A genetic and molecular analysis of flowering time in *Arabidopsis thaliana* using natural variation

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A genetic and molecular analysis of flowering time in *Arabidopsis thaliana* using natural variation

Een genetische en moleculaire analyse van bloeitijd in *Arabidopsis thaliana* gebruikmakend van natuurlijke variatie

Proefschrift

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Propositions

1- Nature, as an abundant source of different life aspects, can be studied in many different ways. The geneticists succeeded to use the "natural" genetic variation in order to analyze the function of different genes.
This thesis; Alonso-Blanco and Koornneef, M. (2000), Trends in Plant Science 5, 22-29

2- The possibility of generating populations segregating for only one gene (Mendelizing), allows efficient molecular analysis of individual QTL.
This thesis; Alonso-Blanco and Koornneef, M. (2000), Trends in Plant Science 5, 22-29

3- The natural genetic variation detected as QTL can be due to a single amino acid change.
This thesis

4- Genetic variation for photoreceptors may be present within a species and may play a role in its adaptation to a specific ecological environment.
El-Assal et al., 2001 Nature Genetics 29, 435-440; Maloof et al., 2001 Nature Genetics 29, 441-446; Pepper et al., 2002 Plant, Cell and Environment 25, 591-600

5- The photoperiod and autonomous pathways of flowering show interaction not only at the level of the downstream target genes.
This thesis

6- Photoperiod responses may be controlled through the effect on protein stability of the regulatory proteins.
This thesis

7- Variation in nature, which may reflect adaptation to specific environments may be relevant for plant breeding, because also varieties require an adaptation to specific ecological environments. Therefore, they can help in the identification of agronomically important genes.

8- Also the Egyptian pharos already used the laws of nature in building the Giza pyramids.

Propositions accompanying the thesis: "A genetic and molecular analysis of flowering time in *Arabidopsis thaliana* using natural variation" by Salah El-Din S. M. El-Assal, Wageningen, May 7, 2002.

In memory of my parents

To my wife and my daughters

Contents

Chapter 1

General introduction

Genetic control of flowering time in Arabidopsis

Arabidopsis, the model for plant research

Arabidopsis thaliana (L.) Heyhn. is a small weed plant belonging to the mustard family (Brassicaceae or Cruciferae). The species can be found in nature almost everywhere in the Northern Hemisphere in ruderal sites, such as sandy patches along roads etc. Arabidopsis has been found from sea level up to high in the Himalayas and from northern Scandinavia to Africa, including the Cape Verde Islands at 16° latitude. It also grows in North America, probably following introduction from Europe.

Arabidopsis was first suggested as a suitable model for plant biological studies, and especially genetics, in the 1940s, because of its small size, its self-fertilization habit and the short generation time of many accessions (isolates), which are often called ecotypes in Arabidopsis (Meinke et al., 1998). In greenhouse or in climate chamber conditions, 6-8 weeks is sufficient time to complete the entire life cycle from germination until seed set for many of the laboratory accessions. Furthermore, Arabidopsis has one of the smallest genomes among higher plants, approximately 130 megabase in size, divided over 5 chromosomes, which is now completely sequenced (Arabidopsis Genome Initiative, 2000). These factors and the ability to efficiently transform the plants, have made it the favorite plant model for molecular genetic studies to date. The Arabidopsis genome is estimated to contain ~25000 protein-coding genes, of which it is thought that nearly 40% have unknown cellular roles, and only ~5% have an established phenotypic function on the basis of mutant phenotypes.

Two different sources of genetic variation can be used for the functional analysis of Arabidopsis genome

Currently, the functional analysis of Arabidopsis genes and the dissection of complex traits are based largely on the phenotypic characterization of mutants selected by forward and reverse genetics from three rapid-cycling laboratory accessions: Landsberg *erecta* (*Ler*), Columbia (Col) and Wassilewskija (Ws) (Meinke et al., 1998). The possibility of identifying genotypes with an insertion in a gene of known nucleotide sequence, independently of the presence of a phenotype is called reverse genetics, and has led to large-scale projects for disrupting most of the Arabidopsis genes (Meinke et al., 1998).

As an alternative to generating laboratory-induced mutants, another source of genetic variation can be found among and within naturally occurring populations of Arabidopsis which are collected from different geographical regions (Rédei, 1970; Alonso-Blanco and Koornneef, 2000; Barton and Keightley, 2002). This geographic distribution embraces substantial variation in growth environments, hence, phenotypic variation among accessions is expected to reflect genetic variation that is important for adaptation to specific conditions. Considerable variation has been found for potentially adaptive traits, such as:

- Resistance to biotic stresses, including insects, fungi, bacteria and viruses (Kunkel, 1996).
- Tolerance to abiotic stress parameters, such as high temperature, freezing, drought, heavy metals, carbon dioxide and ozone (reviewed by Alonso-Blanco and Koornneef, 2000).
- Developmental traits, such as flowering time (Jansen et al., 1995; Mitchell-Olds, 1996; Alonso-Blanco el al., 1998b), plant size (Li et al., 1998), seed size (Alonso-Blanco et al., 1999), trichome number and distribution (Larkin et al., 1996).
- Physiological traits, such as seed dormancy (Kugler, 1951), phosphate uptake (Krannitz et al., 1991), and water-use efficiency (Nienhuis et al., 1994).
- Biochemical traits, such as glucosinolate (Kliebenstein et al., 2001), seed oligosaccharide (Bentsink et al., 2000), epicuticular wax composition (Rashotte et al., 1997) and several enzymatic activities (Mitchell-Olds and Pedersen, 1998).

Mapping loci from quantitative variation

In contrast with the commonly studied mutants, which provide qualitative (discrete) variation, most of the variation among accessions is of a quantitative nature because of the effects of allelic variation at several loci (multigenic inheritance), which, combined with the environmental effect determines a continuous phenotypic distribution of the trait in segregating populations. The genotype at these loci cannot be directly known from the single phenotypic value of a plant determined by the various loci and the environment, but this can be inferred indirectly from linked marker loci. The detection and location of the loci underlying this quantitative variation, which are called Quantitative Trait Loci (QTL) requires first the generation of a segregation population and its characterization for molecular markers to obtain a

genome-wide genetic map. Secondly, after scoring the trait(s) of interest, associations between the molecular genotypes and the phenotypes of the trait are searched by means of specific statistical methods. The number of QTL detected and the accuracy of their map position and effect estimates depends, among others, on the overall heritability of the trait, the magnitude and location of the QTL and the amount of observed recombination in the segregating population (Kearsey and Farquar, 1998; Doerge, 2002). In addition, it is affected by the following manipulable experimental parameters:

- Size and type of mapping population.
- Coverage of the molecular genetic map.
- Statistical QTL mapping method employed.

Comparison of map positions among the QTL identified and the mutant or genes known to affect a trait have indicated that some of the QTL correspond to loci of previously unknown function (Van der Schaar et al., 1997; Swarup et al., 1999). In particular, the identification of loci at novel map positions has allowed the identification of new function loci for traits that are not exhaustively analyzed and for which only a few mutants have been previously isolated.

To characterize an individual QTL it must be separated from the rest of the segregating loci. To achieve this genotypes should be obtained that will give rise to monogenic segregation in subsequent progenies. Commonly, this process is referred to as "Mendelization" of a QTL. The "Mendelization" of a QTL is best accomplished by constructing near-isogenic lines (NILs), ideally differing only for the alleles in a small genomic region spanning a few cM around the QTL of interest. Once NILs with monogenic segregation are obtained, their comparison enables the phenotypic and genetic characterization of a QTL in a similar way to that performed with mutants.

The molecular characterization of alleles responsible for the naturally occurring variation requires that the respective genes are cloned. Isolation of these loci can be achieved mainly by using chromosome walking approaches, which, with the availability of the complete physical map of large genomic inserts (Yeast Artificial Chromosomes (YACs) and Bacterial Artificial Chromosomes (BACs) and the complete nucleotide sequence of the five linkage groups has become a routine procedure in Arabidopsis (Lukowitz et al., 2000).

In the past few years, several genes involved in disease resistance have been isolated using the existing variation among accessions (Kunkel, 1996).

The transition to flowering

The analysis of flowering time variation in the naturally late-flowering accessions has complemented the mutagenic approach to genetically dissect this, particularly in identifying repressors of the floral transition. A number of genes *FRIGIDA (FRI), Flowering Locus C (FLC),* and quantitative trait loci (QTL) that are not represented in

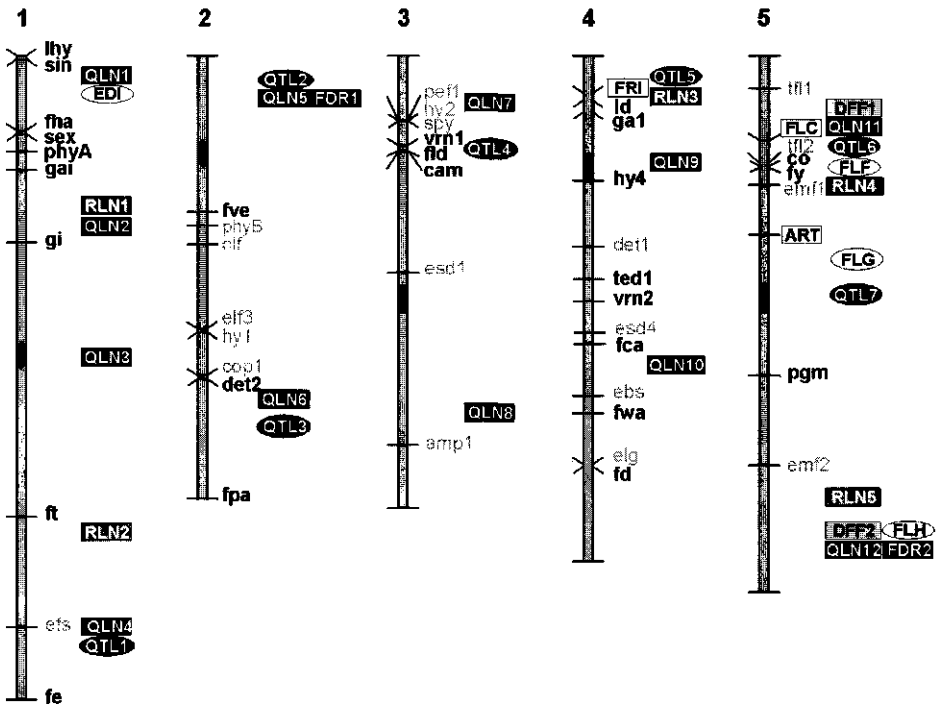

Figure 1-1. Arabidopsis genetic map showing the mutant loci and different flowering time QTLs. Loci in black are the late, while in grey are the early flowering mutants. FLC, FRI and ART loci identified from the natural populations, and are indicated with white boxes. Black and grey boxes represents the approximate position of putative QTLs identified in different crosses; DFF1-2, QTLs in a Hannover/Münden F2 population; RLN1-5, QTLs in a Ler x H51 F2/F3 population; QLN1-12 in Ler x Col RIL population; FDR1-2 in the same Ler x Col RIL population; QTL1-7 in a backcross to Limburg-5, with selective genotyping, from F1 Limburg-5, with selective genotyping, from F1 Limburg-5 x Naantali; EDI, FLF, FLG and FLH in a Ler x Cape verde Island (Cvi) RIL population. (Adapted from Koornneef et al., 1998b).

the mutant collections have been identified using this approach (reviewed in Koornneef et al., 1998b, see Fig. 1-1).

Arabidopsis thaliana has a distinct vegetative phase during which the shoot apical meristem (SAM) produces lateral meristems that develop into leaves subtending an axillary bud. The nodes do not elongate, resulting in the formation of a rosette. Flowering transition is marked by the establishment of a floral fate in these meristems, which replaces leaf identity of the meristem by floral identity. A bi-directional development has been shown in this transition, with flowers being initiated acropetally. After floral initiation and following a basipetal direction, the axillary buds of the leaf primordia mostly develop into secondary shoots, which are also called paraclades or co-florescences (Hempel and Feldman, 1994). In specific genotypes, they replicate the fate of the vegetative meristems by forming axillary rosettes. Following the fate changes of these lateral meristems, internode elongation takes place (bolting). The elongated stem or inflorescence bears cauline leaves at higher internodes. The part of the inflorescence with leaves, which was called early inflorescence (Haughn et al., 1995), 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.

Arabidopsis is a facultative long-day (LD) plant, which means that plants flower earlier under LDs than under short days (SDs), but a LD treatment is not an absolute requirement for flowering. When plants of the commonly used early laboratory genotypes are of sufficient age and have achieved competence for flowering, one LD is sufficient to induce flowering (Corbesier et al., 1996; Hempel et al., 1997). This treatment has been used to monitor the morphological (Hempel and Feldman, 1995) and molecular changes (Hempel et al., 1997) involved in the transition of the meristems.

The photoperiodic control of flowering is thought to be mediated by a mechanism that measures the length of the daily light period and might involve the interaction with photoreceptors, such as phytochrome and cryptochrome, and a clock mechanism or circadian rhythm. Photoreceptors play a role in setting 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 in promoting flowering than red (R) light (Brown and Klein, 1971; Eskins, 1992). Besides, the sensitivity of plants to light quality itself depends on

a circadian rhythms (Lin, 2000). 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 (Roldán et al., 1999). A higher irradiance also promotes flowering probably by its effect on carbohydrate supply (Bagnall, 1992; King and Bagnall, 1996).

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 (Napp-Zinn, 1969). Furthermore, an increase in temperature also affects flowering as measured not only by flowering time but also by leaf number (Araki and Komeda, 1993), which should correct for temperature effects on growth.

Moreover, many chemical treatments have been shown to promote flowering (Martinez-Zapater et al., 1994) of which the application of gibberellins (GAs) (Wilson et al., 1992; Blazquez et al., 1998) and base analogues (Martinez-Zapater et al., 1994) has attracted most attention, because of their relatively large effects.

Genes regulating the floral transition through different pathways

A summary of genes considered to play a role in flowering time control, identified using the genetic variation present among naturally occurring ecotypes that vary in flowering time and induced mutations that result in either early or late flowering, has been recently compiled (Levy and Dean, 1998). Taken together, there are currently about 80 genes and loci in Arabidopsis known to affect flowering time. Mutations at these loci are either specific for flowering because no pleiotropic effects have been described or affect several responses including flowering timing. Examples of the latter are photoreceptor, plant hormone and carbohydrate mutants.

The study of how flowering time mutants respond to the environmental treatments, such as vernalization and photoperiod, has shown the existence of multiple pathways that control flowering time in Arabidopsis (Martinez-Zapater et al., 1994; Koornneef et al., 1998a; Simpson et al., 1999), summarized in Figure 1-2. The photoperiod promotion pathway integrates daylength into the flowering decision through a series of genes that sense and respond to the regular day-to-night transition. The vernalization pathway promotes flowering in many late-flowering ecotypes in response to an extended period of cold temperature. It is now known that the role of

this pathway is to suppress *FLC* expression, which is a floral repressor (Sheldon et al., 1999; Michaels and Amasino, 1999a, 2000). The repression of *FLC* is inhibited by a number of genes that represent the so-called autonomous pathway. The mutants in these genes flower late in LDs and SDs, and have an increased sensitivity to vernalization. In Arabidopsis, mutants defective in gibberellin (GA) biosynthesis flower late, especially in SD.

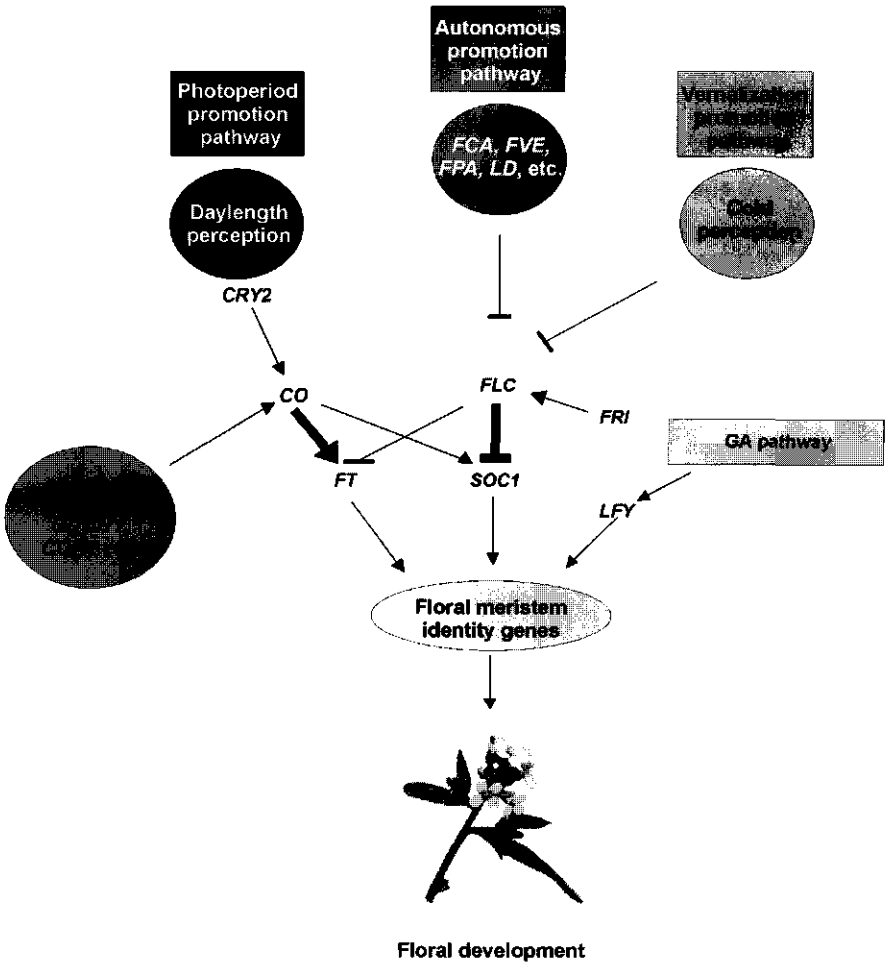

Figure 1-2. A model representing the genetic interaction between the different flowering time genes. Different groups of genes have been grouped according to their genetic and physiological behaviour, are shown in boxes. pathways are indicated. "→" denote a promotive effect; "⊥" depicts inhibitory effect.

The genetic analysis of the epistatic relationships between GA mutants and other flowering time mutants showed that those GA mutants promote the flowering time through a separate promotion pathway than the others (Blazquez et al., 1998; Reeves and Coupland, 2001).

The final players are genes such as *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*), that integrate the various pathways and induce the expression of meristem identify genes such as such as *LEAFY (LFY)* and *APETALA1 (AP1)* (Weigel, 1995). As floral induction pathways act upstream of these genes in the initiation of flowering, constitutive expression of meristem-identity genes might bypass the requirement for these upstream flower-inducing pathways. Hereafter the various pathways are described in more detail.

The vernalization promotion pathway

Vernalization is a long cold temperature treatment that accelerates flowering in many species. Naturally occurring late-flowering accessions of Arabidopsis and in mutants of the autonomous promotion pathway (Wilson and Dean, 1996) respond strongly to this treatment in contrast to the early accessions L*er* and Col.

Two genes, *FRI* and *FLC* interact to create the winter annual habit in Arabidopsis and both genes have recently been cloned. The *FLC* gene encodes a MADS-domain-containing transcription factor (Michaels and Amasino 1999a; Sheldon et al., 1999). *FRI* encodes a protein without significant identity to proteins of known function (Johanson et al., 2000), and promotes expression of *FLC*. Early-flowering, wild-type laboratory strains such as Col are *fri* null mutants (Johanson et al., 2000) in which *FLC* expression is hardly detected (Michaels and Amasino 1999a; Sheldon et al., 1999). In the presence of *FRI* or with an autonomous pathway mutation, *FLC* mRNA levels are up-regulated. Vernalization down-regulates *FLC* expression, which low level of expression is maintained after the plants have been transferred to normal temperatures. The strong negative correlation between *FLC* transcript levels and flowering suggests that the cause of the late-flowering in the non-vernalized genotypes is the elevated level of *FLC*, acting as a floral repressor, which is confirmed by the observation that over-expression of *FLC* very much delays flowering (Sheldon et al., 2000).

However, because after 60 days of cold exposure in short days, *flc*-null mutations flowered with fewer than half the number of leaves as non-cold-treated

plants they still show a clear vernalization response. Thus down-regulation of *FLC* does not totally account for the vernalization response (Michaels and Amasino, 2001) in Arabidopsis.

The *VERNALIZATION* (*vrn1* and *vrn2)* mutants were isolated on the basis of their reduced vernalization response in the late flowering vernalization-responsive *fca-1* mutant background (Chandler et al., 1996). The *VRN2* gene was found to encode a nuclear enc-localized zinc finger protein with similarity to Polycomb group proteins and apparently functions by maintaining the low level of *FLC* expression, because in the *fca-1 vrn2* double mutant *FLC* expression increases again when the plants are returned to normal; temperatures, whereas these levels remain low in the vernalized *fca1* mutant. (Gendall et al. 2001).

The autonomous promotion pathway

This pathway is represented by a group of mutants at six loci, *fca, fy, fpa, fve, ld* and *fld,* that flower late in LDs and SDs and the late flowering phenotype can be suppressed by either vernalization treatment or light conditions with a low red to far-red photon ratio. These genes function in the so-called autonomous pathway, which in early accessions is functionally redundant with the vernalization pathway. The severity of the late-flowering phenotype of these mutants is affected by the genetic background in which they were isolated: *ld* (Lee et al 1994a) and *fld* (Sanda and Amasino, 1996b) appear only late in backgrounds bearing a strong *FLC* allele.

The *FCA* gene has been cloned (Macknight et al., 1997), and encodes a protein with two copies of a well-characterized RNA-binding domain, the so-called RNP motif (Macknight et al., 1997). *FCA* also contains a WW protein-protein interaction domain. Interestingly, *FPA*, which was recently cloned by Schomburg et al., (2001) encodes also a protein bearing RNP motifs. The presence of RNA-binding domain in these two proteins raises the possibility that post-transcriptional events figure largely in the autonomous promotion pathway.

LUMINIDEPENDENS (LD) gene was the first flowering time gene to be cloned (Lee et al., 1994a) and encodes a homeodomain containing protein. The expression analysis of both *FCA* (Macknight et al., 1997) and *LD* (Aukerman et al., 1999) reveals a pattern that is not restricted to the SAM around the time of floral induction.

In the double mutants that combine autonomous pathway mutants and the *flc-3* null allele, the late-flowering phenotype of autonomous pathway mutations was completely eliminated, except in *fpa* where the double mutants was slightly later flowering (Michaels and Amasino, 2001). Thus, *FCA*, *FPA*, *FVE*, and *LD* are likely to act upstream of *FLC* and promote flowering by inhibiting *FLC* expression.

