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Photoperiodic flowering of *Arabidopsis*: integrating genetic and physiological approaches to characterization of the floral stimulus

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ABSTRACT

In many plants the transition from vegetative growth to flowering is controlled by environmental cues. One of these cues is day length or photoperiod, which synchronizes flowering of many species with the changing seasons. Recently, advances have been made in understanding the molecular mechanisms that confer photoperiodic control of flowering and, in particular, how inductive events occurring in the leaf, where photoperiod is perceived, are linked to floral evocation that takes place at the shoot apical meristem. We discuss recent data obtained using molecular genetic approaches on the function of regulatory proteins that control flowering time in *Arabidopsis thaliana*. These data are compared with the results of physiological analyses of the floral transition, which were performed in a range of species and directed towards identification of the transmitted floral singals.

Key-words: Arabidopsis; floral signals; flowering; flowering time gene; phloem; photoperiodic floral induction.

INTRODUCTION

Seasonal changes in light, temperature and rainfall have strongly influenced the evolution of life on earth. For example, many plants and animals living at latitudes above the equator often alter their behaviour or developmental programmes in response to environmental signals such as day length or temperature (Hastings & Follet 2001). The role of day length, or photoperiod, in controlling seasonal responses was originally proposed by Tournois (1912) and Klebs (1913) at the beginning of the twentieth century, but Garner & Allard (1920, 1923) were the first to show clearly that flowering and other developmental responses could be controlled by exposure to long days (LDs) or short days (SDs) depending on the plant species. They demonstrated that the duration, rather than the quantity, of light in the daily cycle was a major factor in regulating plant development and introduced the term photoperiodism, which is defined as the response to the length of the day that enables living organisms to adapt to seasonal changes. Plants were then classified into photoperiodic groups based on their responses to day lengths (reviewed in Thomas & Vince-Prue 1997). LD plants flower only, or most rapidly, when exposed to more than a certain number of hours of light in the daily cycle, which is referred to as the critical day length, SD plants flower only, or most rapidly, if the day length is shorter than a critical day length, and day-neutral plants flower at the same time irrespective of the photoperiodic conditions.

Later, Bünning (1936) proposed that plants might use the same time-keeping mechanism that regulates daily rhythms in leaf movements to measure day length and, thereby, control seasonal responses. Indeed, a mechanism by which plants measure the duration of a photoperiod is a prerequisite for the photoperiodic control of flowering time. Thus Bünning hypothesized that the mechanism controlling daily movements in leaves or petals, subsequently named the circadian clock, is also the basis of photoperiodic time measurement. This hypothesis was extended with the proposal that a daily rhythm that controlled the photoperiodic response and was sensitive to light at particular times of the day could explain photoperiodic time measurement (Pittendrigh & Minis 1964). This process, referred to as the external coincidence model, has been reviewed extensively recently, and received strong support from the study of genes controlling flowering time of *Arabidopsis* (Yanovsky & Kay 2003; Searle & Coupland 2004).

Soon after the discovery of photoperiodism, day length was shown to be perceived in the leaves, although floral development occurs at the shoot apex (reviewed in Thomas & Vince-Prue 1997). The first experiments that demonstrated this were based on exposure of different parts of the plant to distinct day lengths. Exposing only the foliage of spinach plants to LDs of 15 h light induced rapid flowering, while exposing the apex of the shoot to LDs while the foliage was exposed to SDs would not induce flowering (Knott 1934). This led to the conclusion that the role of the leaves in the induction of flowering in response to day length is 'in the production of some substance, or stimulus, that is transported to the growing point'. The conclusion that leaves are the source of a floral stimulus was strengthened by generating grafts between plants exposed to different conditions (see next section).

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Using experimental systems in which flowering could be strictly controlled, the flowering process was subdivided into successive steps. 'Induction' mechanisms determining flowering time occur in the leaf and precede floral 'evocation' which consists of the events occurring in the shoot apical meristem (SAM) that commit it to form flower (Evans 1969). In photoperiodic species, signals moving from the leaves to the SAM through the phloem are an important link between 'induction' and 'evocation' and movement of these substances could be timed precisely (Bernier, Kinet & Sachs 1981a). Although transport of the floral stimulus across graft junctions was followed indirectly by its effect on flowering, its identity was difficult to establish despite extensive studies (reviewed in Bernier 1988; Bernier *et al*. 1993). Nevertheless, recent molecular-genetic studies in the facultative LD plant *Arabidopsis thaliana* have made progress in identifying genetic pathways and regulatory proteins associated with the control of flowering time (Mouradov, Cremer & Coupland 2002; Yanovsky & Kay 2003; Boss *et al*. 2004; Jack 2004; Putterill, Laurie & Macknight 2004; Searle & Coupland 2004). Specifically in the photoperiodic control of flowering, models were proposed in which circadian clock control of gene transcription and post-transcriptional regulation of protein stability by light can combine to confer control of a regulating pathway that mediates the induction of flowering by day length (Searle & Coupland 2004). In this article, we focus on the later stage of photoperiodic flowering, and in particular how inductive events in the leaf are linked to floral evocation in the shoot meristem.

THE FLORAL STIMULUS: INSIGHTS FROM PHYSIOLOGICAL APPROACHES

Grafting experiments performed with several photoperiodic species clearly demonstrated that the floral stimulus is produced in the leaves. For example, in *Perilla* grafting of a single induced leaf onto an uninduced shoot was sufficient to induce flowering (Zeevaart 1985). The pattern and velocity of movement of the floral stimulus is similar to that of assimilates, indicating that it is transported through the phloem (King, Evans & Wardlaw 1968; King & Zeevaart 1973). In some species floral inhibitors, which are transmitted across graft junctions and delay flowering, have also been demonstrated (Weller *et al*. 1997b), whereas in other species these do not seem to occur and the inhibitory effect of uninduced leaves appears to be due to them acting as a sink for photosynthate and the contents of the phloem (Zeevaart 1976). In *Perilla*, once induced, a single leaf stably produces the stimulus, and can induce flowering in multiple shoots; repeated grafting of a single induced *Perilla* leaf sequentially triggered flowering in seven shoots over a period of 97 d (Zeevaart 1985).

