* expression and cannot upregulate *FT*. FKF1 protein appears to be a novel blue light photoreceptor that is able to influence *CO* transcript patterns. Interestingly, its activity (like that of the CO protein) relies on coincidence with light in the afternoon in long days.

In summary, the external coincidence model of day-length perception originally predicted the existence of an internal, clock-regulated and light-inducible phase. The *CO* gene appears to largely embody the key features of this component of the model; it is able to integrate light and clock signals and is activated in long days to promote flowering of *Arabidopsis*.

Day-length control of flowering in rice

The flowering time of many economically important crops, including rice, is regulated by day length. In contrast to *Arabidopsis*, rice is a short-day plant, flowering more rapidly in short-day-length conditions than in long days. The adaptive benefit of this response to short days is that it can synchronise flowering and sexual reproduction in rice with the rainy season.

Given the opposite response of rice and *Arabidopsis* to day length, are the molecular mechanisms for day-length detection also completely different? The first evidence that the key players regulating rice photoperiodic flowering are the same as used by *Arabidopsis* has come from a major effort in Japanese laboratories to map and clone QTLs for day-length-sensitive flowering (called heading date) in rice.

Three genes, *Heading date* (*Hd*) *1*, *3a* and *6* have been identified and they all encode genes similar to those found in the *Arabidopsis* long-day pathway (Fig. 2B). *Hd1* is predicted to encode an orthologue of *Arabidopsis CO*,[19] *Hd3a* is similar

to *Arabidopsis FT*[20] and *Hd6* encodes a homologue of the α subunit of a kinase, *CK2*, a regulator of circadian clock activity and flowering time in *Arabidopsis*.[21,22]

Genetic analysis also indicates that rice phytochromes regulate flowering in response to day length; the *photoperiod sensitivity 5* (*se5*) mutant cannot synthesise the phytochrome chromophores and is insensitive to day length, flowering early in both long and short days.[23] A fifth player strongly implicated in rice flowering is the circadian-regulated rice *GI-like* gene (*OsGI*). It is expressed at lower levels than usual in the *se5* mutant, and manipulation of *OsGI* expression in transgenic rice has striking effects on flowering time (see below).[24,25]

This work points to a remarkable conservation of the *Arabidopsis* long-day pathway components in rice and is further supported by bioinformatic analysis of the rice genome sequence, which predicts many additional photoreceptor, clock component and flowering-time genes known from *Arabidopsis* (Fig. 2B).[26]

But if similar players are present in rice and *Arabidopsis*, how do they generate an opposite flowering response to day length? Clues to the order and regulation of genes in the rice pathway have come from recently reported transgenic experiments in rice. Constitutive expression of *OsGI* in transgenic rice resulted in delayed flowering while reducing *OsGI* expression in rice, using RNAi, caused earlier flowering in long days.[25] This work indicated that *OsGI* appeared to have an opposite activity to *GI* in *Arabidopsis*, where *GI* promoted flowering (*gi* mutations cause delayed flowering in long days).

To find an explanation for the late-flowering phenotype of rice plants constitutively expressing *OsGI*, the expression of genes predicted from the *Arabidopsis* pathway to be downstream of *OsGI, Hd1* and *Hd3a* was examined.[25] In these plants, *Hd1* was increased (during the light period), but *Hd3a* expression was greatly reduced. These results, and others from a transient assay system indicating that *Hd1* regulated *Hd3a* expression, suggest that, in long days, *OsGI* promoted *Hd1* expression, but *Hd1* then functioned to repress *Hd3a* expression which led to delayed flowering (Fig. 2B). Consistent with this idea, the *hd1* mutant flowers more rapidly than wild-type plants[27] and has higher levels of *Hd3a* expression in long days.[28]

The exciting result from this work is that, in the pathways controlling photoperiodic flowering of both rice and *Arabidopsis*, the key players and their order of function is the same. But the main change is that, in long days, the regulation of flowering by *Hd1* is different. *Hd1* represses *Hd3a* in rice, but *CO* promotes *FT* expression in *Arabidopsis*. These results from rice also fit well with the prediction of the external coincidence model; in short-day plants, coincidence of light with an internal light-inducible phase (*Hd1)* inhibits flowering.[28]