The flowering time of non-autonomous pathway mutants (*co, gi* and *soc1*) was unaffected by *FLC* and these genes are likely to act in pathways that are downstream or independent of *FLC* (Michaels and Amasino, 2001).

The gibberellin (GA) promotion pathway

In Arabidopsis, signaling mediated GA appears to play a promotive role in flowering that is particularly apparent under non-inductive SD photoperiods, where the gibberellin deficient *ga1-3* mutant does not flower unless provided with GA (Wilson et al., 1992), and the gibberellin-insensitive (*gai*) mutant flowers very late. Furthermore, *spy*, a mutant that exhibits constitutive GA-mediated signal transduction, flowers early (Jacobsen and Olszewski 1993), as do plants constitutively expressing *FPF1*, a gene that appears to be involved in GA-mediated signal transduction or responsiveness to GAs (Kania et al., 1997).

The results of epistatic analyses with the *ga1-3* mutant deficient in GA biosynthesis, combined with mutants in the photoperiod (Putterill et al., 1995) or the autonomous promotion pathway (Reeves and Coupland, 2001) are consistent with GAs functioning in a pathway that is separate from both of photoperiod and autonomous promotion pathways.

To test whether GAs are required for the vernalization response, plants containing *ga1-3 FRI FLC* plants were grown in LD with and without a 45-day cold treatment. Without cold treatment, the *ga1-3FRI FLC* line eventually senesced without flowering after 10 months and after forming more than 100 rosette leaves. However, after the cold treatment the *ga1-3 FRI FLC* plants flowered at the same time than the *ga1-3* monogenic mutant, demonstrating that the late-flowering phenotype of *FRI* and *FLC* is eliminated by vernalization also in the *ga1-3* background (Michaels and Amasino, 1999b). The authors explained the inability of vernalization to promote flowering in the Arabidopsis *ga1-3* mutant in SD by assuming that the block of flowering in SD is too strong to be overcome by vernalization (Michaels and Amasino, 1999b).

In order to understand the role of GAs in activation of the *LFY* gene, Blazquez et al., (1998) analyzed the activity level of *LFY* promoter and recorded that it was lower in *ga1* mutants, and that its up-regulation by LDs is delayed. In contrast, *LFY* activity is slightly higher in a *spy* mutant grown in SDs, correlating with an acceleration of flowering. Moreover, a 35S::*LFY* transgene was also found to partly rescue flowering in *ga1* mutant plants in SDs. Thus GA promotes flowering in Arabidopsis at least in part by activating *LFY* expression.

The photoperiod promotion pathway

Martinez-Zapater et al., (1994) have placed a group of mutants that flower late in LDs but that are not significantly delayed by SDs, in the photoperiod promotion pathway. This group of mutants includes *co, gi* and *fha*, and these genes mediate the promotion of flowering caused by LD photoperiods. *CONSTANS (CO)* encodes a putative transcription factor (Putterill et al. 1995) that mediates between the circadian oscillator and activation of the flowering-time gene *FT* (Súarez-López et al., 2001). The signals generated by the circadian clock are believed to affect the expression of downstream genes that operate in the photoperiod operation in the photoperiod promotion pathway including *GI* and *CO*.

Plants that over-express *CO* in combination with *gi* mutants are early and show that *GIGANTEA (GI)* acts upstream of *CO* (Pineiro and Coupland 1998).

The transcription of *GI*, which has recently been cloned (Fowler et al., 1999; Park et al., 1999), is controlled by the circadian clock, since the analysis of the *GI* transcript levels continued to cycle in a similar phase under LL and DD, (Fowler et al.,1999). The effect of *gi* mutations on two clock-associated genes, *CCA1* and *LHY*, have been tested, and the circadian expression pattern of both genes were altered in both *gi-1* and *gi-2* mutants. Although, the cyclic expression of the *GI* transcript shows that it is also under circadian control, it is unlikely that *GI* is a central oscillator component because the putative null mutation (*gi-2*) does not abolish rhythmicity, but alters period and reduces amplitude (Park et al., 1999).

Since CRY2 was found to be the product of the *FHA* gene (Guo et al., 1998), cryptochromes were shown to be involved in photoperiodic promotion. Plants overexpressing *CRY2* flowered earlier than wild type and had increased levels of *CO* mRNA, suggesting that blue light promotes flowering via cry2 and CO (Guo et al., 1998), and cry2 is a positive regulator of *CO* expression in response to photoperiod.

However, Súarez-López et al. (2001) tested further the relation between CO and cry2 using both the *cry2-1* and *fha-1* alleles and testing them in extended LD, SD as well as true LD, and found that *CO* mRNA abundance was similar in the mutants and the wild type. Nevertheless, 35S::*CO fha-1* plants flowered at the same time as 35S::*CO*, indicating that overexpression of *CO* corrects the delay of flowering due to the *fha-1* mutation.

Genes promoting the floral transition in the downstream part of the floral induction pathway

A group of late-flowering mutants that are not vernalization responsive, have recently been shown to act in the downstream part of the floral-induction pathway and the respective genes partially integrate the various pathways. They act downstream of *FLC* (autonomous pathway) and *CO* (photoperiodic pathway), and function to activate directly the meristem identity genes. This group of mutants includes *ft, fwa* and *soc1* and probably also *fe* and *fd*. The *ft, fd*, and *fe* mutants are all recessive, suggesting that they normally function to promote the flowering.

FT was cloned by T-DNA tagging (Kardailsky et al., 1999; Kobayashi et al., 1999), and found to encode a protein with pronounced similarity to the meristem identity gene, *TFL1* (Bradley et al., 1997). Despite their similarity, *TFL1* and *FT* have opposing functions, with *TFL1* repressing and *FT* promoting flowering. *FT* mRNA was present at higher abundance in 35S::CO plants than in the wild type. The requirement for FT and LFY in the early-flowering phenotype of 35S::*CO* was tested genetically. A severe *lfy* mutant allele (*lfy-6*) did not delay reproductive development of 35S::*CO* plants, whereas *ft* mutations caused a significant delay (Samach et al., 2000). *FT* is therefore required for the extreme early flowering of 35S::*CO* plants. These findings suggest that *FT*, but not *LFY*, is an early target gene of *CO*.

FWA has been cloned and found to encode a homeodomain protein, which is not expressed in wild type due to methylation at its 5' end (Soppe et al., 2000). Increased expression of *FWA* in the *fwa* mutants leads to late flowering. Genetic analyses have placed *FWA* in the epistatic group of genes that promote flowering through the photoperiodic promotion pathway. In particular, *fwa* appeared fully epistatic to *ft*, since the double mutant *fwa ft* does not flower later than the single mutants (Koornneef et al., 1998a). Moreover, the expression pattern of *FT* in an *fwa* mutant background and in wild-type plants is similar, suggesting that *FWA* functions

downstream of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999) acting as a suppressor of *FT* function.

Epistatic analysis by Ruiz-Garcia et al (1997) demonstrated that when *ft* or *fwa* were combined with *lfy*, the severity of meristem identity defects was significantly enhanced, indicating that *ft* and *fwa* function in a parallel pathway to *lfy* to activate other meristem identity genes.

A gene encoding a MADS-box transcription factor that was first designated *AGAMOUS-LIKE 20* (*AGL20*) and later renamed *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), plays a critical role in integrating the *CO* and vernalization (*FLC* mediated) pathways. The *soc1* mutation partially suppresses the early-flowering phenotype caused by 35S::*CO*. *SOC1* expression responds to long photoperiods in wild-type plants. *SOC1* mRNA was not detected in sections of the shoot apex of plants grown under SD, but was present in the shoot apical meristem and leaf primordia 16 hours after the shift to continuous light (Samach et al., 2000). This response is similar to that of mustard *MADS A* gene, which is the *Sinapis alba* homologue of *SOC1* (Borner et al., 2000). These results, taken together with the findings of induction of *SOC1* by glucocorticoid induced CO expression (in the 35S::*CO:GR* transgenic plants) and partial suppression of the 35S::*CO* phenotype by the *soc1* mutation, demonstrated that *CO* promotes flowering in part through activation of *SOC1* (Samach et al., 2000). *SOC1* and *FT* are both regulated by the autonomous pathway, as was shown by the analysis of the expression of both genes in the *fca-1* mutant and *CO* overexpression backgrounds (Samach et al., 2000). The abundance of *SOC1* mRNA was reduced in a 6- and 13 -day-old *fca-1* mutants relative to the wild type, and it was reduced to a similar extent in *fca-1* 35S::*CO* plants relative to 35S::*CO* plants. *FT* expression was also reduced by *fca-1*, but less extreme than for *SOC1*.

The classification of *FD* and *FE* in this group is less clear because they are not fully epistatic to CaMV35S:: *LFY* in contrast to *ft* and *fwa*. Because these genes have only been cloned recently (C. Alonso-Blanco and T. Araki personal communication), their expression in various genetic backgrounds has not yet been reported. In addition *LFY* expression is down-regulated and delayed in *fd* in contrast to *ft*, *fwa*, and wild type Arabidopsis (Nilsson et al., 1998).

Floral meristem identity genes

The decision to flower involves a dramatic change in plant architecture, which is governed by the interplay of genes that promote shoot identity, such as *TFL1* and *TFL2* and those that promote floral identity, such as *LFY*. These genes are at the border between flowering time and meristem identity, and mutations in these genes affect both processes. For instance, *tfl1* mutations confer early flowering and premature transformation of the shoot meristem into a flower, and a reduction in *LFY* activity causes delayed flowering and inability to form normal flowers. The similar phenotype of *tfl1* and *tfl2* coincides with an increased expression of *FT* in both mutants (Kardailsky et al., 1999), suggesting that the effect of the wildtype *TFL* alleles is suppression of *FT* expression.

Mutants in the *LFY* gene result in the conversion of first formed flowers into shoot-like structures subtended by a leaf. Later primordia form more flowers-like structures but these lack petals or stamens (Weigel et al., 1992). Over expression of *LFY* (35S::*LFY*) causes early formation of determinate floral meristems (Weigel and Nilsson, 1995), indicating that *LFY* is sufficient to determine floral fate.

UNUSUAL FLORAL ORGANS (*UFO*), is another meristem identity gene. *UFO* carries a functional F-box, indicating that it is involved in the targeting of other proteins, possibly transcription factors, for ubiquitin-mediated degradation (Lee et al., 1997). The *ufo* mutations cause phenotypes that are similar to those caused by partial loss-of-function *lfy* alleles. Over-expression of *UFO* does not rescue the *lfy* mutant phenotype, thus indicating that *UFO* does not act as a simple mediator between meristem and floral organ identity genes (Lee et al., 1997).

The *ap1* mutation results in the formation of shoots at the first few positions normally occupied by flowers, with later-forming primordia producing flowers, with no petals or petals that are either leaf-like or stamenoid, and containing secondary flowers in the axils of the outer organs (Bowman et al., 1993). The double mutant *lfy ap1* shows a much more sever phenotype than either *lfy* or *ap1* single mutants, with shoot-like structures formed at almost all the primordia (Bowman et al., 1993; Weigel et al., 1992). This demonstrates that *LFY* and *AP1* have partially redundant functions.

Simon et al. (1996), have applied dexamethasone treatments that induce CO expression by activation of the glucocortinoid receptor in *CO:GR* plants to test the effect of *CO* expression on *AP1* and *LFY* transcription and compared this with the effect of exposing wild-type plants to LDs. As expected, transcripts of *AP1*, *LFY* and *TFL* were absent from time-zero control plants. The transcripts of LFY and TFL were

present in *CO:GR* plants 24 h after application of dexamethasone, irrespective of whether the plants were grown in SDs or LDs, while the *AP1* transcript was present 72 h after treatment of LD-grown *CO:GR* plants. (Simon et al., 1996). In response to LDs, the *AP1* transcripts therefore appears after the *LFY* transcript, which is in agreement with genetic data demonstrating that *AP1* acts after *LFY* in conferring floral meristem identity (Weigel and Nilsson, 1995). However, in SD-grown *CO:GR* plants, there was a further delay in the appearance of *AP1* transcript, indicating that *AP1* was expressed more slowly in response to *CO* activation than in response to LDs (Simon et al., 1996).The previous investigations suggested that *CO* act within a genetic pathway that activates the transcription of *TFL* and *LFY* in response to LDs. However, this pathway is not sufficient to activate *AP1* as rapidly as does exposing plants to LDs. Simon et al (1996), proposed that another flowering-time pathway is activated by LDs and is required to promote *AP1* transcription. A candidate for this activation is FT because, double mutants of *ft* and *fwa* with *lfy* virtually lack floral initiation and do not show *AP1* mRNA in the inflorescence apex (Ruiz-Garcia et al., 1997).

Plant photoreceptors

Plants are able to perceive and monitor changes in light quality and quantity. The primary photosensory receptors of higher plants are the red/far-red light-absorbing receptors called phytochromes (phy) and the blue/UV-A light absorbing receptors called cryptochromes (cry) (Kendrick and Kronenberg, 1994). In addition, there are some other photoreceptors: phototropin, formerly called *NON-PHOTOTROPIC HYPOCOTYL* (*nph1*) and a yet unidentified UV-B photoreceptor(s) (Casal, 2000), that modulate growth and development. Signals from the photoreceptors are considered to entrain components of the circadian clock, which in turn regulates the expression of effector genes including *CO* (Suarez-Lopez et al., 2001). However, not all photoreceptors regulate flowering time exclusively through the circadian clock (Koornneef et al., 1995; Miller et al., 1995).

Phytochromes

Just over 50 years ago, the first signaling photoreceptor in plants, a photo-reversible pigment, called phytochrome was discovered. Phytochromes are dimeric

chromopeptides with monomers of 120-130 kDa that possess two photoconvertible forms: Pr (red light absorbing phy) and Pfr (far-red light absorbing phy) (Kendrick and Kronenberg, 1994). Synthesis of phytochrome is in the Pr form that upon light absorption (peak in red light region of the spectrum) is transformed to Pfr, which is considered the physiologically active form. In turn, light absorption of far-red light transforms Pfr back to Pr. There are five phytochromes (phyA through phyE) in Arabidopsis whose apoproteins are encoded by different genes (Sharrock and Quail, 1989; Clack et al., 1994). The chromophore, apparently common to all phytochromes, is a linear tetrapyrrole. Different phytochromes regulate either distinct light responses or similar responses under different light conditions, but also have overlapping functions. Taking the well-characterized light-inhibition of hypocotyl elongation as an example (Quail et al., 1995), the *phyA* mutant is impaired in hypocotyl inhibition in far-red light, but not in red light. Conversely, the *phyB* mutant loses the ability to inhibit hypocotyl elongation in red light, but not in far-red, suggesting that although phyA and phyB both mediate light inhibition of hypocotyl elongation, phyA functions primarily in far-red light, whereas phyB acts mainly in red light.

Cryptochromes

Cryptochromes are photoreceptors of blue light and UV-A radiation. Two members of this family, cryptochrome 1 and 2 (cry1 and cry2) have been identified in Arabidopsis (Ahmad and Cashmore, 1993; Lin et al., 1998). Cryptochromes are flavoproteins that share over 30% amino acid sequence similarity to the prokaryotic DNA-repair enzyme DNA photolyase. DNA photolyases are flavo-enzymes that catalyze the blue/UV-A light-dependent DNA photo-repairing reaction through an electron-transfer mechanism (Cashmore et al., 1999). However, the plant cryptochrome proteins had no photolyase activity and contained a C-terminal extension not found in the photolyases.

The isolation of an Arabidopsis mutant, *hy4*, which showed greatly reduced sensitivity to blue-light-induced inhibition of hypocotyl elongation (Koornneef et al., 1980), played a crucial role in our understanding of cryptochromes and allowed the cloning of the first cryptochrome gene (*CRY1*) (Ahmad and Cashmore, 1993). The irradiance Arabidopsis *hy4* (= *cry1*) mutant is insensitive to blue light, especially high-irradiance blue light and transgenic Arabidopsis plants over-expressing *CRY1*, show enhanced blue light sensitivity (Lin et al., 1996). Analysis of CRY1 N-terminal

photolyase-homology domain expressed in *Escherichia coli* showed that the CRY1 protein is associated with a flavin adenine dinucleotide chromophore (FAD), which primarily absorbs blue and UV-A light (Lin et al., 1995). In addition to FAD, a pterin (5,10-methenyltetrahydrofolate) was also found to bind to the expressed protein, suggesting that like photolyase, cry1 might contain pterin as a second chromophore. The second Arabidopsis cryptochrome gene, *CRY2*, was cloned using the *CRY1* cDNA as the hybridization probe (Lin et al., 1998). CRY1 and CRY2 are about 50% identical, but most of the sequence similarity is concentrated in the N-terminal photolyase like domain, whereas the C-terminal domains are quite different (Lin et al., 1998). The CRY2 protein shows rapid degradation by increasing irradiances of blue light but are not affected by red light, and this is not the case of *CRY1* (Lin et al., 1998; Guo et al., 1999). Transgenic plants over-expressing *CRY2* were hypersensitive to blue light (Lin et al., 1998).

Phototropin

In 1988, Gallagher et al., reported that blue light could activate the phosphorylation of a plasma membrane protein from the growing regions of etiolated seedlings, and there was strong evidence that this protein was not only the photoreceptor and kinase for its own phosphorylation but a photoreceptor for phototropism as well. Using the Arabidopsis *nph1* mutant the gene was cloned and subsequently named phototropin. Phototropin is a 120-kDa flavoprotein, containing two PAS domains, that mediates phototropic responses to the direction of blue light, UV-A or even green light. This flavoprotein is a serine-threonine protein kinase able to phosphorylate its serine and threonine residues (Christie et al., 1998).

The role of photoreceptors in regulating flowering time

In searching for photoreceptors regulating photoperiodic responses, action spectra have been extensively analyzed in different plants to investigate how light quality affects flowering time. Arabidopsis plants grown in continuous red light flower significantly later than those grown in continuous blue light (Guo et al., 1998), suggesting that at least for Arabidopsis, far-red light and blue light promote flowering, whereas red light is often inhibitory. The studies of photoreceptor mutants have allowed to assign specific functions of individual photoreceptors in the regulation of

flowering time. In Arabidopsis, phyA, phyB, cry1 and cry2 photoreceptors play a more obvious role compared with the other photoreceptors in the control of flowering-time

Phytochrome A (phyA)

The Arabidopsis *phyA* mutant flowers later than wild-type plants in LD, indicating that phyA promotes flowering (Johnson et al., 1994; Lin et al., 1998; Neff and Chory, 1998). Consistent with a promotive role in flowering, transgenic Arabidopsis plants over-expressing phyA flowered earlier than the wild type in both SD and quasi-LD (LD extension with far-red rich light and/or night break methods (Bagnall et al., 1995). The phyA mutant (*fun1*) in pea also flowers late in LD, which together with grafting experiments suggested that phyA signaling may suppress the biosynthesis of a floral suppressor (Weller et al., 2001).

Phytochrome B (phyB)

The Arabidopsis *phyB* mutant flowers earlier than the wild type in both LD and SD conditions, but the early-flowering phenotype of the phyB mutant is more pronounced in SD than in LD conditions. This indicates that *PHYB* gene plays an inhibitory role in floral initiation (Goto et al., 1991; Mockler et al., 1999). However, the function of phyB in floral initiation may be more complex than simply as a floral inhibitor. For example, transgenic Arabidopsis plants overexpressing phyB also flowered earlier than wild type, which could not be easily explained (Bagnall, et al., 1995).

Cryptochrome 1 (cry1)

The mode of action of CRY1 in floral initiation remains unclear, because, in contrast of other photoreceptors, there is a great deal of inconsistency in the flowering time date for the *cry1* (= *hy4*) mutant (Goto et al., 1991; Mozley and Thomas, 1995; Bagnall et al., 1996 and Mockler et al., 1999).

Cry1 may have a promotive effect on flowering because the *hy4* mutant (in Landsberg *erecta* background) was shown to flower slightly later than wild type under SD conditions (Mozley and Thomas, 1995). Also the *hy4* mutant in the Col background flowered late in both SD and quasi-LD conditions with either day extensions or night breaks, and the night breaks with blue light had a stronger effect than night breaks with white light or red light (Bagnall et al., 1996).

Cryptochrome 2 (cry2)

That *CRY2* clearly promotes flowering, became clear when it was discovered that *cry2* mutants are allelic to the photoperiod-insensitive late-flowering *fha* mutant. The *cry2* alleles in the Col background had a stronger phenotype than the *fha* alleles in the L*er* background, which flower late in LD but not in SD (Guo et al., 1998 ; Koornneef et al., 1991). Transgenic plants over-expressing *CRY2* flowered early in SD but not in LD conditions. Therefore, either a mutation or an over-expression of the *CRY2* gene resulted in the reduced sensitivity to photoperiods. Guo et al (1998) and Mockler et al (1999) showed that the *cry2* mutant flowered at the same time as the wild type in continuous blue light or red light, while the late-flowering phenotype of *cry2* mutant in white light could be phenocopied in blue-plus-red light. Therefore, the flowering promotion function of *CRY2* is dependent on both blue and red light.

The *cry2 phyB* double mutant grown in LD flowered significantly earlier than the *cry2* monogenic mutant. The *cry2 phyB* double mutant grown in SD flowered at about the same time as the *phyB* monogenic mutant, and both flowered significantly earlier than the wild type, proposed that *CRY2* removes the red light inhibition by *PHYB* (Guo et al., 1998; Mockler et al., 1999).

The flowering time of the *cry1 cry2* double mutant was very similar to that of the *cry2* monogenic mutant in both LD and SD photoperiods, suggesting no apparent interaction of *CRY1* and *CRY2* in photoperiodic flowering (Mockler et al., 1999). Interestingly, although the *cry1* and *cry2* monogenic mutations flowered at about the same time as the wild type in both red and blue light, the *cry1 cry2* double mutant plants flowered significantly later than the wild type or the *cry1* and monogenic *cry2* mutants parents under blue light.

The circadian clock and flowering

The circadian clock is an internal oscillator, which can be broadly defined as the signaling system that is made up of three functional components: an internal oscillator (or central pacemaker) that generates the circadian oscillation, an input pathway that resets (entrains) the peacemaker according to the environmental cues, such as light, and an output pathway that renders oscillations of the pacemaker to overt circadian rhythms (Dunlap, 1999; Somers, 1999).