The phenomenon of indirect induction of flowering suggests that at least in some species the floral stimulus has wider significance than triggering floral development at the shoot apex. In *Xanthium strumarium*, *Silene armeria* and *Bryophyllum daigrementianum* shoots induced to flower by grafting to donor shoots can themselves act as donors in subsequent grafts (Zeevaart 1976). This suggests that the floral stimulus can act in the leaves of these species to trigger its own synthesis. However, this phenomenon may not be widespread, since other species, such as *Perilla*, do not exhibit indirect induction of flowering. Nevertheless, grafting experiments performed with plants showing different photoperiodic responses and even originating in different genera suggest that the identity of the floral stimulus might be highly conserved. The SD plant *X. strumarium* acted as a donor of the floral stimulus when grafted to the LD plant *S. armeria* (Wellensiek 1970). Although, in many cases grafts between species from different genera failed this could be due to difficulties in establishing tissue connection in grafts, in genetic differences in responsiveness to the stimulus or in differences in the amount of stimulus produced by different species rather than in the identity of the stimulus (Zeevaart 1976).

Perhaps the central question addressed by physiological approaches to long-distance signalling in the floral transition is the nature of the leaf-generated signals that control the floral program at the SAM. The success of interspecies grafts led Chailakhyan (1937) to propose that the floral stimulus is a universal, unique and specific hormone called 'florigen'. Lang (1965) suggested that non-induced leaves may also produce 'antiflorigen' and, that in plants producing both florigen and antiflorigen, floral evocation is caused when the balance of these two factors at the SAM is shifted in favour of florigen. Gibberellins (GAs) were long considered to be florigen, however, GAs are florigenic in some species like *Arabidopsis* but failed to induce flowering in other species such as caulescent plants, which have an elongated stem at the vegetative stage (Zeevaart 1983). The limits of this florigen/antiflorigen theory are reviewed in Bernier (1988) and, despite extensive studies, these universal hormones were never isolated.

Alternative hypotheses for the identity of the long-distance signal were then proposed. Karege, Penel & Greppin (1982) suggested that at least part of the floral stimulus exported by induced leaves could be a fast-moving electrophysiological signal. However, this does not appear compatible with the demonstration based on grafting experiments that the signal moves with assimilates in the leaves, is inhibited by sink leaves, and can take up to 72 h to be exported from the leaves (King & Zeevaart 1973; Zeevaart 1976). Floral induction was proposed to be a means of modifying the source/sink relationships within the plant so that the shoot apex receives a higher concentration of assimilates, mainly sugar, than under non-inductive conditions (Sachs & Hackett 1969). This 'Nutrient Diversion hypothesis' is based on the observation that treatments that increase photosynthate levels at the meristem accelerate flowering. These treatments included exposure to conditions that favour photosynthesis (high irradiance, high atmospheric CO_2 concentration) and removal of sinks (roots, young leaves, shoot branches) that compete with the SAM for assimilates. In fact, the sucrose content of the SAM and/or the phloem sap reaching the SAM increases

in several LD or SD species following floral induction (Bernier, Kinet & Sachs 1981b; Bernier *et al*. 1998).

Following the observation that treatments of vegetative plants by substances such as sugars, plant growth regulators and their antagonists can induce in the SAM events specific to floral evocation, Bernier proposed the 'Multifactorial control of flowering' (Bernier 1988; Bernier *et al*. 1993). In this hypothesis, the floral transition occurs if all factors are present in the SAM at appropriate concentrations and times. While assimilates and hormones are generally present in most plants, some of these compounds may be absent or not present at optimal levels and the floral induction process would correct this. Nevertheless, in some species, the floral transition appears to be under the control of a single substance, such as in the LD plants *Samolus parviflorus, Rudbeckia bicolor* and *Lolium temulentum*, which can be induced to flower in SDs by treatment with GA_3 (Bernier *et al*. 1981b; King *et al*. 2001).

All these theories propose that floral evocation results from the translocation of signals to the SAM in response to day lengths flowering (Kinet 1993). These signals would be transmitted through phloem sap, and to a lesser extent through the xylem. They might be identified by sap collection and comparison of the composition of the sap between vegetative and floral-induced plants. The best species to use in these studies are those in which floral induction is rapid and can be synchronized. Examples are species that can be induced to flower by a single LD, such as *Sinapis alba* or *L. temulentum*, or a single long night like *X. strumarium* or *Pharbitis nil*. In several of these physiological model plants, extensive studies of the composition of the sap were performed (reviewed in Bernier 1988; Bernier *et al*. 1993, 1998; Kinet 1993; Levy & Dean 1998). Among the factors controlling the photoperiodic floral transition in *S. alba*, a caulescent plant belonging to the same family of the *Brassicaceae* as *Arabidopsis*, roles for sucrose, glutamine, cytokinins, auxin and putrescine were demonstrated, while GAs seem to play a minor role (reviewed in Bernier *et al*. 1993, 1998; Corbesier *et al*. 2004). In later sections, we focus on the photoperiodic control of flowering in *Arabidopsis* and try to integrate the regulatory proteins identified by molecular genetics with the leaf-generated signals proposed from physiological observations.

PEA AND MAIZE AS GENETIC MODEL SYSTEMS TO APPROACH THE FLORAL STIMULUS

Mutations or natural-genetic variation that alter flowering-time have been described in many species. Studies in pea plants were enhanced by the availability of extensive genetic stocks, and the ability to readily graft different genotypes so that the effect of this variation on long-distance signalling could be assessed (Weller *et al*. 1997b). For example, *gigas* (*gi*) mutants flower later than wild-type plants, but their flowering is accelerated by grafting a *gi* shoot onto a wild-type stock (Beveridge & Murfet 1996). This suggests that the pea *GI* gene may be involved in the synthesis or transport of the floral stimulus. Flowering of wild-type pea plants is accelerated in response to LDs and is delayed by exposure to SDs. The *gi* mutant flowers later under both conditions, and often never flowers under SDs. This suggests that the floral stimulus controlled by *GI* is not part of the response to day length, but is expressed under all environmental conditions tested.

