Time measurement and the control of flowering in plants

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Summary

Many plants are adapted to flower at particular times of year, to ensure optimal pollination and seed maturation. In these plants flowering is controlled by environmental signals that reflect the changing seasons, particularly daylength and temperature. The response to daylength varies, so that plants isolated at higher latitudes tend to flower in response to long daylengths of spring and summer, while plants from lower latitudes avoid the extreme heat of summer by responding to short days. Such responses require a mechanism for measuring time, and the circadian clock that regulates daily rhythms in behaviour also acts as the timer in the measurement of daylength. Plants from high latitudes often also show an extreme response to temperature called vernalisation in which flowering is repressed until the plant is exposed to winter temperatures for an extended time. Genetic approaches in *Arabidopsis* have identified a number of genes that control vernalisation and daylength responses. These genes are described and models presented for how daylength might regulate flowering by controlling their expression by the circadian clock. *BioEssays* 22:38–47, 2000.

Introduction

Plants and animals that live at high latitudes often alter their behaviour or development in response to the changing seasons. These alterations include the initiation of reproduction in birds, mammals, and plants, the onset of diapause in many insects, variations in wing colour in butterflies, and migratory restlessness in birds. Environmental signals, often changes in daylength and temperature, trigger these responses. The role of daylength in controlling seasonal responses was originally proposed by Garner and Allard[1] when they recognised that daylength controlled the initiation of flowering in many plant species. They showed that some plants would flower only in daily cycles in which the light period was longer than a particular threshold length, referred to as the critical daylength, while other plants would flower only if daylength was shorter than a critical daylength. In these plants, named long-day and short-day species, respectively, the duration of the critical daylength varies between species and also between varieties of the same species adapted to different latitudes.[2] Varieties of the same species grown at different latitudes flower in response to different photoperiod lengths; for example, varieties of cocklebur found in Florida flower when the daylength reaches 14 hours, but further north in Michigan they require 16-hour long days.[3]

Around 15 years after Garner and Allard's original description of photoperiodism, Bünning[4] proposed that plants might use the same time-keeping mechanism that regulates daily rhythms in leaf movements to measure daylength and, thereby, control seasonal responses. General acceptance that the daily timer (the circadian clock) controls daylength responses came much later, however, and stemmed from experiments in which plants were exposed to cycles longer than the daily cycle of 24 hours (Ref. 5; reviewed in detail in Ref. 2). The most striking of these involve growing plants under long periods of darkness and then inducing or repressing flowering by transient exposure to light at different times within the dark period. This demonstrates that the effect of these light treatments on flowering varies dramatically depending on when they are given within a 24-hour cycle, and that peaks in sensitivity to the treatments occur in a 24-hour cycle. For example, growth of the short-day plant *Chenopodium rubrum* in continuous light prevents flowering and flowering is induced by exposure to one dark period of 72 hours. Disruption of the dark period, however, with a 4-minute flash of light prevents flowering if given 36 or 60 hours into the dark period, but not if given 20 or 44 hours into it.[6] The 24-hour periodicity of the effect of light suggests that the light flashes prevent flowering by interacting with an underlying circadian rhythm. Similar results have been observed using a range of plant species with different responses to daylength, as well as in insects and birds. Furthermore, *Arabidopsis* mutants were described recently in which both circadian rhythms and flowering time control are disrupted and it is now generally accepted that the

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Abbreviations: LD; long day: SD; short day: Pr; form of phytochrome after exposure to far-red light: Pfr; form of phytochrome after exposure to red light.

For those unfamiliar with gene nomenclature in *Arabidopsis*, throughout this article the names of wild-type genes are given in upper case italics, while gene names of their mutant counterparts are referred to in lower case italics.

circadian clock provides the time-keeping mechanism for photoperiodic responses (reviewed in Ref. 2).

In plants, temperature is also important in controlling seasonal responses. Perhaps the most dramatic of these effects is the initiation of developmental events in response to extended exposures to low temperatures. In this response, termed vernalisation, developmental processes such as the initiation of flowering are repressed until the plant has been exposed to an extended period of low temperature similar to those experienced in winter. Flowering of many plant species that grow at high latitudes is repressed until they have been exposed to such conditions, and this repression of flowering occurs even if the plant is growing under photoperiods that would otherwise promote flowering. For example, although exposure to long days (LDs) promotes early flowering in many varieties of *A. thaliana*, varieties that require low temperature treatments will not flower early even in LD conditions unless they have been previously exposed to low temperatures.[7–10] The most successful treatments require exposure to low temperatures for a few weeks, suggesting that vernalisation ensures that flowering occurs in the spring following exposure to winter conditions. Thus, flowering in the spring or early summer occurs through a combination of exposure to the preceding winter, causing vernalisation, plus longer photoperiods that actively promote flowering.

Finally, the age of a plant can determine whether it will flower in response to environmental stimuli. For example, the life cycle of many plants includes a juvenile phase during which they will not flower even in environmental conditions that would induce flowering in older plants. The duration of this juvenile phase varies dramatically between species and, in trees, it may last many years. Transition from the juvenile to adult phase can often also be observed in the organs that form at different stages during vegetative development and, in some species, these changes are related to the acquisition of the ability to flower. For example, in *Arabidopsis,* mutations that accelerate the transition between phases of vegetative development and cause early flowering have been identified.[11] In contrast, in maize, mutations that extend the juvenile phase of vegetative development do not affect the age at which the plants can be induced to flower.[12] These phases demonstrate that there is a mechanism for generating age-related changes in the development of the plant shoot that can also influence flowering time. These developmental phases are outside of the scope of this article, but have been reviewed recently.[11,13]

In this article, we mainly review recent progress in understanding the response to daylength and its role in the control of flowering time. We also briefly describe recent advances in understanding vernalisation. Much of the recent progress has come from molecular genetic approaches in *Arabidopsis*, and we will largely concentrate on these experiments and on the role of the genes that they have identified. More general reviews describing the control of flowering time have appeared recently.[14–16]

Vernalisation: the promotion of flowering by extended exposures to low temperature

Vernalisation is most effective when plants are exposed to low temperatures for prolonged periods lasting several weeks. For example, exposure of *Arabidopsis* seedlings to 4°C for 6 weeks results in a maximal response, while treatment for 2 weeks gives a much lesser effect.[17] This response can be considered a form of biological timer that ensures that flowering is repressed until the end of the winter months.

