GENETIC CONTROL OF FLOWERING TIME IN ARABIDOPSIS

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ABSTRACT

The timing of the transition from vegetative to reproductive development is of great fundamental and applied interest but is still poorly understood. Recently, molecular-genetic approaches have been used to dissect this process in Arabidopsis. The genetic variation present among a large number of mutants with an early- or late-flowering phenotype, affecting the control of both environmental and endogenous factors that influence the transition to flowering, is described. The genetic, molecular, and physiological analyses have led to identification of different components involved, such as elements of photoperception and the circadian rhythm. Furthermore, elements involved in the signal transduction pathways to flowering have been identified by the cloning of some floral induction genes and their target genes.

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INTRODUCTION

In order to achieve successful sexual reproduction, plants must be able to flower under favorable environmental conditions, and the proper timing of flowering is, therefore, supposed to have an important adaptive value for plants. The transition from vegetative to reproductive development is controlled by both environmental and endogenous factors. Plant physiologists have studied this important process by changing environmental factors and analyzing the subsequent morphological, physiological, and biochemical consequences of these treatments. More recently, genetics has been used to study the mechanism of flower initiation by analysis of genetic variation in species, such as pea and Arabidopsis. Especially in Arabidopsis, the possibility to pursue the genetic analysis down to the molecular level is attractive. This topic and aspects of it have been reviewed (4, 33, 51, 72, 91, 105, 138). In the present review, we summarize the current progress made in the analysis of the transition to flowering using the genetic and molecular approaches as they have been applied to Arabidopsis.

The Transition to Flowering—Meristem Fate Changes

*Arabidopsis thaliana* has a distinct vegetative phase during which the apical meristem produces lateral meristems developing into leaves subtending an axillary bud. The nodes do not elongate, resulting in the formation of a rosette. Floral transition is marked by the establishment of a floral fate in these meristems and by the suppression of leaf production.

A bi-directional development has been shown in this transition, with flowers being initiated acropetally and leaf primordia being suppressed basipetally (53). After floral initiation and following this basipetal direction, the axillary buds of the leaf primordia mostly develop into a secondary shoot (or paraclades or coflorescences). In specific genotypes, they replicate the fate of the initial meristem by forming axillary rosettes. Following the fate change of these lateral meristems, internode elongation takes place (bolting). The elongated stem or inflorescence bears cauline leaves and flowers that are not subtended by leaves at higher internodes. The part of the inflorescence with leaves, which was called early inflorescence by Haughn et al (51), should be considered as part of the vegetative phase. As a consequence of this, total leaf number together with time to flowering are the best quantitative parameters to monitor flowering initiation. Although the appearance of flowers is the final and most dramatic result of the phase change, other changes occur earlier. These are somewhat gradual and can be observed in leaf morphology (93) and in the gradual appearance of trichomes at the abaxial side of the leaves and their gradual disappearance at the adaxial side (27, 131). It has been proposed that phase changes involve a decrease of a floral repressor (130), called a *controller of phase switching* (COPS), which at critical low levels leads to the activation of the *floral initiation process* (FLIP).
The latter is controlled by the so-called Floral Meristem Identity or FLIP genes, such as LEAFY (LFY), APETALAI and 2 (AP1, AP2), AULIFLOWER (CAL), and UNUSUAL FLORAL ORGANS (UFO) (51).

Environmental and Endogenous Control of Flowering

Arabidopsis is a facultative long-day (LD) plant, which means that plants flower earlier under LDs than under short days (SDs). When plants of the common early laboratory genotypes are of sufficient age, indicating a certain competence for flowering, one LD is sufficient to induce flowering (32, 55, 99). This treatment has been used to monitor the morphological (54) and molecular changes (55) involved.

The photoperiodic control of flowering is thought to be mediated by the interaction of photoreceptors, such as phytochrome and cryptochrome, and a clock mechanism or circadian rhythm. Photoreceptors play a role to set the phase of the circadian rhythm, but they can also affect flowering directly, thereby involving light quality in the control of this process. Blue (B) light and far-red (FR) light are known to be more effective to promote flowering than red (R) light (16, 41). Besides, the sensitivity of plants to light quality itself depends on a circadian rhythm (20). The importance of light quality in flowering is determined by the mechanism of light perception, since the ratio red:far-red (R:FR) determines the phytochrome status in the plant. Nevertheless, light is not a prerequisite for flowering, since flowering occurs rapidly in complete darkness when sufficient carbohydrates are provided to the growing shoot meristem (88, 115). A higher light intensity also promotes flowering probably by its effect on carbohydrate supply (7, 67).

Another important treatment promoting flowering is vernalization, which is a transient exposure to low temperatures. The effectiveness of vernalization depends on the stage of the plant, the length of the treatment, and the temperature employed (100, 102). Furthermore, the growing temperature also affects flowering as measured not only by flowering time but also by leaf number (6), which should correct for differences in temperature effects on growth.

In Arabidopsis, the effect of (sensitivity for) the environmental factors strongly depends on the genotype (see below). These environmental factors are thought to modulate certain endogenous components, thus affecting and controlling flowering. Many chemical treatments have been shown to promote flowering (91), of which the application of gibberellins (GAs) (7, 143) and base analogues (91, 114) has attracted most attention, because of their relatively large effects.

GENES AFFECTING FLOWERING TIME

The genetic differences present among ecotypes and, mainly, the genetic variation induced by mutagenic treatments are very important for the analysis of
flowering time in Arabidopsis. Many mutants with an early- or late-flowering phenotype have been described that affect genes controlling both environmental and endogenous factors that influence the transition to flowering. Besides, some cloned genes of unknown function are involved in flowering through their constitutive expression in transgenic plants. Furthermore, the regulation of gene expression through DNA methylation changes has been suggested to play a role in this process.

