

**THE MOLECULAR BIOLOGY AND BIOTECHNOLOGY
OF FLOWERING**
Second Edition

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THE MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF FLOWERING

Second Edition

Edited by

Brian R. Jordan

*Lincoln University
Canterbury, New Zealand*



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Preface

In 1993 *The Molecular Biology of Flowering* was published. At that time the physiological events associated with flowering had been well characterized and yet the underlying molecular mechanisms remained unknown. This book captured the spirit of molecular research that was beginning to provide a greater understanding of the flowering process. In the late 1980s and early 1990s, homeotic genes such as *floricaula* (*flo*) had been isolated and established as playing important roles in the transition of the vegetative to the floral apex. Furthermore, genes that are involved in organogenesis had been identified and provided insight for the development of the ABC model of floral morphogenesis. The advent of molecular approaches opened new avenues of research and seemed to provide the 'Dawn of a New Age'.

This second edition of the book (with slightly modified title) documents the progress that has been made since those early days. In developing the overall theme for the book, I have tried to update the research for subject areas covered by the original book and, whenever possible, involve the same authors. Using this approach I hope to allow the reader to appreciate the developments that have taken place in the last 12 years. Two examples will emphasize this point. In Chapter 1, Brian Thomas *et al.* cite recent molecular studies that provide convincing evidence that FT mRNA induced in the leaf is then transferred to the apex where it initiates flowering. Has the molecular mechanism of florigen or anti-florigen action now been discovered after 80 years of investigation? In Chapter 11, Rod Scott *et al.* state 'Twelve years ago little was known about genetic control of stamen development beyond specification of stamen primordia by floral homeotic genes. New footholds have been established in several areas, notably patterning of the microsporangium, regulation of meiosis and anther dehiscence'. This increase in knowledge, both in breadth and depth, is clearly reflected in these reviews. In addition, it is very apparent that since 1993, molecular genetics has been applied to a much wider range of related areas. Thus, topics such as the evolution of flowers, floral senescence and

apomixis have been included in the new edition. These reviews add different perspectives and provide further stimulus to investigate and enhance our understanding. To some extent I feel research on flowering has not had the prominence it deserves; molecular biology and the commercial opportunities of biotechnology have started to redress this situation. There is still much to learn, but I believe that this volume will contribute substantially to increase the understanding of this vitally important process.

Most importantly, I would like to thank the authors for their time and considerable effort in producing these excellent reviews. In a period when scientists are under considerable work pressure, with a variety of demands on their time, I am very appreciative of their willingness to contribute their knowledge and expertise to this volume. It is also particularly rewarding for me to read the contributions from colleagues working on flowering from all parts of the world. Thanks, once again, to Tim Hardwick of CABI for his support.

Finally, I would like to thank my invaluable assistant, Bronwyn Hamilton, without whose patient endeavours the smooth preparation of this book would not have taken place.

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September 2005

I

External and Internal Regulation of Flowering

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1

Photoperiodism and Flowering

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Introduction

Photoperiodism can be defined as the response to changes in daylength that enables plants (or any other living organism) to adapt to seasonal changes in their environment. Except at the equator, the passage of the year is marked by a continuous but highly reproducible variation in the length of the day. In order to locate the time of year accurately, a timekeeping mechanism operates with precision as part of the plant's photoperiodic sensing mechanism in a way that is insensitive to less predictable variations in the environment such as temperature. Photoperiod alone is not an unambiguous signal as any particular daylength occurs twice in an annual cycle. Progressive changes in daylength, which are at their greatest around the equinoxes in spring and autumn, do, however, provide a certain environmental signal for the passage of the seasons (Thomas and Vince-Prue, 1997). The seasonal range and rate of change of daylength is lower in the tropics than at higher latitudes and photoperiodic mechanisms need to be sufficiently precise and flexible to operate across the entire range of daylengths.

The ability to detect seasonal change and respond to it confers a selective advantage to plants because it provides a means of anticipating, and consequently preventing, the adverse effects of a particular seasonal environment. Photoperiodic plants are common, even in tropical latitudes where the seasonal daylength changes are small, and daylength is used to synchronize reproductive or other activities with seasonal events such as dry or rainy periods. Coincident flowering in members of a population increases the chances of outbreeding and hence genetic recombination. For this reason, synchronization of floral initiation through photoperiodic sensitivity can confer advantages independently of whether reproduction is matched with a particular favourable environment. A further potential benefit of photoperiodic responses is that they can enable organisms to occupy an ecological niche in space and time. For example,

a response to short days can enable a woodland plant to flower and seed before the dense leaf canopy is formed in the spring.

Photoperiodic control of flowering is also important in agriculture and horticulture. Breeding to extend latitudinal range or altered timing of flowering involves understanding and exploiting variation in the photoperiodic responses of a particular species. Daylength manipulation in order to schedule flower production is also a common practice.

This chapter deals with mechanisms underlying the photoperiodic control of flowering and is divided into three sections: (i) the first describes the physiological background to molecular and genetic studies of the photoperiodic control of flowering; (ii) the second details recent progress in understanding the genetic regulatory networks controlling flowering in response to daylength; and (iii) the third covers the molecular and genetic basis of the underlying timekeeping mechanisms for plant photoperiodism.

Physiology of Photoperiodism

Discovery and variation

The response of plants to the daily duration of light was proposed independently by Julien Tournois and Hans Klebs at the beginning of the 20th century. It was the American physiologists Garner and Allard (1920), however, who first saw clearly that flowering and many other responses in plants could be accelerated either by long days (LDs) or by short days (SDs), depending on the plant. They were led to their discoveries by studies on 'Maryland Mammoth' variety of tobacco that failed to flower and grew large in summer, while plants grew under glass during winter and early spring, and soybean, in which for a particular variety, flowering tended to occur at the same time in the field, irrespective of planting date. After experimentally eliminating temperature and light intensity as causal factors they concluded that the tobacco and soybean plants would only flower if the duration of the daylight period was sufficiently short.

They introduced the terms photoperiod and photoperiodism and classified plants into the photoperiodic groups in use today. Short-day plants (SDPs) are those that flower or in which flowering is accelerated by days which are shorter than a critical daylength. Long-day plants (LDPs) are plants that flower or in which flowering is accelerated when the daylight period exceeds a critical daylength. Plants that flower at the same time irrespective of the photoperiodic conditions are called day-neutral plants (DNPs). Plants that respond to daylength can be further subdivided into obligate (or qualitative) types, where a particular daylength is essential for flowering, or facultative (or quantitative) types, where a particular daylength accelerates but is not essential for flowering. Many important crop species are potentially photoperiodic, e.g. many cereals such as wheat and barley are LDPs, while SDPs include rice and soybean (see Table 1.1). The model plant for molecular genetic studies, *Arabidopsis thaliana*, is a typical facultative LDP under this classification.