Vernalisation has been analysed genetically in *Arabidopsis* by comparing naturally occurring varieties that either do or do not respond to low temperature treatments. In varieties that do respond, exposure to low temperatures causes the plants to flower much earlier than they would if not exposed. These two response types differ at two major loci, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*),[7–10] which act together to delay flowering. The *FLC* gene was cloned recently and encodes a MADS box transcription factor that represses flowering when expressed at high levels[18,19] (Fig. 1). Low temperature treatment of plants for 3 weeks, however, reduces the expression of *FLC*, and this correlates with early flowering.[18,19] This suggests that during vernalisation low temperature treatments reduce the transcription of genes that repress flowering such as *FLC*. Similarly, expression of *FRI* or mutations in the *FCA* gene increases *FLC* levels and so delays flowering unless the plant is exposed to low temperatures[18,19] (Fig. 1). The kinetics with which *FLC* expression is reduced during low temperature treatments is not known, and it will be interesting to assess whether increasing cold exposure from a few days to 3 weeks causes progressively lower levels of *FLC* expression. This is suggested by analysis of *FLC* mutant alleles that are expressed at higher levels than wild-type, which in turn repress flowering more dramatically. Plants with these mutant *FLC* alleles require longer low temperature treatments to flower early, as exposure to low temperature does not reduce *FLC* as rapidly as wild-type and this correlates with an incomplete effect of vernalisation on the acceleration of flowering.[18]

How is transcription of *FLC* progressively reduced in response to exposure to low temperatures over a period of several weeks? One possibility is that progressive changes in gene methylation during the course of vernalisation change the pattern of *FLC* expression, and perhaps also the expression of other flowering time genes.[18,20] Evidence supporting this comes from the observation that in some plants reduction of methyl transferase activity causes early flowering. These plants also have reduced expression of

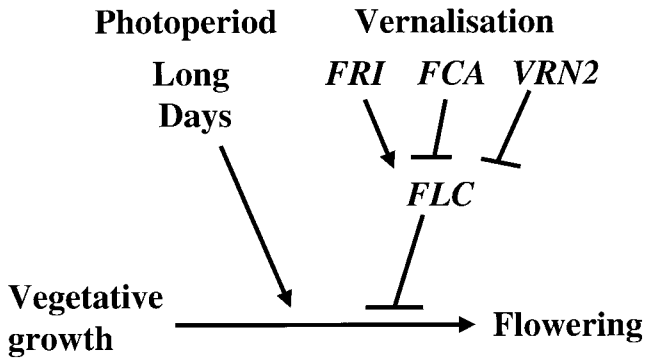

Figure 1. Genetic and molecular interactions involved in vernalisation in *Arabidopsis*. The *FLC* gene encodes a MADS box transcription factor that represses the transition from vegetative growth to flowering. Reduction in *FLC* mRNA in response to low temperature is proposed to play a central role in vernalisation. *FRI*, *FCA,* and *VRN2* genes regulate *FLC* expression; *FRI* gene increases *FLC* expression; *VRN2* acts during vernalisation to reduce *FLC* expression in response to low temperature treatments; the flowering time gene *FCA* represses *FLC* expression, so that *fca* mutants are late flowering but this can be corrected by low temperature treatments. An independent genetic pathway, described in Figure 3, promotes flowering in response to photoperiod.

FLC.[18] Genetic screens have also been performed for mutations that prevent vernalisation,[21] and at least one of the mutations identified, *vernalisation 2* (*vrn2*), blocks the decrease in *FLC* expression that normally occurs during low temperature treatment.[18] Therefore, the processes required for vernalisation should be amenable to genetic analysis.

Daylength measurement involves the circadian clock

A mechanism by which plants measure the duration of a photoperiod is a prerequisite for the photoperiodic control of flowering time. The circadian clock that controls daily rhythms in gene expression and behaviour has been proposed to act as the timer in photoperiodic response as described above. Circadian rhythms have been widely studied in insects, fungi, plants and mammals[22] (Fig. 2). Features of these rhythms are that the duration of one cycle is approximately 24 hours, that they are entrained to (or synchronised with) the day/night cycle by environmental changes in light/dark or in temperature, and that the rhythm persists when organisms are shifted from light/dark cycles to continuous conditions of light or dark. The circadian clock that controls these rhythms is often considered in three parts: a central oscillator that creates the 24-hour periodicity, input pathways to the oscillator that synchronise the oscillation to the day/night cycle, and outputs from the oscillator that are overt rhythms in gene expression and behaviour.[22,23] Figure 2 illustrates these processes schematically in relation to the *Arabidopsis* circadian clock and they are discussed in more detail in following sections.

In plants, entrainment of the clock to daily cycles of light and dark is controlled by light receptors, including the phytochromes (red/far-red light receptors) and cryptochromes (blue light receptors). These will be discussed in detail later. The proteins that form the central oscillator of the plant circadian clock are unknown, although candidate genes and proteins are discussed in the next section. In other systems, such as *Drosophila*, *Neurospora,* and mammals, the proteins required for the central oscillator form a negative feedback loop based on the control of transcription and translation of central clock molecules. For example, in *Drosophila* the proteins dCLOCK and CYCLE activate expression of the *Period* (*Per*) gene. As PER protein accumulates in the cytoplasm it heterodimerises with TIMELESS (TIM); when this heterodimer reaches a threshold concentration it is imported into the nucleus where it prevents the activation of *Per* by dCLOCK/CYCLE (reviewed in Ref. 22). This cycle of negative feedback control of PER on its own expression takes approximately 24 hours, and this cycle time is largely due to the time taken for the TIM/PER heterodimer to accumulate before it is imported into the nucleus. In Figure 2 it is assumed that the plant circadian clock is also based on such a negative feedback loop, and candidate proteins for oscillator components are described in the following section.

Output pathways in *Arabidopsis* control expression of a wide range of clock-controlled genes that peak in expression in different phases of the cycle, such as *COLD AND CIRCADIAN REGULATED 2*, *GIGANTEA* (*GI*), *CHLOROPHYLL A/B BINDING PROTEIN 2*, and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*).[24–27] These pathways also control overt rhythms in leaf movement, stomatal opening, and hypocotyl elongation[28–30] and, in addition, the photoperiodic control of flowering is also likely to represent an output pathway. How these output pathways are controlled by the oscillator is unknown but, because components of the central oscillator are themselves transcription factors (at least in *Drosophila*), they may directly activate genes involved in particular output pathways.