The pea *LATE FLOWERING* (*LF*) gene is proposed to encode a target of the floral stimulus at the apex of the plant; dominant alleles at this gene delay flowering and the effect is not influenced by grafting of an *LF* shoot onto a wild-type stock (Murfet 1971, 1985). *LF* is a homologue of the *Arabidopsis* gene *TERMINAL FLOWER1*, which was also shown to repress flowering in *Arabidopsis* (Foucher *et al*. 2003). Expression of *LF* does not change during floral induction, suggesting that the LF protein may modulate the response to the floral stimulus at the meristem rather than be a direct target of it. In addition to the floral stimulus controlled by *GI*, there is evidence for a long-distance inhibitory signal-regulating flowering-time of pea plants. Mutations in the *STERILE NODES* (*SNE*), *DIE NEUTRALIS* (*DNE*) or *PHOTOPERIOD* (*PPD*) genes cause early flowering, and flowering of the shoots of these plants can be delayed by grafting onto a rootstock of a wild-type plant (King & Murfet 1985; Weller, Murfet & Reid 1997a; Weller *et al*. 1997b). Plants in which these genes are mutated are almost day-length insensitive, flowering at the same time under both LDs and SDs, indicating that the photoperiod response is largely caused by production of an inhibitor under SDs. These experiments suggested a model in which the timing of the transition to flowering at the apex of pea plants is determined by a balance between long-distance promotive and inhibitory signals, so that, when the ratio of stimulus to inhibitor exceeds a certain level, flowering occurs (Weller *et al*. 1997b).

Analysis of the *INDETERMINATE* (*ID*) gene of maize provided the first molecular information on a gene that appear to regulate the floral stimulus. Mutations in *ID* dramatically delay the transition to flowering, so that many more leaves are formed than in wild-type plants (Colasanti, Yuan & Sundaresan 1998). Eventually *id* mutants do flower, but the reproductive structures develop abnormally and show vegetative characteristics. *ID* mRNA which encodes a putative transcriptional regulator was detected in young, immature leaves, but not in the SAM or in mature leaves. The expression of *ID* in the leaves, but not the SAM, indicated that it acts to regulate long-distance signals that influence the transition to flowering of the meristem. The expression of *ID* appears to occur in sink leaves, which receive nutrients from photosynthetically active source tissues, and not to be expressed in source leaves (Colasanti & Sundaresan 2000). This observation led to the suggestion that ID may not promote the production of the floral stimulus, but rather acts in the developing leaves to regulate its flow. However, the mechanism by which ID regulates flowering requires further knowledge of the identity and function of the genes whose expression it regulates.

MOLECULAR-GENETICS IN *ARABIDOPSIS*: IDENTIFICATION OF A REGULATORY HIERARCHY THAT CONTROLS FLOWERING

The genetic control of flowering has been most extensively studied in *Arabidopsis*. The behaviour of mutants exhibiting a severe delay in flowering was first described in detail by Redei (Redei 1962), and this analysis was later broadened and extended by Koornneef (Koornneef, Hanhart & Van Der Veen 1991; Koornneef *et al*. 1998). More recently a large number of mutants and natural accessions showing either later or earlier flowering have been described (Mouradov *et al*. 2002).

Environmental conditions influence flowering time of *Arabidopsis*. As a quantitative LD- and vernalization requiring-plant, flowering of *Arabidopsis* is promoted by exposure to LDs and delayed under SDs, whereas vernalization treatments promote flowering (Martinez-Zapater *et al*. 1994). In addition to these seasonal cues, less dramatic changes in ambient conditions also strongly influence flowering time. Exposure to lower temperatures (16 °C) delays flowering compared to the effect of growing plants at typical growth temperatures of 20–24 °C, and exposure to the high ratios of far-red to red light associated with shading conditions accelerates flowering (Blázquez, Ahn & Weigel 2003; Cerdan & Chory 2003).

The genes identified by mutagenesis and by allelic variation between accessions were placed in pathways based on genetic criteria and their effect on the response of flowering time to different environmental cues (Koornneef *et al*. 1998). The major features of this model were later confirmed by cloning of the genes and analysis of their expression patterns in wild-type and mutant plants (Mouradov *et al*. 2002; Simpson & Dean 2002).

Within this model four major pathways control flowering time and converge to regulate the expression of genes that integrate the information received from the different pathways (Fig. 1). One pathway controls the response to vernalization. In response to extended exposures to low temperature this pathway reduces the abundance of the mRNA encoding the MADS box transcription factor FLOWERING LOCUS C (FLC), which is a potent repressor of flowering (Michaels & Amasino 1999; Sheldon *et al*. 1999). Therefore, vernalization accelerates flowering by reducing *FLC* expression. Mutations in the second pathway, the autonomous pathway, delay flowering under both LDs and SDs, and cause an increase in *FLC* mRNA levels (reviewed in Boss *et al*. 2004). This second genetic pathway also regulates *FLC* expression but independently of vernalization so that the high *FLC* mRNA levels observed in these mutants can be corrected by vernalization. Mutants affected in this pathway also show an altered flowering time in response to ambient temperatures (Blázquez *et al*. 2003). The autonomous pathway appears to represent protein complexes involved in histone modification and RNA processing (He, Michaels & Amasino 2003; Simpson *et al*. 2003; Ausin *et al*. 2004), and probably also has a more general role than the regulation of *FLC* expression. Thirdly, application of the growth regulator GA_3 promotes flowering of *Arabidopsis*, and mutations that affect genes required for GA biosynthesis delay flowering, particularly under SDs (Wilson, Heckman & Somerville 1992). Finally, the photoperiodic pathway controls the response to day length, and specifically promotes flowering in response to