Table 1.1. Examples of LDP and SDP, including some important crop species. (Modified from Thomas and Vince-Prue, 1997.)

SDP (qualitative or absolute)	SDP (quantitative)
<i>Coffea arabica</i>	<i>Cannabis sativa</i>
<i>Fragaria</i> × <i>ananassa</i>	<i>Cucumis sativus</i> (some cvs)
<i>Glycine max</i> Biloxi	<i>Glycine max</i>
<i>Hibiscus cannabinus</i> ; <i>esculentus</i>	<i>Gossypium</i>
<i>Humulus japonicus</i> ; <i>lupulus</i>	<i>Helianthus annuus</i> ; <i>tuberosus</i>
<i>Kalanchoë blossfeldiana</i>	<i>Nicotiana tabacum</i>
<i>Nicotiana tabacum</i> Maryland Mammoth	<i>Oryza sativa</i>
<i>Oryza sativa</i>	<i>Rhododendron</i> spp. (florist's azalea)
<i>Perilla</i> (red)	<i>Ricinus communis</i>
<i>Pharbitis nil</i>	<i>Rosa gallica</i> ; <i>rugosa</i>
<i>Xanthium strumarium</i>	<i>Sesamum indicum</i>
	<i>Sorghum bicolor</i> ; <i>halepense</i>
	<i>Zea mays</i>
LDP (qualitative or absolute)	LDP (quantitative)
<i>Avena sativa</i> (spring strains)	<i>Allium ampeloprasum</i>
<i>Brassica juncea carinata</i> ; <i>pekinensis</i>	<i>Antirrhinum majus</i>
<i>Delphinium elatum</i> , garden hybrids	<i>Arabidopsis thaliana</i>
<i>Fuchsia hybrida</i>	<i>Dianthus carthusianorum</i> Napoleon III; <i>caryophyllus</i> (glasshouse carnations)
<i>Hyoscyamus niger</i> (annual)	<i>Eustoma grandiflorum</i>
<i>Jasminium grandiflorum</i>	<i>Hemerocallis fulva</i>
<i>Lemna gibba</i> ; <i>minor</i>	<i>Hordeum vulgare</i> (spring strains)
<i>Lolium temulentum</i> Ceres	<i>Linum usitatissimum</i>
<i>Nicotiana sylvestris</i>	<i>Lolium temulentum</i> Ba 3081
<i>Papaver somniferum</i>	<i>Medicago sativa</i>
<i>Silene armeria</i> ; <i>coeli-rosa</i>	<i>Petunia hybrida</i>
<i>Trifolium pratense</i> (English Montmorency)	<i>Pisum sativum</i>
	<i>Secale cereale</i> (spring cvs)
	<i>Solanum tuberosum</i>
	<i>Triticum aestivum</i> (spring cvs)
	<i>Vicia faba</i>

The length of the day and night are mutually linked within the 24-h daily cycle. Photoperiodic responses could therefore be theoretically determined by either the length of the day or the length of the night. Classic experiments with SDP *Xanthium* revealed that flowering only occurred if the night length was greater than 8.5 h, irrespective of the relative durations of light and darkness in the experimental cycle (Hamner and Bonner, 1938). SDs did not cause flowering if they were coupled with short nights but when the night was sufficiently long, flowering occurred even when the accompanying light periods were long. However, although a sufficiently long dark period appeared to be the decisive factor for flowering to occur, the level of flowering was also affected by the length of the light period. This indicated that the interaction between light and darkness formed part of the daylength-sensing mechanism. If a long night is

interrupted by a short (e.g. 30 min) period of light (or night break, NB) near the middle, SDPs respond as if they have been exposed to an LD. For LDPs, such NBs are only effective if given in combination with daylengths that are just longer than those needed to permit flowering or if they are of several hours duration. Also, in LDPs, unlike in SDPs, the amount and spectral composition of the light given during the day period, especially in the latter part, has a large effect on flowering. If the response to daylength depends primarily on the length of the dark period, the plants are called dark-dominant and conversely, if the light period is the main influence, they are called light-dominant (Thomas and Vince-Prue, 1997). In general, most SDPs are dark-dominant and most LDPs are light-dominant.

Transmissible signals

A common feature of photoperiodism appears to be that daylength perception is a separate process from the response to photoperiod. When either the leaves or the shoot tips of photoperiodically sensitive plants are exposed to different daylengths, flowering depends on the daylength given to the leaves and not to the apex (Knott, 1934). In several instances, leaves from plants, which have been given a daylength treatment that initiates flowering have been grafted on to plants that have not been exposed to permissive daylengths, with the result being flowering in the receptor plants. Daylength therefore is perceived in leaves and results in a localized change in the properties of that leaf. Flowering then occurs as a result of a signal transmitted from the leaves to the apex. The change in the leaf is termed induction, while the response at the apex leading to the initiation of flowering is sometimes called evocation.

From grafting experiments, it is known that in some species, induced leaves are independently capable of generating a flowering stimulus over many days or even weeks while in others, favourable cycles must be continued until the apex has become recognizably floral, indicating the need for a continual supply of a floral stimulus (presumably from the leaf) if flower development is to be sustained. An intact and functioning nutrient transport system is a requirement for communication between the sites of perception in the leaves and the sites of response. Daylength response is lost when the pathway is disrupted by removing the source leaf, inhibiting transport by localized heat or cold treatments applied at intermediate points in the transport path or stem girdling. Also, when leaves taken from an induced plant are grafted on to a receptor plant, promotion of flowering or other responses occurs only when a graft union has developed.

It was proposed by Chailakhyan (1936) more than half a century ago that the signal passing between leaves and response sites is a specific flowering hormone: florigen. This idea was based on a series of experiments showing that grafting of leaves from one donor species to a separate receptor species could cause flowering (see Table 1.2). This strongly suggested the participation of common signals in different species. Other grafting experiments suggested that other substances inhibiting flowering may be involved; the appropriate day-

Table 1.2. Examples of successful transfer of flowering stimulus between species or genera following grafting. (After Thomas and Vince-Prue, 1997.)

Donor	Response type	Receptor	Response type
Intraspecific grafts			
<i>Glycine max</i> Agate	DNP	<i>G. max</i> Biloxi	SDP
<i>Chenopodium rubrum</i> 60° 47 N	SDP*	<i>C. rubrum</i> 34° 90 N	SDP
<i>Pisum sativum</i> various genetic lines	DNP or LDP*	<i>P. sativum</i> line G	LDP
Interspecific grafts			
<i>Gossypium hirsutum</i>	DNP	<i>G. davidsonii</i>	SDP
<i>Nicotiana tabacum</i> Delcrest	DNP	<i>N. sylvestris</i>	LDP
Intergeneric grafts			
<i>Blitum virgatum</i>	LDP	<i>Chenopodium rubrum</i>	SDP
<i>Chenopodium polyspermum</i>	SDP	<i>Blitum capitatum</i>	LDP
<i>Cucumis sativus</i>	DNP	<i>Sicyos angulatus</i>	SDP
<i>Centaurea cyanus</i>	LDP	<i>Xanthium strumarium</i>	SDP

length would then lead to removal of an antiflorigen rather than (or in addition to) synthesis of a floral hormone.