Genes that alter or disrupt circadian regulation in *Arabidopsis*

In *Arabidopsis*, three mutations and one transgene that disrupt or alter normal circadian output have been described. Three of the relevant genes have been recently cloned and two of these, *LATE ELONGATED HYPOCOTYL* (*LHY*)[31] and *CCA1*,[27] encode very similar proteins that contain a single

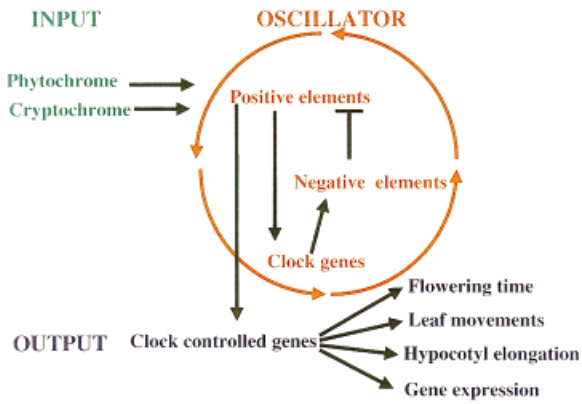

Figure 2. A schematic model of circadian clock control in *Arabidopsis*. Light signals entrain the circadian clock to the daily cycle of night and day. Phytochrome and cryptochrome photoreceptors convey the "input" signals to the central oscillator. The oscillator generates the 24-hour rhythm, and is proposed to be a negative feedback loop with properties similar to those described in other systems.[18] In this scheme the expression of clock genes is promoted by "positive elements." As the abundance of clock gene products rises, they repress their own synthesis by antagonising the function of the "positive elements." The abundance of clock gene products then falls and the function of the "positive elements" is restored. This regulation produces peaks in clock gene expression that are approximately 24 hours apart. The "positive elements" might also regulate the expression of clock-controlled genes that direct overt rhythms or output from the oscillator. In *Arabidopsis,* these include leaf movements, hypocotyl elongation, the expression of many genes and the photoperiodic control of flowering time.

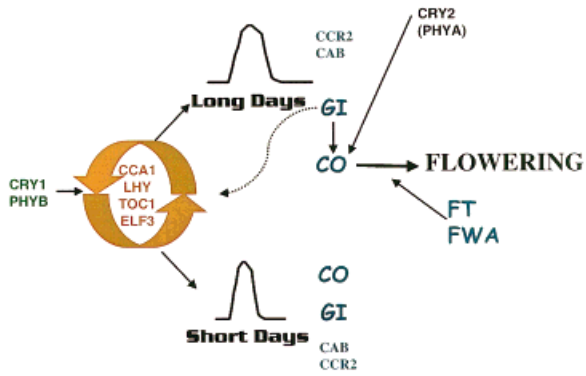

Figure 3. Genetic and molecular interactions involved in photoperiod response in *Arabidopsis*. Four genes (*CCA1*, *LHY*, *TOC1*, and *ELF3*, shown in red) are proposed to act within the central oscillator of the circadian clock or to act at positions in input or output pathways that are closely associated with oscillator function. The oscillator is entrained to the day/night cycle by light receptors, and *CRY1* and *PHYB* (shown in green) play an important role in this entrainment under high fluence light conditions. The phase of expression of genes regulated by the circadian clock differs in response to long or short daylengths, although not all of these genes have a role in flowering regulation. The phase of expression of flowering time genes that occurs under long days is proposed to trigger the promotion of flowering through the *GI* and *CO* genes. GI also affects the amplitude of LHY and CCA1 expression.[25] CRY2 and PHYA photoreceptors promote flowering in response to photoperiod, and CRY2 increases the abundance of *CO* mRNA. Whether PHYA (shown in brackets) acts in a similar way is not known. The *FT* and *FWA* genes act downstream of *CO*.

MYB repeat which suggests that these proteins bind DNA and regulate transcription. Indeed, the CCA1 protein does bind to specific DNA sequences.[32] Expression of both *LHY* and *CCA1* mRNAs have a diurnal rhythm in plants grown in light/dark cycles, with peak levels occurring at dawn. A similar pattern of expression has been shown for the CCA1 protein. When moved to constant light conditions, plants express both genes in a circadian rhythm, and *LHY* also maintains a circadian rhythm of expression in constant darkness.

Circadian patterns of *LHY* and *CCA1* expression may be required to maintain all circadian outputs. Constant high levels of either transcript in transgenic plants disrupts all rhythmic outputs examined, both in constant light and in constant dark. Furthermore, overexpression of either gene causes a loss of rhythmicity in the expression of their own and each others transcript.[25,27,31]

Although these studies show that overexpression of *LHY* and *CCA1* disrupts clock function, they do not prove that the genes are required for normal clock function. Recent characterisation of a *CCA1* loss-of-function mutant, however, demonstrates that *CCA1* is required for circadian clock regulation with a normal period.[33] Entrained plants transferred to constant light showed a reduction of 3 hours in the period of expression of two clock-regulated genes that normally peak at the same time as *CCA1*. Genes that normally peak later in the day also had an abnormal expression pattern that might also be explained by a shorter period. Whether *CCA1* is also required for normal rhythms in constant dark or in light/dark conditions has not yet been reported. The phenotype of the *CCA1* mutant demonstrates that, although *CCA1* and *LHY* are closely related in sequence, *LHY* cannot completely compensate for the loss of *CCA1*, although it may do so partially. Characterisation of a *LHY* loss-of-function mutant is in progress (K. Wheatley and G. Coupland, unpublished). At this time, attempting to place either *LHY* or *CCA1* in the input, output, or within the oscillator itself is premature.

Constant overexpression of either *LHY* or *CCA1* causes late flowering in long days, and *LHY* overexpression causes

the flowering time of plants to become insensitive to daylength. The correlation between the flowering-time phenotype and circadian clock function suggests that, in *Arabidopsis,* the response to photoperiod requires a functional circadian clock. The basis of this may be the regulation of expression of flowering-time genes which have reduced and abnormal diurnal patterns of expression in plants that overexpress *LHY* (see below).