In Arabidopsis, the circadian clock regulates the expression of many genes, and the clock-regulated expression of genes encoding chlorophyll-*a/b*-binding protein (*CAB2*) and catalases (*CAT2* and *CAT3*) have been most extensively studied (McClung and Kay, 1994; Somers et al., 1998). Several genes in the long day flowering pathway affect both circadian rhythms and flowering time in Arabidopsis. Mutations in the *EARLY FLOWERING3* (*ELF3*) locus result in the loss of both photoperiod sensitivity and circadian regulation, making *ELF3* a candidate for linking circadian clock function with the photoperiodic induction of flowering. The *elf3* mutant plants flower early and at the same developmental time in both LD and SD conditions (Zagotta et al., 1996). The long hypocotyl phenotype of the *elf3* mutant suggests a defect in light reception or the transduction of light signals (Zagotta et al., 1996). In addition, leaf movements and circadian clock-regulated gene expression are arhythmic in *elf3* mutants in constant light condition but not in constant dark conditions, suggesting that a circadian clock remains functional in the absence of wild-type *ELF3* function (Hicks et al., 1996). This model is supported by recent results showing that ELF3 is required to gate light input to the circadian oscillator, altering the sensitivity of the central oscillator to light at a particular point in the circadian cycle (McWatters et al., 2000; Covington et al., 2001).

Three genes have been suggested as components of the oscillator: *LATE ELONGATED HYPOCOTYL* (*LHY*) Schaffer et al., 1998, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) Wang and Tobin, 1998, and *TIMING OF CAB 1* (*TOC1*) Somers, 1999). *LHY* and *CCA1* encode highly conserved single-MYB signal transduction factors, which, when expressed at high and constitutive levels, disrupt the normal functioning of the clock (Schaffer et al., 1998; Wang and Tobin, 1998). The third gene *TOC1*, was initially identified as the *toc1-1* mutant, which is a short period mutant with altered clock function through the entire life cycle of the plant. Moreover, the effect is independent of light quantity, suggesting a role for *TOC1* in the core of the oscillator (Strayer et al., 2000). Late flowering plants resulted from over-expression of either of *CCA1* and *LHY*, suggesting that the target of these genes may encode repressors of flowering or that *CCA1* and *LHY* may inhibit transcription of floral promoters (Schaffer et al., 1998; Wang and Tobin 1998). In both genotypes, circadian clock-controlled expression of several genes was disrupted. Moreover, over-expression of the *LHY* gene also disrupted circadian clock of leaf movements in Arabidopsis (Schaffer et al., 1998). The potential interactions between *TOC1* and the

MYB genes *LHY* and *CCA1* have been studied (Alabadi et al., 2001). They investigated the *TOC1* expression patterns in *lhy* mutant (over-expressing *LHY*) and *CCA1*-over-expressing plants. The *TOC1* mRNA oscillated with high amplitude in the wild-type parental lines in constant light (LL). The *TOC1* mRNA level was constant in both the *lhy* mutant and the *CCA1*-overexpressing plants under the same light conditions, as expected for plants in which the oscillator function is mostly disrupted. The *TOC1* transcript level in these mutants was similar to or lower than its expression in wildtype. *LHY* and *CCA1* therefore appear to be negative regulators of *TOC1*. The fact that *TOC1* transcript oscillates 12 h out of phase with both the *LHY* and *CCA1* transcripts in wild-type plants further supports this idea (Alabadi et al., 2001).

The *GIGANTIA* (*GI*) transcript levels are under the control of the circadian clock, as shown by the behavior when plants entrained in LD were transferred to either continuous light (LL) or continuous dark (DD). Mutants of *GI* gene assigned this gene to the LD promotion pathway on the basis of its lateness in LD and its epistatic interactions (Koornneef et al., 1998a, Fowler et al., 1999). To investigate further how the circadian clock controls *GI* expression, the effect of two circadian clock controlled genes that affect flowering time on *GI* expression was studied in *CCA1* overexpressers. The results show that the rhythmic pattern of *GI* expression was disrupted as *GI* transcripts were detected all over the day, indicating that the circadian rhythm of *GI* expression in LL is disrupted by constant expression of *CCA1* (Fowler et al., 1999). *GI* gene expression in LD cycles is also affected in *lhy* plants, and does not show its characteristic peak in expression. (Fowler et al., 1999). Analysis of the circadian rhythms of the expression of a *CAB2-LUC* (luciferase) fusion gene showed that blue light and red light could accelerate the pace of the circadian clock (Millar et al., 1995). For example, *CAB2*-promoter activity had a 24-25 h period length in continuous blue light or red light, compared with the period length of ~30-36 h in continuous dark, suggesting that photoreceptors might shorten the period length (Millar et al., 1995). It might be expected that the mutation in a photoreceptor would cause the circadian clock to run more slowly in the relevant wavelength of light. Indeed it has been shown that mutations of photoreceptor genes *PHYA*, *PHYB* and *CRY1* causes the circadian rhythm of *CAB2* promoter activity to oscillate with a longer period length than that of the wild type under various light conditions (Somers et al., 1998). The *cry1* mutant displayed a longer period than wild type at both low and high fluence rates of blue light but showed a wild-type period length at

intermediate fluence rates of blue light (Somers et al., 1998). Further research by Devlin and Kay (2000) showed that the *phyA* mutant was deficient in the perception of low-fluence-rate blue light, displaying a longer than wild-type period length in those conditions. The *phyB* mutant, however showed a wild-type response to blue light and shortens the expression period. The *phyA phyB* double mutant showed a lengthening of period at low fluence rates that was consistent with the loss of phyA but indicating no phyB function in blue light, suggesting that phyB plays no role in blue-light input to the clock. Moreover, the length of the free running period of the *CAB2* transcription rhythm was examined in *cry1*, *cry2* and *cry1 cry2* mutant seedlings in blue light (Devlin and Kay, 2000). The *cry1 cry2* double mutant exhibited a long period of *CAB2::LUC* oscillation in all fluence rates of blue light, indicating a role for both cry1 and cry2 in perception of blue light in the control of the period length of the endogenous clock. Moreover, cry1 and cry2 act with complete redundancy at intermediate fluence rates of blue light, in the control of circadian period and are not essential for circadian rhythmicity; this is, loss of both photoreceptors is required to see a change in phenotype over this range (Devlin and Kay, 2000; Somers et al., 1998).

Since the discovery of plant cryptochromes, this type of photolyase-like pigment has also been found in animals. For example, human and mouse each have two cryptochrome genes (*hCRY1* and *hCRY2* for human (Todo et al., 1996) and *mCRY1* and *mCRY2* for mouse (Hsu et al., 1996), and *Drosophila* has one cryptochrome (*dCRY*, Emery et al., 1998).

Obviously the plant *cry1 cry2* double mutant still shows a strong circadian rhythm of *CAB2::LUC* expression in blue light. This is distinct from the phenotype of the mouse $mCRY1^{-/-}$ $mCRY2^{-/-}$ double mutant, which is arhythmic in constant conditions (Van der Horst et al., 1999). The mouse cryptochromes mCRY1 and mCRY2 form part of a transcriptional feedback loop that makes up the central circadian oscillator in mammals, and loss of both mCRY1 and mCRY2 stops the clock (van der Horst et al., 1999). The plant cryptochromes clearly do not act within the clock mechanism itself, which means that their role is distinct from that of the mammalian cryptochromes. This is consistent with a phylogenetic analysis of the animal and plant cryptochromes that suggests that cryptochromes arose independently in plants and animals (Cashmore et al., 1999). This analysis suggests that the plant cryptochromes diverged from the type II photolyases before the divergence of plants

and animals, whereas the animal cryptochromes diverged more recently from the 6-4 photolyases. Animals are therefore presumed subsequently to have lost the cryptochrome sequence related to the type II photolyases. Apparently, the Drosophila cryptochrome dCRY is closer to that of the plant cryptochromes. Although dCRY interacts directly with the components of the central oscillator, it is not essential for the running of the clock, and its role is purely one of light input to the clock (Ceriani et al., 1999).

Scope of the thesis

The induction of flowering in higher plants leads to a change in the fate of the apical meristem that results in the formation of flowers. Environmental factors such as daylength and temperature have a strong influence on the process.

In addition to genetic variation generated by mutations, natural variation in traits such as flowering time can be used to identify the respective genes. In Arabidopsis natural variation for flowering time is very common, and the occurrence of Arabidopsis in environmental conditions as different as Northern Europe, and central Asia needs a physiological-genetic adaptation to the local environments. This kind of variation can be investigated using QTL analysis and the identified loci might be studied more clearly when the respective genes are cloned.

This thesis described the genetic and molecular characterization of flowering time QTL identified using the progeny of Cvi/L*er*. In chapter 1 a description is given of the recent progress in locating and isolating Arabidopsis genes including those that account for naturally occurring variation among accessions. In addition, an overview is given of the present knowledge on the genetic, environmental and molecular control of flowering in Arabidopsis. Chapter 2 described the genetic analysis of flowering time that segregates in the Recombinant Inbred Lines (RIL) population derived from the cross between the Arabidopsis accessions L*er* and Cvi, which resulted in the identification of four main QTLs, called *EDI, FLF, FLG*, and *FLH* respectively. In chapter 3, the map based cloning of the *EDI*-QTL is described, which led to the molecular identification of a novel allele of the *CRY2* gene in which specific amino-acid change was shown to affects the protein stability in SD conditions, resulting in altered flowering phenotype. Chapter 4 gives a description of the genetic relation between the *EDI*-QTL and other genes controlling different flowering time pathways,

using a group of different double and triple mutants and expression analysis of a number of target genes of the floral induction pathway. Finally, in chapter 5 the work presented in this thesis is summarized and discussed.

Chapter 2

Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana*

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Abstract

We have analyzed the flowering behaviour of two Arabidopsis ecotypes: the laboratory strain Landsberg *erecta* (L*er*) and an ecotype from the tropical Cape Verde Islands (Cvi). They differ little in their flowering phenotypes and in their responses to photoperiod length changes and to vernalization treatment. However, segregating populations derived from crosses between them showed a much larger variation. An approach of *q*uantitative *t*rait *l*ocus (QTL) mapping in recombinant inbred lines (RILs) grown under three environments differing in day-length and/or vernalization treatment, has been used to detect and locate flowering loci. Four main QTLs were identified, designated *earlu daylength insensitive* (*EDI*), *flowering F, G, and H* (*FLF*, *FLG*, and *FLH* respectively), to which most of the flowering behaviour differences could be attributed. To further characterize the individual loci, near isogenic lines were constructed by introgressing Cvi early alleles of *EDI* and *FLH* into the L*er* genetic background. *EDI*-Cvi alleles produce earliness under both long- and short-day photoperiods, rendering L*er* plants almost daylength neutral. In addition, RILs were selected to analyze *FLF* and *FLG*. These loci interact epistatically and RILs carrying late alleles at *FLF* and *FLG* were very responsive to vernalization and showed an increased response to photoperiod length changes. The possible role of these loci for the control of flowering is discussed in the context of the current Arabidopsis model.

Introduction

To reproduce successfully plants must flower under favorable environmental conditions and therefore the time of flowering is likely to have an important adaptative significance (Murfet, 1977). The transition from the vegetative to the reproductive phase is influenced by environmental factors such as photoperiod length and temperature, indicating that plants detect fluctuations in these parameters. The model plant *Arabidopsis thaliana* is being extensively used to dissect this developmental process genetically (reviewed in Martinez-Zapater et al., 1994; Coupland, 1995; Amasino 1996; Koornneef et al., 1998b). A large number of mutations affecting initiation of flowering, mostly in a quantitative manner, have been artificially generated. The genetical and physiological characterization of these mutations has shown that the regulation of this

developmental switch in meristem fuction is complex. Several elements controlling the perception and transduction of light quality and daylength such as the phytochromes A and B (Goto et al., 1991; Whitelam and Harberd,1997), the cryptochromes (Bagnall et al., 1996; Guo et al., 1998) and components of the circadian clock, like the *ELF3* and *LHY* genes (Hicks et al., 1996; Schaffer et al., 1998) have been identified. Other genes, like the *VRN* loci, seem to control the cold signalling involved in the flowering response to vernalization (Chandler et al., 1996). The environmental factors are thought to modulate the action of several endogenous signalling components such as gibberellins (Bagnal, 1992; Wilson et al., 1992) and sucrose (Roldán et al., 1997). Furthermore, several loci that might be involved in the signal transduction pathways that lead to flowering have been identified. Some of these have already been cloned and encode putative transcription factors such as *LD* (Lee et al. 1994b) and *CO* (Putterill et al., 1995) or an RNA-binding protein like *FCA* (Macknight et al., 1997), indicating that the regulation of flowering involves the sequential activation of genes.

In addition to induced mutations, genetic variation for flowering time has been found among natural populations (ecotypes) of Arabidopsis (Laibach, 1951; reviewed in Napp-Zinn 1969,1987). Arabidopsis has a wide distribution throughout the Northern hemisphere (Rédei, 1970) and differences found among ecotypes grown under the same environmental conditions are considered to reflect adaptations to different natural environments. Karlsson et al. (1993) analysed 32 ecotypes in several environments with different photoperiod length and vernalization treatments, and they have shown that genotype by environment (G x E) interactions are very significant, which illustrates the diversity of responses found in nature. The identification of the loci responsible for this natural variation has been attempted for over 40 years (Napp-Zinn, 1957; Van der Veen, 1965). The advent of molecular markers and the development of genetic maps has facilitated the localization and characterization of some of the large effect alleles. Thus, the flowering behaviour difference between very late ecotypes that respond to vernalization and the early ecotypes (classified under longday (LD) light conditions) has been shown to involve 2 epistatic loci : the *FRI* locus mapped on the top of chromosome 4 (Clarke and Dean, 1994; Lee et al., 1993; Burn et al., 1993) and *FLC* located on chromosome 5 (Koornneef et al., 1994; Lee et al., 1994a). Dominant alleles at both loci confer the lateness and vernalization requirement of late ecotypes. Moreover, these late alleles respond strongly to photoperiod changes, causing facultative LD strains to behave as "obligate" LD strains when they are not vernalized (Lee and Amasino, 1995). The

identification of natural allelic variation of smaller effect has required the combination of genetic maps with statistical methods to locate quantitative trait loci (QTLs). Flowering QTL analyses have been performed in crosses between late and early ecotypes (Clarke et al., 1995; Kuittinen et al., 1997), as well as between early ones (Kowalski et al., 1994: Jansen et al., 1995; Mitchell-Olds, 1996). The distinct number of QTLs detected in different crosses, varying between 2 and 12, does not fairly reflect the different number of segregating loci, but rather differences in the QTL detection power through the coverage of the corresponding molecular maps, the type and size of mapping population and the statistical approach. The combination of recombinant inbred line (RIL) populations and statistical methods that take into account the effect of multiple QTLs is particularly powerful (*m*ultiple *Q*TL *m*odel -MQM- mapping, Jansen and Stam, 1994; or *c*omposite *i*nterval *m*apping - *cim* (Zeng, 1994), and allows the separation of linked flowering loci (Jansen et al., 1995; Kuittinen et al., 1997). The analysis of QTL by environment (QTL x E) interactions in these populations enables the detection of loci causing the G x E interactions (Clarke et al., 1995; Jansen et al., 1995). Furthermore, epistasis has been detected among some QTLs (Clarke et al., 1995; Kuittinen et al., 1997). All of these studies have shown the wealth and complexity of the natural genetic variation that is available, but most of them were restricted to determine the number and approximate location of segregating loci. With the exception of the *FRI* and *FLC* loci no further analysis of this allelic variation has been reported. The genetical and physiological characterization of QTLs requires the introgression of the new alleles in a genetic background similar to the laboratory strains used to generate artificial mutations. By constructing near isogenic lines (NILs) comparisons of allele effects, allelism tests and fine mapping can be performed. Consequently, the loci at which the natural variation occurs might be determined and eventually, their characterization at the molecular level will be achieved.

In the present study we have analysed the allelic variation affecting flowering time in two early ecotypes: the laboratory strain Landsberg *erecta* (L*er*) and an ecotype originating from the Cape Verde Islands (Cvi). A QTL mapping approach in RILs has been used to identify and locate the loci responsible for the flowering variation in three environments differing in photoperiod length and/or vernalization treatment. The four largest effect QTLs have been further characterized genetically and physiologically in relation to the flowering responses to daylength and vernalization. For that, NILs containing Cvi early alleles in a L*er* genetic background and several selected RILs

carrying Cvi late alleles have been analyzed. The possible role of these loci for the control of flowering is discussed in the context of the current Arabidopsis model.

Materials and Methods

Plant material

A set of 162 recombinant inbred lines (RILs) derived from crosses between the laboratory strain Landsberg *erecta* (L*er*) originating from Northern Europe (Rédei, 1992) and the ecotype Cvi, from the tropical Cape Verde Islands (Lobin, 1983) was used to identify flowering QTLs. These lines have been previously characterized for AFLP and CAPS markers (Alonso-Blanco et al., 1998a).

Selected RILs were crossed with the following late-flowering genotypes, in a predominantly L*er* genetic background: i) the *FRI*-M73 introgression line containing the *FRI* locus from the genotype M73 (Koornneef et al., 1994) and ii) the *ld* introgression line with the *ld-1* mutation originally generated in Columbia (Col) background (Koornneef et al., 1994). All crosses were performed using the L*er* background plants as female parents.

Construction of NILs

As a first step to construct near isogenic lines (NILs), early flowering Cvi alleles were introgressed into L*er* genetic background by phenotypic selection under LD conditions. Selection was basically performed to introgress non-recessive Cvi alleles with relative large effect. Three early-flowering inbred lines were obtained with 4 backcross generations, and 3 final selfing generations. These lines were genotyped using 370 AFLP and CAPS markers. One line, referred to as S10, appeared to be completely L*er* for chromosomes 2, 3 and 4, and contained Cvi introgressions at three genomic regions: top and bottom of chromosome 1 (genetic segments of approximately 25 and 20 cM, respectively), and bottom of chromosome 5 (10 cM approximately). This line was backcrossed to L*er* and an F2 was genotyped for CAPS markers in the segregating regions. Two different F2 plants for each of the 3 different homozygous introgression genotypes were selected as the final NILs. These lines are designated *EDI*-Cvi, *FLH*-Cvi and *EDI*-Cvi,*FLH*-Cvi, because they contain Cvi alleles at the loci *EDI* or/and *FLH*, respectively. Lines containing Cvi alleles at the

bottom of chromosome 1 were constructed, but they were removed from the analysis because no significant effect on flowering could be detected.

Growth conditions

In experiments without vernalization treatment, seeds were sown in Petri dishes on water-soaked filter paper and incubated during three days in a growth chamber at 24° C with 16 h light (for long day -LD- light conditions) or 8 hours light per day (for short day -SD- light conditions). The vernalization treatment was given as described in Koornneef *et al.* (1994). For that, seeds were sown on Murashige-Skoog medium supplemented with 1% sucrose (MS-10). Subsequently, Petri-dishes were incubated in a cold room at 4° C during three weeks and then transferred to a climate chamber (24° C, with 8 or 16 h light per day) during two days before planting. LD experiments were performed in an air-conditioned greenhouse supplemented with additional light from middle September until the beginning of April providing a day-length of at least 14 h. SD experiments were carried out in a single climate chamber with 8 h light as described by Koornneef et al. (1995).

RIL evaluations: The complete set of RILs, parental lines and reciprocal F1 hybrids were evaluated for flowering under three different environmental conditions: LDs with and without vernalization treatment, and SDs without vernalization. RILs were grown under both LD conditions, with and without vernalization treatment, in the same experiment and therefore the non-vernalized seeds were also sown on MS-10 medium. Twelve plants for each RIL and 24 for the parental lines and F1 hybrids, were grown per treatment in a two blocks design. Blocks were divided in rows of 12 plants, and the six plants of each genotype per block were grown in half a row, lines being completely randomized. For the SD experiment, 12 plants per line were grown in two pots sorted in a two blocks design. Lines were completely randomized within the blocks.

NIL evaluations: The early flowering near-isogenic lines, parents and F1 hybrids were evaluated under four different environments, namely LD and SD conditions either with and without vernalization treatment. The vernalized and non-vernalized treated lines were grown together and therefore all seeds were sown on MS-10 medium. The design was basically similar to that described above for the RIL experiments, but 24 plants per genotype and treatment were grown.

Evaluations of F1 hybrids and F2 populations involving selected RILs, FRI-M73 and ld: The F1 hybrids and F2 populations involving the L*er*/Cvi RILs 40, 104 and 130, the parental lines and the introgression lines *FRI*-M73 and *ld* were grown under LD condition experiments. For the F1 hybrids, 24 plants per genotype were grown in a two blocks design as described above for the RIL evaluations. This experiment was repeated and similar flowering data were collected on both occasions. Only data from the most complete experiment are presented. The six different derived F2 populations were grown together in a single LD experiment. Each population consisted of 100-120 plants. Twenty four plants of each parental line were grown in every experiment.

Measurement of flowering

The flowering phenotype was measured following two criteria: flowering time (FT) and total leaf number (TLN). FT was recorded as the number of days from the date of planting until the opening of the first flower. TLN was scored as the number of rosette leaves (RLN) plus the number of cauline leaves (CLN).

Statistical and QTL analyses

To map QTLs using the RIL population, a set of 99 markers covering most of the Arabidopsis genetic map was selected from the RIL L*er*/Cvi map (Alonso-Blanco *et al.*, 1998). These markers spanned 482 cM, with an average distance between consecutive markers of 5 cM and the largest genetic distance being 12 cM. The phenotypic values recorded were transformed ($\log_{10}$) to improve the normality of the distributions and the values of ten plants per RIL were used to calculate the line means for each of the four traits (FT, TLN, RLN and CLN) and the three environments (LD, SD and LD with vernalization). The line means were used to perform the QTL analyses. Every trait was analysed separately for each environment. All the statistical comparisons shown were based on the transformed data, but none of the conclusions was changed when using the original data. Therefore, results are presented in figures with the original scale. The computer program MapQTL (tm) version 3.0 (Van Ooijen and Maliepaard, 1996a and b) was used to identify and locate QTLs linked to the molecular markers using both interval mapping and multiple-QTL-model (MQM) mapping methods. In a first step, putative QTLs were identified using interval mapping. Thereafter, one marker at each putative QTL (between 3 and 9 depending on trait and environment) was selected as a cofactor and the selected

markers were used as genetic background controls in the approximate multiple QTL model of MapQTL. To refine the mapping and to identify linked QTLs, different cofactor markers were tested around the putative QTL positions (Van Ooijen and Maliepaard, 1996a and b), selecting as final cofactors the closest marker to each QTL, i.e. those maximising the LOD score. A LOD score of 2.4 was used as the significance threshold to declare the presence of a QTL, in both interval and MQM mapping, based on thresholds previously obtained by simulation with Arabidopsis mapping data of another RIL population (Jansen et al., 1995; Van der Schaar et al., 1997). In the final MQM model the additive genetic effect was estimated at each QTL and 2-LOD support intervals were established as an approximate 95% confidence level (Van Ooijen, 1992).