Natural Variation
Genetic variation for flowering time has been described within and among Arabidopsis natural populations (ecotypes) since the earliest research (76, 77, 102, 114). Arabidopsis has a wide range of distribution in the Northern hemisphere (114), and the differences found when growing different ecotypes under the same laboratory conditions are supposed to reflect particular adaptations to different natural environments. To illustrate this genetic variation, Karlsson et al (66) analyzed 32 ecotypes under SD and LD light conditions, with and without a vernalization treatment. Interactions between the three parameters—ecotype, photoperiod, and vernalization—were found. The first genetic analyses of Arabidopsis flowering time made use of this natural variation to establish the minimum number of genes involved in particular crosses. These early studies often showed the segregation of one or two major genes (65, 102, 132). However, because different parental combinations were analyzed it is not clear whether the same genes were segregating in those populations. Furthermore, segregation of genes with relatively small effects (minor genes) escaped detection in such studies. Napp-Zinn (100, 102) studied in detail the flowering time differences and vernalization requirement between the late ecotype Stockholm and the early Limburg-5 and isolated genotypes with single major flowering time gene differences. This analysis showed that at least four genes were involved and that alleles at the loci with larger effect were more or less epistatic to the alleles with smaller effect. At the locus FRIGIDA (FRI), the dominant allele produced a large delay in flowering time, and at the KRYOPHILA (KRY) and the JUVENALIS (JUV) loci the recessive alleles did so with a smaller effect. Vernalization overcame most of the effects of these late alleles (101).

The advent of molecular markers and the development of genetic maps has facilitated the localization in the genome and the characterization of some of the major loci controlling flowering time differences between very late and very early ecotypes. Napp-Zinn’s FRI gene has been mapped on top of chromosome 4 (30). It has been shown that the extreme lateness present in several ecotypes is due to dominant alleles at a locus mapping at a similar position, which is probably FRI (18, 46, 81, 103, 123). The late-flowering phenotype of FRI is very much suppressed under LDs by the Landsberg erecta (Ler) allele at locus Flowering Locus C (FLC) mapping on top of chromosome 5 (69, 82), likely at
Figure 1  Arabidopsis genetic map showing the mutant loci and polymorphic QTLs identified affecting flowering time. Loci in bold correspond to genes with late-flowering mutant phenotype; otherwise the mutant is early. FLC, FRI, and ART loci, identified from natural populations, are indicated with white boxes. Black and gray boxes correspond to the approximate position of putative quantitative trait loci (QTLs) identified in different crosses; DFF1-2, QTLs in a Hannover/Münden F2 population (73); RLN1-5, QTLs in a Ler × H51 F2/F3 population (31); QLN1-12 in Ler × Col RIL population (60); FDR1-2 in the same Ler × Col RIL population (97); QTL1-7 in a backcross to Limburg-5, with selective genotyping, from F1 Limburg-5 × Naantali (74); QFT 1-5 in a Ler × Cape Verde Island RIL population (3).}

a different position than any of the known flowering mutant loci (see Figure 1). Therefore, the flowering time differences between late and early ecotypes are largely determined by these two loci, each one by itself having a small effect and requiring dominant alleles at both to produce extreme lateness. So far, only the laboratory strains Ler and C24 (69, 120) have been found to contain early FLC alleles. The late-flowering phenotype of FRI and FLC, present under both LD and SD conditions, is reduced by FR-enriched light and eliminated by vernalization; saturation of vernalization abolishes a further effect of FR light (79). The Ler early FLC alleles also suppress the lateness of mutant alleles at several loci (see below) such as ld (69, 82) and fld (121), which were isolated in Col background but not in Ler.

A third locus, Aerial RosetTe (ART), located on chromosome 5, has been identified by analyzing another very late ecotype, Skye (46). The dominant
ART allele in combination with dominant alleles at another gene located on chromosome 4, probably FRI, delays the transition from vegetative to reproductive in the axillary meristems, giving rise to aerial rosettes under LDs. ART alone seemed to produce lateness, but taking into account the close location to FLC, it is unclear how much of the ART late phenotype comes from FLC and whether late FLC alleles are also necessary to produce the aerial phenotype. Epistatic analysis shows that aerial rosettes are produced by combining ART not only with FRI but also with the late-flowering mutants fca, fve, fpa, ld, fwa, co, and gi (see below) (47). Thus, ART might act downstream in the flowering pathways, and in a late-flowering background it would produce a prolonged insensitivity to the floral evocation signals in the axillary meristems.

To find other natural alleles of smaller effect has required the combination of molecular genetic maps with statistical methods to map quantitative trait loci (QTLs) (59). QTL analyses have been performed using crosses between late and early ecotypes (31, 74) and between early ones (3, 60, 73, 97) (Figure 1). Multiple QTLs have been found in all the crosses and therefore differences in behavior of flowering mutant alleles in different genetic backgrounds cannot be directly attributed to a single gene differing between ecotypes. Further analyses are needed to detect the interacting genes in each particular case. The spectrum of natural variation is different from the spectrum of flowering-time variants obtained by mutational analyses. This is at least due to the limitations of the reduced number of ecotypes used to generate mutants, and to the possible deleterious pleiotropic effects of some of the induced mutations. For example, no mutant allele has been identified for the FRI locus. Some dominant late-flowering mutants such as McKelvie’s florens (F) mutant (95) and the M73, L4, L5, and L6 mutants (133) were reported allelic to FRI, but it was unclear whether they were mutants or contaminant natural variants (69). Some of the putative QTLs locate at mutant gene positions, and therefore it is expected that part of the natural variants will correspond to alleles of mutant flowering genes. However, there are known mutant flowering genes scattered all over the genome (see Figure 1), and complex situations such as very closely linked QTLs might be expected. As an example, several analyses have detected QTLs on top of chromosome 5, a region enriched for mutant flowering genes, and at least some of these QTLs are likely to correspond to a different locus than FLC (3, 74). Therefore, the identification of the individual alleles controlling this variation is necessary.