The phenotype of loss-of-function *early flowering 3* (*elf3*) mutants suggest that *ELF3* is required to maintain circadian output under certain conditions.[34] Circadian output in *Arabidopsis* is often investigated using a fusion between the promoter region of the circadian clock-controlled gene *CAB2* and the luciferase marker gene (*CAB:LUC*). *elf3* mutant plants entrained in light/dark cycles and moved to continuous white light showed arrhythmic *CAB:LUC* expression, as well as arrhythmic leaf movements and *LHY* expression.[31,34] On the other hand, when moved into constant dark loss of *ELF3* had no significant effect on *CAB:LUC* rhythmicity. Plants entrained to different light/dark cycles suggest that *CAB:LUC* expression is normal in short days but becomes abnormal as the photoperiod lengthens. In addition, in *elf3* mutant plants grown in short-day (SD) conditions, there are significant changes in the expression of genes that normally peak later in the day than CAB:LUC[25] (P. Suarez-Lopez and G. Coupland, unpublished), suggesting that the effect of *elf3* in SDs may be more dramatic on these genes compared to CAB:LUC. This may be important in explaining the flowering phenotype of *elf3*, since it is under these conditions that *elf3* shows its major flowering time phenotype.[35] The *ELF3* gene was cloned recently and encodes a polypeptide with no significant sequence resemblance to known proteins.[36]

Another gene, *TIMING OF CAB 1* (*TOC1*)[37,38] is also thought to play an important role in circadian regulation. In continuous light, plants that express the semi-dominant *toc1-1* mutant have a shorter period for several markers[37–39] and, in some strains of *Arabidopsis,* this results in early flowering.[38] The mutation probably causes the central oscillator to run at an inherently faster pace. The behaviour of *toc1-1*-mutant plants in different light/dark cycles has not been described, but under a 24-hour temperature entrained cycle the abnormal waveform of *CAB:LUC* in plants indicates that *TOC1* is required for correct processing of the entraining signals. This indicates that entrainment to a cycle (in this case a 24-hour temperature cycle) that differs greatly from the period of the endogenous clock (21 hours for *toc1*) results in a distortion in the normal rhythmic pattern of gene expression. Indeed, in *Drosophila* expression of different mutant alleles of *PER* causes short or long periods under free-running conditions and results in a shift in the timing of behavioural patterns and gene expression under light/dark cycles.[40]

Photoreceptors in *Arabidopsis* and their role in circadian clock entrainment

Two types of light receptors have been analysed in detail in *Arabidopsis*. The first of these, cryptochromes, are flavoproteins that function as blue light receptors. *CRYPTOCHROME 1* (*CRY1*)[41] encodes a soluble protein that is expressed at similar levels in dark- and light-grown seedlings.[42] Loss-of-function mutants show reduced sensitivity to blue light[41] and transgenic gain-of-function mutants exhibit increased photosensitivity.[42] *CRYPTOCHROME 2* (*CRY2*, also known as *FHA*)[43,44] encodes a soluble light-labile protein.

The second group of light receptors, phytochromes, are encoded by a small family of five genes in *Arabidopsis* (*PHYA* to *PHYE*).[45,46] Each phytochrome exists in two forms. The Pr form is converted to the Pfr form by exposing plants to red light, and the reverse reaction occurs in far-red light. Physiological and mutational analyses have shown that PHYA and PHYB have distinct yet overlapping functions.[47] The *PHYA*[48] gene encodes a protein that is abundant in dark-grown seedlings and, whereas the Pr form of PHYA is stable in the cell, the Pfr form is subject to rapid degradation, which results in very low PHYA levels in red light. Analysis of *phyA* mutants has revealed that PHYA is the primary, if not the only, phytochrome responsible for de-etiolation in continuous far-red light.[48,49] On the other hand *PHYB*[50,51] encodes a light-stable protein that is the primary phytochrome responsible for de-etiolation in response to red light and is a major contributor to the shade avoidance-response.[52]

The effects of the cryptochromes and phytochromes on entrainment of the circadian clock have been investigated by analysing the effect of mutations on *CAB:LUC* expression, and mutations in genes encoding CRY1, CRY2, PHYA, and PHYB influence circadian clock-entrainment under specific conditions. However, under conditions of high light intensity (high fluence) CRY1 and PHYB are the most important light receptors in circadian clock entrainment.[53,54] Mutations in *CRY1* caused a lengthening in period under high fluence blue light, while mutation in *phyB* has similar effects under high fluence red light. Although mutations in *CRY2* and *PHYA* had no effect under high fluence conditions, *phyA* lengthened period under low fluence red or blue light and *cry2* caused a slight shortening of period under low fluence blue light and had no effect on period length under white light. Clearly, therefore, there is redundancy between light receptors that entrain the plant circadian clock.[54] It is striking, however, that *phy*A and *cry*2, which have marked effects on the photoperiodic control of flowering (see below), only affect clock entrainment under specialised low fluence conditions. This suggests that these genes do not affect daylength responses by influencing circadian clock entrain-

ment but by modulating other aspects of the daylength response.[54]

It is not known how these effects relate to the expression of genes such as *LHY* and *CCA1* proposed to be involved in the regulation of circadian rhythms. It is known, however, that *CCA1* expression responds rapidly to exposure of etiolated seedlings to light, and that loss of function of both *PHYA* and *PHYB* causes an 8-hour delay in red light induction of the *CAB* gene.[47] If these loss-of-function mutations were to have similar effects on *CCA1* and *LHY* expression, one could envisage a mechanism by which the phase of all circadian outputs could be altered. When plants are grown in the dark, CRY1 and PHYA proteins also modify gene expression of some circadian-regulated genes. For example, expression of the *CAT3* gene increases and oscillations are rapidly lost when plants are moved to constant dark, and functional CRY1 and PHYA proteins are required for this response.[55] Recently, evidence of physical interactions between CRY1 and PHYA proteins has been reported,[56] which suggests that CRY1 and PHYA might work together in the input of blue light in very low fluence rates and in regulation of gene expression of clock-regulated genes.