For every trait and environment the contribution of each QTL to the phenotypic variance was estimated by analysis of variance components. For each analysis, the closest linked markers to the corresponding detected QTLs were used as random factors in ANOVA (the same markers used as cofactors in the MQM mapping with MapQTL). Since for all traits and environments the two markers corresponding to the QTLs located in the upper arm of chromosome 5 showed a highly significant interaction, and none of the remaining two-way interactions among the QTL markers was significant ($P>0.005$), the interaction term between these two factors was included in the linear models. Thus, the contribution of this interaction was also estimated.

For FT and TLN a search for interactions between QTLs was performed using the computer program EPISTAT (Chase et al., 1997). Two-way interactions were searched among all pair wise combinations of the 99 markers using as significance threshold a log-likelihood ratio equivalent to $P<0.005$. Ten thousand trials were used in the Monte Carlo simulations performed with EPISTAT to establish the statistical significance of the log-likelihood ratios of the interactions detected (Chase et al., 1997).

The overall genotype x environment (G x E) interaction was tested for each trait by a two-factor ANOVA using genotypes (RILs) and environments as classifying factors. For each trait and for each putative QTL, QTL x E interaction was tested by ANOVA using the corresponding marker and the environment as between and within (repeated measurements) classifying factors, respectively ($P<0.005$). The General Linear Model module of the statistical package SPSS version 7.5 was used for the

ANOVAs and for the variance component analyses from the Type III sum of squares ANOVA.

Molecular markers

The introgresion lines containing early flowering Cvi alleles were genotyped using amplified fragment polymorphism (AFLP) marker analysis, which was performed according to Vos et al. (1995). About 350 polymorphic bands amplified with the fourteen primer combinations previously used to build the L*er*/Cvi molecular map (Alonso-Blanco et al., 1998a) were scored for absence and presence. The genetic location of AFLP bands was therefore previously known and covered most of the genetic map.

CAPS and microsatellite markers previously mapped in the L*er*/Cvi RILs and/or the Ler/Col RILs (Alonso-Blanco et al., 1998; *At*DB) were used to genotype genomic regions containing flowering loci, in the introgression lines and in the backcross-like and F2 populations. CAPS markers were analysed according to Konieczny and Ausubel (1993) and microsatellite markers according to Bell and Ecker (1994). The following PCR markers were used: PVV4, AXR1, PhyA and g2395 for locus *EDI* (present work); ANL2 and GA1 for the loci *FRI* and *ld* (Clarke and Dean; 1994; Lee et al., 1994b), the two CAPS markers being linked at a genetic distance of 12 cM and flanking both flowering loci; nga158 and nga151 for the loci *FLF* (present work); nga139 for locus *FLG* (present work) and g2368 for locus *FLH* (present work).

Results

Flowering behaviour of Ler, Cvi and the RI lines

The flowering phenotype of the parental ecotypes L*er* and Cvi, the reciprocal F1 hybrids, and a set of 162 L*er*/Cvi RILs was evaluated under three different environmental conditions: LD photoperiod, with and without vernalization treatment, and SDs (Fig. 2-1 and Table 2-1). Comparison of the flowering phenotypes between the SD and LD environments provided an estimate of the response to photoperiod length, and comparison of LD conditions with and without vernalization treatment provided an estimate of the vernalization response. Both ecotypes flower at rather

similar times under LD conditions and can be considered as early flowering. The later flowering time of L*er* under SD, indicates that Cvi responds less than L*er* to photoperiod length changes. In contrast, Cvi shows a more pronounced response to the vernalization treatment. The F1 hybrids flower earlier or similar to the earliest parent (Table 2-1), although the FT means of the nonvernalized reciprocal F1s grown under LD conditions were significantly different ($P<0.001$; which was observed consistently and was even more pronounced in two other experiments not shown). Reciprocal differences have been previously observed in crosses between other Arabidopsis ecotypes suggesting a certain influence of maternal factors on flowering (Westerman, 1970; Clarke and Dean, 1994), but they have not been further analyzed.

Although the flowering differences between L*er* and Cvi are small, transgressive variation in both directions was observed in the RIL population under the three environments indicating the presence in the two parental lines of alleles increasing and reducing flowering time (Fig. 2-1; Table2-1). A large amplification of the flowering range was observed in the RIL population when grown under SDs, and three major classes of flowering time appeared. In contrast, a reduction in the flowering range occurs when vernalizing the RILs (Fig.2-1; Table 2-1). The G x E interactions were highly significant ($P<0.001$) when the flowering responses to vernalization or to photoperiod length were compared in the RIL population. This indicates the presence of allelic variation whose effect is expressed differentially with the environments to control the different responses of the RILs to photoperiod length changes and to vernalization treatment.

The flowering phenotype was measured as FT and as TLN. As shown in Fig. 2-2 both traits are tightly correlated in the RIL population and therefore both are expected to be mostly under the same genetic control as was previously observed with mutant genotypes (Koornneef *et al.*, 1991).

Mapping loci that control the flowering behaviour differences between Ler, Cvi and the RILs

To identify and locate the loci controlling the flowering behaviour differences between L*er* and Cvi, the phenotypic values of the 162 RILs collected under the 3 environments were used for QTL analysis. Four flowering related traits, (FT, TLN, RLN, and CLN) were analyzed separately for each environment (LD with and without vernalization treatment, and SD) using the MQM method of MapQTL (see Materials

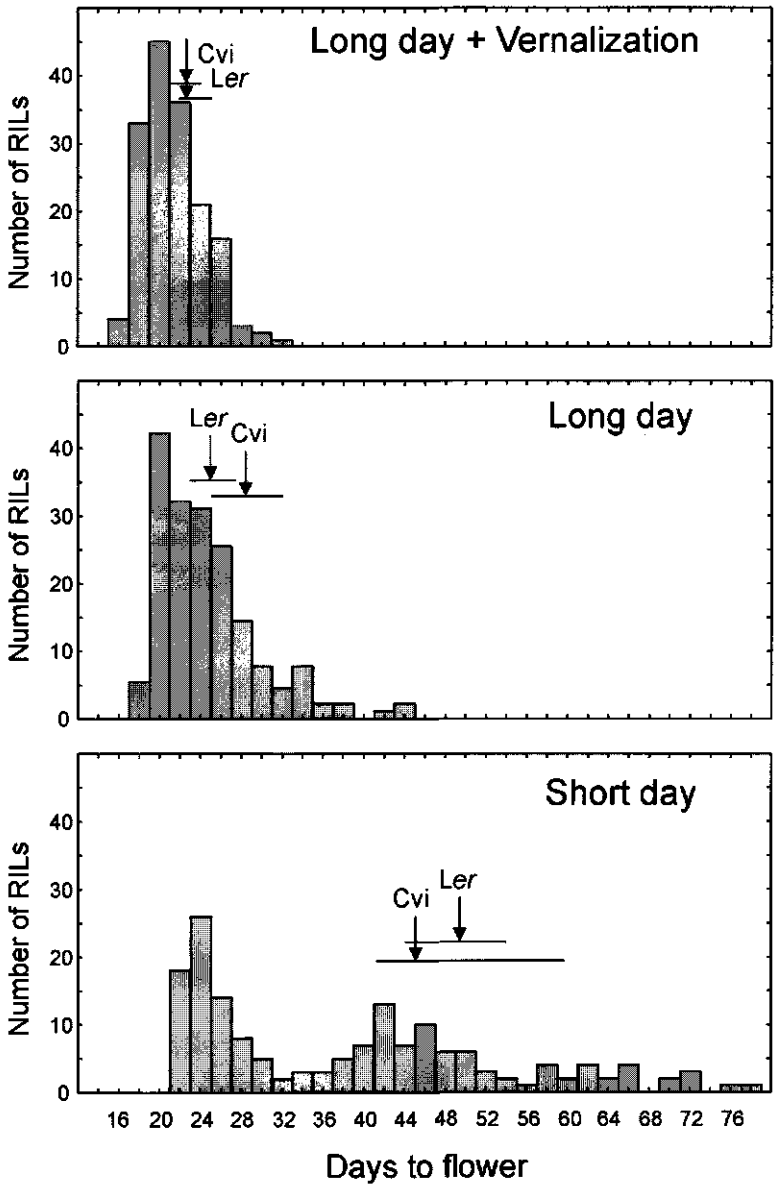

Figure 2-1. Frequency distributions of flowering time means of the L*er*/Cvi RILs grown under three environments with different photoperiod length and/or vernalization treatment. Arrows correspond to the parental line means (20 plants per parent) and the horizontal bars represent their ranges of variation.

Table 2-1.
Phenotypic values for flowering traits of the parental lines, reciprocal F1 hybrids, and the RIL population grown in three different environments (10 plants were used per RIL; 20 plants for the rest of lines).

	Long day	Long day + Vernalization	Short day
Flowering Time (days)			
Ler	25.0 ± 1.0	21.6 ± 1.0	49.4 ± 2.9
Cvi	28.0 ± 1.8	21.6 ± 0.9	45.4 ± 6.6
F1 *Ler* x Cvi	25.1 ± 0.9	21.1 ± 0.9	-
F1 Cvi x *Ler*	22.2 ± 1.9	19.8 ± 0.7	-
RIL mean	24.8 ± 5.1	21.4 ± 2.9	38.4 ± 14.7
Min.-Max. RIL mean	18.1 - 44.8	16.3 - 32.0	21.1 - 78.0
RIL LSD	1.7	1.7	4.4
Total Leaf Number			
Ler	10.4 ± 1.0	9.1 ± 1.0	32.5 ± 2.4
Cvi	11.6 ± 1.3	8.1 ± 1.0	27.0 ± 8.9
F1 *Ler* x Cvi	10.9 ± 0.8	8.7 ± 0.6	-
F1 Cvi x *Ler*	10.2 ± 1.7	9.5 ± 0.8	-
RIL mean	10.5 ± 5.2	8.6 ± 2.3	20.8 ± 14.0
Min.-Max. RIL mean	5.7 - 32.1	5.4 - 18.1	5.4 - 55.6
RIL LSD	1.9	0.6	4.1

Values are means ± SD. RIL mean, minimum and maximum, and least significant differences at $P \leq 0.01$ (LSD) for mean RIL comparisons are also shown.

and Methods). The use of cofactors strongly improved the mapping accuracy of linked QTLs which could not be separated with interval mapping. Figure 2-3 shows the QTL likelihood maps obtained for TLN under the 3 environmental conditions, indicating the genetic intervals where the putative QTLs were mapped. A total of 11 QTLs were

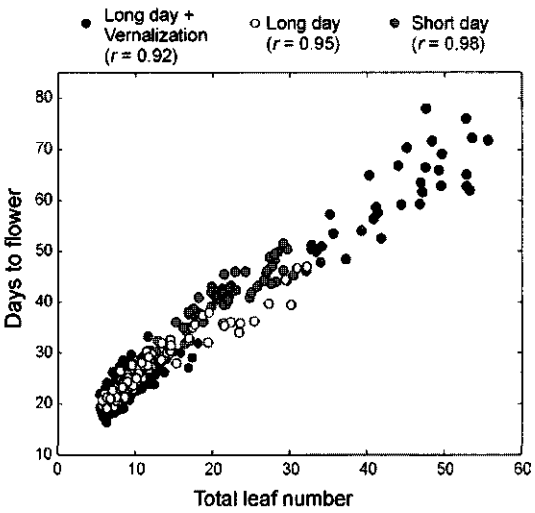

Figure 2-2. Relationship between flowering time mean and total leaf number mean in the RIL population.
r, correlation coefficient

detected along the 5 linkage groups. However, a clear distinction can be made between large effect (major) and small effect (minor) loci (Tab. 2-2).Allelic variation at 4 loci mapping on top of chromosome 1, and on top, middle and bottom of chromosome 5 respectively, had a large effect on both TLN and FT (15% of the phenotypic variance could be attributed in at least one environment). We have named them *EDI* (*e*arly *d*ay-length *i*nsensitive; see later), and *FLF*, *FLG* and *FLH* (for *fl*owering F, G and H) respectively. Cvi alleles produce earliness at *EDI* and *FLH* and lateness at *FLF* and *FLG*, this allelic variation accounting for nearly all the RIL phenotypic variance in the 3 environments and for the parental phenotypes (see Fig. 2-4 in which *FLH* has not been included but its effect is in agreement with the phenotypes of L*er* and Cvi). The remaining 7 QTLs had small additive effects (in general less than 5% of the variance could be attributed to each one) and were detected only under the LD with vernalization.

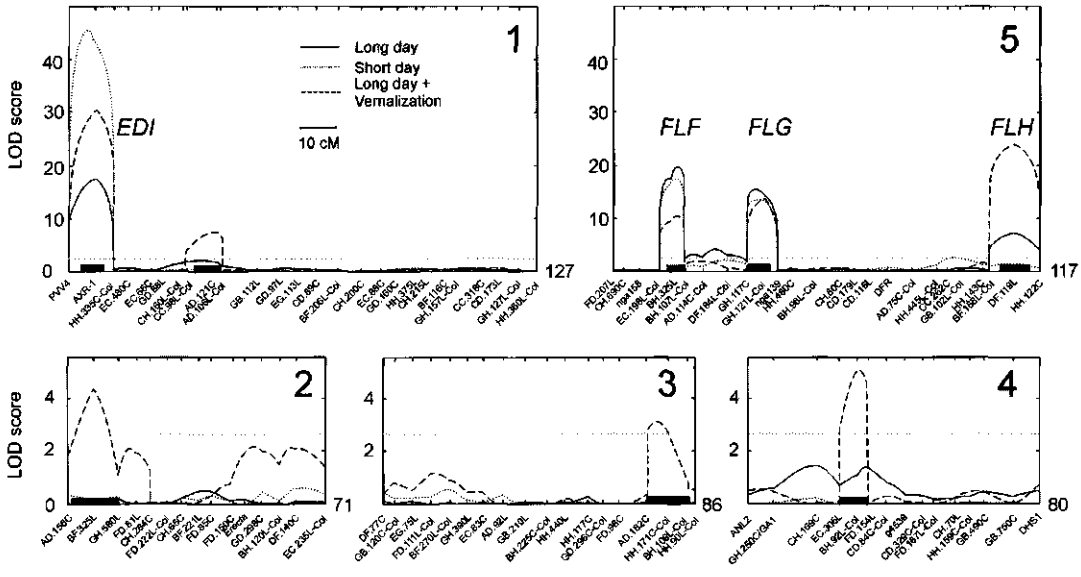

Figure 2-3. QTL likelihood maps for total leaf number in the three different environments. The abscissas correspond to the genetic maps in cM, the linkage group number being indicated in the right upper corner of each map. Horizontal dashed line corresponds to the LOD score threshold of 2.4. Two LOD support intervals for the significant QTLs are shown as solid bars along abscissas. The largest effect QTLs have been named as *EDI*, *FLF*, *FLG* and *FLH*.

The QTLs detected for FT and TLN were in most cases mapped in the same intervals, indicating pleiotropy at these loci. The 4 main QTLs showed comparable contributions to the phenotypic variance of both traits (Tab. 2-2). However, two small effect QTLs on chromosome 2 appeared as significantly affecting FT, but not TLN (markers FD.81 and DF.140C) and two others as significant for TLN, but not for FT (BF.325L and HH.171C-Col). These putative QTLs were considered either significant or not on the basis of the 2.4 LOD threshold, but the likelihood values for both traits always increased around the corresponding positions (see for instance chromosome 2 on Fig.2-3). In agreement with this, one of the small QTLs affecting FT, but not TLN (DF.140C) was significant for RLN. Only the QTL located at the bottom of chromosome 4 (around DHS1) appeared to affect CLN but not RLN and FT in the LD conditions. Therefore, most of the QTLs identified affected FT and TLN, although small differences might exist in their relative effect on both traits, or for their relative contribution to RLN or CLN.

Epistasis between QTLs was analyzed by performing a genome-wide search for two-way interactions. The two major QTLs located on the top and middle of chromosome 5 (*FLF* and *FLG*) show very significant synergistic interaction for all traits and all environments ($P<0.0001$; see Tab. 2-2 and Fig. 2-4). These loci have relatively small additive effects individually (*FLF* shows practically no effect while *FLG* has small effect), and lateness in the 3 environmental conditions is mainly observed when both Cvi alleles are present. Interactions were also detected between these regions and markers at the bottom of chromosome 1. However, because pseudolinkage is observed in the RIL population between markers at the bottom of chromosome 1 and the top of (22% recombination frequency due to the lack of RILs with recombinant genotypes) these interactions were rejected as not true epistasis. Another significant epistatic interaction was detected between the QTL linked to BF.325L on chromosome 2, and the marker HH.440L on chromosome 3, which had not been previously associated with flowering.

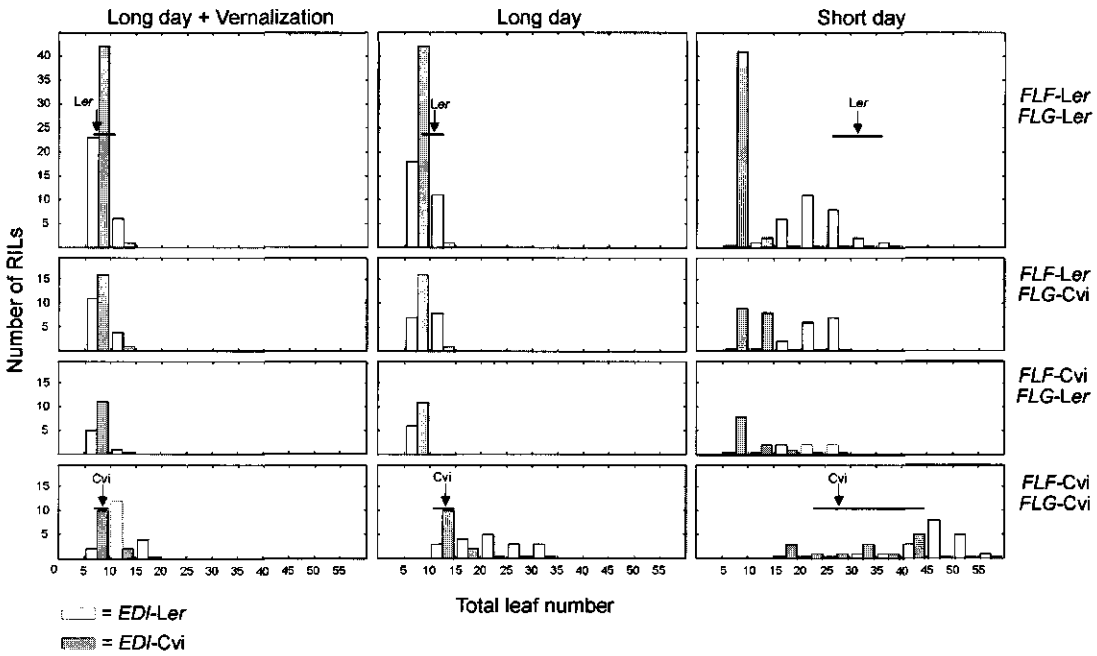

Figure 2-4. Frequency distributions of total leaf number means of the RILs grown in three environments with different photoperiod length and/or vernalization treatment. The RILs have been classified according to their genotype at the closest markers to the loci *EDI*, *FLF* and *FLG*. The four distributions within each environment (vertical) correspond to the distributions of the four RIL classes obtained according to their genotypes at *FLF* and *FLG* (legend in the right part of the figure). Within each graph, the RILs are classified in relation to the genotype at *EDI* and the two distributions are overlaid. Arrows indicate the parental line means (20 plants per parent) and the horizontal bars represent their ranges of variation.

The significant interaction of the 3 environments with *EDI*, *FLF* and *FLG* (Table 2) indicates that these are the loci responsible for the different flowering responses in the RILs. The QTL on chromosome 1 around AD.121C also showed significant Q x E interaction but it was due to its genetic linkage with EDI, since it was not significant when analysing the interaction of both QTLs simultaneously. The remaining QTLs did not show significant interactions with the environments and

therefore were not considered as environment specific. The overall effect of the 3 major loci on the flowering responses was examined. For that, the responses of each RIL were quantified as the difference in TLN between the LD and SD conditions (photoperiod length response) and between the LD and the LD with vernalization treatment (vernalization response). Figure 2-4 shows the TLN frequency distributions of the RILs classified according to these 3 loci under the 3 environments. Several conclusions can be summarized as:

1) *EDI, FLF and FLG* are the loci controlling the differences in photoperiod length response. RILs carrying late alleles at *EDI*, or at *FLF* and *FLG*, not only flower later but responded more to photoperiod length than the RILs carrying early alleles at these loci. An extremely low response was shown by the genotypes *EDI*-Cvi, *FLF*-L*er*, *FLG*-L*er*, which led to the naming of this locus as *e*arly *d*aylength *i*nsensitive (*EDI*). Therefore, to "abolish" the photoperiod response in the L*er*/Cvi RILs required early alleles at the 3 loci.

2) *FLF* and *FLG* are the main loci controlling the differences in vernalization response. The *FLF* and *FLG* effects are much smaller under vernalization conditions than in normal LDs. In other words, the lateness observed under LDs in RILs carrying *FLF*-Cvi, *FLG*-Cvi alleles, is very much diminished by a 3 weeks vernalization treatment. It is expected that a longer vernalization treatment would have reduced even more the effect of these loci, since saturation of the vernalization response in late flowering responsive genotypes requires longer treatments (Lee and Amasino, 1995).

Characterization of Cvi early alleles: the loci EDI and FLH

Near isogenic lines containing Cvi alleles at *EDI*, and/or *FLH* in a L*er* genetic background were constructed by phenotypic and genotypic selection (see Fig. 2-5 and Material and Methods). Only the introgression line containing Cvi alleles in the *EDI* region was used for further genetic mapping, analyzing an F2 population under SD conditions where the flowering segregation could be classified qualitatively and behaved in monogenic manner. The location of *EDI* was narrowed to a segment smaller than 10 cM comparable to the 2 LOD support interval established in the QTL analysis (data not shown). The genetic length of the introgression segment in the monogenic *FLH*-Cvi NIL (10 cM approximately) confirmed the *FLH* position obtained in the MQM analysis of the RILs.