Genes Controlling Floral Initiation

Pathways to flowering in *Arabidopsis*

Flowering is generally regarded as a default process that will occur at some point in the plant's life. The time that a plant flowers, however, is affected by many environmental and endogenous factors and consequently there are numerous *genetic* pathways that are involved in the control of flowering time. These pathways interact in different ways depending upon endogenous signals and the environmental conditions thus enabling the plant to flower in the most favourable conditions. Our understanding of the genetic and signalling pathways controlling flowering has increased dramatically over the last decade, based largely on the analysis of the flowering responses of winter- and spring-annual genotypes of *A. thaliana*, a facultative LDP.

The predominance of the different pathways changes with the developmental state of the plant. Early on in the life cycle of the plant flowering is actively repressed to enable the plant to grow sufficiently large to be able to support the development of flowers, fruits and seeds. As the plant develops this repression is gradually lifted by what have been termed floral-enabling pathways such as the vernalization and autonomous pathways (Boss *et al.*, 2004). In certain environments there is also activation of floral-promotion pathways such as the photoperiodic, gibberellin (GA), ambient temperature and light-quality pathways. At some stage the point is reached when promotion is

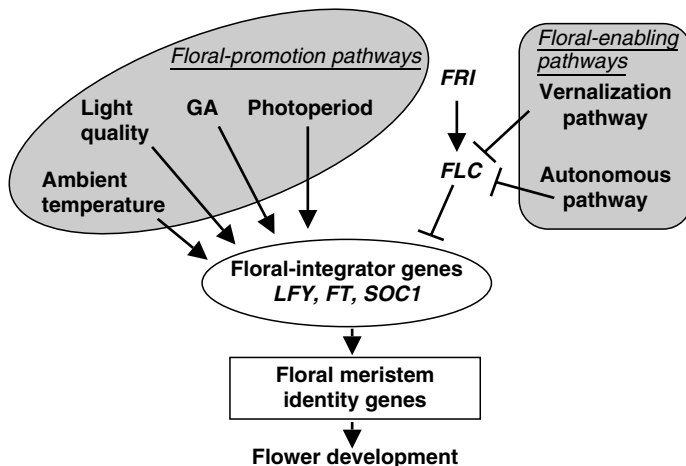


Fig. 1.1. Integration of the photoperiodic flowering pathway with other environmental and developmental pathways to flowering in *Arabidopsis*.

greater than repression and the flowering transition occurs. All the different flowering pathways converge on a small group of genes called floral-pathway integrators. These genes are *FLOWERING LOCUS T (FT)*, *LEAFY (LFY)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, and they are responsible for the activation of the floral meristem identity genes that direct floral organ formation (see Fig. 1.1). Activation of the floral integrators will thus directly result in flowering.

Expression of the floral-integrator genes is actively repressed by the floral inhibitor *FLOWERING LOCUS C (FLC)*. Consequently flowering is prevented until this repression is lifted by the floral-enabling pathways, thus allowing activation of the floral-pathway integrators by the floral-promotion pathways. The levels of *FLC* are maintained at a high level by the *FRIGIDA (FRI)* gene. The activity of the *FRI* gene and consequently the resulting levels of *FLC* are major determinants in flowering time in *Arabidopsis*. Mutations in the *FRI* gene are responsible for most of the variation in flowering time observed in different ecotypes of *Arabidopsis* (Johanson *et al.*, 2000). Loss-of-function mutations in the *FRI* gene are found in early flowering ecotypes of *Arabidopsis*, such as Landsberg *erecta* and Colombia. These mutations result in low *FLC* levels and only mild repression of the floral-pathway integrators. This low-level repression can be directly overridden by activation of a floral-promotion pathway, e.g. the photoperiodic pathway, without the need for a floral-enabling pathway to first lift the repression by *FLC*. In addition to *FRI* other genes are involved in the upregulation of *FLC*. These include *EARLY IN SHORT DAYS 4 (ESD4)*, *PHOTOPERIOD INDEPENDENT FLOWERING 1 (PIE1)*, *EARLY FLOWERING IN SHORT DAYS (EFS)* and *VERNALIZATION INDEPENDENCE (VIP)* genes. The mechanism of action of these genes is currently poorly understood.

Floral-enabling pathways

Two floral-enabling pathways result in the downregulation of the *FLC* repressor, the vernalization pathway and the autonomous pathway (Fig. 1.1). Vernalization, resulting from exposure to low temperatures (between 0°C and 10°C), is a quantitative response and causes progressive downregulation of the *FLC* gene until the response is saturated after several weeks (Chapter 2, this volume for further details). Once the vernalized state has been established it is stable throughout the life of the plant even if returned to warmer temperatures. It is thus mitotically stable through numerous cell divisions indicating that the repression of *FLC* expression is epigenetic. This epigenetic silencing of *FLC* is mediated by the *VERNALIZATION 1 (VRN1)* and *VERNALIZATION 2 (VRN2)* genes, which are involved in histone methylation and the formation of mitotically stable transcriptionally silent heterochromatin. Mutations in these genes prevent the stable repression of the *FLC* gene, and in the *vrn1* and *vrn2* mutants *FLC* expression increases back to normal as soon as the plant is returned to warm temperatures. The initial decrease in *FLC* expression is mediated in part by the *VERNALIZATION INSENSITIVE 3 (VIN3)* gene (Sung and Amasino, 2004). The *VIN3* gene is induced by long periods of cold treatment and is thought to be an early step in the vernalization process.

The autonomous pathway is the other floral-enabling pathway that acts to reduce the levels of *FLC* expression. There are several genes in this pathway, *FCA*, *FLOWERING LOCUS D (FLD)*, *FPA*, *FVE*, *FY*, *LUMINIDEPENDENS (LD)* and *FLOWERING LOCUS K (FLK)*, which act in different ways to repress *FLC*. *FVE* is thought to act together with *FLD* in a histone deacetylation complex and repress *FLC* expression by deacetylating *FLC* chromatin. *FCA*, *FPA*, *FY* and *FLK* all appear to have roles in RNA processing although there is no direct evidence that they directly affect *FLC* RNA. Active *FCA* mRNA levels are low at germination but increase significantly in meristems 4–5 days after germination, at a time when the floral-enabling pathways are likely to become active (Macknight *et al.*, 2002).