Photoperiodic response in *Arabidopsis* and the role of photoreceptors

Arabidopsis plants grown in LD conditions flower earlier and with fewer leaves then those grown under SDs.[57,58] A response to LDs can be detected soon after germination; seedlings grown in LDs and shifted to SDs when they are 8–10 days old will flower at a similar time to plants grown continuously in LDs.[59] Furthermore, older *Arabidopsis* plants respond rapidly to longer daylengths and exposure to a single LD can be sufficient to induce flowering.[60–62] Short exposures to light in an otherwise noninductive long dark period can also, under certain conditions, promote flowering of *Arabidopsis*, and far-red and blue night breaks are the most efficient in eliciting this response.[58] Thomas and Vince-Prue[2] reported a circadian rhythm in the response to night break in *Arabidopsis*.

Photoreceptors which seem to be specifically required for promotion of flowering in *Arabidopsis* under LD conditions are CRY2 and PHYA. Loss-of-function *CRY2* mutants have no flowering-time phenotype in SDs and are late-flowering in LDs,[59] although they still respond weakly to changes in photoperiod.[63] The flowering phenotype is more pronounced in some varieties (e.g., Columbia) than others (e.g., Landsberg *erecta*). A mutation in *CRY2* delays flowering of plants grown in constant red, red-plus-blue or white light,[43,65] and transgenic gain-of-function mutants are early flowering in SDs.[43] As described earlier, *cry2* does not affect circadian clock entrainment and probably alters flowering time by directly influencing the expression of flowering time genes. Although *cry1* mutations affect flowering time, and most alleles cause late flowering in SDs, they do not affect flowering time in response to extended short days, night break treatments,[58,66] nor constant blue light treatments,[43] suggesting that CRY1 is not involved in LD promotion of flowering.

The *phyA-1* mutant is late-flowering in extended SD conditions and has a reduced response to night break treatment.[47,67] Overexpression of *PHYA* causes early flowering in SDs as well as photoperiod insensitivity.[68] As with *cry2,* the effect of *phyA* on the response to photoperiod is probably not due to direct effect on circadian clock itself, but is more likely to be a result of an independent PHYA signalling pathway.[54] It is interesting that both PHYA and CRY2 are light-labile, the absence of either results in late flowering in LDs, and neither is required for normal period length of the circadian clock in high irradiance conditions. We propose a possible role for these receptors in modulating the expression or function of flowering time genes.

Genes that affect flowering in response to photoperiod in *Arabidopsis*

In addition to *cry2* and *lhy* discussed above, mutations in other genes, *constans* (*co*), *gigantea,* (*gi*); *ft*; *fwa*, that disrupt photoperiodic responses have also been described (reviewed in Refs. 14, 16). These mutations delay flowering under LDs but have no, or only slight, effects under SDs, and are considered as defining genes required to promote flowering in response to LDs.[64,68–74] As double mutants, carrying combinations of *cry2*, *co*, *gi,* or *lhy,* flower at approximately the same time as the later of the single mutants it has been proposed that they all function in the same pathway, called the long-day pathway[14,71] (Schaffer, Wheatley, and Coupland, unpublished). The *ft* and *fwa* mutations have different genetic interactions compared to the other four genes in this pathway, for example, when *ft* and *fwa* are combined with mutations in the floral meristem identity gene *LEAFY* the double mutants almost completely lack floral tissues.[75]

This suggests that they may act in a different aspect of the photoperiodic response. *CRY2* and *LHY* were described in earlier sections, and most of the other genes in this group have now been cloned. *CO* encodes a zinc-finger protein that is localised to the nucleus and is most likely a transcription factor[68] (Costa, Pineiro, and Coupland, unpublished). *GI* encodes a large protein with several possible membrane-spanning domains.[25] The *FT* gene encodes a putative phosphatidylethanolamine-binding and nucleotide-binding protein[76,77] similar to *TERMINAL FLOWER* (*TFL*),[78] although the mode of action of these proteins is still unknown. FWA is a gain-of-function mutant, and the gene has not yet been cloned.

The order with which these genes act within the pathway has been analysed at the molecular level by comparing their

expression in mutant and wild-type plants, and by creating transgenic plants which overexpress individual genes in wild-type and mutant backgrounds. Transgenic plants in which *CO* is overexpressed from the 35S viral promoter flower very early and the plants are insensitive to daylength.[79] This gain-of-function *CO* transgene is epistatic to *gi*, *cry2*, and *lhy* mutations but *ft* and *fwa* delay flowering of *35S:CO* (Igeno, Robson, Onouchi, Wheatley, Coupland, unpublished). These genetic interactions suggest that *CO* acts downstream of *GI*, *CRY2,* and *LHY*, but upstream or parallel to *FT* and *FWA* in the LD promotion pathway. Similarly, expression of *CO* is reduced in late-flowering *cry2* mutants and increased in early-flowering plants that overexpress *CRY2*,[43] suggesting that *CO* acts downstream of *CRY2*. A loss-of-function mutation in *ELF3* and gain-of-function mutations in *LHY* and *CCA1* affect expression of *GI* transcript, as expected for a circadian-regulated gene.[25] The reduction in *LHY* and *CCA1* expression seen in *gi* mutants, however, suggests that these genes do not act in a straightforward linear way, but that they influence each other's expression.[25] The expression patterns of several of the genes in this pathway are regulated by the circadian clock and are altered by daylength, suggesting that transcriptional regulation may activate this pathway under LD conditions. For example, at certain times of the daily cycle *CO* expression is higher in long than short days[68] (Suarez-Lopez and Coupland, unpublished). The recent analysis of *GI* expression also suggests how changes in photoperiod might influence flowering time. Levels of the *GI* transcript are regulated by the circadian clock with peak expression occurring 8–12 hours after dawn.[25] The timing and duration of this peak is influenced by daylength: it is broader and extends into the night in longer days. It is possible, therefore, that alterations in the precise timing or structure of the circadian peak in expression may explain how this pathway is activated by daylength (also see following section).

A working model for time measurement in the control of flowering in response to changes in photoperiod

Two general models have been proposed to explain how daylength is measured in the control of developmental responses, such as flowering (reviewed in Ref. 2). One of these models, often called the external coincidence model, proposes that an underlying circadian rhythm is sensitive to light at particular phases of the rhythm, and if the plant is exposed to light at that time flowering will be either promoted in a long-day plant or repressed in a short-day plant. The second model, the internal coincidence model, proposes that two underlying rhythms are out of phase under conditions that do not induce flowering but are brought into phase under conditions that are inductive.