Table 2-2.
QTLs detected for four flowering related traits in three environments differing in photoperiod length and/or vernalization treatment

				Long-day + Vernalization		Long-Day		Short-Day		
Trait	QTL	Map position		% of variance	Additive allele effect	% of variance	Additive allele effect	% of variance	Additive allele effect	QTL x E Interaction
Flowering Time				89.3		90.5		93.2		
	AXR-1 (*EDI*)	1 - 7.5		43.9	-3.5	27.5	-4.6	56.2	-18.1	*
	AD.121C	1 - 40.5		3.8	1.1	NS		NS		*
	BF.325L	2 - 7.2		NS		NS		NS		
	FD.81L	2 - 18.7		1.5	1.0	NS		NS		NS
	DF.140C	2 - 62.3		3.0	1.0	NS		NS		NS
	HH.171C-Col	3 - 78.4		NS		NS		NS		
	BH.92L-Col	4 - 30.2		0.8	-0.7	NS		NS		NS
	DHS1	4 - 80.2		NS		NS		NS		
	BH.325L (*FLF*)	5 - 15.7		2.9		5.1		3.9		*
	GH.121L-Col (*FLG*)	5 - 41.5		14.8		23.2		10.6		*
			FLF x *FLG*	4.0	3.2	31.4	8.6	22.5	21.7	
	DF.119L (*FLH*)	5 - 110		14.5	-2.0	3.3	-1.7	NS		NS
Total Leaf Number				89.5		90.7		91.9		
	AXR-1 (*EDI*)			33.6	-2.4	20.1	-3.8	54.7	-15.8	*
	AD.121C			4.9	1.1	NS		NS		*
	BF.325L			1.5	-1.0	NS		NS		NS
	FD.81L			NS		NS		NS		
	DF.140C			NS		NS		NS		
	HH.171C-Col			2.1	-0.6	NS		NS		NS
	BH.92L-Col			2.5	-0.8	NS		NS		NS
	DHS1			NS		NS		NS		
	BH.325L (*FLF*)			4.6		6.8		6.5		*
	GH.121L-Col (*FLG*)			13.8		18.2		11.0		*
			FLF x *FLG*	5.3	2.8	38.7	8.7	19.7	22.2	
	DF.119L (*FLH*)			21.2	-1.9	6.9	-2.4	NS		NS

Continued

Trait	QTL	Map position	Long-day + Vernalization		Long-Day		Short-Day		QTL x E Interaction
			% of variance	Additive allele effect	% of variance	Additive allele effect	% of variance	Additive allele effect	
Rossette Leaf Number			90.7		91.1		91.8		
	AXR-1 (*EDI*)		33.3	-1.8	19.4	-3.1	54.4	-12.6	*
	AD.121C		7.6	0.9	1.9	1.0	NS		*
	BF.325L		1.5	-0.7	NS		NS		NS
	FD.81L		NS		NS		NS		
	DF.140C		2.3	0.4	NS		NS		NS
	HH.171C-Col		NS		NS		NS		
	BH.92L-Col		3.8	-0.8	NS		NS		NS
	DHS1		NS		NS		NS		
	BH.325L (*FLF*)		3.5		4.9		4.9		*
	GH.121L-Col (*FLG*)		13.9		22.8		13.0		*
		FLF x *FLG*	3.6	2.0	36.7	7.1	19.5	18.3	
	DF.119L (*FLH*)		21.4	-1.5	5.4	-1.7	NS		NS
Cauline Leaf Number			72.1		79.7		86.6		
	AXR-1 (*EDI*)		26.7	-0.5	23.7	-0.9	53.4	-3.2	*
	AD.121C		NS		NS		NS		
	BF.325L		3.0	-0.2	NS		NS		NS
	FD.81L		NS		NS		NS		
	DF.140C		NS		NS		NS		
	HH.171C-Col		NS		NS		NS		
	BH.92L-Col		NS		NS		NS		
	DHS1		5.6	-0.2	3.0	-0.4	NS		NS
	BH.325L (*FLF*)		8		13.5		12.0		*
	GH.121L-Col (*FLG*)		4.7		11.9		3.7		*
		FLF x *FLG*	5.6	0.5	20.6	1.8	17.6	3.9	
	DF.119L (*FLH*)		18.5	-0.4	7.0	-0.5	NS		NS

The closest marker to each QTL is shown and its location is indicated by the linkage group number followed with its map position. Only QTLs with LOD score > 2.4 are reported. The QTLs with the largest effects have been designated as EDI, FLF, FLG, and FLH and are indicated between parentheses. For each trait and environment, the relative contribution of each QTL was estimated by analysis of variance components. Because of the epistatic interaction between FLF and FLG (see text) the relative contribution of their interaction the relative contribution of their interaction was included in the model and it is shown (*FLF* x *FLG*). The additive allele effects of *FLF* and *FLG* have been added together. The additive allele effects are the estimated mean differences between the two RIL genotypic groups carrying the Cvi and L *er* alleles (a positive value implies Cvi allele increasing the corresponding phenotypic value; a negative value, Cvi allele decreasing). Allele effects are shown in the original scale of measurement (days for flowering time and number of leafs for leaf numbers). For each trait, the QTLs interacting with the environments are indicated by * ($P<0.005$); NS, not significant.

The NILs and the line S10, from which they were derived, were analyzed under LD and SD photoperiod conditions, with and without vernalization treatment (Fig. 2-5). The Cvi allele of *EDI* was largely dominant, which was particularly manifest under SD conditions where L*er* plants flowered on average with 18.9 more leaves than the *EDI*-Cvi plants. *EDI*-Cvi plants flowered with almost the same TLN under both photoperiod length conditions, thus behaving as an almost daylength neutral genotype. These plants show little response to vernalization, and are comparable to L*er*. At the *FLH* locus, the slight earliness produced by the Cvi allele behaved on average codominantly. However, its effect was almost absent under LD conditions without vernalization, differing from the effect estimate obtained in the RIL population. This suggests *FLH* might be involved in some undetected epistatic interaction, or that some introgressed fragment not detected in the extensively genotyped lines affected flowering time. In contrast, under SDs, *FLH*-Cvi plants flower on average with 3.4 fewer leaves than L*er* plants, an effect not detected in the QTL analysis. These plants responded to photoperiod in a comparable way to L*er*. However, it is remarkable that they responded more than L*er* to vernalization, an effect that was mainly observed under SD conditions. The allelic effects at *EDI* and *FLH* were basically additive since plants of the *EDI*-Cvi, *FLH*-Cvi line flowered earlier than the monogenic introgression lines in all environments.

Characterization of Cvi late alleles: the loci FLF and FLG

Three RILs were selected on the basis of their genotype as being L*er* at *EDI* and *FLH* (and as much as possible in the rest of the genome), but carrying Cvi alleles at *FLF* and/or *FLG*. RIL 130 was selected as genotype *FLF*-Cvi, *FLG*-Cvi; RIL 104 as *FLF*-Cvi, *FLG*-L*er* and RIL 40 as *FLF*-L*er*, *FLG*-Cvi (the chromosome 5 regions of RILs 40 and 104 are not overlapping). To confirm the presence of two linked flowering loci we performed a reconstruction experiment, under LD conditions, to obtain the expected late flowering genotype when the homozygotes *FLF*-Cvi and *FLG*-Cvi are combined. For that, an F1 hybrid between the genotypes *FLF*-Cvi (RIL 104) and *FLG*-Cvi (RIL40) (heterozygote in repulsion for both loci) was crossed with the line *FLF*-Cvi, *FLG*-Cvi (RIL 130) (Fig. 2-6). This population was partially genotyped for the microsatellite markers nga158 and nga139, closely linked to the support intervals established for *FLF* and *FLG* in the QTL analyses respectively (Fig. 2-3). Indeed, 10 out of the 13 latest plants of this population originated

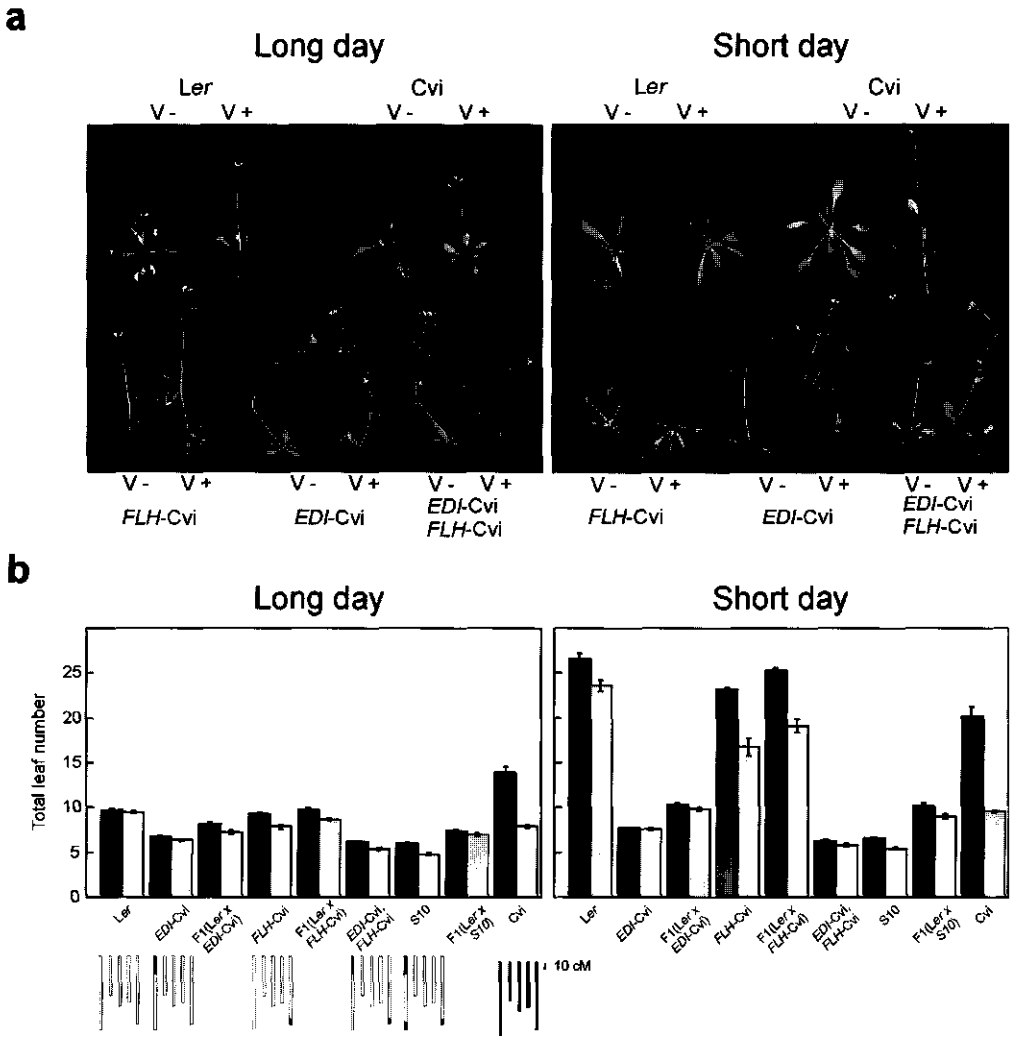

Figure 2-5. (**a**)Phenotype of the parental lines L*er* and Cvi, and the introgression lines in Ler genetic background containing Cvi alleles at *EDI* and/or *FLH*. Plants were grown in four different environmental conditions: **a**) LD photoperiod (left side); SD photoperiod (right side); V-): without vernalization treatment; V+) with vernalization treatment. Plants were photographed 25 days after planting. (**b**) Total leaf number of the parental lines L*er* and Cvi, the introgression lines in L*er* genetic background containing Cvi alleles at *EDI* and/or *FLH*, and the F1 hybrids with L*er*. The line S10 from which the introgression lines were derived is also included (see Materials and Methods). Plants were grown in four different environmental conditions: LD photoperiod (left side); SD photoperiod (right side); without vernalization treatment (dark columns); with vernalization treatment (light columns). Graphical genotypes of the lines are shown in the left lower side, each of the five bars corresponding to one linkage group. Total leaf numbers are the mean of 20-24 plants and the standard errors are represented by error bars.

from recombinant gametes between both markers, thus confirming the presence of 2 flowering linked loci at a genetic distance of at least 15 cM. The flowering phenotypes of the different genotypic classes of this population and of the F1 hybrids between these RILs and Ler (Figs. 2-6 and 2-7), indicate that late Cvi alleles at both *FLF* and *FLG* behave additively (codominantly), i.e. their allelic effects are dossage dependent.

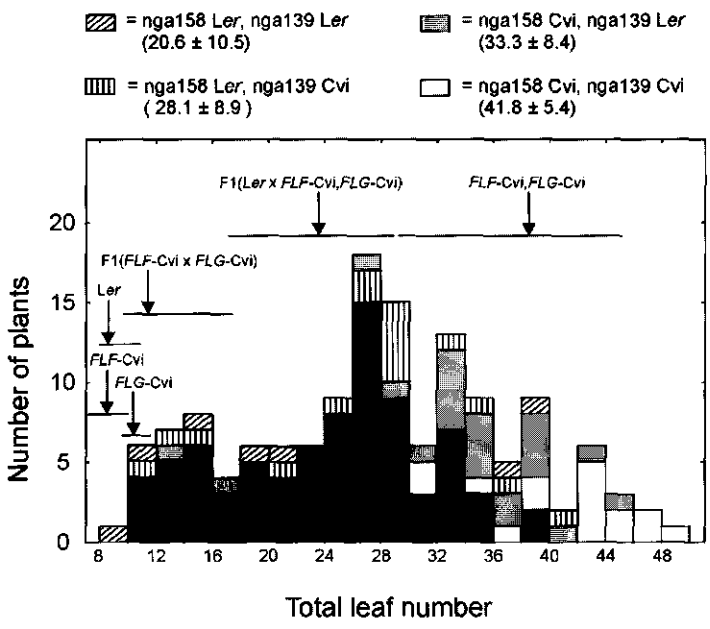

Figure 2-6. Frequency distribution of total leaf number in the backcross like population derived from the cross RIL *FLF*-Cvi, *FLG*-Cvi x F1(RIL *FLF*-Cvi x RIL *FLG*-Cvi). Plants were grown under LD light conditions. The genotypes at markers closely linked to *FLF* (*nga158*) and *FLG* (*nga139*) were determined for 64 out of the 142 plants of the population (not filled columns). The symbols for genotypes at these markers in the segregating gametes are indicated in the upper part. The TLN mean ± standard deviation of the four genotypic classes are also shown. Arrows indicate the line means of the parents and some hybrids; the horizontal bars represent their ranges of variation.

Another locus, *FLC*, at which natural allelic variation has been previously reported, maps in the region of *FLF* (Koornneef et al., 1994; Lee et al., 1994a). FLC-Ler alleles are known to be early in relation to most other tested ecotypes, and late FLC alleles interact synergistically with late *FRI* alleles and with mutant alleles at the LD locus. The *FRI* locus maps on top of chromosome 4, where no QTL was identified in the Ler/Cvi material, and it is very closely linked to LD (Clarke and Dean, 1994; Lee et al., 1994b).

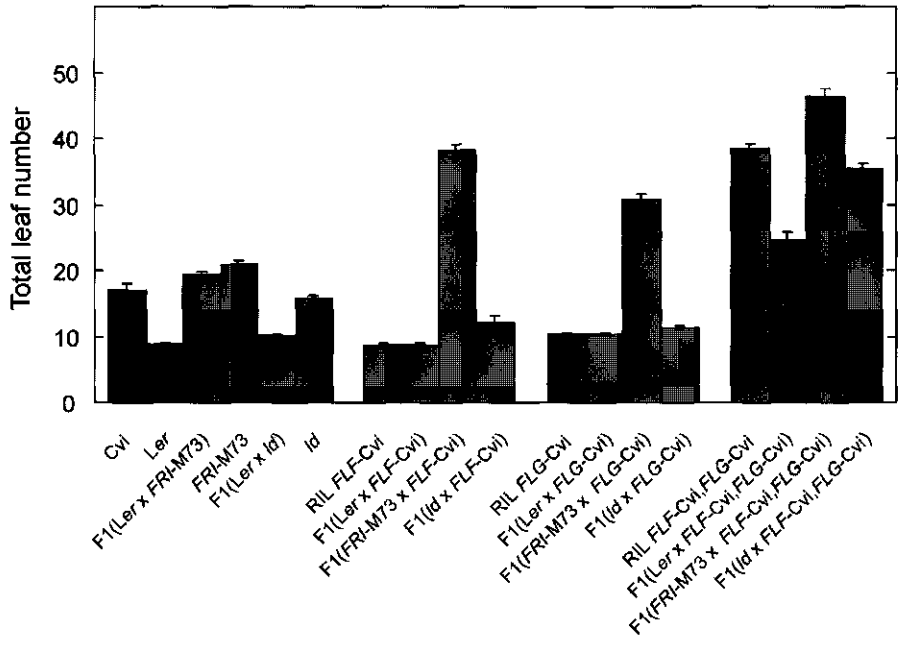

Figure 2-7. Total leaf number means and standard errors of F1 hybrids involving L*er*, the three RILs selected as RIL *FLG*-Cvi, RIL *FLF*-Cvi, and RIL *FLF*-Cvi,*FLG*-Cvi, and the late flowering introgression lines *FRI*-M73 and *Id*. Plants were grown under LD light conditions

To determine whether *FLF* might be *FLC*, we studied the genetic interactions between *FLF* and *FLG* and the loci *FRI* and *Id*. For that, we analysed the flowering phenotype of F1 hybrids and derived F2 populations between the three selected RILs and the late-flowering introgression lines in L*er* genetic background, FRI-M73 and Id (Figs. 2-7 and 2-8). F1 and F2 populations were grown under LD conditions in different experiments and therefore they are not directly comparable. Transgression over the latest parent was observed in all F2 populations indicating the effect of Cvi late alleles. The latest flowering plants of each F2 population were genotyped for molecular markers closely linked to *FLF*, *FLG*, *FRI*, and *Id* (Fig. 2-8; see Material and Methods). Thus, it was confirmed that the late-flowering phenotype was due to the effects of *FLF*-Cvi and/or *FLG*-Cvi and not to interactions of *FRI*-M73 or Id with Cvi alleles in other genomic regions (either detected in the QTL analysis or not) that might be segregating. Taking together the flowering phenotype of the

F1 hybrids and of the latest F2 plants, and the proportion of towards lateness transgression in these populations, several conclusions can be summarized:

1) *FLG*-Cvi behaves additively with ld to produce lateness and shows a weak synergistic interaction with *FRI*-M73. The phenotypes of the corresponding F1 hybrids and F2 populations were in agreement, confirming that both *FLG*-Cvi and *FRI*-M73 are partly dominant and *ld* is recessive.

2) *FLF*-Cvi behaves as a late allele of *FLC* in its synergistic interaction with *FRI*-M73, and with *ld*, although it must be a weaker allele than *FLC*-Sf2 or *FLC*-Col when comparing with TLN's previously reported (Koornneef et al., 1994; Lee et al., 1994a). The phenotypes of the corresponding F1 hybrids and F2 populations were again in agreement with *FLF*-Cvi and *FRI*-M73 being partly dominant and *ld* recessive. Therefore, it is likely that *FLF* and *FLC* are the same locus.

Discussion

In the present study we have analysed the flowering behaviour of two early Arabidopsis ecotypes: the laboratory strain Landsberg *erecta* originating from Northern Europe (Rédei,1992) and the ecotype Cvi, from the tropical Cape Verde Islands (Lobin, 1983). They hardly differ in their flowering phenotype (measured as both TLN and FT) and in their responses to photoperiod length and vernalization treatment. However, segregating populations derived from crosses between these ecotypes show a much larger variation than observed in other crosses between early ecotypes such as Landsberg erecta and Columbia (Jansen et al., 1995). The flowering behaviour differences between the Ler/Cvi lines can be mainly attributed to 4 loci referred to as *EDI*, *FLF*, *FLG*, and *FLH*. Cvi alleles at *EDI* and *FLH* produce earliness while at *FLF* and *FLG*-Cvi alleles produce lateness, thus explaining the similar behaviour of the parental lines and the transgression in the RILs. Another 7 putative minor QTLs might contribute secondarily to these differences, but since they were only found in the environment with the lowest phenotypic variation further confirmation of this is necessary. This is, at least partly, due to the limitations for detecting minor QTLs in small populations where several QTLs with large effects are segregating, as seen, for instance, with the effect of *FLH* which was not detected under SD conditions in

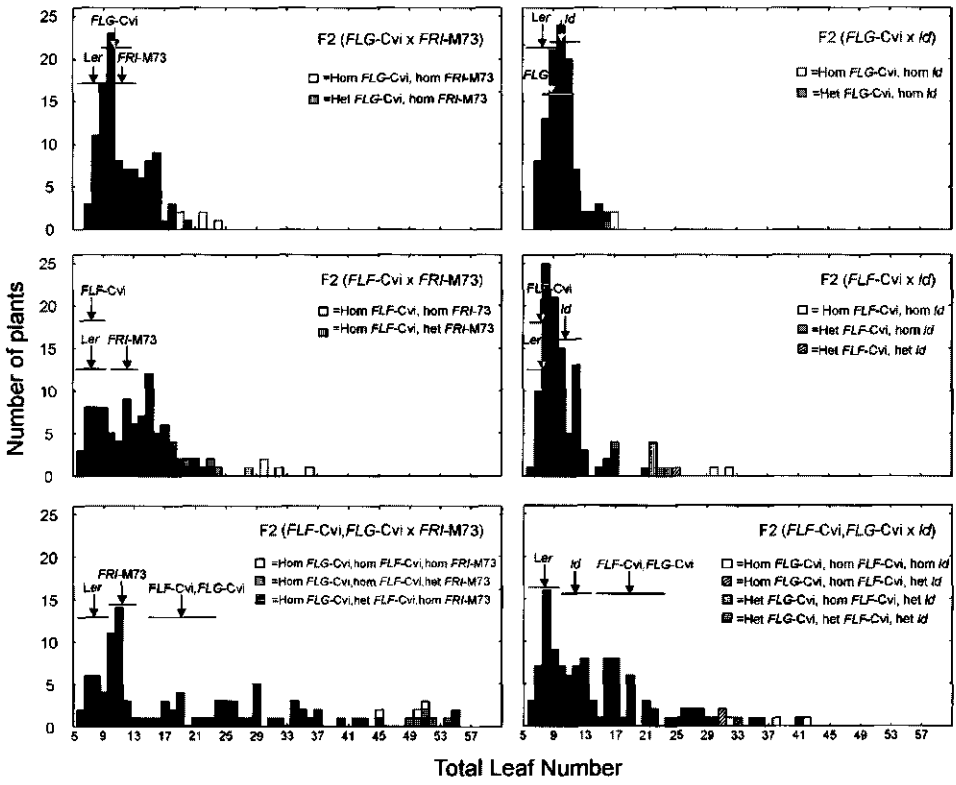

Figure 2-8. Frequency distributions of total leaf number in F2 populations derived from crosses between the three RILs selected as *FLG*-Cvi, (upper part) RIL *FLF*-Cvi (middle part) and RIL *FLF*-Cvi, *FLG*-Cvi (lower part) and the three late flowering introgression lines *FLC*-Sf2 (left side), *FRI*-M73 (middle side) and *Id* (right side). Plants were grown under LD light conditions. The genotypes at molecular markers closely linked to *FLF*, *FLG*, *FLC*, *FRI* and *Id* were determined for the latest flowering plants of each F2 (not filled columns). In each graph, the cross involved and the symbols for the genotypes at the corresponding flowering loci are indicated in the right upper corner. Arrows indicate the parental line means and the horizontal bars represent their ranges of variation. Hom, homozygote; het, heterozygote.

the RIL population but it was present in the L*er* genetic background NILs. Alleles with major effect at the loci *FRI* and *FLC* have previously appeared responsible for most flowering differences between several very late, vernalization responsive ecotypes and the early ones (classified according to their flowering behaviour under long-day light conditions; Napp-Zinn 1969; Burn et al., 1993; Koornneef et al., 1994; Lee et al., 1994b;

Clarke et al., 1995; Kuittinen et al., 1997; Sanda et al. 1997). It was shown before that large allelic effects can also be present in crosses between some early ecotypes (Van der Veen, 1965; Kowalski et al., 1994; present work). Furthermore, it can be predicted that strong effect alleles will probably segregate in crosses between late ecotypes, since some of them carry large effect late alleles with different genetic behaviour to the allelic variation at *FRI* and *FLC* (Burn et al., 1993). Therefore, major effect mutations seem to contribute frequently to the natural flowering variation observed among Arabidopsis ecotypes, although how many loci are involved is still unknown.