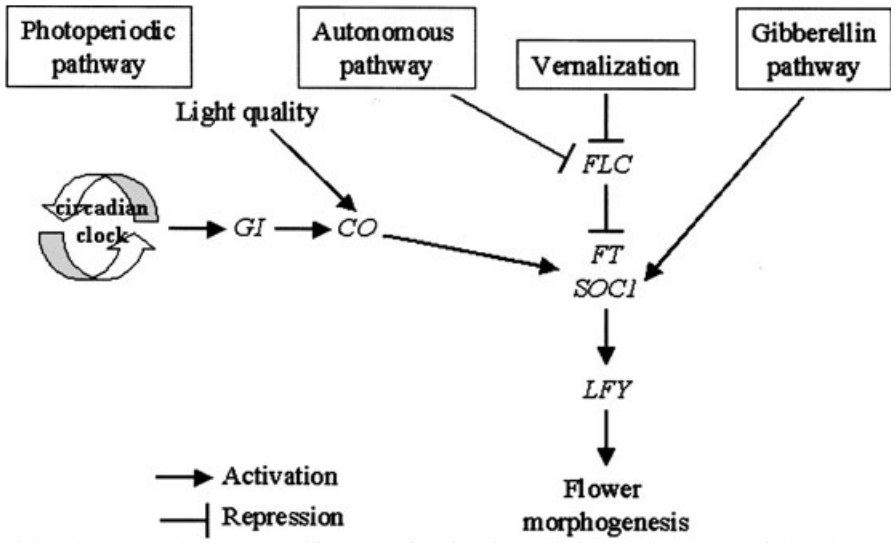

Figure 1. Simple model of the four pathways controlling flowering time in *Arabidopsis*. The photoperiod pathway promotes flowering specifically under LDs. The transcription of the *GI* and *CO* genes is regulated by the circadian clock, whereas the light quality regulates CO protein abundance. The autonomous pathway negatively regulates the abundance of the mRNA of the floral repressor FLC. *FLC* mRNA are also repressed by vernalization but independently of the autonomous pathway. Finally gibberellin promotes flowering of *Arabidopsis*, particularly under SDs. All four pathways appear to converge on the transcriptional regulation of the floral integrators genes *FT* and *SOC1* which promote *LFY*, a gene required to confer floral identity on developing floral primordia. The data underlying this model are described in detail in the text.

LDs (Yanovsky & Kay 2003; Hayama & Coupland 2003; Searle & Coupland 2004). Mutations in this pathway can either delay flowering under LDs or accelerate flowering under SDs. The last gene that is specifically involved in this pathway is *CONSTANS* (*CO*), which encodes a zinc finger protein that promotes transcription of downstream flowering-time genes (Putterill *et al*. 1995; Robson *et al*. 2001). This photoperiodic pathway probably also plays a role in the effect of light quality on flowering, because high ratios of far-red to red light promote flowering and stabilize the CO protein (Valverde *et al*. 2004), although the flowering response to light quality also involves a CO-independent pathway (Cerdan & Chory 2003).

These distinct genetic pathways finally converge to regulate the expression of a small group of downstream genes, sometimes described as floral integrators (Mouradov *et al*. 2002; Simpson & Dean 2002). This group includes two genes that promote flowering, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *LEAFY*, a gene encoding a transcription factor required to confer floral identity on developing floral primordia. *FT* encodes a protein with similarity to RAF kinase inhibitors of animals (Kardailsky *et al*. 1999; Kobayashi *et al*. 1999) whereas *SOC1* encodes a MADS box transcription factor (Borner *et al*. 2000; Lee *et al*. 2000; Samach *et al*. 2000). Mutations in each of these genes delay flowering, whereas their overexpression from the viral CaMV 35S promoter causes extreme early flowering. The expression of *SOC1* and *FT* is increased by CO and reduced by FLC, indicating that they are downstream of the point of convergence of the vernalization and photoperiod pathways (Samach *et al*. 2000; Hepworth *et al*. 2002). Furthermore, the expression of *SOC1* is increased by treating plants with GA, suggesting that it acts downstream of all three pathways (Moon *et al*. 2003).

POSITIONING THE FLORAL STIMULUS WITHIN THE REGULATORY NETWORK

A major problem in the genetic analysis of the floral transition has been in elucidating in which tissues of the plant the expression of particular flowering-time genes must occur to enable floral induction to proceed (Périlleux & Bernier 2002). Therefore, in most cases it has been unclear whether specific pathways regulate the function of long-distance signals expressed in the leaves or respond to these signals in the meristem. Simply analysing the spatial pattern of expression of these genes did not help to address this problem, because many flowering-time genes are expressed broadly.

For example, classical physiological experiments suggested that vernalization acts in the meristem to promote flowering (Michaels & Amasino 2000). Initial observations were based on exposing only the leaves or only the apices of celery plants to vernalization treatments, and demonstrating that vernalization of the meristem was sufficient to induce flowering. The vernalization pathway is therefore likely to act in the meristem to reduce *FLC* expression and thereby induce flowering, and this may also be true for the autonomous pathway. Consistent with vernalization acting in the meristem, *FLC* is expressed specifically in the shoot and root meristems in young seedlings, although in older plants it is also expressed in expanded leaves (Michaels & Amasino 2000; Sheldon *et al*. 2002; Noh & Amasino 2003; Bastow *et al*. 2004).

Concerning the photoperiodic induction of flowering, existence of the floral stimulus was originally demonstrated by inducing flowering with appropriate day lengths and grafting experiments (see Introduction and next section). Therefore the photoperiod pathway of *Arabidopsis* might be expected to include long-distance signalling components analogous to the floral stimulus. A molecular hierarchy within the photoperiod pathway has been defined. Two flowering-time genes specific to this pathway are *GIGANTEA* (*GI*) and *CO*. The *GI* gene encodes a large protein of 1173 amino acids that is present in the nucleus and is highly conserved among the angiosperms but has no animal homologues (Fowler *et al*. 1999; Park *et al*. 1999). The biochemical function of GI is unknown, but *gi* mutations cause severe late flowering (Redei 1962), whereas overexpression of *GI* causes early flowering (Wright & Coupland unpublished). GI regulates flowering time at least in part by the regulation of *CO* mRNA abundance; *gi* mutants contain less *CO* mRNA (Suarez-Lopez *et al*. 2001) while *GI* overexpressors show higher *CO* mRNA abundance. The abundance of *GI* and *CO* mRNAs is circadian clock regulated. Under LDs of 16 h light, in which these genes promote early flowering, *GI* mRNA abundance peaks around 10–12 h after dawn, whereas *CO* mRNA abundance rises around 12 h after dawn and stays high throughout the night until the following dawn (Fowler *et al*. 1999; Park *et al*. 1999; Suarez-Lopez *et al*. 2001). *CO* mRNA abundance is therefore high when plants are exposed to light at the end of a LD. *CO* expression is also regulated at the post-transcriptional level, so that the cryptochrome and phytochrome A photoreceptors act at the end of the day to stabilize the CO protein (Valverde *et al*. 2004), whereas in darkness the protein is rapidly degraded, probably as a consequence of being ubiquitinated. Under SDs the *CO* mRNA is only expressed in the dark, and so the protein would be predicted never to accumulate. In agreement with these data, in wild-type plants *FT* is activated by CO under LDs, but not under SDs (Suarez-Lopez *et al*. 2001; Yanovsky & Kay 2002). Therefore, the combination of circadian clock mediated regulation of *CO* mRNA abundance, and stabilization of CO protein by exposure to light can explain why CO promotes *FT* expression and, thus, flowering only under LDs.