Late-Flowering Mutants
Late-flowering mutants with a strong effect but with no other obvious pleiotropic effects were described for the first time by Rédei (113). He isolated the constans (co), gigantea (gi), and luminidependens (ld) mutants in Col background. Later
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on, more mutant alleles at these and 11 other loci in Ler were isolated and described by Koornneef et al (70) and in Wassilewskija (Ws) by Lee et al (80). Thus, the loci LD, CO, GI, FE, FT, FD, FY, FCA, FHA, FPA, FVE, and FWA have been considered the classical late-flowering genes (Figure 1). They have been physiologically characterized, and epistatic relationships have been examined in relation to early, late, and meristem identity genes (50, 68, 119). Koornneef et al (68) constructed 42 double mutants among 10 of these loci. The epistatic interactions proved to be complex, but groups of loci similar to the ones established on the basis of their physiological behavior were identified. A major epistatic group could be identified corresponding to the group of mutants co, fd, fe, fha, ft, fwa, and gi. These mutants are late mainly under LD conditions, i.e. they show little or no response to daylength, and they have a low response to FR supplementary light and to vernalization treatments. In contrast, the epistatic behavior of the mutants that are much more responsive to these environmental factors (fca, fpa, ld, fve, and fy) is more complex. Combining the FLC-Col allele with late-flowering mutants in Ler background, Sanda & Amasino (122) showed that the mutants fca, fpa, and fve, of the same group, all have very enhanced late phenotypes like those of ld, FRI, and fld. Flowering locus D (fld) is another late-flowering mutant without apparent pleiotropic effects (121). This mutant retains its response to photoperiod, and its flowering time can be reduced by cold treatment and low R:FR ratio.

Five of these late-flowering genes, LD, FCA, CO, FT, and FHA, have been cloned. LD encodes a glutamine-rich nuclear protein containing a possible homeodomain (80). Its function remains unknown.

FCA encodes a protein containing two RNA-binding domains and a WW protein interaction domain, suggesting that it is functioning in the posttranscriptional regulation of transcripts involved in flowering (87). An interesting characteristic of this gene is that the transcript is alternatively spliced; four different FCA transcripts have been found, the full-length transcript being only one third of the total amount. The WW domain, which is only present in the full-length transcript, seems essential for the flowering time effect.

The CO gene was found to encode a protein with similarity to GATA-1–type transcription factors (111). Constitutive expression of CO leads to earliness (127), thereby confirming that this gene has flowering promoting properties. Besides, transgenic plants with extra copies of CO flower earlier than wild type, suggesting that CO activity is limiting flowering time (111). The CO mRNA appears more abundant in plants grown under LDs than under SDs, in agreement with the role of this gene in promotion of flowering under LDs. It is interesting to note that two homologues of the CO gene, CONSTANS LIKE 1 (COL1) and COL2, have been described recently (78, 110), and although quite similar in structure, their role in flowering has not yet been demonstrated.
The FT and FHA genes have been recently cloned. The FT gene shows strong homology to the TERMINAL FLOWER 1 (TFL1) gene (5; D Weigel, personal communication). The FHA gene encodes the CRY2 protein (85; C Lin, personal communication) and is thought to be involved in blue-light perception.

Late mutants have been identified that are involved in light perception or transduction. The mutants long hypocotyl 4 (hy4) and phytochrome A (phyA) correspond to the blue-light photoreceptor CRY1 (1) and phytochrome A (phyA) (142), respectively. An elongated hypocotyl is also shown by the late hypocotyl (lhy) mutant (126). This mutant is daylength insensitive and lacks circadian rhythms for leaf movement (R Schaffer, I Carré & G Coupland, personal communication). It is suggested that this Myb-like transcription factor (R Schaffer & G Coupland, personal communication) might be a component of the circadian clock.

Mutants deficient in GA biosynthesis, like ga1, or action, like gibberellin insensitive (gai), show a late phenotype under SD conditions (143).

Late-flowering mutants have also been identified as defective in starch metabolism, such as phosphoglucomutase (pgm) (22) and ADP glucose pyrophosphorylase 1 (adp1) (86), which lack leaf starch and flower late, mainly under SD conditions. In contrast, starch excess 1 (sex1) (23) and carbohydrate accumulation mutant 1 (cam1) (40), which also flower late, have increased starch content in leaves. This characteristic was also observed in the late mutant gi (6, 40). In the pgm and sex1 mutants, the late-flowering phenotype could be suppressed by a vernalization treatment (11). The late-flowering phenotype observed in these mutants is not due to the defect in starch accumulation and the slow growth but more to the inability to mobilize the stored carbohydrates (11, 40). Nevertheless, it remains unclear how carbohydrate metabolism affects flowering time in Arabidopsis.

Additional mutants that show lateness either under specific conditions and/or with more pronounced pleiotropic effects are de-etiolated 2 (det2), ted1 (a suppressor of det1) (108), ethylene insensitive (ein) (39), ethylene responsive (etr1) (14), short integument (sin) (112), and vernalization (vrn) (25). Several of these genes have been cloned and are known to encode steps in brassinosteroid biosynthesis (DET2) (84) and ethylene action (EIN, ETR1) (39).