Floral-promotion pathways

Of the floral-promotion pathways, the photoperiodic pathway is probably the best understood. Key elements include a gene named *CONSTANS*, or *CO*, which encodes a nuclear protein with two zinc fingers at the amino terminus and a conserved carboxyl-terminal domain, known as the CCT domain for the three plant proteins in which it was identified (*CO*, *COL*, *TIMING OF CAB1 (TOC1)*). *CO* is regulated both transcriptionally and post-transcriptionally. Transcription of the *CO* mRNA is controlled by the circadian clock and it is also upregulated by the nuclear protein *GIGANTEA (GI)*. *CO* protein stability is affected by the action of different photoreceptors. Under SD conditions, *CO* protein levels remain low at all times, but under LD conditions, the combined action of the blue light photoreceptor *CRYPTOCHROME 2* and of the far-red light photoreceptor *PHYTOCHROME A (PHYA)* promotes accumulation of

the CO protein at the end of the light period. This in turn activates transcription of the floral-pathway integrators *FT* and *SOC1* (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). The mechanism by which accumulation of the CO protein is modulated by daylength will be described in further detail below.

Recently evidence has emerged for the existence of a light-quality pathway that acts independently of CO and the photoperiodic pathway. This pathway acts through *PHYTOCHROME B* (*PHYB*) and a nuclear protein called *PHYTOCHROME AND FLOWERING TIME 1* (*PFT1*) to upregulate *FT* levels in response to low red light to far-red light (R/FR) ratios (Cerdan and Chory, 2003). The effects of light quality on the activation of *FT* (either acting through CO or *PFT1*) can explain the promotion of flowering by vegetative shade which is part of the shade-avoidance response observed when plants are grown in environments with low R/FR ratios.

Temperature has also been shown to affect *PHYB*-mediated control of flowering. At 22°C *PHYB* is the predominant photoreceptor mediating the repression of flowering by red light, but at 16°C this repression is mediated through *PHYTOCHROME E* (*PHYE*) (Halliday *et al.*, 2003). *PHYA*-mediated promotion of flowering through the photoperiodic pathway is also temperature-dependent and *PHYA* promotes flowering at 23°C but not at 16°C (Blazquez *et al.*, 2003).

The other floral-promotion pathway is the GA pathway. In *Arabidopsis* GA promotes flowering, and mutations in genes involved in GA biosynthesis or response, such as *gibberellic acid 1* (*ga1*) or *gibberellic acid insensitive* (*gai*), respectively, cause late flowering in SDs. The effect of these mutations is not observed in LDs because the photoperiodic pathway is actively promoting flowering in these conditions, however in SDs the GA pathway is the major floral-promotion pathway (Reeves and Coupland, 2001). It is thought that GA acts by inducing the expression of a MYB-like transcription factor AtMYB33 that binds to a motif in the *LFY* promoter and induces *LFY* expression. This GA-responsive element is distinct from a photoperiod-responsive element, which is also present in the *LFY* promoter. Thus, *LFY* acts to integrate signals from the GA and from the photoperiodic-response pathways. GA may regulate the expression of the other floral-integrator genes *SOC1* and *FT* as well. Interestingly, the expression of GA 20-oxidase, an enzyme involved in GA biosynthesis, is reported to be higher in LDs than SDs; and genes encoding both GA 20-oxidase and 3 β -hydroxylase (which catalyses a later step in the pathway) are induced by red light and downregulated by far-red light. Whether the influence of photoperiod and light quality on GA biosynthesis results in altered control of flowering by the GA pathway is not yet known.

Floral-promotion pathways do not act in isolation but interact with floral-enabling pathways. The photoperiodic pathway promotes flowering through activation of the floral-integrator genes *FT* and *SOC1*, but this is not possible when high levels of *FLC* are present. Thus, activation of the *SOC1* gene by overexpression of CO can be blocked by overexpression of *FLC*. The *FLC* protein binds to an element in the *SOC1* promoter that presumably prevents induction of the gene by CO (Hepworth *et al.*, 2002). *FLC* also directly downregulates the photoperiodic pathway as high levels of *FLC* repress

CRY2 expression thus preventing the *CRY2*-mediated promotion of CO activity (El-Assal *et al.*, 2003). The photoperiodic pathway can thus only induce flowering once the FLC-mediated repression has been alleviated by the floral-enabling pathways, or in early flowering ecotypes where mutations to the *FRI* gene result in only low levels of *FLC* expression. This interaction between FLC and the photoperiodic pathways provides a mechanism for plants such as winter annual or biennials to delay flowering until winter has passed (and vernalization has reduced the levels of FLC) and the warm weather of late spring or summer has arrived (when LDs will activate the photoperiodic-promotion pathway).

Mechanism of Photoperiodic Time Perception

The circadian clock as the timing mechanism

The timing mechanism used in photoperiodism seems in most cases to be based on endogenous circadian rhythms in light sensitivity, as first postulated by Bünning (1936). He suggested that the circadian clock consisted of two half cycles, photophil and scotophil. When light was received in the scotophilic phase, the daily cycle was perceived as an LD, but the absence of light during the scotophilic phase produced an SD response. This idea was further refined by Pittendrigh and Minis (1964) to form the external coincidence model. In this model, a signal was produced when an environmental signal (light) coincided with the sensitive phase of an endogenous circadian rhythm of photoresponsiveness.

Support for these circadian clock-based models was initially provided by physiological studies (reviewed in Thomas and Vince-Prue, 1997). Thus, responsiveness to NBs was rhythmic in SD species and oscillated with a period of approximately 24 h. Results obtained with LDPs also supported this model, but suggested that the characteristics of the interaction of light and circadian rhythms were not the same in SDPs and LDPs. SDPs often exhibit a qualitative requirement for inductive photoperiods and can frequently be induced to flower in response to a single inductive light–dark cycle. In such plants the circadian rhythm of responsiveness to NBs is entrained by the dusk signal, so that the photoinducible phase always occurs at about the same time in darkness (Lumsden *et al.*, 1982). Light received during the photoinducible phase (dawn or an NB) prevents the SD response. Thus it is easy to think that the circadian rhythm in light sensitivity provides the timing base that allows the length of the dark period to be distinguished by the plant.

For LDPs or light-dominant plants the situation is not quite as straightforward. Because sensitivity to NBs is much reduced, it is very difficult to do NB experiments. Rhythms in response to an NB have been described in the LDPs but the pattern of response varies with the duration of the experimental dark period (Perilleux *et al.*, 1994) suggesting an interaction with the subsequent light period. A circadian rhythm in light sensitivity in constant light can be seen when far-red light is added to a background of white fluorescent or red light

(Deitzer *et al.*, 1982) indicating that the rhythm in light sensitivity continues to run in constant light.