The observation that CO is a major part of the molecular mechanism by which *Arabidopsis* discriminates between LDs and SDs suggests that CO may act in the leaf to regulate the transition to flowering at the apex. The *CO* mRNA is present at very low abundance, but is expressed widely. *In situ* hybridizations and reverse transcriptase

(RT)-polymerase chain reaction (PCR) detected the *CO* mRNA in the meristem, young leaf primordia and whole seedling (Putterill *et al*. 1995; Simon, Igeno & Coupland 1996). A more refined expression pattern was identified using fusions of the *CO* promoter to the *GUS* marker gene (Takada & Goto 2003; An *et al*. 2004). In *CO:GUS* plants, *GUS* expression was most strongly detected in the phloem of cotyledons, leaves and stems, but also in the protoxylem, young leaves and meristem.

Several recent observations suggest that CO acts in the vascular tissue and not the meristem to promote flowering. In wild-type plants, the CO target gene, *FT*, is expressed in the phloem, as detected using *FT::GUS* reporter constructs. Furthermore, *FT* expression is increased in the early flowering *terminal flower 2* (*tfl2*) mutant, and in particular is expressed at higher levels in the vascular tissue, suggesting that CO may activate its target gene in these tissues (Takada & Goto 2003). Consistent with this conclusion, *FT* expression was reduced in *tfl2 co2* plants compared to *tfl2* mutants. Ayre & Turgeon (2004) showed recently that specifically triggering the expression of *CO* in the companion cells of the minor veins of the phloem of the mature leaves, using the promoter of a gene from melon encoding galactinol synthase (GAS) complemented the *co1* mutation. Independently, An *et al*. (2004), using the phloem companion cell-specific promoter of the *SUCROSE TRANSPORTER 2* (*SUC2*) gene of *Arabidopsis*, obtained similar results and showed that expression of *CO* from meristem-specific promoters had no effect on flowering. Therefore, *CO* appears to act specifically in the vascular tissue to regulate the synthesis or transport of a long-distance signal that initiates floral development at the apex.

Some proteins can move through the phloem from source to sink tissues, but CO protein itself is unlikely to be the long-distance signal. The *SUC2* promoter is specific to the companion cells of the phloem of source tissues, mature leaves and stem, and expression of GUS enzyme from this promoter produced staining specifically in the vasculature of these tissues (Truernit & Sauer 1995). In contrast, expression of GFP from the same promoter produced fluorescence both in source and sink leaves, indicating that GFP is downloaded from the companion cells into the phloem sieve elements and transported to sink leaves (Imlau, Truernit & Sauer 1999). However, CO is approximately 20 kDa larger than GFP and expression of GFP:CO from the *SUC2* promoter complemented the *co2* mutation, but GFP fluorescence was only detected in the vascular tissue and not in the meristem or leaf epidermal cells (An *et al*. 2004). After grafting of *co1* mutant scions onto *GAS::CO* Columbia stocks, Ayre & Turgeon (2004) were not able to detect *CO* mRNA by PCR in the *co1* scions. This, together with the observation that *CO* does not promote flowering when expressed in the meristem, suggests that CO acts in the phloem of the mature leaves to promote flowering and is not transported to other cells.

The mechanism by which CO acts to promote flowering in the phloem partially involves the *FT* gene. *FT* mRNA abundance was increased in the phloem of *SUC2::CO* plants, and *ft* mutations strongly suppressed the early flowering of *SUC2::CO* (An *et al*. 2004). Furthermore, expression of *FT* in the phloem from the *SUC2* promoter complemented the *co* mutation. However, in contrast to CO, FT promoted flowering when expressed in the meristem and the epidermal layer, as well as the phloem (An *et al*. 2004). No data are available so far on the movement of the FT protein between cells. However, FT is a small protein of 23 kDa (Kardailsky *et al*. 1999; Kobayashi *et al*. 1999) and is therefore smaller than GFP, suggesting that it may be able to move freely between cells. Furthermore, the observation that *FT* can promote flowering when expressed in the meristem is consistent with the idea that the protein could move from the phloem to the meristem where it acts to promote flower development. However, these data could also be explained if FT can act in almost any cell type to trigger the synthesis of a small molecule that induces flowering and is able to move freely between cells.

The biochemical function of FT is unknown. It is a member of a small protein family in *Arabidopsis* and shares homology with characterized proteins in other species. These proteins are referred to as CETS, after CENTRORADIALIS (CEN) of *Antirrhinum majus*, TERMINAL FLOWER 1 (TFL1) of *Arabidopsis* and SELF PRUNING (SP) of tomato (Bradley *et al*. 1997; Pnueli *et al*. 1998; Kardailsky *et al*. 1999; Kobayashi *et al*. 1999). CETS proteins share homology to RAF kinase inhibitor proteins of mammals (Kardailsky *et al*. 1999; Pnueli *et al*. 2001), and the crystal structure of CEN is similar to that of RAF kinase inhibitors (Banfield & Brady 2000). In the yeast two-hybrid system, the interaction of SP with a NIMA-like kinase, bZIP transcription factors and a 14-3-3 protein led to the suggestion that CETS proteins act as adapters in a variety of signalling pathways (Pnueli *et al*. 2001). How these interactions relate to the role of FT in promoting flowering is not known. Combining *ft* mutations with mutations affecting flower development identified a strong genetic interaction between *ft* and mutations in the floral organ identity gene *LEAFY* (*LFY*) (Ruiz-Garcia *et al*. 1997). The *ft lfy* double mutant failed to produce any mature floral organs, and resembled a *lfy apetala1* (*ap1*) double mutant. This indicates that the role of FT in promoting flowering might involve the activation of *AP1*, which is expressed exclusively at the meristem in floral primordia. One possibility is that in wild-type plants, *FT* mRNA is also expressed in the meristem, although so far this has not been detected. Alternatively, FT might activate a long-distance signal in the phloem that leads to *AP1* activation in the meristem, or FT protein either alone or together with a physiologically defined component of the floral stimulus such as sucrose might move to the meristem where it activates *AP1*. The involvement of sucrose is supported by suggestions that it is required together with GA to activate *LFY* expression (Blázquez *et al*. 1998). Similarly, Roldan *et al*. (1999) showed that addition of sucrose to the growing medium of

co3 mutant grown either in light or in darkness complemented the *co* mutation whereas this sugar treatment was insufficient to correct the *ft-1* mutation.