If rice *Hd1* acts as a repressor of flowering in long-day conditions, what function, if any, does it have in floral inductive short days? *Hd1* appears to have a different role in short days; it promotes flowering and upregulates *Hd3a* transcript levels.[19,20,25,28] Similarly when *OsGI* transcript levels were reduced in rice using RNAi, the transgenic plants flowered later in short days, indicating that *OsGI* normally promotes flowering of rice in short days.[25] These results support a model where, in short days, *OsGI* promotes *Hd1*, which in turn, upregulates *Hd3a* expression to promote rice flowering (Fig. 2B).

Great progress has been made on understanding the molecular mechanisms regulating photoperiodic flowering in rice and the current results raise several new questions. What changes *Hd1* from an activator of rice flowering in short days, to a repressor of flowering in long days? Are *Arabidopsis CO* and rice *Hd1* functionally interchangeable despite having opposite effects on flowering in long days? While the latter question remains to be tested, it has been recently reported that the *Hd1* orthologue from the long-day grass, wheat, can complement *Hd1*-deficient rice.[29] Similarly, a *CO* gene from a short-day plant *Pharbitis nil* can complement the *Arabidopsis co* mutation.[30] This suggests that long-day plant and short-day plant *CO/Hd1* genes may not be structurally different.

Regulation of flowering time by extended exposure to cold temperatures

In addition to day length, the exposure to prolonged periods of cold, winter temperatures, is an important signal used by some plants to ensure that flowering occurs in the spring. This process is called vernalisation. *Arabidopsis* from regions with cold winters adopt a reproductive strategy known as a winter-annual life cycle. These plants germinate in the summer, grow vegetatively over winter and only flower in response to the long days of spring, after being exposed to one to three months of cold temperatures. In contrast, *Arabidopsis* with a rapid-cycling summer-annual life cycle, lack a vernalisation requirement and germinate and flower in the same summer.

To discover the genes responsible for conferring the vernalisation requirement, winter- and summer-annual *Arabidopsis* plants were crossed and their progeny analysed using molecular markers. It was found that dominant alleles of two genes, *FLC* and *FRI*, act synergistically to confer this vernalisation requirement. When the *FLC* and *FRI* genes were sequenced from *Arabidopsis* found in different regions throughout the world, it was discovered that all the *Arabidopsis* plants with a summer-annual life cycle (i.e. lacking a vernalisation requirement) had mutations in the *FRI*-coding region and/or mutations in the *FLC* gene that reduce its expression. A variety of mutations were found in these genes, suggesting that the summer annuals evolved from winter annuals independently a number of times.[31–33] Thus, allelic differences within the *FRI* and *FLC* genes are key to *Arabidopsis* adapting to grow in warmer regions.

A breakthrough in our understanding of vernalisation came with the molecular identification of the *FLC* gene, and thus the

ability to track the expression of this gene.[1] Vernalisation leads to reduced *FLC* mRNA and protein levels, thereby removing the *FLC*-mediated repression of flowering (Fig. 1). However, the presence of a functional *FRI* allele causes increased expression of the *FLC* floral repressor, explaining why *FRI*-containing plants flower later in the absence of vernalisation (Fig. 5).

The major role of *FLC* is to repress flowering by inhibiting the expression of *FT* and *SOC1*.[1] This is opposite to the function of the long-day floral promoter, *CO*, which activates these genes (Fig. 1). So far, there is evidence for antagonistic regulation of the transcription of *SOC1* at its promoter by FLC and CO.[34] FLC binds to a specific sequence (a CArg box) within the *SOC1* promoter in vitro, and this sequence is involved in *FLC* repression of *SOC1* expression in vivo.[34] It is proposed that recruitment of CO, via another region of the *SOC1* promoter, is opposed when FLC is also bound. Thus, until vernalisation and the autonomous pathway genes (see below) decrease the levels of *FLC* in winter-annual *Arabidopsis*, the ability of CO to activate *SOC1* expression to trigger flowering is compromised.