Recent molecular data have supported the potential for these types of models. As described earlier, genes that are required for the photoperiodic response of *Arabidopsis* are circadian-regulated and their phase of expression changes in LDs compared to SDs. An effect of photoperiod on the phase of overt rhythms was originally described for the eclosion rhythm in *Drosophila*[80] and has been demonstrated for the pattern of expression of the circadian clock-regulated gene *CAB2*.[81] Furthermore, regulation of flowering time by the phase of expression of flowering time genes may explain why mutations such as *toc1* that alter the phase of expression of circadian clock controlled genes are also altered in flowering time.[38]

Alterations in the temporal pattern of expression in response to photoperiod indicate that a model similar to the internal coincidence model might operate. For example, two rhythms regulated by the circadian clock may cycle in similar but distinct phases and overlap in expression under some, but not other, photoperiods. These rhythms could be in two flowering time genes, such as *GI* and *CO*, or in one flowering time gene product and a modulator of protein activity. One candidate for such a modulator is the level of free calcium in the cytosol. There is a circadian rhythm in cytosolic calcium concentration in *Arabidopsis*, with a peak occurring shortly after dawn.[82] Oscillations of free calcium within the range reported will affect the function of many cellular systems, and possibly flowering, and a time-dependent effect of calcium on flowering has been proposed in photoperiodic induction in the SD plant *Pharbitis nil*.[83]

Alternatively, the alterations in the rhythmic expression patterns detected under long and short days also provide support for the external coincidence model. For example, the phase of expression of flowering time genes might be altered by the light in long but not short days, and it is possible that light is needed to activate the proteins or their target genes. In this scenario, the light receptors PHYA and CRY2 are likely candidates to mediate this interaction, since both are required for LD perception. Both proteins are light labile, which suggests that they might act in dark or at dawn.

Conclusions

Genetic approaches in *Arabidopsis* have provided access to genes whose products control flowering in response to seasonal changes in temperature and daylength. The analysis of these genes has emphasised the role of the circadian clock in regulating the response to daylength. For example, the *lhy* and *elf3* mutations or the overexpression of *CCA1* disrupt circadian clock control and the photoperiodic response, while the expression of the *GI* gene, which promotes flowering in response to LDs, is regulated by the circadian clock. This has led to the formulation of models whereby the pho-

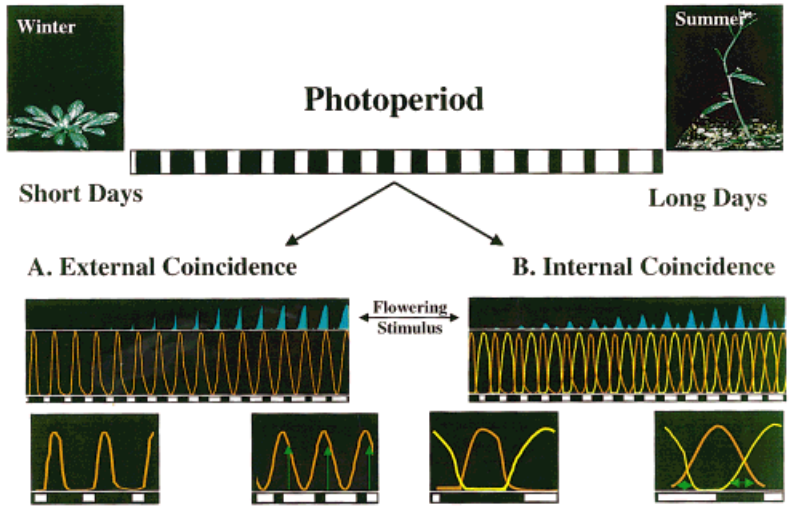

Figure 4. Interpretations of the external and internal coincidence models as applied to the photoperiodic control of flowering in *Arabidopsis*. *Arabidopsis* flowers late under short daylengths associated with winter conditions (top left) and early under long daylengths associated with spring or summer conditions (top right). Two general models have been proposed to account for this, these are **A**: the external coincidence model and **B**: the internal coincidence model. External coincidence model: The circadian rhythm is represented in red, and the flowering stimulus that promotes flowering in blue. Under short days (**A**, bottom left) the peak of the circadian rhythm in gene expression is narrower than under long days (**A**, bottom right). Therefore, under long days the plant is exposed to light during the peak of expression (represented by the green arrows in **A**, bottom right) and this could activate protein function leading to an increase in floral stimulus and flowering. Internal coincidence model: In this model, there are two circadian rhythms in different phases (represented in yellow and red). In short days the rhythms do not significantly overlap (**B**, bottom left); however, in long days they do (**B**, bottom right). This overlap (represented by the green arrows **B**, bottom right) is required to increase expression of the floral stimulus and promote flowering.

toperiodic response is controlled by modulating the expression of flowering time genes in response to both the circadian clock and daylength.

The genes that have been identified so far provide a general outline of the processes that regulate the photoperiodic response, but major questions remain. For example, we propose that inductive photoperiods promote flowering by changing the phase of expression of flowering time genes (Figs. 3, 4). This model is supported by the effects of different photoperiods on the pattern of expression of GI,[25] but direct experimental evidence that this influences flowering time is required. This may also lead to the identification of factors that activate flowering time gene proteins in one phase of expression but not in another. The potential role of CRY2 and PHYA in this area is intriguing, since they have an effect on the photoperiodic response but not circadian clock entrainment.[54] Furthermore, the analysis of flowering time mutants has identified genes that are important in the generation of circadian rhythms. Whether these genes act within the central oscillator is not clear but can be tested using approaches similar to those used in other systems.[22] The identification of genes that underlie the photoperiod response in *Arabidopsis* will also provide probes to address how the function of these genes changes in plants with different response types, such as SD plants whose flowering response is activated by entirely different photoperiod regimes.

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Note added in proof

A second paper describing the isolation of GIGANTEA appeared after submission of this review. Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG. Control of Circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science 1999; 285:1579–1582.