The small size of the FT protein suggests that it may move from the phloem to the meristem and directly trigger changes in gene expression. Symplastic downloading of proteins from the sieve elements into the sink tissues of the apex through plasmodesmata has been proposed (Ruiz-Medrano, Xoconostle-Cazares & Lucas 2001), suggesting that FT may move directly by this mechanism into apical cells and induce flowering. Furthermore, the size exclusion limit or selectivity of plasmodesmata that allow downloading from the sieve elements into the meristem have been shown to change around the time of flowering (Gisel *et al*. 2002) as well as their frequency (Ormenese *et al*. 2000), suggesting that this might be an important regulatory step. If FT does move from the companion cells of the leaf to the meristem, nothing is known of the mechanisms underlying its export from the leaf and import to the crucial cells of the meristem.

IDENTIFICATION OF THE FLORAL STIMULUS IN *ARABIDOPSIS*: INTEGRATING MOLECULAR-GENETIC AND PHYSIOLOGICAL APPROACHES

At present, a divide exists between the work performed in physiological model species, in which no regulating network has been described, and work on *Arabidopsis* in which very few studies have explored the flowering behaviour of mutants and transgenics affected in metabolism, transport or transduction of putative floral signals, such as sucrose and growth regulators. *Arabidopsis*, as a small rosette plant, is not ideal for biochemical analyses. In addition, although it is a LD plant, some of the accessions commonly used in the laboratories flower very rapidly, even under SDs, rendering difficult the establishment of a growing system allowing the control and the synchronization of the floral transition.

So far, only the Columbia ecotype has been used successfully in this respect. Corbesier *et al*. (1996) designed a growing system in which, after a period of vegetative growth in non-inductive 8 h SDs, Columbia plants were synchronously induced to flower by exposure to a single LD of 22 h or to a single 8 h SD shifted 10 h later within a 24 h cycle. In these conditions, the export of the floral stimulus out of the mature leaves starts 20 h after the beginning of the LD and lasts for approximately 12 h. Using grafting, Turnbull and colleagues also showed effective transmission of floral signals in *Arabidopsis* between shoots differing in mutations for photoperiod pathway genes or differing in photoperiod treatment (An *et al*. 2004; Ayre & Turgeon 2004; Turnbull & Justin 2004).

Carbohydrate metabolism has been related to flowering in *Arabidopsis* using mutants, as previously shown for *S. alba* using physiological approaches (Bernier *et al*. 1993). Eimert *et al*. (1995) observed that the late-flowering *gi* mutant assigned to the photoperiodic pathway accumulates excess starch in the leaves and the stem during the photoperiod. They also showed that the *carbohydrate accumulation mutant1* (*cam1*), an *Arabidopsis* mutant accumulating starch at higher levels than the wild type just before the onset of flowering, was late-flowering in continuous light and in SD conditions. However, the accumulation of starch *per se* does not seem to be the direct cause of the late-flowering phenotype in these mutants since the flowering-time defect conferred by *gi* and *cam1* was not rescued by crossing them with mutants lacking starch.

Studies with the *phosphoglucomutase1* (*pgm1*) mutant, which is deficient in starch biosynthesis (Caspar, Huber & Somerville 1985), show that sucrose plays a critical role in the floral transition (Corbesier, Lejeune & Bernier 1998). A strong increase in the export of sucrose from mature leaves could be observed transiently between 16 and 24 h after the start of the inductive LD. On exposure of the *pgm1* mutant to the displaced SD, in which the time of exposure to light but not the duration of light is changed, flowering did not occur in the absence of increased export of sucrose. In this system, both photosynthesis and starch mobilization are important for flowering to occur similar to what has been previously shown for *S. alba* (Bernier *et al*. 1993, 1998). Yu *et al*. (2000), using another *Arabidopsis* mutant deficient in starch synthesis also described the important role played by sugar metabolism in floral initiation. Interestingly, addition of sucrose to the growing medium of *co3* mutants grown either in light or in darkness complemented the *co* mutation while this treatment was not effective with the *ft-1* mutant (Roldan *et al*. 1999; Ohto *et al*. 2001). These results suggest that sucrose might act downstream of *CO* in the photoperiodic pathway, but upstream or in parallel to *FT*. A careful analysis of sugar metabolism and sucrose transport in these mutants may also help in understanding the potential link between *CO*, sucrose and *FT*. Sucrose may also be involved in the transcriptional regulation of *LFY*. Addition of 1% sucrose in the growing medium of transgenic *LFY::GUS* plants enhanced the expression of the transgene in vegetative plants (Blázquez *et al*. 1998). This increase was potentiated by simultaneous incubation with GA_3, although incubation with GA_3 alone did not have a noticeable effect.

Changes in the amino acid content of the phloem sap of *Arabidopsis* at floral transition have not really been investigated, even although amino acids are the second most prevalent compound (behind carbohydrates) found in this sap (Peoples & Gifford 1990; Lam *et al*. 1995). In *Arabidopsis*, Corbesier *et al*. (2001) showed that both LD and displaced SD inductive treatments were correlated with an increased export of glutamine or asparagine in the phloem sap. This export occurs at the same time as that of sucrose and at a time compatible with the export of the floral stimulus out of the leaves. In parallel, the C/N ratio of the phloem sap, mainly the sucrose/glutamine ratio, increases suggesting that the relative availability of C and N at the level of the SAM could be of critical importance (Corbesier, Bernier & Périlleux 2002 and unpublished results). However, although the comparative analysis of *S. alba* and *Arabidopsis* gave consistent results, whether the changes

observed are causally related to flowering cannot be asserted.