The vernalisation pathway: epigenetic control of flowering

The identification of the *FLC* gene has also helped answer a long-standing question in flowering-time research: how do plants that have been vernalised remember this signal and flower maybe months later? The perception of cold occurs in the cells of the growing tip of the plant, the shoot apex, and, after an extended exposure to cold, a vernalised state is induced in these cells. This state can be passed on through mitotic cell divisions even in the absence of cold, but is lost after meiosis. The recent identification and molecular analysis of two genes, *VRN1* and *VRN2*, and their effects on *FLC* expression (Fig. 5), suggests that epigenetic changes in chromatin structure at the *FLC* locus are the basis of this cellular memory of vernalisation.

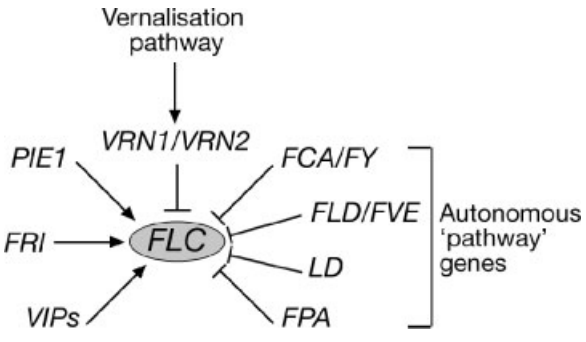

Figure 5. Multiple genes control *FLC* expression. The *FLC* gene is an important repressor of flowering and many different genes, including those in the vernalisation and autonomous pathways, regulate its expression. Lines with arrows indicate upregulation of gene expression, lines with bars indicate gene repression.

Although the *vrn1* and *vrn2* mutants are able to perceive cold and respond by downregulating *FLC* mRNA levels, they are defective in their ability to remember the cold, as once exposed to warm temperatures, *FLC* mRNA returns to pre-vernalisation levels.[35] Good clues to the mechanism of action of *VRN2* have come from cloning the gene. *VRN2* encodes a nuclear-localised zinc-finger protein with similarity to the *Drosophila* Polycomb Group (PcG) protein *SU(Z)12*.[35] PcG proteins are components of complexes that repress gene expression by maintaining the chromatin in a state incompatible with transcription. This state is maintained after mitotic but not meiotic cell divisions. It is therefore proposed that *VRN2* functions in a similar manner to other PcG proteins, to maintain the vernalisation-induced repression of *FLC*.

VRN1 may also function in a chromatin-modifying complex as it encodes a protein with B3 DNA-binding domains and, in vitro, binds DNA in a strong, but non-sequence-specific manner.[36] Non-sequence-specific binding in vitro has been observed for other PcG proteins, and it may be that *VRN1* interacts with DNA more specifically in vivo and perhaps targets VRN2-containing PcG complexes to the *FLC* gene. Deletion analysis of the *FLC* gene has shown that part of the first intron is needed for the maintenance of *FLC* repression.[37] Interestingly, different regions of the *FLC* gene are required for the initial vernalisation-induced repression, again suggesting that different mechanisms are involved in the repression by cold and maintaining the repressed state.

Given that vernalisation results in *FLC* chromatin being transcriptionally inactive, other genes might be expected to have the opposite function and maintain *FLC* chromatin in an active state before vernalisation. A number of early-flowering *Arabidopsis* mutants have been identified that have reduced *FLC* expression and the corresponding genes are candidate activators of *FLC*. One of these, *PIE1*, encodes a protein with homology to the ATP-dependent chromatin-remodelling proteins of the ISWI family.[38] In *Drosophila*, proteins in this family function in complexes, called the trithorax group (trxG), that activate transcription by promoting an open and active chromatin conformation.