The link between circadian rhythms and the circadian clock has been firmly established by recent studies using *Arabidopsis*, a facultative LDP. First, abnormal function of the circadian clock in *Arabidopsis* mutants was correlated with defective floral responses to photoperiod. For example, the short-period mutant *timing of cab 1-1 (toc1-1)* flowered earlier than wild-type under SD conditions, whereas the long-period mutant *zeitlupe (zt1)* exhibited delayed flowering under LD conditions (Somers *et al.*, 1998b, 2004). The complete arrhythmia of the *lhy-1* and *elf3* mutants, observed in constant light, was correlated with daylength-insensitive flowering (Hicks *et al.*, 1996; Schaffer *et al.*, 1998). Yet the causal link between the circadian clock defect and the flowering-time phenotype remained unclear as seemingly equivalent circadian phenotypes were in some cases associated with opposite effects on flowering times. In order to address this question, Roden *et al.* (2002) investigated the effects of experimental conditions that artificially altered the phase of circadian rhythms relative to the light and dark portion of the environmental cycle. One of the fundamental properties of the circadian clock is that it will advance or delay its phase (that is, shift its rhythm forward or back relative to dawn and dusk) when entrained to light–dark cycles that are either longer or shorter than 24 h, respectively. When *Arabidopsis* plants were exposed to a range of these atypical light–dark cycles, floral responses were not determined by the number of light or dark hours within a cycle but reflected how much transcription of the clock-controlled gene *CO* coincided with light. These results were consistent with a model in which expression of *CO* under the control of the circadian clock mediates rhythmic changes in photoresponsiveness and perception of external coincidence with light.

Clock mechanism

Circadian rhythms (from *circa*, approximately, and *dies*, day) have been described in a wide range of organisms ranging from cyanobacteria to mammals and at every level of organization. These rhythms all share the same fundamental properties: (i) their ability to become entrained, or synchronized, to diurnal changes in environmental conditions; (ii) persistence upon transfer to constant conditions; and (iii) a constant period over the physiological range of temperatures. In plants, the circadian clock controls expression of approximately 6% of the transcriptome (Harmer *et al.*, 2000). This includes genes encoding components of all major metabolic pathways as well as genes involved in hormone biosynthesis, photoreceptors and floral regulators such as *CO*, *FT* and *GI*.

The circadian oscillator of higher plants comprises transcriptional–translational feedback loops similar to those described earlier for fungal and animal clocks (Young and Kay, 2001). In *Arabidopsis*, the central oscillator comprises three key components named *LATE ELONGATED HYPOCOTYL (LHY)*, *CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1)* and *TIMING OF CAB1 (TOC1)*. The *LHY* and *CCA1* genes encode single MYB transcription

factors with largely overlapping functions. Expression of the *LHY* and *CCA1* mRNA levels oscillates and levels of both transcripts peak shortly after dawn. The *LHY* and *CCA1* proteins also are synthesized rhythmically with a lag of approximately 2 h after their cognate mRNAs. Both transcription factors bind to a promoter element known as the evening element and act to inhibit transcription of evening-specific genes, including *TOC1*. In the evening, the level of both repressors declines and transcription of the *TOC1* gene resumes. Accumulation of the *TOC1* protein at night promotes transcription from the *LHY* and *CCA1* promoters, thus initiating a new cycle (Alabadi *et al.*, 2001). It is not clear at this point how *TOC1* performs this function as the *TOC1* protein does not comprise a DNA-binding domain and does not exhibit features typical of any transcription-factor family. The *TOC1* protein comprises an N-terminal domain similar to response-regulator proteins of plant and bacterial two-component signalling systems, but lacks an aspartate residue that is required for phospho-transfer (Makino *et al.*, 2000; Strayer *et al.*, 2000). Another domain known as the CCT-domain is thought to play a role in protein–protein interactions since in yeast it mediated binding of CO and *TOC1* to the transcriptional regulator *ABI3* (Kurup *et al.*, 2000). *TOC1* may regulate transcription through its interaction with *ABI3*. It has also been shown to interact with a number of basic helix-loop-helix (bHLH) transcription factors including the phytochrome-interacting protein *PIF3* (Makino *et al.*, 2002; Yamashino *et al.*, 2003). This is interesting because *PIF3* binds a G-box motif (CCACTG?) within the promoters of the *LHY* and *CCA1* genes (Martinez-Garcia *et al.*, 2000). *TOC1* may therefore regulate expression of the *LHY* and *CCA1* mRNAs by modulating the activity of a light-responsive transcription factor, thus placing the oscillator very close to light-response mechanisms.

Disruption of the *LHY/CCA1/TOC1* feedback loop severely affected the ability of the oscillator to free-run in constant conditions. Thus, *lhy cca1* double mutants became gradually arrhythmic upon transfer to constant light or darkness, whereas plants in which expression of *TOC1* was inhibited using RNA interference (RNAi) technology also became arrhythmic in constant darkness and in constant red light but not under blue or white light (Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002; Mas *et al.*, 2003a). These results suggest that the *LHY/CCA1/TOC1* feedback loop functions as part of the oscillatory mechanism of the clock, but that its importance for self-sustained rhythmicity may vary with light conditions.

Evidence is accumulating that the plant circadian clock comprises additional interlocking feedback loops, similar to those described in animal and fungal clocks (Young and Kay, 2001). A number of other elements of the circadian systems have been identified that contribute to the positive regulation of *LHY* and *CCA1* expression. The *ELF3*, *ELF4* and *GI* transcripts are expressed at night with phases similar to *TOC1* (Fowler *et al.*, 1999; Hicks *et al.*, 2001; Doyle *et al.*, 2002). The *ELF3* gene is particularly interesting because its function is essential for rhythmicity in constant light, but not in constant darkness. *ELF3* has been proposed to negatively regulate light signals to the clock and to act to dampen effects of light at times when its effects on the clock might be deleterious (Covington *et al.*, 2001). A set of four rhythmically expressed

pseudo response-regulator proteins related to TOC1 has also been proposed to compose a regulatory feedback loop within the oscillator mechanism (Matsushika *et al.*, 2000). Expression of the *PRR9* transcript peaks shortly after dawn, followed at approximately 3-h intervals by *PRR7*, *PRR5* and *PRR3*. The *TOC1* transcript, also described as *PRR1*, describes the latest wave of gene expression. An increasing body of evidence suggests that all of the *PRR* genes function as part of the clock mechanism. Mutations in each of these genes caused relatively subtle effects on circadian period (Eriksson *et al.*, 2003; Michael *et al.*, 2003). However, plants lacking function of both *PRR7* and *PRR9* exhibited very long-period phenotypes in constant light and severely dampened rhythmicity in constant darkness (Farré *et al.*, 2005). Expression of *PRR7* and *PRR9* was increased in *LHY*- and *CCA1*-overexpressing plants and decreased in double loss-of-function mutants, suggesting a positive effect of *CCA1* and *LHY* on *PRR7* and *PRR9* expression levels. Despite its dramatic long-period phenotype the *prr7 prr9* double mutant exhibited nearly normal levels of *LHY*, *CCA1* and *TOC1* expression. Further work is therefore required to determine the organization of the multiple feedback loops within the plant circadian clock.