References

1. Garner WW, Allard HA. Effects of the relative length of night and day and other factors of the environment on growth and reproduction in plants. J Agric Res 1920;18:553–606.
2. Thomas B, Vince-Prue D. Photoperiodism in plants. San Diego: Academic Press; 1997.
3. Moore PD. Opening time by degrees. Nature 1995;375:186–187.
4. Bünning E. Die endonome tagesrhythmik als grundlage der photoperiodischen reaktion. Ber Deutsch Bot Ges 1936;54:590–607.
5. Hamner KC. Photoperiodism and circadian rhythms. Cold Spring Harbor: Symposia on Quantitative Biology 1960;25:269–277.
6. Cumming BG, Hendricks SB, Borthwick HA. Rhythmic flowering responses and phytochrome changes in a selection of Chenopodium rubrum. Can J Botany 1965;43:825–853.
7. Burn JE, Smyth DR, Peacock WJ, Dennis ES. Genes conferring late flowering in *Arabidopsis* thaliana. Genetics 1993;90:147–155.
8. Clarke JH, Dean C. Mapping *FRI*, a locus controlling flowering time and vernalisation response in *Arabidopsis thaliana*. Mol Gen Genet 1994;242:81–89.
9. Koornneef M, Blankestijn-de vries H, Hanhart C, Soppe W, Peters T. The

phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg erecta wild-type. Plant J 1994;6:911–919.
10. Lee I, Michaels SD, Masshardt AS, Amasino RM. The late flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of *Arabidopsis*. Plant J 1994;6:903–909.
11. Telfer A, Poethig RS. *HASTY*: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. Development 1998;125:1889–1898.
12. Bassiri A, Irish EE, Poethig RS. Heterochronic effects of *teopod-2* on the growth and photosensitivity of the maize shoot. Plant Cell 1992;4:497–504.
13. Lawson RJR, Poethig RS. Shoot development in plants — time for a change. Trends Genet 1995;11:263–268.
14. Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W. Genetic control of flowering time in *Arabidopsis*. Annu Rev Plant Physiol Plant Mol Biol 1998; 49:345–370.
15. Pineiro M, Coupland G. The control of flowering time and floral identity in *Arabidopsis*. Plant Physiol 1998;117:1–8.
16. Levy YY, Dean C. The transition to flowering. Plant Cell 1998;10:1973–1989.
17. Martinez-Zapater JM, Somerville CR. Effect of light quality and vernalization on late flowering mutants of *Arabidopsis thaliana*. Plant Physiol 1990;92: 770–776.
18. Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES. The FLF MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalisation and methylation. Plant Cell 1999;11:445–458.
19. Michaels SD, Amasino RM. Flowering locus C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 1999;11:949–956.
20. Burn JE, Bagnall DJ, Metzger JD, Dennis ES, Peacock WJ. DNA methylation, vernalization, and the initiation of flowering. Proc Natl Acad Sci USA 1993; 90:287–291.
21. Chandler J, Wilson A, Dean C. *Arabidopsis* mutants showing an altered response to vernalization. Plant J 1996;10:637–644.
22. Dunlap J. Molecular bases for circadian clocks. Cell 1999;96:271–290.
23. Kay SA, Millar AJ. New models in vogue for circadian clocks. Cell 1995;83: 361–364.
24. Carpenter CD, Kreps JA, Simon AE. Genes encoding glycine-rich *Arabidopsis thaliana* proteins with RNA-binding motifs are influenced by cold treatment and an endogenous circadian rhythm. Plant Physiol 1994;104:1015–1025.
25. Fowler S, Lee K, Onouchi H, Richardson K, Samach A, Morris B, Coupland G, Putterill J. Molecular characterisation of *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis*. EMBO J 1999;18:4679–4688.
26. Millar AJ, Kay SA. Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. Plant Cell 1991;3:541–550.
27. Wang ZY, Tobin EM. Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell 1998;93:1207–1217.
28. Engelmann W, Simon K, Phen CJ. Leaf movement in *Arabidopsis thaliana*. Z Naturforsch 1994;47:925–928.
29. Webb AAR. Stomatal rhythms. In: Lumsden PJ, Millar AJ, editors. Biological rhythms and photoperiodism in plants. Oxford: BIOS Scientific; 1998.
30. Dowson Day MJ, Millar AJ. Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis*. Plant J 1999;17:63–71.
31. Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre I, Coupland G. The late elongated hypocotyl mutation in Arabidopisis disrupts circadian rhythms and the photoperiodic control of flowering. Cell 1998;93:1219–1229.
32. Wang ZY, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM. A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* Lhcb gene. Plant Cell 1997;9:491–507.
33. Green RM, Tobin EM. Loss of the *Circadian clock associated 1* protein in *Arabidopsis* results in altered clock regulated gene expression. Proc Natl Acad Sci USA 1999;96:4176–4179.
34. Hicks KA, Millar AJ, Carre IA, Somers DE, Straume M, Meeks-Wagner DR, Kay SA. Conditional circadian dysfunction of the *Arabidopsis early-flowering 3* mutant. Science 1996;274:790–792.
35. Zagotta MT, Hicks KA, Jacobs CI, Young JC, Hangarter RP Meeks-Wagner DR. The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. Plant J 1996;10:691–702.
36. Liu XL, Meeks-Wagner DR. ELF3 in the flowering signal transduction pathway. Keystone meeting, interactions and intersections in plant signalling pathways. 1999;(Abstract)218.
37. Millar AJ, Carre IA, Strayer CA, Chua NH, Kay SA. Circadian clock mutants in *Arabidopsis* identified by Luciferase imaging. Science 1995;267:1161–1163.
38. Somers DE, Webb AAR, Pearson M, Kay SA. The short-period mutant, toc1-1, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development 1998;125:485–494.
39. Kreps JA, Simon AE. Environmental and genetic effects on circadian clock-regulated gene expression in *Arabidopsis*. Plant Cell 1997;9:297–304.
40. Hamblen MJ, White NE, Emery PTJ, Kaiser K, Hall JC. Molecular and behavioral analysis of four *period* mutants in *Drosphila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 1998; 149:165–178.
41. Ahmad M, Cashmore AR. HY4 gene of A. thaliana encodes a protein with characteristics of a blue-light photoreceptor. Nature 1993;366:162–166.
42. Lin C, Ahmad M, Cashmore AR. *Arabidopsis* cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. Plant J 1996;10:893–902.
43. Guo H, Yang H, Mockler TC, Lin C. Regulation of flowering time by *Arabidopsis* photoreceptors. Science 1998;279:1360–1363.
44. Lin C, Yang H, Guo H, Mockler T, Chen J, Cashmore AR. Enhancement of blue-light sensitivity of *Arabidopsis* seedlings by a blue light receptor cryptochrome 2. Proc Natl Acad Sci USA 1998;95:2686–2690.
45. Sharrock RA, Quail PH. Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes Dev 1989;3:1745–57.
46. Whitelam GC, Devlin PF. Roles of different phytochromes in *Arabidopsis* photomorphogenesis. Plant Cell Environ 1997;20:752–758.
47. Reed JW, Nagatani A, Elich TD, Fagan M, Chory J. Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol 1994;104:1139–1149.
48. Dehesh K, Franci C, Parks BM, Seeley KA, Short TW, Tepperman JM, Quail PH. *Arabidopsis* HY8 locus encodes phytochrome A. Plant Cell 1993;5: 1081–1088.
49. Nagatani A, Reed JW, Chory J. Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol 1993;102: 269–277.
50. Somers D, Sharrock R, Tepperman J, Quail P. The hy3 long hypocotyl mutant of *Arabidopsis* is deficient in phytochrome B. Plant Cell 1991;3:1263–1274.
51. Reed JW, Nagpal P, Poole DS, Furuya M, Chory J. Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell 1993;5: 147–157.
52. Smith H, Whitelam GC. The shade avoidance syndrome: multiple responses mediated by multiple phytochromes. Plant Cell Environ 1997;20:840–844.
53. Millar AJ, Straume M, Chory J, Chua NH, Kay SA. The regulation of circadian period by phototransduction pathways in *Arabidopsis*. Science 1995;267: 1163–1166.
54. Somers DE, Devlin PF, Kay SA. Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. Science 1998;282:1488–1490.
55. Zhong HH, Resnick AS, Straume M, McClung CR. Effects of synergistic signaling by phytochrome A and cryptochrome1 on circadian clockB regulated catalase expression. Plant Cell 1997;9:947–955.
56. Ahmed M, Jarillo JA, Smirnova AR, Cashmore AR. The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A in vitro. Mol Cell 1998;1:939–948.
57. Brown JAM, Klein WH. Photomorphogenesis in *Arabidopsis* thaliana (L.) Heynh, threshold intensity and blue-far-red synergism in floral induction. Plant Physiol 1971;47:393–399.
58. Goto N, Kumagai T, Koornneef M. Flowering responses to light-breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long-day plant. Physiol Plant 1991;83:209–215.
59. Mozley D, Thomas B. Developmental and photobiological factors affecting photoperiodic induction in *Arabidopsis thaliana* Heynh. Landsberg erecta. J Exp Botany 1995;46:173–179.
60. Hempel FD, Feldman LJ. Bi-directional influorescence development in *Arabidopsis thaliana*: acropetal initiation of flowers and basipetal initiation of paraclades. Planta 1995;192:276–286.
61. Corbesier L, Gadisseur I, Silvestre G, Jacqmard A, Bernier G. Design in