**Early-Flowering Mutants**

Early-flowering mutants were described later than the late ones, probably due to the use of early ecotypes growing in LD conditions, which makes the effects of early mutants less pronounced.

The early-flowering mutants with the most dramatic phenotypes are embryonic flower 1 and 2 (emf1 and emf2). The emf mutants do not produce a normal rosette after germination, but they make only a few cauline leaves
followed by floral buds. In addition, their flowers are usually abnormal and incomplete (130). The phenotype indicates that most of the normal vegetative phase is bypassed, and EMF genes are therefore likely to play a central role in the COPS mechanism (51, 145). Double-mutant analyses indicated that the emf is epistatic to both early- and late-flowering mutants (145), although differences have been found among double mutants of emf with several late-flowering mutants (146). Interactions between EMF and genes regulating inflorescence meristem development and floral organ identity were revealed in the analysis of double mutants between emf and terminal flower (tfl) and agamous (ag). It has been proposed that the EMF genes play a role during the different phase transitions of the plant by a gradual reduction in its activity (145).

Several early-flowering mutants are involved in light perception and light signal transduction pathways. Among these, long hypocotyl 1 and 2 (hy1 and hy2), which are defective in phytochrome chromophore biosynthesis (106), and phytochrome B (hy3 = phyB), deficient in phytochrome B (128), are daylength sensitive (45). Overexpression of phytochrome B also leads to early flowering (9), suggesting that the balance between different phytochromes is important for the proper timing of transition to flowering. Furthermore, phytochromes A and B are not the only phytochromes influencing this transition because phyA phyB double mutants still respond to increases in the proportion of FR light by flowering early (37).

The phytochrome-signaling early-flowering (pef1) mutant shows a similar phenotype to hy1 and hy2 but cannot be rescued by the chromophore precursor biliverdin, which can rescue hy1 and hy2. This suggests that pef1 has a mutation in a signaling intermediate interacting with all the phytochrome family members (2). The pef2 and pef3 mutants more closely resemble phyB mutants. Therefore, they may have lesions early in the signaling pathway primarily mediated by phyB and/or some of the other phytochrome gene family members (phyC, D, E) (2).

The sucrose-uncoupled 2 (sun2) mutant has an early-flowering phenotype, at least under LD conditions, and shows a long hypocotyl and reduced fertility (38). This mutant was initially isolated as showing reduced repression by sucrose of a transgenic plastocyanin promotor. These phenotypes suggest an interaction between carbohydrate metabolism repression and light signaling in the flowering process.

Some of the mutants influence the circadian rhythm. The early-flowering 3 (elf3) mutant lacks rhythmicity in circadian-regulated processes under constant light conditions (56), while the cop1 and det1 show shorter circadian period lengths in constant darkness (96). The elf3 mutant flowers early under both LDs and SDs, is photoperiod insensitive, and has a long hypocotyl (most noticeably in blue and green light). Double mutant analysis with hy4 and hy2 indicates
that \textit{ELF3} is involved in blue light–regulated photomorphogenesis (147). In contrast, the \textit{cop1} and \textit{det1} mutants are early flowering in SDs and also have a constitutive photomorphigenic phenotype. \textit{DETI} encodes a novel nuclear-localized protein, suggesting that it controls cell type–specific expression of light-regulated promotors (109). \textit{COP1} encodes a protein with both a zinc-binding motif and a G\_\beta homologous domain (35). Double mutant analysis with \textit{hy1} and \textit{hy4} suggests that \textit{COP1}, together with other \textit{COP} and \textit{DET} genes, acts downstream of phytochrome and the blue-light photoreceptor (28, 75). The \textit{DET}/\textit{COP} protein complex formed in darkness negatively regulates transcription of certain genes involved in photomorphogenesis (134). It is thought that light signals mediated by multiple photoreceptors can be transduced to inactivate the pleiotropic \textit{COP}/\textit{DET} regulators and thus release the repression of seedling photomorphogenesis. Nevertheless, since the \textit{cop/det} mutants also have a clear phenotype in light-grown plants, these genes may also function in other pathways (94) that are not directly related to photomorphogenesis.

Cytokinins, applied to wild-type plants, result in a phenocopy of \textit{det1} mutants (29). Consistent with this is the altered meristem program 1 \textit{(amp1 = pt = hpt = cop2)} mutant, which has high levels of cytokinin, shows a constitutive photomorphigenic phenotype, flowers early, and is daylength insensitive, like the \textit{det1} mutant (26). This suggests a role for cytokinins in the light signal transduction. Nevertheless, this mutant shows a strongly altered growth and leaf formation rate rather than altered flowering time. Other mutants, like \textit{spy}, show the role of GAs in the transition to flowering. The \textit{spy} mutant has the phenotype of wild-type plants treated with GAs and is therefore early flowering. The \textit{SPY} gene is probably involved in the GA signal transduction pathway (58).

The early-flowering elongated \textit{(elg)} mutant shows a pleiotropic phenotype that suggests a disruption of phytochrome and/or GA function. However, it has been shown that \textit{ELG} acts independently of phytochrome and GA action (49).

Another group of mutants involves genes whose function in the transition to flowering has not yet been determined. Two of these mutants, \textit{early-flowering 1} and 2 \textit{(elf1} and \textit{elf2)}, do not show clear pleiotropic phenotypes and have a daylength response (148). In contrast, \textit{early in short days 1} \textit{(esd1)} (JM Martínez-Zapater, C Gómez-Mena, L Ruiz-García & J Salinas, personal communication) and 4 \textit{(esd4)} (126; G Murtas, P Reeves & G Coupland, personal communication) \textit{early bolting in short days} \textit{(ebs = speedy)} (JM Martínez-Zapater, C Gómez-Mena, M Pineiro & G Coupland, personal communication), and \textit{early flowering in short days} \textit{(efs)} (129) have a reduced daylength response and show pleiotropic phenotypes such as reduced fertility and/or plant size. Double mutant analysis indicated that these mutants might interact with some
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of the late-flowering mutants (126, 129). The ESD4 gene has recently been cloned but did not show homology to other genes of known function, although related sequences were found in a range of other organisms (117).