Synchronization of circadian clocks to light–dark cycles (often described as entrainment) is mediated by the action of multiple photoreceptors. In *Arabidopsis*, this includes at least four of the five phytochromes (PHYA, B, D and E) and both cryptochromes (CRY1, 2) (Somers *et al.*, 1998a,b; Devlin and Kay, 2000). In most organisms, effects of light on the clock are mediated by light-induced changes in the level of one of the oscillator components. Plants are unusual in this respect because light affects the oscillator at several levels. First, light promotes transcription of the *LHY* and *CCA1* genes in the morning (Martinez-Garcia *et al.*, 2000; Kim *et al.*, 2003). Expression of the LHY protein is boosted further by light-stimulated translation of the *LHY* mRNA (Kim *et al.*, 2003). Light also modulates the accumulation of the TOC1 protein through the effect of the F-box, Kelch-repeat LOV-domain protein ZEITLUPE (ZTL) (Somers *et al.*, 2000; Mas *et al.*, 2003b). The LOV domain of the ZTL protein is related to that found in the blue light photoreceptors CRYPTOCHROMES and PHOTOTROPINS and is thought to bind a flavin chromophore. F-box proteins act to target specific molecules for ubiquitination and degradation by the 26S proteasome. ZTL promotes degradation of the TOC1 protein in darkness but not in the light, suggesting that light may act to inhibit ZTL activity. It is likely that light modulates proteolytic degradation of additional clock molecules through the effects of DEETIOLATED 1 (DET1) and CONSTITUTIVELY MORPHOGENETIC 1 (COP1), since plants lacking either of these activities exhibit short-period phenotypes and since both of these proteins participate in the proteasome-mediated degradation of positive effectors of morphogenesis in the dark (Millar *et al.*, 1995; Schwechheimer and Deng, 2000).

Photoperception

The multiple effects of light on the timing mechanism are important determinants of photoperiodic responses as they serve to set the phase of the

photoperiodic-response rhythm. Yet they must be distinguished from separate effects of light mediating the floral response downstream of the clock. Two types of photoreceptors have a role in the latter: (i) phytochromes, which have maximum sensitivity in the red and far-red parts of the spectrum; and (ii) cryptochromes, the blue light photoreceptors.

There are five members of the phytochrome gene family in *Arabidopsis* and equivalent gene families in other species (Kendrick and Weller, 2004). Phytochromes are chromoproteins that contain identical tetrapyrrole chromophores (Lagarias and Rapoport, 1980). Mutants or transgenic plants in which chromophore biosynthesis is impaired will be incapacitated with regard to all of the functional phytochromes. Such plants show altered daylength responses, usually flowering earlier than wild types (Montgomery *et al.*, 1999, 2001; Sawers *et al.*, 2002). Different phytochromes have different roles in controlling plant development and this is the case for the photoperiodic-perception process. Thus, PHYA is required for normal daylength perception in *Arabidopsis* and in the LDP, pea (Johnson *et al.*, 1994; Weller *et al.*, 2001). PHYB is also essential for daylength perception in barley since the BMDR-1 mutant of barley, which contains a defective PHYB is insensitive to photoperiod (Hanumappa *et al.*, 1999). In contrast, *Arabidopsis* mutants deficient in PHYB flower earlier than wild type in both SD and LD but retain sensitivity to daylength (Reed *et al.*, 1994). PHYB, along with PHYD and PHYE promotes early flowering in *Arabidopsis* in response to low-red to far-red ratios (Halliday and Whitelam, 2003). This response plays a role in shade avoidance and is distinct from the role of PHYB in photoperiodism, which is inhibitory (Mockler *et al.*, 2003).

The cryptochromes are flavoproteins that mediate plant responses to blue light (Kendrick and Weller, 2004). Two members of the cryptochrome gene family (CRY1 and CRY2) are present in *Arabidopsis*. CRY2 is thought to be the major blue photoreceptor for flowering in *Arabidopsis*, although *cry1 cry2* double mutants flowered earlier in blue light than the single mutants, indicating that both cryptochromes play a role to promote flowering (Mockler *et al.*, 1999). Further evidence for a role for CRY2 comes from a study by El-Assal *et al.* (2001) in which a quantitative trait loci (QTL) for flowering time in *Arabidopsis* was accounted for by an allele of CRY2. The early flowering phenotype resulted from a single amino acid substitution that reduces the light-induced turnover of the CRY2 protein under short photoperiods. The participation of cryptochromes in the control of flowering in *Arabidopsis* is consistent with physiological studies, which have shown that blue light has a promotive effect on flowering for LDP of the *Cruciferae*, however, this is not necessarily true for other families (Thomas and Vince-Prue, 1997; Runkle and Heins, 2001). There is no physiological evidence for a specific role for blue light, and by inference cryptochromes, in SDP, but this remains to be confirmed in genetic and comparative genomic studies.

Perception of external coincidence

One of the genes whose expression is under control of the circadian clock is *CO*, which is a key regulator of the photoperiodic pathway (Suarez-Lopez *et al.*,

2001). The rhythmic expression of *CO* cycles such that low levels of transcripts are observed during the day. Expression begins to increase approximately 8 h after dawn, followed by a broad peak between 12 and 16 h after dawn (Fig. 1.2). In SD conditions this increase coincides with the beginning of the night, however, in LD the *CO* transcript accumulates to relatively high levels in the light. *CO* directly activates transcription of the floral integrators *FT* and *SOC1*, but only when expression of its mRNA coincides with the light period under LD conditions (Fig. 1.2). This is because the *CO* protein is subject to post-transcriptional regulation and is ubiquitinated and degraded by the 26S proteasome in the dark (Valverde *et al.*, 2004). Consequently, the protein does not accumulate when expression of the *CO* transcript takes place in darkness and *FT* transcription is not induced. Accumulation of the *CO* protein is promoted under monochromatic far-red and blue light by the action of *PHYA*- and *CRY2*-mediated pathways, respectively, but is prevented in red light via a *PHYB*-dependent pathway. Thus under natural conditions of white light where all of these photoreceptors are stimulated at the same time, the balance between the activity of these different pathways must determine the floral response. This is in good agreement with mutant analyses, which suggested that far-red light acting through *PHYA* and blue light acting through *CRY1* and *CRY2* may act to antagonize the repression of flowering mediated by *PHYB* (Mockler *et al.*, 2003).