Consistent with the idea that *PIE1* activates *FLC* expression by promoting an active chromatin structure, *PIE1* is required for the activation of *FLC* expression by both *FRI* and the late-flowering autonomous pathway mutants. A group of seven other genes, *VIP1* to *VIP7* are also involved in activating *FLC*.[39,40] Two of these genes have been cloned, but while their molecular function is as yet unclear, it is possible that some of these VIP genes encode proteins that function with *PIE1* in a trxG-like chromatin-modifying complex. *PIE1*-mediated *FLC* activation maybe replaced after vernalisation

by a repressive VRN1–VRN2 protein complex that maintains *FLC* in an inactive chromatin state.[38]

Cold perception: a black box

A major unanswered question is how do plants perceive cold? Detection of cold is not only required for vernalisation and flowering, but for the induction of freezing tolerance (cold acclimation). Vernalisation and cold acclimation appear to share at least one component in common, the negative regulator *HOS1*.[41] Mutant *hos1* plants have increased freezing tolerance, flower early and have decreased expression of the floral repressor *FLC*. A family of CBF transcription factors is upregulated in *hos1* and overexpressing just one member, CBF1, in transgenic plants causes increased freezing tolerance.[42] However, increased *CBF1* expression alone does not affect *FLC* expression or flowering.[42] Therefore, it appears that, although cold may be perceived by a common mechanism, cold acclimation and vernalisation may use different downstream signal transduction pathways.

Fine tuning the vernalisation response

Although *FLC* is the major target of the vernalisation pathway, the fact that *flc* null mutants still respond, albeit weakly, to vernalisation suggests that there are additional target(s).[43] A number of *FLC*-related transcription factors including *MAF1* to *MAF5* and *AGL24* are regulated in response to vernalisation, independently of *FLC*.[44–47] These genes probably play an important role in modulating the vernalisation response and, in particular, the *MAF2* gene is required to prevent flowering after short cold spells.[48] *MAF2*, like *FLC*, represses the expression of the floral integrator *SOC1* and is downregulated after vernalisation.[48]

Further fine-tuning of the vernalisation response might involve *AGL24*. *AGL24* has a similar function to *SOC1* and promotes flowering. Interestingly, there is evidence of cross-talk between *SOC1* and *AGL24*, with both being able to upregulate each other's expression.[46,47] Since *AGL24* is upregulated by vernalisation, this provides an *FLC*-independent route for vernalisation to regulate flowering.

The autonomous pathway

In addition to environmental factors, internal signals regulate flowering. The autonomous pathway comprises a group of six genes (*FCA*, *FY*, *FLD*, *FVE*, *FPA* and *LD*) that, when mutated, produce late-flowering phenotypes.[1] Since these mutants are not defective in their ability to respond to day-length and vernalisation signals, it has been suggested that they might respond to an internal developmental signal. However, this remains to be proved and, in fact, the notion that the 'autonomous pathway' is a typical linear pathway by which a signal leads to a response is now questioned because both genetic and molecular analyses suggest that the autonomous genes function in different subgroups to promote flowering (Fig. 5).

Like the vernalisation pathway genes, *PIE1* and *VIP*, discussed above, the major target of the autonomous genes is the floral repressor *FLC* (Fig. 5). The autonomous mutants have higher levels of *FLC* mRNA and their late-flowering phenotype is abolished when combined with a null *flc* mutant.[43] Thus, the autonomous genes promote flowering by repressing *FLC* expression. Interestingly, recent experiments have suggested that different autonomous genes repress *FLC* expression using different mechanisms.

Recent work suggests that two of the autonomous genes, *FCA* and *FY*, function together to control gene expression by regulating polyadenylation site selection. *FCA* encodes an RNA-binding protein with a WW protein-interaction domain. This WW domain is essential for its flowering-time function and for it to interact with FY, which turns out to be a polyadenylation factor. The FCA–FY partnership functions in a negative feedback loop that limits the amount of functional FCA protein produced[49] (Fig. 6). This is achieved by alternative transcript