Late alleles at 2 of the major loci identified in the L*er*/Cvi population, *FLF* and *FLG*, interact synergistically. A similar type of interaction has been previously shown to occur between natural late alleles at *FRI* and *FLC* (Koornneef et al., 1994; Lee et al., 1994a) and in addition, *FLF*-Cvi and *FLG*-Cvi interact synergistically with late alleles at *FRI*. Epistasis has also been detected in two previous crosses where it has been analysed (Clarke et al., 1995; Kuittinen et al., 1997) and therefore, epistasis among natural alleles might account for an important proportion of the phenotypic variation, as shown among alleles of mutant loci (Koornneef et al., 1998a), and among induced and natural alleles (Sanda and Amasino, 1996a,b).

The Cvi ecotype shows a slightly reduced response to photoperiod length changes and a more pronounced vernalization response than the L*er* ecotype. The three major loci, *EDI*, *FLF* and *FLG* control most of the response differences to photoperiod and vernalization, as shown by their strong QTL by environment interactions. Early alleles at these loci not only reduced flowering time but also diminished the response to photoperiod length. In fact, as shown with the near isogenic line *EDI*-Cvi in L*er* genetic background, the combination of *EDI*-Cvi alleles with *FLF*-Ler, *FLG*-Ler is able to render Arabidopsis practically day-length neutral in its flowering behaviour. On the other hand, *FLF*, *FLG* accounted for much of the vernalization response, the late-flowering effect of Cvi alleles being eliminated by a 3 weeks vernalization treatment. In agreement with these results, the Cvi ecotype flowered almost at similar times under LD and SD conditions when vernalized, i.e., Cvi eventually behaved as almost daylength neutral when the effect of *FLF*-Cvi, *FLG*-Cvi was physiologically removed by the vernalization treatment. In other Arabidopsis populations where QTL x E interactions have been analysed, also the largest effect QTLs showed significant interaction (Clarke et al., 1995; Jansen et al., 1995). In addition, allelic variation at *FLC* and *FRI* is differentially expressed depending on the

vernalization treatment (Koornneef et al., 1994; Lee et al., 1994a; Lee and Amasino, 1995). Therefore major effect loci controlling the flowering differences among Arabidopsis populations seem to interact with the environment, which might be an important factor for maintaining natural genetic variation (Mitchell-Olds, 1995).

Many of the Arabidopsis flowering loci have been characterized genetically and physiologically in relation to the vernalization and photoperiod responses and a model for the control of the transition from the vegetative to the reproductive phase is being developed (reviewed in Martinez-Zapater et al., 1994; Coupland, 1995; Amasino, 1996; Koornneef et al., 1998b). Three major flowering promotion pathways with partly additive and partly redundant functions have been defined, namely, the autonomous (also called constitutive or endogenous), the photoperiod (or long-day) and the vernalization pathways. The vernalization flowering promotion is thought to act on certain targets common to the autonomous pathway, and it has been suggested they might involve gibberellin metabolism or sensitivity. Mutants of loci involved in the autonomous flowering promotion pathway (*fca, fld, fpa, fve, fy* and *ld*) are more responsive to day-length and vernalization than the L*er* wild type, whereas mutations in the photoperiod pathway (*co, fd, fe, fha, ft, fwa* and *gi*) are less responsive to day-length changes. The analysis of *EDI* suggests it might be involved in the photoperiod flowering promotion pathway given the lack of photoperiod response observed in the *EDI*-Cvi NIL in L*er* genetic background. The dominance associated with the flowering behaviour of *EDI*-Cvi allele indicates that its product might promote flowering (or repress the vegetative phase) and this function the would be reduced in the *EDI*-L*er* allele. The *EDI*-Cvi line flowers earlier than L*er* under both LD and SD conditions and somehow resembles the phenotype of transgenic lines carrying the *CO* gene under control of a 35S-promoter (Simon et al., 1996), suggesting that the photoperiod promotion pathway is over-functioning under both day-length conditions leading to the earliness and day-length insensitivity observed. In other words, EDI function could be controlled by photoperiod length when encoded by the L*er* allele but might be expressed independently of daylength when encoded by the Cvi allele.

Late alleles at the *FLF* and *FLG* loci are very responsive to vernalization and confer a more pronounced response to photoperiod length, as seen from the behaviour of the *EDI*-L*er*, *FLF*-Cvi, *FLG*-Cvi RILs, features also shared with the late alleles at *FRI* and *FLC* (Lee and Amasino, 1995). The similar physiological behaviour of the *FLF*-Cvi and *FLG*-Cvi alleles and the late mutant alleles of the autonomous flowering promotion

pathway suggest that they act in the same pathway. Given the co-dominance of these Cvi late alleles it is not possible to speculate whether they might promote or repress the flowering process. However, recessive early alleles at the *FLC* locus have been obtained by mutagenesis (Sanda and Amasino, 1996a) and candidate mutant alleles at the positions of *FRI* and *FLG* are not known, which might indicate that their gene products play a role inhibiting the flowering process. The similar physiological and genetical behaviour of late alleles at the *FLF* and *FLC* loci, together with their matching map positions, suggests they are probably the same locus. In addition, the similar genetic and physiological characteristics of *FLF*-Cvi and *FLG*-Cvi and the late alleles at *FRI*, and the fact that they are partly interchangeable in their genetic interactions, suggest they have certain redundant functions repressing flowering within the autonomous promotion pathway. As proposed by Lee et al. (1994a) and Sanda and Amasino (1996a), the effect of *FLC/FLF* would be counteracted by the autonomous pathway mutant genes, such as LD, given their epistatic interaction. Since *FLG*-Cvi does not interact with *ld*, LD might act directly on *FRI, FLC/FLF* but probably not on *FLG*.

Considering together the behaviour of the 3 loci *EDI*, *FLF* and *FLG*, it is worth noting that RILs *EDI*-Cvi, *FLF*-Cvi, *FLG*-Cvi respond to photoperiod length, in contrast to the *EDI*-Cvi NILs. Under the discussed model, in such genotypes the photoperiod pathway would be promoting flowering at the same level in both daylengths. This photoperiod response would therefore imply that under SDs there is also an inhibition (or lack of promotion) of the autonomous flowering pathway, which would operate through *FLF*, *FLG*. In agreement with this, similar genetic behaviour has been observed in double mutants between non-responsive and responsive loci, which show mostly an intermediate, additive, day-length response (Koornneef et al., 1998a).

The allelic variation at the *FLH* locus has a rather mild effect on flowering, Cvi alleles responding like L*er* to daylength changes. The additive behaviour of *EDI* and *FLH* together with the more pronounced response of *FLH*-Cvi alleles to a vernalization treatment, suggest that *FLH* might be involved in the autonomous flowering promotion pathway. However, opposite to *FLF,FLG* and to other vernalization responsive loci, at *FLH* it is the early allele which increases the response, i.e. *FLH*-Cvi early alleles make L*er* more vernalization responsive. This might suggest its role in the control of the vernalization response.

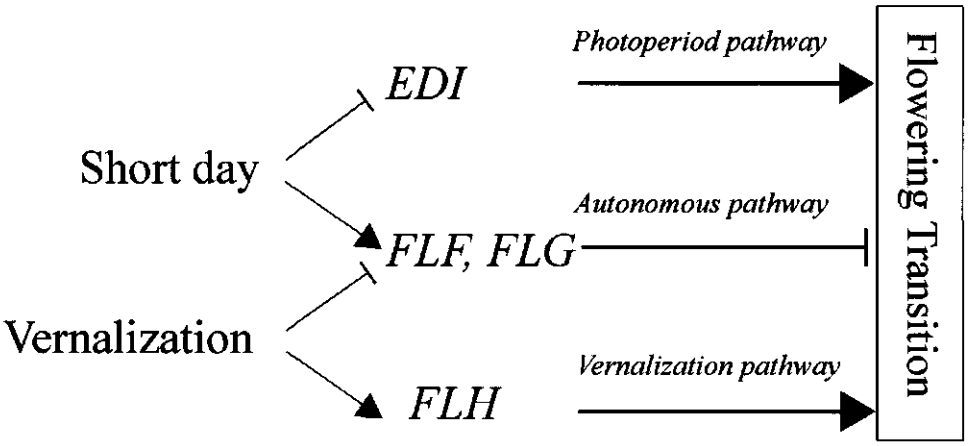

Figure 2-9. A model for the control of flowering by *EDI, FLF, FLG* and *FLH*. The pathway affected by them and the effect of environmental conditions on these genes and pathways is indicated. →, promotive effect; ⊥, inhibitory effect.

Figure 2-9 shows a scheme of the current general model for the control of flowering initiation (Koornneef et al., 1998b) where the possible role of *EDI, FLF, FLG* and *FLH* is indicated.

We have shown that the L*er*/Cvi allelic variation probably concerns loci involved in different flowering pathways. The comparison of map positions between the identified QTLs and the mutant loci might suggest putative candidate genes at which this natural variation occurs. Nevertheless, cautions must be taken given the inaccuracy of the QTL mapping and the large number of known mutant loci affecting flowering behaviour which appeared scattered over the 5 linkage groups (Koornneef et al. 1998b). Similar considerations must be taken when comparing QTL positions in different populations, and fine-scale mapping and allelism tests are required to determine the locus (or tightly linked loci) involved in the corresponding allelic variation. Two mutant loci, *LHY* and *FHA* (Simon and Coupland, 1996; Koornneef et al. 1991), assigned to the photoperiod flowering promotion pathway, have been mapped on chromosome 1 in the *EDI* region, although preliminary fine mapping has left *LHY* out. Furthermore, a flowering QTL has been

mapped on this genomic region in the cross between Landsberg *erecta* and Columbia ecotypes (Jansen et al., 1995). The *FLF* locus maps in the same region as *FLC*, and the similar physiological and genetical behaviour of late alleles at both loci suggests they are probably the same locus. Several other loci have been identified by mutagenesis close to *FLC*, such as *FY* and *CO* (Koornneef et al., 1994). Allelic natural variation has been also assigned to this region in all crosses previously analyzed (Kowalski et al., 1994; Clarke and Dean, 1994; Jansen et al., 1995; Kuittinen et al., 1997). It is unknown whether this ecotype variation belongs only to the *FLC* locus, which would indicate the existence of multiple alleles with different flowering effects, or to several closely linked loci. One natural variant, ART-Sy0, has been mapped in the region of *FLG* (Grbic and Bleecker, 1996; Grbic and Gray, 1997). ART-Sy0 gives rise to aerial rosettes when combined with late alleles at another locus on chromosome 4, probably *FRI*. In addition, it seemed to produce lateness in the absence of late alleles at the chromosome 4 locus, but taking into account the genetic linkage to *FLC* it is unclear whether this lateness involved *FLC* and whether late *FLC* alleles are also necessary to produce the aerial rosette phenotype. Nevertheless, aerial rosette phenotypes were not observed in late plants of the crosses *FLG*-Cvi and *FLF*-Cvi, *FLG*-Cvi with *FRI*-M73. No known mutant locus maps at the *FLH* position, although QTLs have been identified in this region in most crosses previously analysed (Kowalski et al., 1994; Clarke et al., 1995; Mitchell-Olds, 1996; Jansen et al., 1995).

It is expected that part of the natural variation will correspond to alleles of mutant flowering genes. Nevertheless, it is evident that the spectrum of natural genetic variation will be different from the spectrum obtained by artificial mutational analyses, partly due to the limitations of the small number of ecotypes used to generate mutants. Some alleles might not be functional in some ecotypes, as is likely to be the case for *FRI* alleles in many early ecotypes (Koornneef et al. 1994), and the epistatic interactions hamper the detection by mutagenesis of flowering loci, such as was previously shown with *LD* or *FLD* which interact with *FLC* (Koornneef et al. 1994; Lee et al., 1994a; Sanda and Amasino, 1996b) and therefore their mutations were detected in Col but not in L*er*. Further analysis of the loci identified in the present work and in other populations is to come and the final identification of individual natural alleles at the molecular level is still needed. This will provide tools not only for the developmental and physiological dissection of the flowering

process, but also for understanding the molecular mechanisms and the ecological and evolutionary significance of this quantitative natural variation.

Acknowledgements

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Chapter 3

A QTL for flowering time in *Arabidopsis* reveals a novel allele of *CRY2*

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Introduction

Variation of flowering time is found in natural populations of many plant species. The underlying genetic variation, mostly of quantitative nature, is presumed to reflect adaptations to different environments contributing to their reproductive success. Analysis of natural variation for flowering time in *Arabidopsis thaliana* has identified several quantitative trait loci (QTL) (Koornneef et al., 1998b), which have yet to be characterized at the molecular level. A major environmental factor that determines flowering time is photoperiod or daylength, the length of the daily light period, which changes across the year differently with geographical latitude (Thomas and Vince-Prue, 1997).

Plants of the model species *Arabidopsis thaliana* flower earlier when grown LD than under short-day (SD) conditions, although substantial variation in response to photoperiod can be found among populations (Karlsson et al., 1993). In particular, the flowering response to the photoperiod is reduced in the *Arabidopsis* accession Cvi compared with the laboratory strains L*er* and Columbia (Col). Among 5 QTL accounting for most of the differences in flowering time between Cvi and L*er*, one locus at the top of chromosome 1 (Alonso-Blanco et al., 1998) is the main one found to be responsible for the reduced daylength sensitivity; this has been named "early, daylength insensitive" (*EDI*).

We identified the *EDI* locus as a QTL partly accounting for the difference in flowering response to photoperiod between two *Arabidopsis* accessions; the laboratory strain Landsberg *erecta* (L*er*), originating in Northern Europe, and Cvi, collected in the tropical Cape Verde Islands (Alonso-Blanco et al., 1998b). Positional cloning of the *EDI* QTL showed it to be a novel allele of *CRY2*, encoding the blue-light photoreceptor cryptochrome-2 that has previously been shown to promote flowering in long-day (LD) photoperiods (Guo et al., 1998). We show that the unique *EDI* flowering phenotype results from a single amino acid substitution that reduces the light-induced down regulation of the CRY2 in plants grown under short photoperiods, leading to early flowering.

Materials and Methods

Plant material and growth conditions

The following previously described *Arabidopsis* genotypes were used: L*er* and Cvi accessions, and NIL45, which carries about 25 cM of Cvi from the top of chromosome 1 introgressed in a L*er* genetic background (described as *EDI*-Cvi in Alonso-Blanco et al. (1998b). and as NIL45 in Swarup et al. (1999); *EDI*-NIL was derived from NIL45 and carried about 7 cM of Cvi on top of chromosome 1; the *fha-1* mutant is in a L*er* background (Koornneef et al., 1991). Growth conditions under LD and SD photoperiod were as previously described in Koornneef et al., (1995) and flowering time was measured as the number of leaves (Koornneef et al., 1991). Blue light treatments for protein expression analyses were carried as described in Van Tuinen et al., (1995), and hypocotyl elongation were carried out as previously documented in Peters et al., (1992). Transgenic plants were generated after transformation of L*er* plants using the vacuum transformation procedure (Bechtold et al., 1993) and the *Agrobacterium tumefaciens* strain AGLO (Bechtold et al., 1993). The presence of a single locus of the transgene was shown by the monogenic segregation of kanamycin resistance.

Mapping, cloning and sequence analyses of the EDI/CRY2 locus

A mapping population was generated from a cross between the *cer1-1* mutant (in an L*er* background) and NIL45. Among the 1,822 F2 plants 1,401 exhibited early flowering under the SD photoperiod, indicating monogenic inheritance and dominance of the Cvi allele.

For mapping, 6 molecular markers were used between the *cer1* and *PHYA* loci (three SSLPs: F21B7, F20D22, T1G11, and 3 CAPS markers in the *PVV4, AXR1* and *PHYA* genes, which were tested for L*er*/Cvi polymorphisms) using 575 F2 plants from the genotypes *EDI-cer1*, *edi-CER1* and *edi-cer1*. Only 4 recombinants were found between *EDI* and both sides of BAC F19P19 (Genbank accession number AC000104); thus seven new CAPS markers were generated from predicted ORFs in this BAC, which included the *CRY2*. No recombinants between *EDI* and the *CRY2* marker were found.

A 4.6-Kb genomic DNA fragment containing the *CRY2* was PCR-amplified from L*er* and Cvi using *Pfu* DNA polymerase (Promega) with *CRY2*-F1 (5'-

AGGGTACCAGTGTCTGATGTTAAGAACG-3') and the *CRY2*-R1 (5'-TGGTCGACCTCAAACTAAACAACTCAGAT-3') primers. PCR fragments were subcloned in pBluescript (Stratagene) and two independent colonies were sequenced from both strands. These 4.6-Kb fragments were cloned into the *KpnI* and *SalI* restriction sites of the binary vector pCAMBIA 2300 (CAMBIA, Canberra, Australia), and used for plant transformation.

For the construction of the reciprocal chimeras, containing a single nucleotide substitution, a 860-bp DNA fragment between amino-acid residues 207 and 445 was isolated from both L*er* and the Cvi *CRY2* with the restriction enzymes *Hpa*I and *Bam*HI and reciprocally subcloned into the Cvi and L*er* *CRY2*. Chimeric constructs were further used for plant transformation.

The presence of the *CRY2* transgene was confirmed after PCR amplification using the primers *CRY2*-F2 (5'-CTGGAGACAAGGCAGGACCGGTTA-3') and pCAMBIA pC-R, (5'TAACGCCAGGGTTTTCCCAGTCAC-3'). Confirmation of the transgene sequence and the single nucleotide change between L*er* and Cvi was carried out using the *CRY2*-F3 (5'-CGTTGTTGAGTCATCTTCG-3') and pC-R primers for PCR amplification and sequencing.

A dCAPS specific marker for the mutation causing the V367M transition in *CRY2*-Cvi was developed based on the method previously described (Neff et al.,1998). The primers EDI-F (5'-CGGGGAAATAAGCGTCAGACACGT-3') and EDI-R (5'-CATTTCCATGGAAGGAGAAGAAACTTCC-3') were used to amplify a 369 bp PCR fragment, which was cleaved by the *BfaI* restriction enzyme in Cvi but not in L*er*.

CRY2 mRNA and protein expression analysis

Total RNA was isolated from seedlings of the genotypes: L*er*, *EDI*-NIL and *fha-1* using the RNAeasy plant kit (Quiagen) according to manufacturer's instructions. Northern-blot analyses were carried out according to Amersham-Pharmacia protocol using 25 μg total RNA. *CRY2* mRNA was detected using the full-length *CRY2* cDNA as a probe (provided by J.A. Jarillo -INIA, Madrid, Spain). The constitutively expressed cyclophylin gene *ROC5* (Chou et al., 1997) was used as a control.

Antibodies against CRY2 were generated in rabbits against the carboxyl-terminal region of CRY2. The 519-bp 3' end of *CRY2* cDNA was subcloned upstream of glotathion S-transferase (GST) in the PGEX-4T-1 vector (Amersham-Pharmacia).

The fused protein (CRY2-GST) was purified as described by (Guan and Dixon, 1991), and used for raising antibodies in rabbits.

For western-blot analyses, proteins from seedlings of the genotypes L*er*, *EDI*-NIL, *fha-1*, and of transgenic *Ler* lines *CRY2*-Ler-367M and *CRY2*-Cvi-367V were isolated and 45 μg per sample being separated on SDS-PAGE gel. Western-blot analyses were carried out according to Amersham-Pharmacia protocol, the detection of CRY2 antibodies being carried with ECL-plus chemiluminescence (Amersham-Pharmacia). The RUBISCO large subunit was detected with Ponceau-S (Sigma) staining and was used as a loading control. Quantification of blots was performed using IQMac v1.2 software (Molecular Dynamics, Inc.)

The amount of CRY2 protein was normalized to a control protein, which was nonspecifically recognized by the anti-CRY2 antibody (star symbol in fig. 3-4*c*). The amount of this unknown protein did not change during treatments and was correlated to the amount of the RUBISCO large subunit.

Whole mount immuno-histochemistry

Anti-CRY2 was purified using CRY2-GST protein (Sessa et al.,1995). The whole-mount immunohistochemistry protocol was modified from the whole mount in situ hybridization technique (Raz et al., 1999). The specificity of CRY2 immuno-localization was determined using, as controls, a pre-immune serum, four different antibodies, and the *fha-1* mutant. Briefly, Seedlings were fixed for 30 min in paraformaldehyde fixation buffer, dehydrated and the wax layer was removed during incubation with xylene. Following rehydration, driselaze treatment (Sigma) was carried out in a vacuum to allow antibody penetration. The detection of anti-CRY2 antibodies was carried out with donkey anti-rabbit alkaline phosphatase antibody (Promega), which was pre-absorbed onto plant material. The detection of the alkaline phosphatase was carried with NBT and BCIP substrates (Roche), which gave a purple color to the tissue.

GenBank accession numbers

Cvi-*CRY2* mRNA, AY057440. CRY proteins: *A. thaliana* CRY1 (HY4), S66907; *A. thaliana* CRY2, U43397; *Sinapis*, X72019; tomato CRY1, AF130423; tomato CRY2,

AF130425; rice, AB024337; *Adiantum* CRY1, AB012626; *Adiantum* CRY2, AB012627.