A surprising observation is that plants that expressed the *CO* mRNA at a constant level from a heterologous promoter (*35S::CO*) only accumulate high levels of the *CO* protein towards the end of the day (Valverde *et al.*, 2004). This pattern was proposed to result from a gradual shift in the balance of different photoreceptor pathways through the course of the day so that *PHYB* promoted the degradation of *CO* in the morning but this effect was antagonized about 12 h onwards through the effects of *CRY2* and *PHYA*, thus allowing the accumulation of *CO* protein in the light. This gradual shift in the

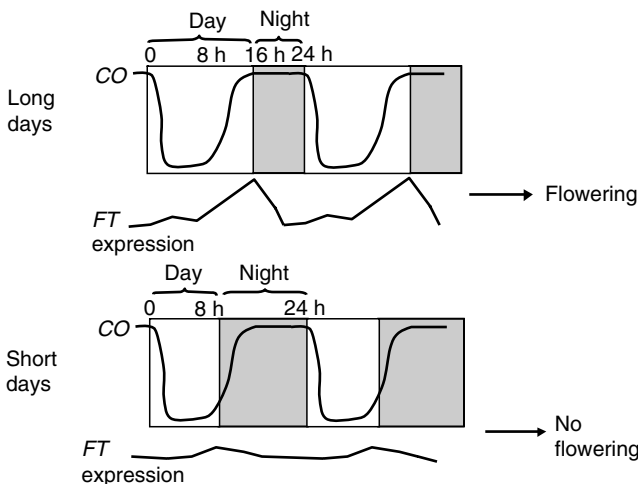


Fig. 1.2. Proposed rhythmic expression of *CO* under long- and short-daylight/dark cycles and the resulting expression of *FT*.

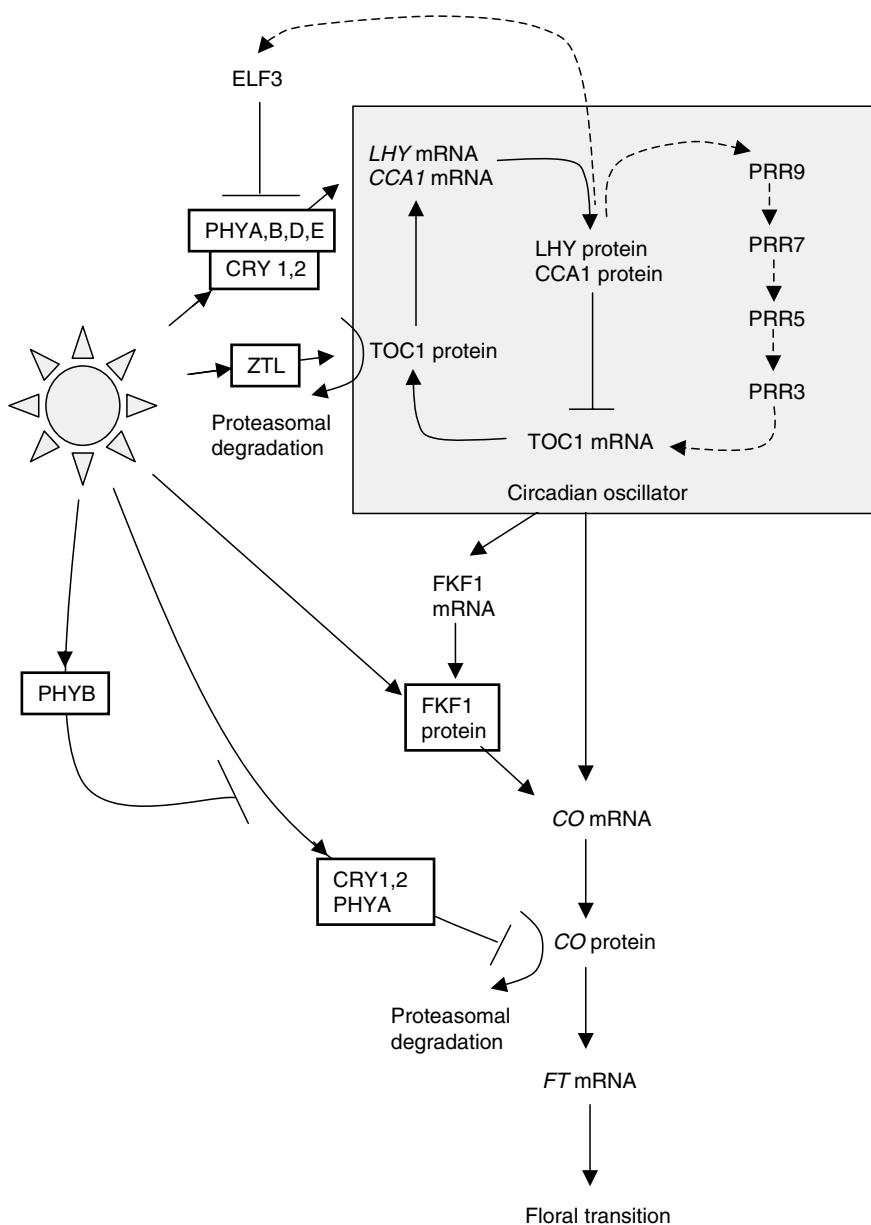
balance of photoreceptor pathways may result from the circadian patterns of expression of the photoreceptors, since expression of the *PHYB* mRNA peaks in the morning whereas that of *PHYA* and *CRY2* mRNAs peak later in the day (Tóth *et al.*, 2001). The levels of the cognate proteins do not oscillate significantly, but it is possible that post-translational modifications alter the properties of these photoreceptors over time and that the older proteins are inactive with regard to photoperiodic induction.

In addition to its effects on CO protein turnover, light promotes expression of the CO mRNA at the end of an LD through the action of a rhythmically expressed protein known as FKF1 (Imaizumi *et al.*, 2003). FKF1 is part of a family of three flavin-binding, Kelch-repeat, F-box proteins (FKF1, ZTL and LKP2) that regulate circadian rhythms by targeting specific proteins for degradation by the proteasome. Interestingly, the FKF1 protein has an LOV domain, which is the light-sensing module of phototropin blue light photoreceptors, and this was shown to bind a flavin-mononucleotide chromophore and to exhibit blue light-induced changes in absorbance. FKF1 thus has properties of a blue light photoreceptor and may be able to perceive blue light. In wild-type plants grown under LD cycles, FKF1 protein levels reach maximum levels before dusk and the peak of FKF1 expression coincides with a shoulder of the broad peak of CO mRNA at the end of the light interval. This shoulder was not detected in a T-DNA insertion allele, *fkf1-2*, indicating that FKF1 activity was required to promote this particular aspect of CO transcription. As a result, the onset of CO transcription was delayed into the dark interval and this correlated with low expression of *FT* and late flowering. The effect of FKF1 on CO transcription required exposure to light, since no difference in CO expression patterns was observed between wild-type and *fkf1* mutant plants upon transfer to shorter photoperiods where FKF1 expression does not coincide with light.