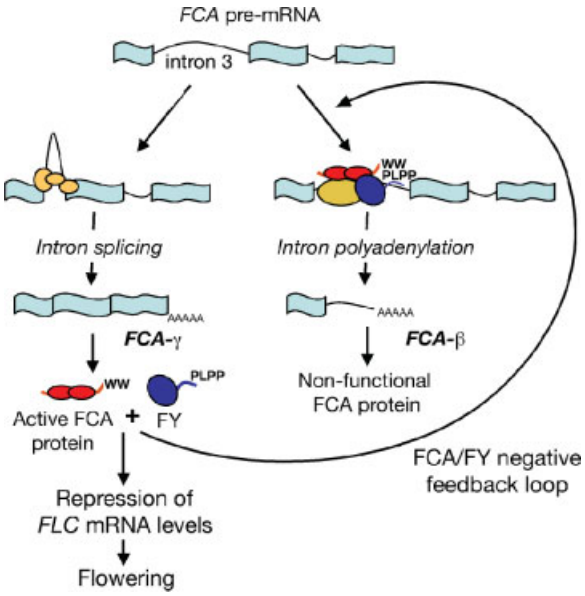

Figure 6. The *FCA* negative feedback loop. Correct splicing of intron 3 of the *FCA* pre-mRNA results in *FCA*γ transcripts that encode the full-length, active FCA protein. FCA protein, via its WW domain, can interact with the PLPP motif of the 3′ end-processing factor, FY. The FCA–FY complex is proposed to feedback to promote the premature polyadenylation of *FCA* pre-mRNA within intron 3 resulting in *FCA*-β transcripts being produced, which encode a non-functional protein. This negative feedback loop limits the amount of active *FCA* produced and therefore controls the degree of repression of *FLC* mRNA levels and flowering time.

processing within the third intron of the *FCA* pre-mRNA. The *FCA-γ* transcript encodes a full-length protein, whereas *FCA-β* is produced after premature polyadenylation and encodes a truncated non-functional protein. Since FCA is an RNA-binding protein, it is likely that FCA binds to its own pre-mRNA and recruits FY, and other components of the polyadenylation machinery, to promote intron 3 polyadenylation.[50] In this way, full-length protein produced from the *FCA-γ* transcript feeds back to negatively regulate its own expression (Fig. 6). This provides a mechanism to tightly regulate the levels of FCA protein and, since increased FCA protein causes earlier flowering, to regulate flowering.

The FCA–FY partnership also regulates *FLC* mRNA levels. When *FCA* autoregulation is bypassed (by over-expressing the FCA-γ transcripts that lacks introns), *FY* is still required for *FLC* downregulation and earlier flowering. These experiments suggest that FCA–FY probably functions to regulate the polyadenylation of *FLC* or an unidentified intermediate. Although no alternative polyadenylated *FLC* transcripts have been detected, it is possible that these are unstable and rapidly degraded.

The recent cloning of another autonomous gene, *FLD*, has revealed a different mechanism by which *FLC* expression is regulated.[51] *FLD* encodes a protein similar to a component of mammalian histone deacetylase complexes. Histone deacetylase complexes can be recruited to target genes, and initiate repression of gene expression by deacetylation of histone residues, leading to a change in chromatin structure. Consistent with the idea that *FLD* represses *FLC* expression via histone deacetylation, in the absence of a functional *FLD* gene, increased acetylation of histones at the *FLC* locus and increased *FLC* expression were observed. Interestingly, another autonomous mutant *fve* showed increased *FLC* histone acetylation (while the *fca, fpa* and *ld* mutants had no effect) suggesting that *FLD* and *FVE* might function in the same complex.[51]

It will be interesting to know if the other autonomous genes, *FPA* and *LD*, regulate *FLC* expression by yet different mechanisms. Like *FCA*, *FPA* encodes an RNA-binding protein suggesting it could also function post-transcriptionally.[52] However, genetic epistasis experiments and the observed synergistic downregulation of *FLC* mRNA levels seen in *fca/fpa* double mutants, indicates they function in parallel.[53,54]

MicroRNAs and regulation of flowering time

As depicted in Fig. 1, many of the flowering-time pathways converge to regulate the expression of *FT* or *SOC1*. The activation of these genes then leads to the upregulation of *AP1* and *LFY*, which trigger flower development. Recent work hints at an extra layer of regulatory complexity between these sets of genes and the involvement of microRNAs in regulating flowering time.[55] MicroRNAs are ~22 bp non-coding RNAs whose important role as negative regulators of gene expression in eukaryotes is only just being realised.[56] They are processed from longer hairpin transcripts, and function within larger complexes to target complementary mRNAs and prevent their expression (either by inhibiting translation or by cleavage of the mRNA).[56]