Arabidopsis thaliana of a synchronous system of floral induction by one long day. Plant J 1996;9:947–52.
62. Hempel FD, Zambryski PC, Feldman LJ. Photoinduction of flower identity in vegetatively biased primordia. Plant Cell 1998;10:1663–1676.
63. Bagnall DJ, King RW, Hangarter RP. Blue-light promotion of flowering is absent in hy4 mutants of *Arabidopsis*. Planta 1996;200:278–280.
64. Koornneef M, Hanhart CJ, van der Veen JH. A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet 1960:229:57–66.
65. Mockler TC, Guo H, Yang H, Duong H, Lin C. Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. Development 1960;126:2073–2082.
66. King R, Bagnall D. Photoreceptors and the photoperiodic response controlling flowering in *Arabidopsis*. Semin Cell Dev Biol 1996;7:449–454.
67. Johnson E, Bradley M, Harberd NP, Whitelam GC. Photoresponses of light-grown phyA mutants of *Arabidopsis*. Plant Physiol 1994;105:141–149.
68. Putterill J, Robson F, Lee, K, Simon R, Coupland G. The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell 1995;80:847–857.
69. Redei GP. Supervital mutants of *Arabidopsis*. Genetics 1962;47:443–460.
70. Martinez-Zapater JM, Somerville CR. Effect of light quality and vernalization on late-flowering mutants of *Arabidopsis* thaliana. Plant Physiol 1990:92: 770–776.
71. Koornneef M, Alonso-Blanco C, de Vries HB, Hanhart CJ, Peeters AJ. Genetic interactions among late-flowering mutants of *Arabidopsis*. Genetics 1998;148:885–892.
72. Araki T, Komeda Y. Analysis of the role of the late-flowering locus *Gl* in the flowering of *Arabidopsis thaliana*. Plant J 1993;3:231–239.
73. Koornneef M, Hanhart C, Van Loenen-Martinet P, de Vries HB. The effect of daylength on the transition to flowering in phytochrome-deficient, late-flowering and double mutants of *Arabidopsis thaliana*. Physiol Plant 1995;95:260–266.
74. Eimert K, Wang SM, Lue WL, Chen J. Monogenic recessive mutation causing both late floral initiation and excess starch accumulation in *Arabidopsis*. Plant Cell 1995;7:71–82.
75. Ruiz-Garcia L, Madueno F, Wilkinson M, Haughn G, Salinas J, Martinez-Zapater JM. Different roles of flowering-time genes in the activation of floral initiation genes in *Arabidopsis*. Plant Cell 1997;9:1921–1934.
76. Kardailsky I, Harrison M, Weigel D. A pair of homologous genes with antagonistic effects on flowering time. 9th International Conference on *Arabidopsis* Research Abstracts 1998;188.
77. Araki T, Kobayashi Y, Kaya H, Iwabuchi M. The flowering-time gene FT and regulation of flowering in *Arabidopsis*. J Plant Res 1998;111:277–281.
78. Bradley D, Ratcliffe O, Vincent C, Carpenter R, Coen E. Inflorescence commitment and architecture in *Arabidopsis* Science 1997;275:80–83.
79. Simon R, Igeno IM, Coupland G. Activation of floral meristem identity genes in *Arabidopsis*. Nature 1996;384:59–62.
80. Pittendrigh CS. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symposia on Quantitative Biology 1960;25: 159–184.
81. Millar AJ, Kay SA. Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in *Arabidopsis*. Proc Natl Acad Sci USA 1996;93:15491–15496.
82. Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A. Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science 1995;269:1863–1865.
83. Friedman H, Goldschmidt E, Halevy AH. Involvement of calcium in the photoperiodic flower induction process of *Pharbitis nil.* response of the Plant Physiol 1990;89:530–534.