Rhythmic transcription of CO under the control of the circadian clock provides the basis for rhythmic responsiveness to light in photoperiodism, since the effects of CRY2 and PHYA on CO protein accumulation can take place only when the CO mRNA is expressed and actively translated. Photoperiodic time perception in *Arabidopsis* involves more than one rhythm of light sensitivity, however, since the peak of FKF1 protein must also coincide with light in order to promote CO transcription. Levels of accumulation of the CO protein have been shown to closely correlate with the transcriptional induction of the floral integrator *FT*. A combination of approaches including grafting and tissue-specific expression have shown that CO acts in the phloem to regulate a systemic flowering signal through cell-autonomous activation of the flowering integrator FT (An *et al.*, 2004). The *FT* gene encodes a 23 kD protein with amino acid sequence similarity to mammalian RAF kinase inhibitor proteins (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). Recent work has provided convincing support for the concept of a systemic flowering signal (florigen or antiflorigen) as described earlier in this chapter. FT mRNA induced locally in the leaf, moves to the apex where the FT protein interacts with the shoot apex-expressed transcription factor FD to initiate flowering (Huang *et al.*, 2005). This strongly suggests that FT mRNA itself constitutes an important part of the floral stimulus (i.e. florigen).

Daylength perception in short-day plants

With the recent completion of its genome project, rice has emerged as a powerful genetic system and an excellent SD plant counterpart to *Arabidopsis*. Genes that control photoperiodic flowering have been identified through QTL analysis of flowering time, also described as heading date. Fourteen loci controlling heading date (labelled *Hd1* to *Hd14*) were identified, five of which were



shown to control photoperiodic responses, including *Hd1*, *Hd2*, *Hd3*, *Hd5* and *Hd6* (Lin *et al.*, 2000; Yamamoto *et al.*, 2000). *Hd6* identified the α -subunit of casein kinase 2 (CK2), a protein that interacts with and phosphorylates CCA1 in *Arabidopsis* and is thought to regulate the function of the circadian clock (Sugano *et al.*, 1998, 1999; Takahashi *et al.*, 2001). *Hd1* was allelic to *Se1*, a flowering-time locus identified through mutant analyses. Map-based cloning of *Hd1* identified a homologue of *CO* in *Arabidopsis*. Another QTL, *Hd3a*, identified a gene related to *Arabidopsis FT* that functions as a positive effector of flowering (Kojima *et al.*, 2002).

The rice counterpart of *CO* (*Hd1* or *Se1*) is expressed rhythmically with a phase similar to that of *CO* in *Arabidopsis*. As in *Arabidopsis*, expression of *Hd3a* and the related rice genes *RFT1*, *FTL* are regulated by *CO*. A key difference, however, is that *Hd1* inhibits expression of *Hd3a* under LDs and promotes it under SD conditions (Izawa *et al.*, 2002; Kojima *et al.*, 2002). Unlike *Arabidopsis CO*, which only plays a role to promote *FT* expression and flowering under LD conditions, *Hd1* may also have differing functions under SD and LD conditions. The *se1* mutant flowered earlier than wild-type under LDs but later than wild-type under SDs, suggesting that the wild-type gene product may be required to delay flowering in one condition while promoting it in the other (Yano *et al.*, 2000).

In addition to these components, which are closely related to genes identified in *Arabidopsis*, *Early Heading Date1* (*Ehd1*) promotes early flowering under SD conditions (Doi *et al.*, 2004). The *Ehd1* gene encodes a protein containing a B-type response-regulator domain, which may be involved in relaying a phosphorylation signal, as well as a GARP DNA-binding domain. No orthologue of *Edh1* was detected in the *Arabidopsis* genome. *Edh1*

Fig. 1.3. Mechanism of photoperiodic timing in *Arabidopsis*. Perception of daylength is mediated by an interaction of a light and a circadian rhythm. The timing mechanism of the clock is composed of multiple transcriptional–translational feedback loops, whose oscillations are entrained to diurnal light–dark cycles through the action of phytochrome and cryptochrome photoreceptors. Light resets the clock through at least two mechanisms, including transcriptional induction of *LHY* and *CCA1* expression and light-induced degradation of the TOC1 protein. Effects of light on the clock are dampened at night through the action of the rhythmically expressed protein ELF3.

Downstream of the clock, expression of the floral regulator *CO* is rhythmic under diurnal light–dark cycles. Light promotes expression of the *CO* mRNA at the end of a long day through the action of the rhythmically expressed photoreceptor FKF1. In addition, the circadian oscillator mediates light-independent expression of the *CO* mRNA at night.

The *CO* protein does not accumulate in the dark because it is subject to proteasomal degradation. Blue light acting through cryptochromes and far-red light acting through PHYA prevent this degradation and allow accumulation of *CO* protein, which can then activate transcription of *FT* and promote the conversion of vegetative meristems to floral meristems. This action of CRY1 and CRY2 photoreceptors is antagonized by red light acting through the PHYB photoreceptor. Thus, photoperiodic induction of flowering in *Arabidopsis* takes place when the circadian rhythm of *CO* transcription coincides with a blue or far-red light signal.

induces transcription of *FT*-like genes independently of *Hd1* and probably represents a second mechanism by which *Hd3a* expression is increased in an SD-specific manner.

The photoreceptor mediating photoperiodic responses in rice is the red/far-red photoreceptor phytochrome. As for *cry2* mutants of *Arabidopsis*, loss of phytochrome (*SE5*) function in rice abolished responses to LDs. The *se5* mutant plants flowered as early under inhibitory LDs and as wild-type under inductive SD conditions (Izawa *et al.*, 2002).

Thus, under LD conditions, coincidence between light and *Hd1* expression may lead to inhibition of *Hd3a* transcription and suppression of flowering. Rhythmic expression of *Hd1* is not altered in the *se5* mutant, suggesting that phytochrome may act downstream to mediate perception of external coincidence (Izawa *et al.*, 2002). The mechanism by which *Edh1* participates in daylength responses independently of *Hd1* remains to be elucidated.

Conclusions

Genetic and molecular studies, largely with the model plant *A. thaliana*, over the last decade have gone a long way to confirm and explain the essential elements of photoperiodism as it applies to flowering in plants. The physiological conclusions that photoperiodic mechanisms involve multiple interactions between photoreceptors and an underlying circadian rhythm in light sensitivity through an external-coincidence model have been borne out. However, the level of complexity in these interactions is much greater than conceived in classical physiology (Fig. 1.3). Homologues of the central genetic elements of the model in the LDP *Arabidopsis* have been shown to affect photoperiodic regulation in SDPs such as rice and *Pharbitis*, implying a common photoperiodic mechanism for all plants. The role of a florigenic signal has also been confirmed and although its exact nature is still unknown, there are prospects of it being discovered in the foreseeable future. We can also soon expect to understand more fully the basis for the different requirements for light quantity and quality in different species. This understanding will be of great benefits to breeders and agronomists in designing and growing plants with flowering properties tailored to the food and ornamental industries and the wider needs of society.

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