Apart from GAs (reviewed in Mouradov *et al*. 2002), little is known concerning the roles of growth regulators in flowering of *Arabidopsis*. Exogenous applications of cytokinins accelerated flowering in various ecotypes (Michniewicz & Kamienska 1965; Besnard-Wibaut 1981; He & Loh 2002). In the Columbia ecotype, exogenous application of cytokinin accelerates flowering, but only when the light irradiance was low, supporting the idea that the cytokinin effect is dependent on the carbohydrate level (Dennis *et al*. 1996). Moreover, high endogenous levels of cytokinins are associated with early flowering in Columbia plants treated with triacontanol, cerium and lanthanum (He & Loh 2002), as well as in the *amp1* mutant (Chaudhury *et al*. 1993; Nogué *et al*. 2000). However, early flowering of *amp1* may not be a direct consequence of cytokinin levels as the *AMP1* gene encodes a putative glutamate carboxypeptidase that is not predicted to be directly involved in cytokinin biosynthesis (Helliwell *et al*. 2001). Nevertheless, the *amp1* mutation rescued the late-flowering phenotype of the *gi* mutant, which regulates the photoperiodic pathway (Dennis *et al*. 1996), formally suggesting that cytokinins could act downstream of *GI*.

Cytokinins of the isopentenyladenine family increase in abundance in both the mature leaves and the phloem sap of *Arabidopsis* leaves at the end of the inductive LD and during the following SD (Corbesier *et al*. 2003). Although these changes occurred later than those of sucrose, they were detected at the time that the floral stimulus moves out of the leaves (Corbesier *et al*. 1996). Immunolocalization approaches also demonstrated that the SAM of LD-induced *Arabidopsis* plants contained more isopentenyladenine and zeatin than vegetative SD controls. These observations are coincident with the observed increase in the rate of cell division in the SAM during flowering (Jacqmard, Gadisseur & Bernier 2003) and with the essential role played by this class of growth regulators in the control of cell proliferation (Francis & Sorrell 2001; Stals & Inzé 2001). A similar situation was reported in *S. alba* apices (Jacqmard *et al*. 2002). Endogenous cytokinins might therefore play a role in the control of cell division during the floral transition in *Arabidopsis* and act as a component of the floral stimulus of leaf origin. However, so far, little is known about their action on flowering gene expression in *Arabidopsis*, although in *S. alba* cytokinins activate expression of *SaMADSA*, the ortholog of *Arabidopsis SOC1* (Bonhomme *et al*. 2000).

Recent experiments support a role for salicylic acid (SA) in control of flowering of *Arabidopsis* (Martinez *et al*. 2004) as proposed in the past for other species (Cleland 1974; Cleland & Ajami 1974; Goto 1981). SA is involved in stress-induced early flowering in *Arabidopsis*, and might interact with the autonomous pathway through an FCA-independent branch and the photoperiod-dependent pathway through a CO-independent process. In late-flowering SA-deficient *Arabidopsis* plants, the levels of *CO*, *FT* and *SOC1* transcripts decreased to around 50% of those found in wild-type plants under LDs (Martinez *et al*. 2004). In contrast, in SD-grown plants, only the level of *FT* was decreased while *CO* transcript abundance was increased and *SOC1* mRNA levels remained unchanged. Plants carrying the *co1* mutation and the *nahG* transgene, which prevents accumulation of SA because it promotes rapid and efficient conversion of SA to catechol (Delaney *et al*. 1994), resulted in plants flowering later than the *co1* mutant in LDs and this late flowering could be corrected by application of SA. The precise role of SA remains however, to be determined.

Further studies may establish more precise relationships between putative floral signals such as sucrose, cytokinins and SA, and the established hierarchy of regulating proteins that control flowering from the phloem.

MICRO-RNAs, NEW CANDIDATES FOR THE FLORAL STIMULUS?

Recently, small RNAs have emerged as another candidate for a component of the floral stimulus. In recent years, evidence has accumulated that micro-RNAs (miRNAs) play a major role in the control of eukaryotic gene expression. miRNAs and related small interfering RNAs are 21- to 25-nucleotides non-coding RNA molecules that regulate the translation and/or stability of protein-coding mRNAs (Reinhart *et al*. 2002; Carrington & Ambros 2003). Recently, several miRNAs have been found to play specific roles in plant development, including the regulation of flowering time and floral organ identity (Aukerman & Sakai 2003; Chen 2004).

Using an activation-tagging approach, Aukerman & Sakai (2003) and Chen (2004) found that *miR172* (Park *et al*. 2002), which is normally expressed in a temporal manner, causes early flowering and disrupts the specification of floral organ identity when overexpressed in *Arabidopsis*. The regulatory target of that miRNA is a subfamily of *APETALA2* (*AP2*) transcription factor mRNAs. The targets of *miR172* are repressed by the miRNA, suggesting that these targets normally act as floral repressors. Indeed, overexpression of *TARGET OF EAT1* (*TOE1*), one of the *AP2*-like target genes, causes late flowering while double mutants for *TOE1* and *TOE2* are early flowering (Aukerman & Sakai 2003). As the levels of *miR172* were not altered in *co* or *luminidependens* mutants and the level of the *MIR172a-2* precursor transcript was identical in LD- or SD-grown plants (Aukerman & Sakai 2003), the positioning of *miR172* within the genetic pathways that control the floral transition in *Arabidopsis* is difficult. Interestingly however, *miR172* displays a temporal expression pattern similar to that of certain flowering-time genes, such as *FT* and *SOC1*, suggesting that these genes could act potentially downstream of *miR172* and *AP2*-like floral repressors (Aukerman & Sakai 2003).