Results and Discussion

To determine the molecular basis of this natural genetic variation, we have isolated the *EDI* locus using a map-based cloning strategy (Fig. 3-1). As the starting point for this process, we generated a near-isogenic line (NIL45) carrying a 25 cM Cvi genomic region on the top of chromosome 1 in a L*er* background (Alonso-Blanco et al., 1998b). This line flowered earlier than L*er* under SD conditions and was practically insensitive to daylength (Alonso-Blanco et al., 1998b). We crossed NIL45 with a *cer1* plant in a L*er* background and screened the derived F2 population for recombination between *EDI* and 6 molecular markers spanning the 10 cM interval between *cer1* and *phyA* (Fig. 3-1*a*, Materials and Methods). This localized *EDI* to BAC F19P19; fine-mapping with 7 additional molecular markers further localized it to a 45-kb region containing 15 open reading frames (ORFs) (Fig. 3-1*b*), including *CRY2*. *CRY2* encodes the blue-light photoreceptor cryptochrome 2, which is known to be involved in the control of flowering time and the perception of daylength (Guo et al., 1998), and we therefore considered *CRY2* to be a candidate gene for *EDI*.

To test this hypothesis, we carried out complementation tests in transgenic plants, and specifically because genetic analysis had shown that the Cvi allele of *EDI* is dominant (see Materials and Methods) we first tested whether a *CRY2* transgene from Cvi could affect flowering of L*er*. We therefore isolated a 4.6 kb genomic fragment containing the complete *CRY2* coding region and 2,260 bp upstream of the translation start site (Fig. 3-1*c*) from L*er* and Cvi, and cloned these fragments into a binary vector for plant transformation of the L*er* background. Transgenic L*er* lines carrying the *CRY2*-Cvi construct showed the same early-flowering, daylength-insensitive phenotype as the near-isogenic line *EDI*-NIL (Fig. 3-2), a L*er* line derived from NIL45, but containing a 7-cM genomic region from Cvi at the top of chromosome 1, including the *CRY2*-Cvi allele. However, plants carrying the *CRY2*-L*er* transgene flowered similarly to the untransformed L*er* controls, and exhibited normal daylength sensitivity (Fig. 3-2). These complementation analyses showed that allelic variation between L*er* and Cvi in

CRY2 accounts for all the variation in flowering behaviour attributable to the *EDI* QTL and we therefore conclude the *EDI* locus is the *CRY2*.

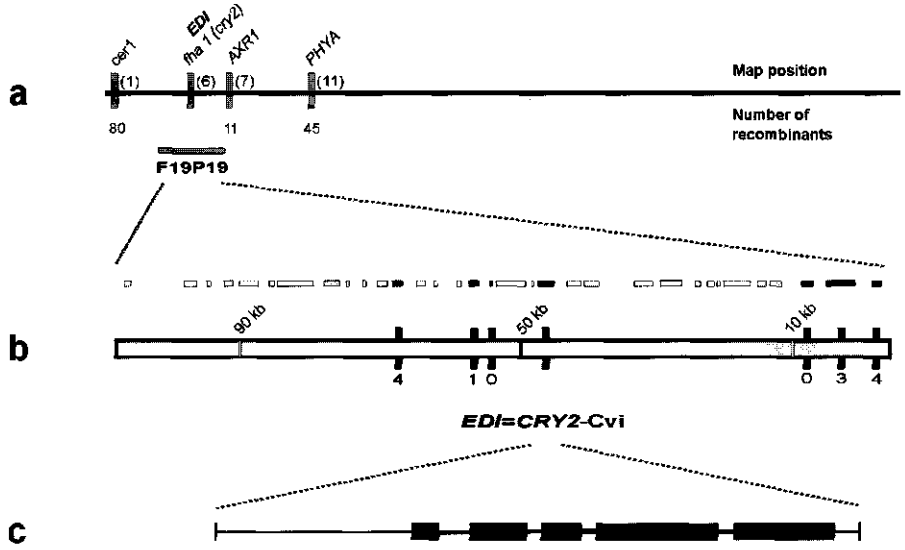

Figure 3-1. Map-based isolation of the Arabidopsis *EDI/CRY2* locus. **a**, Genetic map of chromosome 1 between the *cer1* and *phyA* markers. Map positions are indicated above the map, and the number of recombinants found between the *EDI* locus and the markers is indicated below it **b**, Physical map of the BAC F19P19 containing the *EDI* locus. The Positions of predicted ORFs according to the Arabidopsis Genome Inititative on BAC F19P19 are shown by small boxes. Black boxes represent the ORFs used for the generation of new cleaved amplified polymorphic sequences (CAPS). The number of recombinants found between *EDI* and the markers is shown below the map **c**, Genomic structure of *CRY2*: a schematic representation of the 4.6 kb region of *CRY2* used for complementation assays. The black boxes represent the *CRY2* exons, the thick bars in between represent introns and the thin lines depict the 5' and 3' untranscribed regions.

This was further confirmed by crossing the *CRY2*-Cvi transgene into a *cry2* null allele mutant background (*fha-1*) (Koornneef et al., 1991). As previously reported, *cry2*-null plants were impaired in the promotion of flowering under LD, whereas the *CRY2*-Cvi, *cry2* homozygous plants exhibited an early flowering and daylength insensitive

phenotype (data not shown) indicating that *CRY2*-Cvi complements the *CRY2* loss of function allele.

To determine the molecular basis for the functional difference of the *CRY2* allele from Cvi, we sequenced the 4.6 kb *CRY2* genomic fragment from Cvi and L*er* (Genbank accession numbers are AY057441 and AY057442 for the Cvi and L*er* sequences respectively) and compared it with the published data for the Columbia (Col)

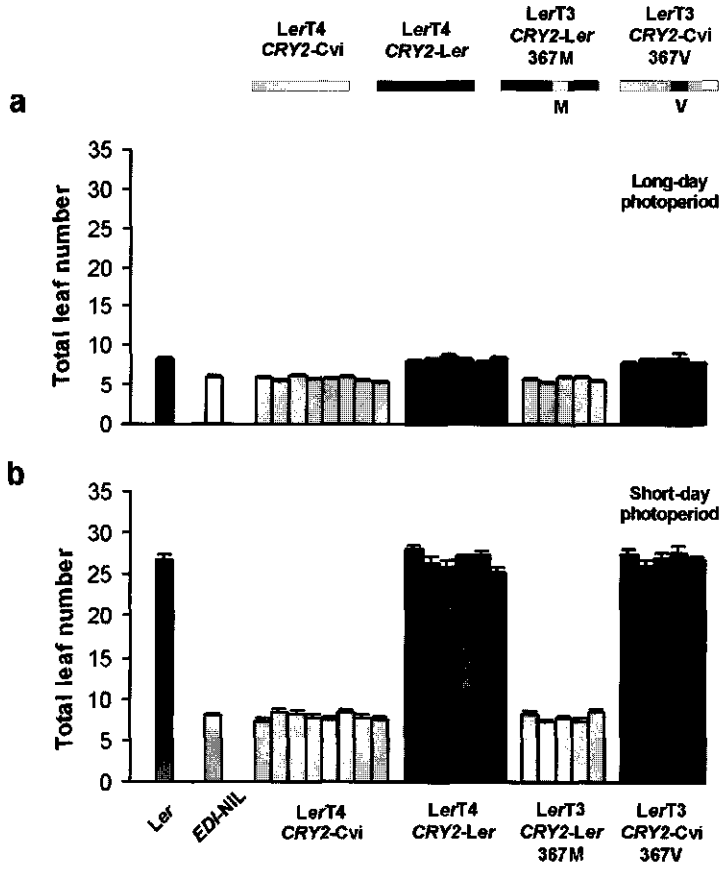

Figure 3-2. Early flowering and daylength insensitivity of *CRY2*-Cvi. **a**, Flowering time (measured as the total number of leaves) of the parental lines L*er* and *EDI*-NIL, as well as the L*er* transgenic lines carrying the *CRY2*-Cvi, *CRY2*-L*er*, *CRY2*-L*er*-367M and *CRY2*-Cvi-367V genomic constructs (graphically depicted in the upper part), grown under LD photoperiod conditions. **b**, The same, with growth under SD photoperiods conditions. Means (± standard error) of 20 plants per line are shown. Between five to eight independent homozygous L*er* transgenic lines per construct are shown. Dark and light shading represent the L*er* and Cvi genotypes, respectively.

accession sequence. We identified a total of 12 single-nucleotide polymorphisms between L*er* and Cvi, 5 of which were located in non-coding regions, of which 4 were in the promoter and one in the 3' untranscribed region. The remaining 7 nucleotide substitutions were located within coding regions and were predicted to give rise to four amino-acid substitutions (Fig. 3-3). For the 2 amino acids at positions 127 and 188, the predicted residue in Cvi differed from that in L*er*, but was the same as in Col. As Col is responsive to photoperiod in a manner similar to L*er* (Jansen et al., 1995), we reasoned that these two differences were not likely to account for the early flowering *EDI* phenotype. The Cvi-specific substitution at position 476 was also considered unlikely to account for the *EDI* phenotype because this residue is poorly conserved across other plant CRY proteins (Fig. 3-3). The other Cvi-specific amino acid difference was the substitution of valine (L*er*) for methionine (Cvi) at position 367.

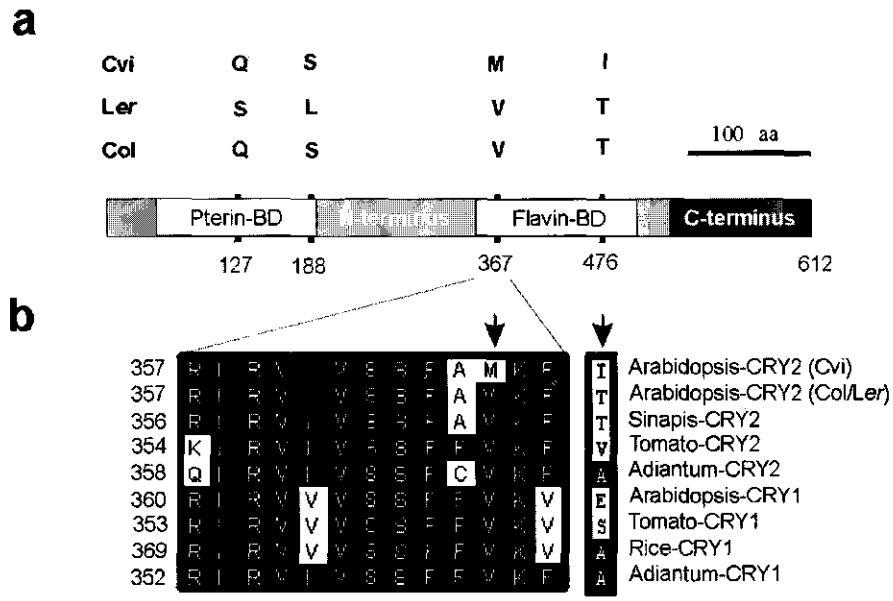

Figure 3-3. Comparison of CRY2 protein sequences. **a**, Schematic representation of the major domains of CRY2 protein according to Cashmore et al. (1999). The amino acids differing between L*er*, Cvi and Col accessions are shown above the bar and their corresponding positions below it. **b**, sequence alignment of the conserved Flavin-BD of CRY proteins from *Arabidopsis thaliana*, *Sinapis*, tomato, rice and *Adiantum* (AB012626 for CRY1 and AB012627 for CRY2) carried out with the CLUSTAL method of MegAlign-DNASTAR software. Identical amino acids are shaded in black, whereas amino-acid substitutions among plant species are shown in white. Arrows indicate the two Cvi-specific predicted amino-acid substitutions, V367 to M and T476 to I.

This valine residue is highly conserved among the CRY proteins from higher (tomato, rice, mustard) and lower plants (ferns) (Fig. 3-3), and we therefore considered this substitution to be the most likely molecular basis for the difference in flowering phenotype.

To investigate the functional significance of this V367M substitution, we exchanged genomic fragments differing only at this position between the L*er* and Cvi *CRY2* alleles and transformed the resulting constructs to L*er*. The resulting transgenic plants thus expressed either L*er* CRY2 with the V367M substitution, or Cvi CRY2 with the reverse M367V substitution. Lines carrying the *CRY2*-L*er*-367M transgene were day-length insensitive, similar to *EDI*-NIL plants, while *CRY2*-Cvi-367V transgenic lines showed a photoperiod response indistinguishable from that of L*er* (Fig. 3-2). These experiments show that a single nucleotide change predicted to give rise to a single V to M substitution at position 367 creates a dominant gain-of-function allele and is sufficient to explain the difference in response to photoperiod between L*er* and Cvi accounted for by the *EDI* locus.

We explored the frequency of this natural *CRY2* V367M substitution causing early flowering and reduced photoperiod sensitivity among other Arabidopsis accessions with a world-wide distribution. We generated a dCAPS marker (Neff et al., 1998) specifically to detect this allelic difference and used it to screen 106 accessions, mostly early-flowering, including 14 collected from sub-tropical regions (below 35°N, from the Canary Islands, India, Japan, Libya and Pakistan), although no accession is as yet available from the low latitude of Cvi. We did not find this allele outside Cvi, showing that it is rare among natural populations and indicating that it might be specific to Cvi. The ecological significance of the *CRY2*-Cvi substitution allele is therefore unclear, and further studies including lower-latitude Arabidopsis accessions and other plant species are needed to confirm its uniqueness.

To understand the molecular mechanisms of the V367M amino-acid (called *EDI*) substitution and how it affects the function of CRY2 and the flowering response to photoperiod, we analysed its effects on *CRY2* expression. It has previously been shown that the *CRY2* transcript level is not strongly regulated, whereas the level of the CRY2 protein is specifically depleted under blue light (Lin *et al.*, 1998; Ahmad et al., 1998). Consistent with these reports, we found that the level of *CRY2* mRNA was similar in light- and dark-grown plants (Fig. 3-4*a*) and that there was also no difference between

L*er* and *EDI*-NIL under either condition (Fig. 3-4*a*). Using anti-CRY2, we also showed that a blue light-induced depletion of the CRY2 occurred normally in both L*er* and the *EDI*-NIL (Fig. 3-4*b*). In addition, we analyzed the dynamics of the CRY2 protein during the dark-to-light transition under different photoperiod lengths and found a photoperiodic regulation. In wild-type L*er* plants grown in SD cycles, CRY2 was depleted shortly after the daily dark-to-light transition (Fig. 3-4*c*). The extent of this SD depletion was substantially reduced, however, in lines carrying the EDI substitution (Fig. 3-4*e*).

We examined this difference in detail by analysing the pattern of CRY2 expression throughout a 24 h SD cycle. In lines containing the L*er* *CRY2* allele (both L*er* itself and L*er* expressing *CRY2*-Cvi containing the M367V substitution), CRY2 was strongly reduced within 2 h after dawn, and remaining at a low level before gradually re-accumulation during the second half of the night (Fig 3-4*e*). In contrast, lines expressing the *CRY2*-Cvi substitution showed a higher level of CRY2 throughout the cycle compared with the level in the L*er* strain. This was evident as a delay in the initial depletion after lights-on, a reduction in the extent of the depletion and a rapid re-accumulation after lights-off. Notably, under LD conditions, a diurnal variation in the level of CRY2 was not observed in plants containing either the L*er* or Cvi *CRY2* allele (Fig. 3-4*f*). In addition, the amount of CRY2 product in darkness was comparable between LD and SD conditions, and thus the light-induced CRY2 down-regulation appears to be specific for SD. This result shows that the early-flowering phenotype under SD conditions conferred by the V to M substitution in *CRY2*-Cvi is associated with a substantial increase in the amount of CRY2 protein, which is particularly evident early in the light period.

We confirmed this difference by whole-mount immunolocalization of CRY2 using a *cry2*-null mutant as a negative control (Fig. 3-4*g*). In L*er* seedlings grown under SD conditions and sampled at the end of the dark period, CRY2 was mostly present in the leaf primordia (Fig. 3-4*g-I*), in agreement with the assumption that leaves are the place of day-length perception and the source of a flowering signal (Bernier et al., 1993). In L*er* seedlings sampled 2 h after lights-on, however, the protein was no longer detectable (Fig. 3-4*g-II*), whereas in *EDI*-NIL seedlings sampled under the same

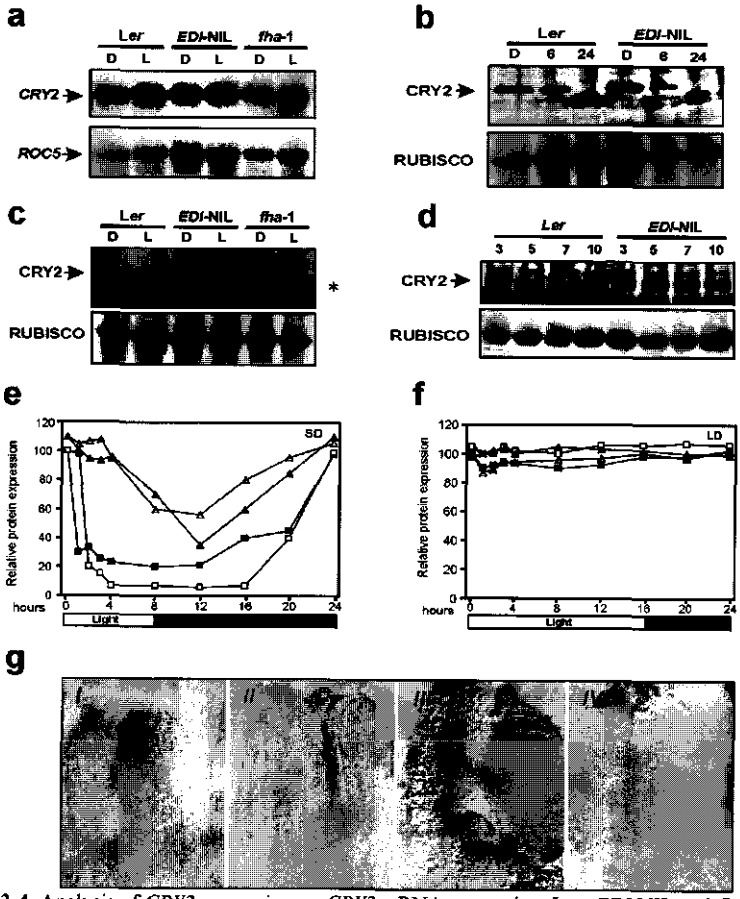

Figure 3-4. Analysis of *CRY2* expression. **a**, *CRY2* mRNA expression. L*er*, *EDI*-NIL and *fha-1* seedlings were grown for 7 days under SD photoperiod conditions and sampled at the end of the dark period (D) or after 2 hours of light (L). Total RNA was extracted and subjected to Northern blot analyses. The upper part shows *CRY2* mRNA, and lower part shows *ROC5* mRNA expression on the same blot. **b**, Blue-light effect on CRY2 protein accumulation. L*er* and *EDI*-NIL seedlings were germinated and grown for 5 days in a dark cabinet, then transferred to continuous blue light (40 µmol m^{-2} s^{-1}). Seedlings were sampled at the end of the dark period (D) and after 6 h or 24 h exposure to blue light. The RUBISCO large subunit was used as a loading control. **c-g**, SD dependent light induced CRY2 depletion. **c**, SD light-induced depletion in 7 days seedlings. L*er*, *EDI*-NIL and *fha-1* seedlings were grown under the same conditions as in (**a**) and subjected to Western-blot analyses using the anti-CRY2. The RUBISCO large subunit stained with Ponceau is shown as a control. **d**, Developmental effect on SD-dependent, light-induced CRY2 depletion. L*er* and *EDI*-NIL were grown under SD photoperiod conditions and 3-, 5-, 7- and 10-day-old seedlings were sampled 2 hours after the light changes and subjected to western-blot analysis. **e** *and* **f**, Time course of CRY2 accumulation during the SD and LD photoperiods. One-week seedlings of the genotypes L*er* wild type (filled squares); *CRY2*-Cvi-367V (open squares); *EDI*-NIL (filled triangles) and *CRY2*-L*er*-367M (open triangles) were grown under SD (C) or LD (D) conditions. Seedlings were sampled at time 0 (before light was turned on) and after 1, 2, 3, 4, 8, 12, 16, 20 and 24 h, and were subjected to western-blot analyses using anti-CRY2. White bars depict the light period, whereas black bars represent the dark period. The value of L*er* at time 0 was taken as 100%. **g**, Spatial localization of CRY2 protein: whole-mount immunolocalization of CRY2 in 4 days L*er* (***I***, ***II***), *EDI*-NIL (***III***) and *fha-1* (***IV***) seedlings grown under SD condition and fixed at the end of the dark period (***I***) or after 2 h of light (***II-IV***). CRY2 detection was carried out using purified anti-CRY2 and substrates for alkaline phosphatase which gave a purple color.

conditions (Fig 3-4*g-III*), CRY2 was present at a similar level and with a similar distribution to that seen in dark-sampled *Ler* seedlings (Fig. 3-4*g-I*).

We also obtained evidence that the light-induced down-regulation of CRY2 is developmentally affected. The extent of SD light-induced CRY2 depletion was small in 3-day-old seedlings but increased significantly in 4-8-day seedlings before gradually diminishing in 10-14-day-old plants (Fig. 3-*4d*). This period of sensitivity for CRY2 depletion corresponds to the developmental phase at which the Col accession of Arabidopsis becomes sensitive to inductive photoperiods or light-quality treatments (Bradley et al., 1997; Mockler et al ., 1999).

In addition to its effects on flowering, CRY2 has also been reported to contribute to the regulation of seedling photomorphogenesis under blue light (Lin et al., 1998). To test whether the EDI substitution also interfered with the photomorphogenic activity of CRY2, we examined the irradiance dependence of hypocotyl elongation in *Ler* and *EDI*-NIL seedlings grown under blue light. *Ler* seedlings showed two phases of response: a weak response at low irradiances (between 0.01 and 0.8 μmol $m^{-2}s^{-1}$) and a stronger response at higher irradiance (0.8-6 μmol $m^{-2}s^{-1}$; Fig. 3-5). *EDI*-NIL seedlings were indistinguishable from *Ler* seedlings in darkness and at the extremes of the irradiance range used, but were significantly shorter under intermediate irradiances (0.08-4 μmol $m^{-2}s^{-1}$; Fig. 3-5). This could represent an extension of the irradiance range for CRY2-Cvi activity towards higher irradiances in *EDI*-NIL. It is clear, however, that this effect is much more subtle than the effect of constitutive *CRY2* over-expression, which conferred a substantial increase in sensitivity for blue-light inhibition of hypocotyl elongation across the entire irradiance range (Lin et al., 1998).