A number of early-flowering mutants are involved in the later stages of floral transition. These genes are regulating the expression of floral meristem identity genes like AP1, LFY, and AG (AGAMOUS). Mutations in TFL1 result in early flowering, replacement of coflorescences by flowers, and determinated growth of the apical meristem, which develops into a flower (125). The tfl mutation shows ectopic expression of LFY and AP1 in the apical meristem (13, 48), agreeing with overexpression in transgenic plants of LFY and AP1, giving a phenotype reminiscent of tfl1 (13, 89, 139). Therefore, it appears that the tfl1 mutant fails in negatively regulating LFY and AP1, thereby promoting early flowering with the formation of a terminal flower (125). The TFL1 gene has been cloned and encodes a putative phosphatidylethanolamine-binding and nucleotide-binding protein (15, 104).

Mutations in the curly leaf (CLF) gene cause a very similar phenotype to the one conferred by constitutive expression of the meristem identity gene AG, showing narrow and upwardly curled leaves as well as early flowering in SDs (98). CLF has been cloned and encodes a protein with homology to polycomb-group genes. CLF is required to repress AG transcription in leaves, inflorescence stems, and flowers (44).

Flowering Time Genes Identified by Constitutive Expression in Transgenic Plants

Constitutive expression of cloned genes is commonly used as a tool to confirm and further analyze the role of genes cloned on the basis of a mutant phenotype. Furthermore, when no mutants are available, the function of cloned genes can be inferred also by analyzing transgenic plants that constitutively express these genes.

For a number of genes of unknown function transgenic plants suggested their role in promoting flowering, although no late mutants were available. The FPF1 gene is expressed immediately after photoperiodic induction. Constitutive expression of this gene leads to early flowering under LDs and SDs and to other associated changes that mimic the effect of GA applications (64). In a search for genes whose products bind to the promotor of the meristem identity gene AP1 (and its Antirrhinum ortholog SQUAMOSA), the SPL3 gene was isolated. Its constitutive expression leads to earliness (19). Although overexpression phenotypes show the sufficiency of these genes to promote flowering, they do not prove that these genes are necessary for the timing of the transition. Therefore, late mutants at these loci may not be found. This is because the function of these genes may be redundant or they may be involved in other related
processes. This is illustrated with the meristem identity genes AP1 (89), LFY (139), and the meristem-organ identity gene AG (98), for which mutants are available without an obvious flowering-time phenotype. However, transgenic plants expressing these genes constitutively do flower early.

Another way by which overexpression may indicate the function of a gene is by providing the endogenous gene with constitutive promoters or enhancers. A transposable element with outward-directing 35S promoter has generated the dominant mutant lhy (34, 126), described above, which constitutively expresses this gene. A phenocopy of the lhy mutant was obtained in transgenic plants with constitutive expression of a related Myb-type gene called CCA1 (136, 137). The protein encoded by this gene binds to phytochrome-regulated promoters, indicating a link with the phytochrome-related long hypocotyl phenotype.

Methylation and Epigenetics

During the past few years, it has become clear that DNA methylation plays an important role during development of eukaryotes. DNA (de)methylation is involved in the control of gene expression during development and differentiation, by either negative or positive regulation (12, 90). An important observation is that mice carrying a homozygous mutation of the DNA methyltransferase gene do not develop beyond a certain embryonic stage (83). Furthermore, there is evidence that DNA methylation is one of the mechanisms to silence foreign DNA in eukaryotes (93).

There is some indication that DNA methylation might be involved in the vernalization response. Arabidopsis plants either cold treated or treated with the demethylating compound 5-azacytidine show reduced amounts of 5-methylcytosine in their DNA. Among the late-flowering mutants there are some, like fca and fy, responsive to vernalization (70), and others such as gi, fd and ft that show little response to this treatment. After treating these mutants with 5-azacytidine (17), earliness was observed in the responsive genotypes but not in the nonresponsive ones, thus imitating the effect of vernalization.

DNA methylation has also been reduced in transgenic plants. Transgenic C24 plants were constructed in which methylation was suppressed by the antisense methyltransferase cDNA MET1 from Arabidopsis thaliana (36, 42, 43). This resulted in a reduction of total genomic cytosine methylation, which induced several developmental effects, and a correlation was found between demethylation and reduction in flowering time. This was particularly clear under SDs where C24 shows a pronounced vernalization response (EJ Finnegan and ES Dennis, personal communication). The authors suggest that demethylation is involved in the process of flower promotion by vernalization. Surprisingly, using the same antisense approach, Ronemus et al (118) found a late-flowering phenotype in Col genetic background.
In addition to effects on flowering time, reduced methylation led to abnormal flowers due to ectopic expression of genes such as AG and APETALA3 (AP3), probably caused by changes in chromatin structure (43). These phenotypes are in some aspects similar to the phenotype of the early-flowering mutant clf, defective in a gene encoding for a polycomb-like protein, which is known to affect chromatin structure (44).