Analysis of global *Arabidopsis* gene expression has identified a large group of potential floral repressors that are downregulated upon floral induction by long days.[55] These include a group of genes, *SMZ, SNZ, TOE1* and *TOE2*, which encode proteins similar to the *AP2* floral organ identity gene. The downregulation of some of these *AP2-like* genes, which normally occurs under long days, is abolished in *co* and *ft* mutants, but unaltered in a *lfy* mutant. This suggests that the *AP2-like* genes might be floral repressors, downstream of *CO* and *FT*, but upstream of *LFY*. Recent results are consistent with the *AP2-like* genes functioning as floral repressors. For example, three of them, *TOE1, SMZ* and *SNZ*, cause late flowering when they are overexpressed.[55,57]

In the first example of a microRNA regulating flowering time, plants overexpressing a gene called *EAT*, whose transcript is processed to produce *microRNA172* (*miR172*), are very early flowering and have floral organ defects similar to those observed in *ap2*.[57] *MiR172* shares partial sequence complementarity with *AP2* and other *AP2-like* genes and has previously been shown to regulate the expression of the *AP2* gene at the translational level.[58] Similarly, overexpression of *miR172* appears to causes early-flowering by downregulating *AP2-like* floral repressors, including *TOE1* and *TOE2*, predominantly at the translational level.

In wild-type plants, *miR172* expression is temporally regulated with levels increasing through development and this would be expected to lead to downregulation of the translation of the AP2-like floral repressors. Once they have decreased to a sufficiently low level, then this may then allow downstream genes such as *AP1* and *LFY* to be expressed and trigger flowering.

Conclusions

In a landmark paper published just over a decade ago, Maarten Koornneef and colleagues reported the characterisation of a group of late-flowering *Arabidopsis* mutants that carried mutations in 11 different flowering-time genes.[59] Since then, considerable effort has been devoted to isolating and analysing these and other newly discovered flowering-time genes. This has led to a detailed model of molecular-genetic control of flowering time in *Arabidopsis* in response to environmental and internal signals. While tremendous progress has been made, much still needs to be done to understand how these genes function at the molecular level. In addition, fundamental questions, such as how *Arabidopsis* detects the floral signals of temperature and maturity, remain to be answered.

Other species of plants are likely to have a variety of flowering-time control mechanisms, but there is evidence that some use the same components as *Arabidopsis*. For example, in rice, the long-day pathway genes are largely conserved but adapted to respond to different day-length cues. Some autonomous pathway genes are also present in other plants, including cereals.[26,50] The function of the downstream floral integrators, such as *LFY*, appears to be conserved in many plants as its constitutive expression in the perennials poplar and citrus caused early flowering.[60,61] However, the vernalisation pathway may have evolved more than once, as wheat[62] and *Arabidopsis* use different genes to prevent flowering until after winter is over.

Apart from its fundamental role in plant development, the trait of flowering time is of great importance to plant breeders and commercial growers as it plays a key role in plant adaptation to growing regions. Ultimately, knowledge of flowering-time genes and how they work should help with the selection of novel plant varieties with altered flowering times. Early-flowering varieties might allow multiple rounds of cropping in a single season, varieties with delayed flowering may increase yields in crops such as sugar beet, pasture grasses, or forestry where vegetative growth is desired, while ornamentals would be induced to flower on command on important dates of the year.

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We apologise to colleagues whose work could not be cited due to limitations of space. We would like to thank people who sent us copies of papers pre-publication and Franz Pichler and Robert Schaffer for their helpful comments on the manuscript.

Note in added proof

Since this review was accepted a number of important papers have been published. Two papers show the epigenetic repression of *FLC* caused by vernalisation, involves changes in the methylation of histones in *FLC* chromatin (Sung and Amasino & Bastow et al., 2004. Nature 427:159–164 & 164–167). The cloning of the autonomous pathway gene *FVE* has been reported by two groups (Ausin et al. & Kim et al., 2004. Nature Genetics 36:162–166 & 167–171) confirming that it encodes a component of a histone deacetylase complex. Finally, the CONSTANS protein is shown to be stabilised by light at the end of the day in long days (Valverde et al., 2004. Science 303:1003–1006.

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