On the other hand, the analysis of global *Arabidopsis* gene expression allowed Schmid *et al*. (2003) to identify a large group of potential floral repressors that are down-regulated upon floral induction by LDs. Among these,

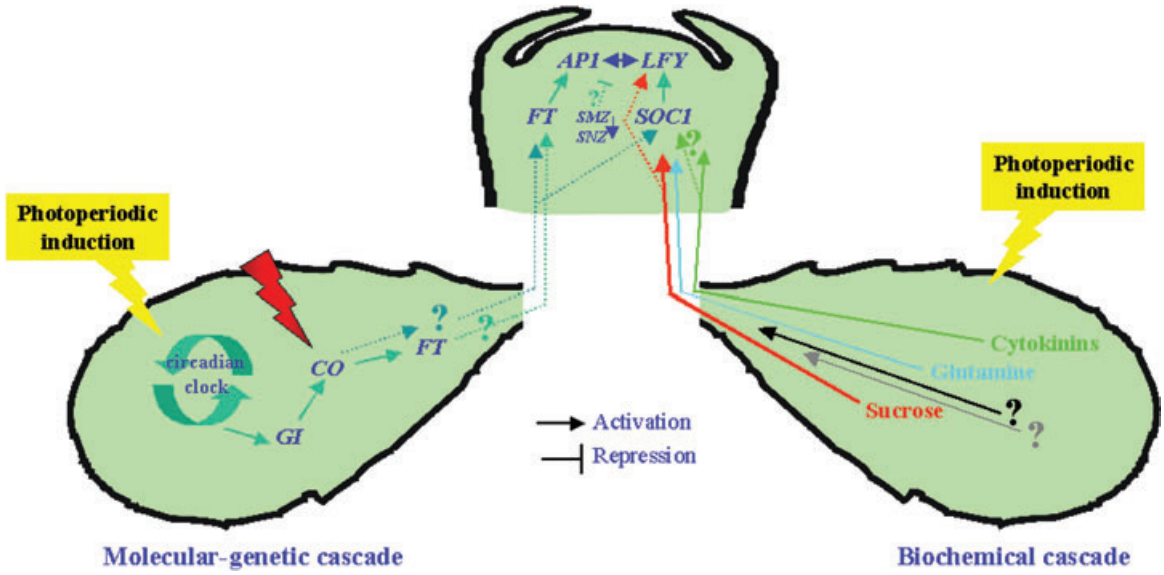

Figure 2. Signalling cascades regulating flowering time by photoperiod in *Arabidopsis*. On the left, the molecular-genetic cascade involving transcriptional activation of genes such as *CO* and *FT* in the leaves through the circadian clock in response to LDs. The result of this gene activation is then transmitted to the SAM where floral morphogenesis takes place. How this signal is transmitted is unknown, but might involve movement of FT protein. On the right, the biochemical cascade in which the LD induction causes increased export of sucrose, glutamine and cytokinins from the leaves towards the SAM. Both the molecular and biochemical changes occurring in the leaves in response to LDs activate a second molecular cascade at the SAM. This leads to the reduction of SMZ and SNZ expression, and to activation of SOC1 expression and finally to the activation of *LFY* and *API*, which induce floral morphogenesis in the SAM. The major unresolved question is how these molecular and biochemical changes interact with each other, both in the leaves and in the SAM. The data underlying this figure are described in detail in the text.

SCHLAFMÜTZE (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*) are two other *AP2*-like genes and potential targets for *miR172*. Although SMZ and SNZ have only a single AP2 domain, phylogenetic analysis showed that they both fall within the same clade as the one defined by TOE1 and TOE2 (Schmid *et al*. 2003). *SMZ* and *SNZ* were repressed upon photoperiod change in Col and L*er* wild type and *lfy* mutants but not in *co* and *ft* mutants, suggesting that these other *AP2*-like genes function as floral repressors and act downstream of *CO* and *FT* but upstream of *LFY*. In their conditions, they found that the *MIR172a-2* precursor was up-regulated after floral induction in a *CO*- and *FT*-dependent manner. Although that seemed to occur at the shoot apex, and may be an indirect effect of *CO* and *FT* function in the phloem.

The *miR159* is also involved in the control of flowering time in *Arabidopsis* (Achard *et al*. 2004). The GA pathway promotes flowering in SDs via a GAMYB-dependent (primarily *MYB33*) activation of *LFY* (Blázquez *et al*. 1998; Blázquez & Weigel 2000; Gocal *et al*. 2001) and, consistent with these ideas, Achard *et al*. (2004) found that transgenic overexpression of *miR159a* resulted in a reduction of the levels of *MYB33* and *LFY* transcripts, and a specific delay in flowering in SDs. The level of *miR159* was also positively regulated by GA and negatively regulated by GAI and RGA proteins, suggesting some complexity in the regulation of *miR159* level.

Although the miRNAs found to play a role in the control of the floral transition have not yet been assigned definitively to a certain genetic pathway, miRNAs, as well as mRNAs and proteins, can move within the phloem sap and between cells allowing them to be considered as potentially a new class of floral signal (Ding, Itaya & Qi 2003). In this respect, Yoo *et al*. (2004) show that small RNA molecules can enter and move through the phloem of several plant species such as *Cucurbita maxima*, *Cucumis sativus*, *Lupinus albus*, *Ricinus communis* and *Yucca filamentosa*. They also found the ortholog of the *Arabidopsis miR159* in cucurbit phloem sap. In addition, a novel protein, *Cucurbita maxima* PHLOEM SMALL RNA BINDING PROTEIN1, that seems to play a role in trafficking of small RNA through phloem and facilitating the movement of small RNA across plasmodesmata, was identified. Finally, heterografting experiments showed that these small RNAs can enter and move with the phloem translocation stream (Yoo *et al*. 2004). Analysis of the miRNAs present in the phloem sap of *Arabidopsis* following the photoperiodic induction of flowering may assess the importance of these molecules as part of the floral stimulus.

PROSPECTS

One of the major goals in understanding the photoperiodic control of flowering is to link the activation of flowering-time genes expressed in the leaf with the export of the floral signal and the role played by these signals at the SAM.

Recent progress has begun to integrate the data derived from the molecular-genetic approach on the regulatory proteins controlling flowering with those based on the physiological analysis of the floral transition and on grafting experiments (Fig. 2). However the nature of the transmitted signal and the full complement of genes activated in the phloem companion cells by regulatory proteins such as CO still remains unclear. Nevertheless, the rate of current progress suggests that a more complete integration of the information derived from the physiological and the genetic approaches may soon be possible.

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