Changes in the DNA methylation state, either by antisense methyltransferase genes or by mutation, also lead to heritable mutant phenotypes. Among these, flowering time phenotypes are relatively abundant. A mutant, designated ddm1 (decrease in DNA methylation) affected in DNA methylation but not exhibiting a flowering time phenotype, has been isolated in Arabidopsis (135). The ddm1 mutation causes hypomethylation up to 70% of the total genomic 5-methylcytosine levels, although these plants exhibit normal methyltransferase activity. The ddm1 mutation induces other heritable mutations after repeated self-pollination (63). Among them, there is a late-flowering mutant designated fts mapped on chromosome 4 at a similar position as fwa (62). The latter late-flowering mutant was described by Koornneef et al (70), and both alleles, fwa-1 and fwa-2, show strong hypomethylation in at least a 5-Mbase region where the gene has been mapped (129). Recently, Jacobsen & Meyerowitz (57) showed that a superman (sup) mutant epi-allele found in antisense cytosine methyltransferase lines is due to highly localized hypermethylation in the SUP gene. The regulation of transcription of certain genes that are involved in the flowering initiation process is apparently either under control or may be influenced by DNA methylation as a component of cell memory.

DISCUSSION: A WORKING MODEL FOR THE CONTROL OF FLOWERING TIME

The complex multigenic control of flowering as revealed by genetic analysis in Arabidopsis (91, 107, 138) and pea (141) indicates that the process is complex and influenced by many factors. This observation supports physiological evidence for a multifactorial control of the transition to flowering (10). Recently, it has been proposed that the transition to flowering is the developmental default state (51, 91, 115, 130, 138). This hypothesis is mainly based on two observations. First, Arabidopsis can flower with very few leaves in complete darkness when sufficient sucrose is provided to the shoot meristem (88, 115). Under these conditions the late mutants, as far as tested, are as early as wild type with the exception of fwa and ft (88). Second, no mutants without flower-like structures have been described, but in contrast, the emf1 and emf2 mutants with hardly any vegetative development have been isolated (130). This default state is then thought to be suppressed by a floral repressor or a promotor of vegetative
development, which may be encoded by the EMF genes (51, 91, 130, 138). During development, the effect of this repressor decreases in accordance with gradients observed for the various parameters related to phase changes (51, 130). The genes identified by the late and early mutants are assumed to play a promotive or repressive role, respectively. Environmental factors such as daylength and vernalization may regulate the flowering time genes.

The genetic and physiological classification of several late mutants has led to group these genes into two different general modifying promotion pathways (Figure 2). The late-flowering genes, FCA, FY, FPA, FVE, LD, and FLD, are assumed to promote flowering constitutively, under LD and SD, and are therefore involved in the so-called constitutive or autonomous promotion pathway. These mutants are highly daylength sensitive, presumably because when this pathway is defective the transition to flowering becomes very dependent on another pathway that is largely regulated by photoperiod. This second pathway has been called the LD promotion pathway, involving the late-flowering genes, CO, FD, FE, FHA, FT, FWA and GI, which are believed to promote flowering mainly under photoperiodically inductive conditions, i.e. LDs. Nevertheless, since the mRNA level of CO, a gene that promotes flowering, is reduced in SD, the effect of LD might be the removal of a hypothetical SD repressor, and therefore this pathway could also be referred to as SD repression.

The reduced responsiveness to vernalization of these LD promotion mutants does not imply that these genes are involved in sensing the cold signal, because long vernalization treatments are effective in these mutants (24) and the parental genotype Ler also has a limited vernalization responsiveness compared with mutants such as fca, even when it flowers late under SD (24, 25). Furthermore, double mutants involving representative genes of the two pathways are sensitive to vernalization, although the absence of the LD promotion cannot be replaced by the vernalization treatment (68). In contrast, the stronger vernalization sensitivity of the constitutive promotion mutants suggests that this pathway and a third one, the vernalization promotion pathway, might converge downstream and are able to replace each other. The candidate genes affecting the sensing or transduction of the cold signal are the VRN genes isolated on the basis of their lack of a vernalization response in an fca mutant background (25).

Analysis of double mutants places the LD promotion mutations with similar phenotypes in the same epistatic group. This study also indicated that the situation for the constitutive pathway mutants is more complex, suggesting parallel subpathways within this group. In particular, the fpa mutant shows a complex behavior and might play a role in both pathways (68).

To place other flowering genes, including those for which the recessive (probably loss of function) phenotype, is earliness, in relation to these two general pathways will be attempted. However, since detailed genetic analyses of double
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Figure 2. A model describing the interactions of flowering time genes in Arabidopsis. Different groups of genes, established according to their genetic and physiological behavior, are shown in boxes. Lines within boxes indicate subgroups. Promotive effects are shown by "→"; repressive effects by "⊣".
mutants are lacking in most cases, this can only be done in a provisional manner. Furthermore, not knowing whether mutants are true null alleles complicates the interpretation of any double mutant analysis (68, 138). In the case of the FRI and FLC genes, which in combination lead to the extreme late-flowering phenotype and vernalization responsiveness observed in many late ecotypes, it is thought that they also affect the constitutive pathway in an inhibitory way. “Double mutant” analyses between the early FLC-Ler allele and the late mutations fld, ld, fca, fve, and fpa flower relatively early in comparison with the late-flowering phenotype observed in these late mutants in an FLC-Col background (122). This suggests that these late genes antagonize inhibitors. A vernalization treatment might have the same effect.

The phenotype of double mutants between late and early mutants has already allowed the placement of some early genes in one of the pathways. The esd4 and efs mutants are epistatic to fve, suggesting that FVE might interact with the inhibiting gene products of these early genes (126, 129).

The analysis of mutants originally not isolated for their flowering phenotype, but having an effect on it mainly either under SDs or LDs, has given indications about the mode of action of GAs and light in the floral initiation process. The extreme lateness of GA-deficient or GA-insensitive mutants, only in SD, suggests that this hormone is required in these conditions, acting through the constitutive pathway and compensating for the absence of the LD promotion pathways. The synergistic effect of the co ga1 and co gai double mutants in LD is in agreement with this (111).

It has been suggested that the outcome of the constitutive pathway is similar to that of vernalization and GAs. In agreement with this, a detailed morphogenetic analysis of fve mutants indicated that they show some symptoms of reduced GA levels or reduced GA action, although these are far less extreme than in ga1 and gai mutants (92). Besides, the implication of GA synthesis in vernalization has been strongly suggested, not only by the work in Thlaspi arvense (52) but also in Arabidopsis by the finding that the ga1-3 mutant does not respond to vernalization in SDs. However, the observation that the fca ga1-3 double mutant responds well to vernalization under continuous light argues against the hypothesis that vernalization acts through GA biosynthesis or through the FCA gene product (25). Nevertheless, GAs have been shown to be crucial for a number of processes associated with flowering, such as internode elongation and the suppression of adaxial trichomes, which indicates that there is a higher GA activity after the transition to flowering, which might be partially due to the promotive effect of LDs on the GA 20-oxidase encoded by the GA5 locus (144). Therefore, the actual sequence in the interaction among the constitutive promotion pathway, GAs, and vernalization remains to be solved, and further research in this area is necessary. Besides, it has been suggested that the vernalization promotion involves modulation of gene
expression through changes in methylation, which needs further confirmation
by the study of the target genes. It is possible that GAs, vernalization, and
the constitutive pathway have a similar target that leads to floral induction.
Therefore, their functions may overlap, and different environmental conditions
may modulate the three promotion pathways in a different way. Whether this
putative common target is at the level of FRI/FLC or downstream is unknown,
but a candidate target gene that probably is specific for GAs is FPF1 (64).

The chromophore and phyB mutations cause early flowering, indicating that
this phytochrome has an inhibitory role in flowering, which seems independent
from the daylength sensing mechanism. The earliness conferred by the hy mu-
tants to the co, gi, and fwa mutant backgrounds under both LDs and SDs (71)
further indicates that early flowering caused by the hy mutations does not act
exclusively through these flowering time genes. However, the hy mutants in the
fca mutant background are late under SDs, suggesting that phyB, apparently,
mainly represses the FCA gene pathway under SD conditions (71). In contrast,
under LDs, hy mutants in the fca background are early, suggesting that under
these conditions another promotion pathway is repressed by phyB. Therefore,
the phyB and other light-stable phytochromes might repress both the constitu-
tive and the LD promotion pathways. Reed et al (116) have shown that phyB
decreases responsiveness to GAs, which suggests that this phytochrome might
repress flowering through this mechanism.

The effect of the light labile phytochrome A is very different and more or less
opposite to that of the light-stable phytochromes. Phytochrome A promotes
flowering, since overexpression of this gene leads to earliness (9), and the
mutant is late when SDs are extended by 8 h of light with a low R:FR ratio (61).
Under LDs provided by “normal” fluorescent lamps, no lateness is observed,
probably because other photoreceptors can compensate for the lack of phyA.
In pea, PHYA-deficient mutants have a much more pronounced late phenotype
under LDs and are photoperiod insensitive (140). In this species, SDs lead to
the production of a graft transmissible inhibitor that is under control of the pea
genes Sn, Dne, and Ppp. Based on grafting studies and the analysis of double
mutants, it was concluded that phyA reduces the level of this inhibitor under
LD conditions (140).

In addition to phytochromes, blue-light receptors, called cryptochromes, play
a role in flowering. As in the case of phytochrome, the different members of this
family of photoreceptor seem to have distinct roles in the transition to flowering.
The promotive role of the cryptochrome I encoded by the HY4 gene seems minor
since the flowering time effect of this mutant is limited (8). The effect of the
cryptochrome II (CRY2) appears more important in LDs, since these mutants
(fha) are clearly late. The similarity in phenotype of these mutants with the
LD promotion pathway mutants strongly suggests that CRY2 and phyA are the
photoreceptors for this pathway.
To measure the length of the photoperiod, apart from photoreceptors, a time measurement mechanism is required, which is probably provided by a circadian rhythm. The relation between daylength and a circadian rhythm affecting leaf movement and \textit{CAB2} gene expression was studied in the \textit{Arabidopsis elf3} mutant, which is early and daylength insensitive (56, 147). The \textit{elf3} mutant lacks these circadian rhythms in continuous light but not in light/dark cycles and continuous darkness, suggesting that \textit{ELF3} is involved in circadian regulation, especially in the transduction of light signals to a component of the clock (20, 56). Two other genes that may affect directly the clock are \textit{LHY} and \textit{CCA1}, both encoding Myb-related proteins. In wild type the \textit{LHY} mRNA is expressed rhythmically (R Schaffer & G Coupland, personal communication). In the presence of the overexpressed copy of \textit{LHY}, transcription from the endogenous \textit{LHY} promotor is repressed, indicating that \textit{LHY} is part of a transcriptional feed-back loop. Both the phenotypic effects and molecular properties of this gene are expected for circadian clock components (21).

In relation to this, the early-flowering phenotype under SD of mutants such as \textit{det1} (108) and \textit{cop1} (138), suggest that the DET1/COP1 proteins suppress flowering under SD, which may be done by repressing floral promotors such as \textit{CO}. The simplest hypothesis to explain this SD inhibition would be through repression by DET1/COP1 in the absence of the LD signal, and this would predict that photoreceptor-deficient mutants, which would not be able to remove the suppression of flowering by DET1/COP1, should be late in LD. Although this might be the case for phyA and blue-light receptor mutants (8, 61), this is not the case for mutants affecting phyB (\textit{phyB = hy3}) and the chromophore (\textit{hy1} and \textit{hy2}), which are relatively early in SD (45) due to the inhibiting effect of phyB discussed above. Nevertheless, analyses of double mutants involving these genes are still needed in order to understand the role of \textit{DET1/COP1} in this process.

Based on grafting studies, daylength is perceived by the leaves, and the signal is then transported to the apical meristem (10). It is not clear whether the crucial target is the apical shoot meristem or the lateral leaf/flower primordia itself. The latter is suggested by the chimeric structures observed by Hempel & Feldman (54) after the transfer of plants from SD to LD. In Arabidopsis, the shoot apical or inflorescence meristem remains undetermined, and to maintain this state the \textit{TFL1} and \textit{TFL2} genes are required. The \textit{TFL1} gene is strongly expressed in a group of cells just below the apical dome of the inflorescence in accordance with a role in this meristem (15). Bradley et al (15) suggested that \textit{TFL1} delays the commitment to flowering during vegetative phase where it is also weakly expressed. In contrast, its \textit{Antirrhinum} ortholog \textit{CEN} is not expressed during vegetative development, and \textit{cen} mutants are not early (15). Double-mutant analyses between \textit{tfl} and the late-flowering \textit{fpa, fve, fwa}, and \textit{co} indicates that to repress flower initiation \textit{TFL} requires the function of the late-flowering loci tested (119).
The floral meristem identity genes \textit{LFY}, \textit{AGL8}, and \textit{AP1} are crucial early targets of the floral promotion process and thereby of the flowering time genes as evidenced from mutant phenotypes, from studies using transgenic plants with constitutive expression, and from expression analyses after flower induction. Elegant studies in which the \textit{CO} function was regulated by the ligand-binding domain of the rat glucocorticoid receptor showed that \textit{LFY} expression increased within 24 h after the activation of \textit{CO} (127) and that \textit{AP1} is expressed later. This sequence of gene expression was also observed after the shift from SDs to LDs (55). Both \textit{LFY} and \textit{AP1} can convert shoot meristems into floral meristems as shown by the early flowering of transgenic plants that constitutively express these genes. However, expression of these genes may only trigger floral development after the main shoot has acquired competence to respond to its activity, since constitutive expression of \textit{LFY} still allows the formation of some leaves (139).

Two lines of evidence indicate that \textit{FT} and \textit{FWA} also have effects in the floral induction process. Double mutants of \textit{ft} and \textit{fwa} with \textit{lfy} virtually lack floral initiation and do not show \textit{AP1} mRNA in the inflorescence apex, indicating the importance of these genes for the initiation of \textit{LFY} and \textit{AP1} expression (119). Furthermore, in contrast to other late-flowering mutants, \textit{ft} and \textit{fwa} still are late in continuous darkness (88). This indicates that their role is not restricted to modifying the level or effect of the light-induced floral repressor only, but instead these genes may work at the meristem level and may be required (also) for the flower initiation process itself. The normal flowers of these mutants show that genetic redundancy exists for the flower initiation program as well as for the control of flowering time (119). The cloning of \textit{FT} (5) revealed strong homology with \textit{TFL1}. The opposite effect of mutations in these genes points to a different role, and the two genes might have in common their interaction with \textit{LFY} and \textit{AP1}.

In what way the promoting flowering environmental signals interact with the flowering genes, how these genes interact, and how they activate their targets is still unknown. The phenotypic and epistatic analyses indicate a complex network and suggest various redundant pathways. Since some of the promotive flowering time genes may act as transcription factors (\textit{LD} and \textit{CO}) or may affect RNA stability (\textit{FCA}), a sequence of gene activation events is a likely mechanism. The combined genetic, physiological, and molecular analyses will provide answers to this just-started and evolving picture of the network.

CONCLUDING REMARKS

Recent genetic, molecular, and physiological analysis of flowering initiation in Arabidopsis has led to the identification of components in this important developmental process. Molecular elements involved in some of the initial steps
such as photoreceptors and components of the circadian clock, in intermediate steps such as some of the cloned flowering genes, and in the target genes of floral induction, are now known. However, many questions remain: how do these elements interact and transmit the signals? Intriguing questions are, for example, how light and clock signals are integrated and how these interact with the flowering genes. The effect of vernalization at the molecular level is not yet understood. Furthermore, although a role for GAs in flowering is strongly indicated, its function remains unclear, as does the role of other hormones such as cytokinins, and factors such as carbohydrates. Besides, the sequence of events and redundancy suggested by the genetics and physiology is not yet understood at the molecular level. However, the molecular and genetic tools are available in Arabidopsis and will further refine and modify the model presented in this review. It will be important to relate and complement these studies in Arabidopsis with those in other plants to identify both the differences and common aspects, as it has been done for flower development between *Antirrhinum* and Arabidopsis. For flowering timing, pea is particularly important because of its similarity with Arabidopsis in the physiological responses and its suitability for grafting studies (140). This may aid in identifying the nature of the floral repressor, deduced thus far only from genetic and physiological studies, and in determining whether any of the flowering time genes encode the elusive graft-transmissible florigen.

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Literature Cited

role of the late-flowering locus, GI in the flowering of Arabidopsis thaliana. Plant J. 3:231–39
47. Grbic V, Gray J. 1997. Aerial rosette 1, ART1, is a new late flowering gene of Arabidopsis thaliana. *Int. Conf. Arabidopsis Res.*, 8th, Madison, Wis. 5-9 (Abstr.)
111. Putterill J, Robson F, Lee K, Simon R, Coupland G. 1995. The *CONSTANS* gene of *Arabidopsis* promotes flowering and
 encodes a protein showing similarities to zinc finger transcription factors. Cell 80:847–57