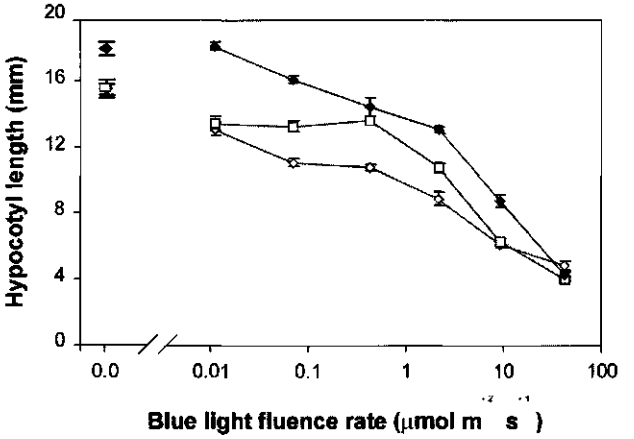

Figure 3-5. Blue-light effect on hypocotyl elongation. Hypocotyl length was determined under different blue-light fluence rates (0.0 (dark); 0.01; 0.07; 0.43; 2.22; 9.48 and 42.5 μmol $m^{-2}s^{-1}$) for seedlings of the genotypes: *Ler* wild type (open squares); EDI-NIL (open diamonds); *fha-1* (filled diamond). Seedlings were germinated and grown for 5 days under the various blue-light fluence rates; means (± standard error) of 15 seedlings per genotype are given.

Our results have shown that there is a light-induced CRY2 down-regulation specific for SD conditions. The post-transcriptional regulation of CRY2 therefore seems to be more complex than just a simple light-dependent depletion involving interactions with additional photoperiod or circadian clock-regulated components. The correlation between the abundance of CRY2 during the light period and the flowering times observed in these genotypes under LD and SD conditions indicates that this photoperiod-dependent, light-induced CRY2 regulation might be part of the mechanism involved in the perception and transduction of the photoperiod-dependent length flowering signal. The transcription of several genes affecting flowering time is now known to be subject to circadian regulation (Mizoguchi et al., 2000), indicating the importance of circadian rhythms in the regulation of flowering time. The *cry2*-null mutants have little effect, however, on circadian period (Devlin and Kay 2000). In agreement with this observation, we found that the activity of the circadian-rhythm-regulated reporter gene *CAB2:LUC* in *EDI*-NIL did not differ significantly from L*er* (data not shown). In addition, the circadian rhythm of leaf movement was not affected in NIL45 (Swarup et al., 1999). These results show that the effect of CRY2-Cvi on flowering time does not result from an interference with clock function. The photoperiod insensitivity of the *cry2* mutant and the CRY2-Cvi alleles is more likely to be caused by alterations in *CRY2*-controlled signaling to floral promoters such as *CONSTANS,* which is modulated by the circadian clock (Súarez-López, et al., 2001).

In the present work, we have identified a novel allele of *CRY2* underlying a major-effect QTL involved in the flowering response to photoperiod of Arabidopsis. This allele is a unique variant not previously isolated in mutational analyses and may help in understanding how plants respond to photoperiodicity. So far, few flowering time genes accounting for the naturally occurring variation have been identified at the molecular level. In the main Arabidopsis *FRI* and *FLC* have been shown to be responsible for much of the flowering natural variation and the response to vernalization (Johanson et al., 2000; Michaels and Amasino 1999). In addition, the *Hd1* and *Hd6*, which are major QTL controlling response to photoperiod in rice were found to be homologues of the Arabidopsis *CONSTANS* and *CK2α* respectively (Yano et al., 2000; Takahashi et al., 2001). Thus, alleles accounting for quantitative natural variation provide an alternative and complementary source of genetic variation for the functional analysis of genomes.

In contrast to previous plant natural genetic variants that were characterized molecularly (Johanson et al., 2000; Michaels and Amasino 1999a; Yano et al., 2000; Takahashi et al., 2001; Wang et al., 1999; Frary et al., 2000; Fridman et al., 2000; Kliebenstein et al., 2001), the *CRY2*-Cvi allele described here provides the first example in which a natural phenotypic variant is demonstrated to be caused by a single amino-acid substitution altering protein function. In addition, single mutation in the red/far-red plant photoreceptor phytochrome A of Arabidopsis is shown to affect hypocotyl elongation (Maloof et al., 2001). Photoreceptors are key regulators controlling various aspects of plant growth and development in response to the environment. These studies show that single-nucleotide mutations in their structural gene sequences might be recruited by nature to engineer their functions while adapting to the environment.

Acknowledgements

We wish to thank Dr. Andrew Miller for providing trangenic seeds expressing the CAB::*LUC* constructs; Dr Jose Jarillo for providing CRY2 cDNA; Dr Wim Soppe for his continuous help and support; Dr. James Weller for his assistance in the blue light experiments and for critical reading of the manuscript; Dr. Hildo H. Offenberg and Dr. Sander van der Krol for their assistance to develop the anti-CRY2 antibodies and the luciferase measurents respectively; to Mariëlle Schreuder and Corrie Hanhart for help with the light cabinets and greenhouse work. This work was supported by a predoctoral fellowship from the government of Egypt to S.E.-D.E.-A.

Chapter 4

The role of cryptochrome 2 in flowering in Arabidopsis

Salah El-Din El-Assal, Carlos Alonso-Blanco, Anton J. M. Peeters, Cornelis Wagemaker, James Weller, and Maarten Koornneef

Introduction

The mechanisms that control the timing of floral initiation have been extensively studied in Arabidopsis by the isolation and characterization of monogenic mutants and by the analysis of 'natural variants' that flower earlier or later than the wild type (reviewed by Martinez-Zapater et al., 1994; Koornneef et al., 1998a; Simpson et al. 1999). Thus, the genetic control of the transition to flowering in Arabidopsis has been shown rather complex, as indicated by the large number of genes known to affect this process. These allelic variants have been classified physiologically on the basis of their responsiveness to environmental factors such as daylength, light quality, and vernalization. Based on this phenotypic analysis and the genetic epistasis among these mutations, flowering-time genes have been grouped into several signal transduction pathways that either suppress or promote floral initiation. These pathways transmit either the developmental or environmental signals that regulate the expression of the floral-meristem-identity genes controlling the formation of the floral meristems (Simpson et al., 1999).

The models for initiation of flowering that have been established include a photoperiod promotion pathway that promotes flowering under long-day (LD) light conditions, an autonomous promotion pathway that promotes flowering independently of the promotive effect of photoperiod and a vernalization promotion pathway that promotes flowering at low temperatures.

The photoperiod promotion pathway (Simpson et al., 1999), also called the LD promotion pathway (Koornneef et al., 1998b), relates photoperiodic timing signals to the floral initiation process. Mutations in genes in this pathway reduce the plant's responsiveness to photoperiods and delay the flowering of Arabidopsis plants grown in LD, but do not alter flowering time of plants grown in short days (SD) (Koornneef et al., 1991). Mutations in genes such as *CONSTANS* (*CO*; Putterill et al., 1995), *GIGANTEA* (*GI*; Fowler et al., 1999), *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999), *FWA* (Soppe et al., 2000), *PHYTOCHROME A* (*PHYA*; Johnson et al., 1994) and *CRYPTOCHROME 2* (*CRY2*; Guo et al., 1998) belong to this class. Cryptochromes (cry1 and cry2) are blue light (BL) photoreceptors and cry2, has been speculated to be the photoreceptor that perceives the LD photoperiod signal in the control of flowering (Guo et al., 1998). This was supported because the cry2 deficient

mutants, which were found to be allelic to the previously isolated photoperiod-insensitive flowering-time mutant *fha1*, flower significantly later than the wild type in LD but not in SD (Koornneef et al., 1991; Guo et al., 1998). In contrast, cry1 has been shown to play a relatively minor role in the regulation of flowering time based on the observation that some *hy4* = *cry1* alleles, with mutations in the *CRY1* gene, are only slightly late flowering in SD and extended-LD (Bagnall et al., 1996; Mozley and Thomas, 1995). In addition to induced mutants, a naturally occurring allele of *CRY2* in an accession from Cape Verdi Islands (Cvi), named as *EARLY DAYLENGTH INSENSITIVE* (*EDI*) has been identified. Plants carrying this variant in a L*er* genetic background flower early in both LD and SD and become daylength insensitive (Alonso-Blanco et al., 1998b). The molecular analysis of this *CRY2*-Cvi allele has shown that is a gain of function allele that in SD conditions leads to a high level of CRY2 protein maintained for longer time after the onset of the light period than in genotypes with the *CRY2*-L*er* allele (El-Assal et al., 2001). This protein stability in SD correlates with early flowering in SD and therefore daylength insensitivity. The role of other photoreceptors such as the red-light (RL) and far red light (FRL) responding phytochromes in daylength perception is less well established and depends on the phytochrome class. Phytochrome A (phyA)-deficient mutants resemble in some aspects mutants of the photoperiod promotion pathway, but phyB deficiency leads to early flowering, indicating that phyB inhibits flowering (Goto et al., 1991). However, because phyB-deficient mutants are still delayed in flowering by SD (Koornneef et al., 1995), they are not exclusively involved in daylength perception.

Mutations affecting the autonomous pathway genes such as *FCA* (Mcknight et al., 1997), *FVE* (Koornneef et al., 1991), *FPA* (Schomburg et al., 2001) and *LD* (Lee et al., 1994a) delay flowering irrespective of photoperiod (Koornneef et al., 1991, 1998b). It is thought that the autonomous pathway promotes flowering by reducing the expression of the *FLC* gene that encodes a repressor of flowering (Michaels and Amasino, 1999a, 2001; Sheldon et al., 1999).

Vernalization, in common with the autonomous pathway, leads to repression of *FLC* expression (Michaels and Amasino, 1999b; Sheldon et al., 1999). The *FRI* gene confers a vernalization requirement in many late naturally-occurring late flowering accessions by elevating the level of *FLC* expression (Michaels and

Amasino, 1999a; Sheldon et al., 1999; Johanson et al., 2000). Ultimately, the photoperiod promotion pathway and the *FLC* mediated pathways seem to converge to the control of the expression of a limited number of target genes, which include *FT* (Kardailsky et al., 1999) and *SOC1* (Samach et al., 2000).

In addition to the genes described above, several other less characterized are associated with flowering, including several whose mutations lead to early flowering such as *EARLY FLOWERING IN SHORT DAYS* (*EFS*, Soppe et al., 1999) and *EARLY BOLTING IN SHORT DAYS* (*EBS*, Gomez-Mena et al., 2001). Mutations in both genes accelerate flowering, mainly under SD photoperiods, by reducing the adult vegetative phase, but they participate in different processes, *EFS* being involved in the autonomous promotion pathway (Soppe et al., 1999) while *EBS* appears regulating *FT* (Gomez-Mena et al. 2001). Furthermore, gibberellin biosynthesis (e.g. *GA1*) and sensitivity (e.g. *GAI*) genes are required to flower under SD (Wilson et al., 1992) and are speculated to participate in an independent-pathway regulating flowering and finally the floral meristem identity genes such as *LEAFY* (*LFY*; Weigel et al., 1992; Blazquez and Weigel, 1999), *APETALA1* (*AP1*; Bowman et al., 1993; Gustafson-Brown et al., 1994; Mandel and Yanofski, 1995) and *TERMINAL FLOWER 1* (*TFL1;* Bradley et al., 1997).

The current models of flowering induction constitute an appropriate framework for the analysis of flowering at the level of mRNA and protein of the genes involved. However, the models are still far from complete and many questions remain, including the interaction of pathways at levels upstream of the common target genes such as *SOC1*. For instance, an indication that the photoperiod pathway may depend on the *FLC* mediated pathways comes from the observation that in the Cvi accession, despite the *CRY2*-Cvi allele, daylength sensitivity is present and is only slightly reduced when compared to L*er*. It was shown that this is genetically due to the presence of Cvi alleles at two other loci identified on chromosome 5, called *FLF* and *FLG* of which *FLF* might be identical with *FLC* (Alonso-Blanco et al., 1998b). These results suggest that the presence of an *FLC*-mediated pathway restores some of the photoperiod responses in lines with the *CRY2*-Cvi allele. Thus far it has been suggested that *CRY2* either controls the expression of *CO* (Guo et al. 1998) or affects its mode of action (Súarez-López et al., 2000) and no effect on the *FLC*-mediated pathways has been proposed.

In this work, we aim to further investigate the role of cry2 in flowering by analyzing the genetic interactions between *CRY2* alleles and mutants of genes involved in the various flowering pathways. For that, the three different *CRY2* alleles currently available were used; the wild-type (WT) L*er* allele, the *fha1-1* mutant, which is an artificially induced null mutant of the *CRY2* gene (Koornneef et al., 1991; Guo et al., 1998), and the *CRY2*-Cvi=*EDI* allele, which is the naturally occurring variant of *CRY2* present in the accession Cvi (Alonso-Blanco et al., 1998b; El-Assal et al., 2001). To investigate the genetic interactions of *CRY2* and the various proposed pathways of flowering, a set of double and triple mutants involving these *CRY2* alleles and mutations in 19 other loci were combined in L*er* genetic background and analyzed. Thus we have studied the effect of various mutations representing the different pathways of flowering in three *CRY2* genetic backgrounds i.e., L*er*, Cvi, and the null mutant *fha1-1*. All genotypes were tested under extended LD and SD light conditions. In addition, the responses to vernalization and R+BL light treatments were tested in a limited set of these genotypes. Furthermore, we analyzed the transcriptional expression of *CO* and *FLC* genes, known to be controlled by environmental factors and *SOC1*, whose expression is regulated by *CO* and *FLC*. These analyses provide new insights on the function of *CRY2* in the regulation of flowering induction, and especially in the control of flowering by photoperiod.

Materials and methods

Construction of genotypes

The following mutant alleles, all in the Landsberg *erecta* (L*er*) genetic background, were used: *co-3, gi-3, fi-1, fwa-1, fca-1, fve-1, fpa-1* (Koornneef et al., 1991): *ld-1* mutation introgressed in L*er* (Koornneef et al., 1994); the early flowering mutants 35S::*CO* (Simon et al., 1996*), efs* (Soppe et al., 1999) and *ebs* (Gomez-Mena et al., 2001); the gibberellin-deficient mutant *ga1-3*; (Koornneef and van der Veen 1980), the floral meristem-identity mutants *ap1-1*, (Bowman et al., 1993; Mandel and Yanofsky, 1995), *lfy-6* (Weigel et al., 1992) and *tfl1-2* (Weigel et al., 1992; Gustafson-Brown et al., 1994; Bradley et al., 1997). The photoreceptor null mutants *hy4-1* = *cry1-1* (formerly called *hy4-2.23N*, Koornneef et al., 1980; Ahmad and Cashmore 1993), *fha1-1* (Koornneef et al., 1991; Guo et al., 1998), *phyA-201*

(formerly *fre-1*, Nagatani et al., 1993) and *hy3-1* = *phyB-1* (former isolation number Bo64; Koornneef et al., 1980; Reed et al., 1993). The line with the *CRY2*-Cvi allele used is called *EDI* and carries about 7 cM of the top of chromosome 1 from Cvi introgressed into a L*er* genetic background (El-Assal et al., 2001). This line is characterized by the first siliques often showing 3 ovaries, which resembles a weak phenotype of *clavata* mutants, and is probably due to a Cvi allele at a closely linked locus to *CRY2* because transgenic plants containing the *CRY2*-Cvi alleles do not show this phenotype (El-Assal et al., 2001). Two introgression lines containing the *FRI-Sf2* or *FLC-Sf2* alleles from the accession Saint Feliu introgressed in L*er* (Lee et al., 1994a) were used as active *FRI* and *FLC* alleles.

Double mutants between *CRY2*-Cvi or *fha1-1* and the above listed alleles were preliminarily selected from the F2 progenies derived from crosses between the single mutants. The selection of plants carrying the *CRY2*-Cvi alleles was assisted by the described fruit phenotype of the *EDI* line. For the isolation of combinations of *CRY2* alleles with other photoreceptor mutants, the selection was performed by growing F3 lines in various broad wavelength color cabinets (Weller et al., 2001) and selecting the lines that were tall in BL for *cry1*, in RL for *phyB*, or in FRL for *phyA* mutants, and thus homozygous lines for the corresponding photoreceptor mutant alleles were obtained. The allele at the *CRY2* locus of all the genotypes used was finally identified by PCR using 2 dCAPS markers specifically designed for the *CRY2*-Cvi and *fha1-1* alleles (Neff and Chory, 1998). Basically DNA was isolated from a few leaves of the candidate lines following the protocol of Bernatzky and Tanksley (1986), and the *CRY2* gene was amplified by PCR using either the *fha1-1* or the *CRY2*-Cvi primers depending on the genotypes; the amplified DNA was then cleaved with the corresponding restricition enzyme and the DNA fragments were separated in 2% metaphore agarose gel (BMA, Rockland, ME USA). For the *CRY2*-Cvi allele marker the primers used were 5'-CGGGGAAATAAGCGTCAGACACGT-3' and 5'-CATTTCCATGGAAGGAGAAGAAACTTCC-3', and the DNA was cleaved with the restriction endonuclease *Bfa*I. For the *fha1-1* marker the primers used were 5'-GACAGTTTTATCCTGGAAGAGCTTCACCAT-3' and 5'-GCTTTGCACAGAGATCCCACGTTCC-3', and the DNA was digested with the NcoI.

Growth conditions and light treatments

Seeds were sown on filter paper (no. 595, Schleicher and Schuell, Germany) soaked with water in plastic Petri dishes, and stored in a climate room during 3 days for germination (25°C, 16 h light for extended-LD experiments; 25°C, 8 h light for SD experiments). Thereafter, seedlings were planted in soil.

Three kinds of photoperiodic light conditions were used: SD, extended LD (both performed in growth chambers) and standard LD (performed in greenhouses). SD and extended-LD experiments were carried out in similar growth chambers, whereby the light treatment provided by fluorescent tubes was the same for the first 8 h in both treatments. However, LD was extended 8 h with four incandescent lamps alone as a source of low-fluence-rate light at the end of the main photoperiod (Koornneef et al., 1995). Ten plants in two 10 x 10-cm pots were used for each genotype/treatment combination. Individual pots were randomized and grown in extended LD and SD cabinets.

For standard LD conditions plants were grown in an air-conditioned greenhouse supplemented with additional light from the middle of September until the beginning of April, providing a daylength of at least 14 h. Day temperature was 22-25°C and night temperature 16-19°C. Two groups of 12 plants were grown in single pots per genotype in a row. En each experiment, plants were grown in two blocks, the genotypes being randomized within the blocks.

Two sorts of light quality conditions were used: either red light (RL) or red light + blue light (R+BL). These conditions were obtained using similar growth cabinets illuminated with only RL tubes (~3 μmol m^{-2} s^{-1}), or with R and BL tubes mixed in order to provide ~7 μmol m^{-2} s^{-1} of each. In R+BL conditions, 3 different times of blue light (BL) exposure were used, either 2 h BL, 8 BL or 24 BL, in all conditions 24 h RL being kept.

For vernalization treatments, seeds were surface sterilized with 20% bleach (4% hypochloride) in 96% ethanol and rinsed twice with ethanol. After drying they were sown on Murashige and Skoog agar medium containing 2% sucrose and stored in darkness at 4°C for 2 or 5 weeks before planting in the climate chambers described above.

Measurement of flowering

The final number of rosette and cauline leaves in the main inflorescence (not including leaves on axillary inflorescences) was counted on the day that the first petals became visible. Total leaf number (TLN) and the time (number of days) from sowing until flowering (flowering time) are tightly correlated traits (Koornneef et al., 1991).

Analysis of gene expression by real time quantitative RT PCR (QPCR)

RNA for QPCR analysis was isolated with the Rneasy plant mini kit from Qiagen (Chatsworth, CA, USA). Total RNA was resuspended in DnaseI buffer and treated with Rnase free DnaseI (GIBCO BRL). For first strand cDNA synthesis, 5 μg of total RNA was used and cDNA synthesis was primed by using the standard $dT_{12\text{-}18}$ adapter primer (GIBCO BRL) and reverse transcribed with M-MLV (GIBCO BRL). Thereafter the cDNAs were diluted to 200 μl with water, and a 5 μl aliquot was quantitatively analyzed for the expression of each gene by the fluorogenic 5'-nuclease PCR assay (Livak et al., 1995). Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) during 40 cycles. Each PCR assay was performed twice. Specific primers and probes for different flowering-time genes were designed by Primer Express software (Applied Biosystems), to avoid detecting homologous genes. Primer sequences used were as follows:

For the *CRY2* gene: forward, 5'-TTGGCGGTTGATGCCAAT-3';

reverse, 5'-TCCAGCCCTAGTTCTTCAATCG-3';

probe, 5'-CAAATCGCTTCAGCCGCTGCAGT-3'.

For the *CO* gene: forward, 5'-AACGACATAGGTAGTGGAGAGAACAAC-3';

reverse, 5'-GCAGAATCTGCATGGCAATACA-3';

probe, 5'-ACGACCCTGTGACACATGCCGGT-3'.

For the *SOC1* gene: forward, 5'-AAATATGAAGCAGCAAACATGATGA-3';

reverse, 5'-TTTTCTCAAGCTGTTGCTCAATCT-3';

probe, 5'-AAGCTTCTAAACGTAAACTCTTGGGAGAAGGCA-3'.

For the *FLC* gene: forward, 5'-ACGCATCCGTCGCTCTTCT-3';

reverse, 5'-GCATGCTGTTTCCCATATCGA-3';

probe, 5'-TCCGCCTCCGGCAAGCTCTACAG-3'.

The expression of each gene is given as the amount of mRNA PCR product. In samples collected at different times the amount of RNA is presented relative to the highest value of the samples after normalization to PCR product of the constitutively expressed *ACTIN2* gene. In samples collected at a single time point (2 h after light turns on) the amount of a gene for each genotype is presented relative to the value of the highest genotype after normalization to the amount of PCR product of the *ACTIN2* gene. Primers for this internal control were: forward, 5'-GCTGAGAGATTCAGATGCCA-3';
reverse, 5'-GTGGATTCCAGCAGCTTCCAT-3';
probe, 5'-AAGTCTTGTTCCAGCCCTCGTTTGTGC-3'.
Primers and probes labeled with 3'Tamra/5'6-FAM were manifactured by Isogen Bioscience (post@isogen.nl). The analysis was repeated two times on one sample of cDNA, which resulted in similar results of which the average was used.

Results

In order to understand the role of cry2 in flowering and daylength perception we investigated the effect of the three *CRY2* alleles on flowering time in combinations with various mutants affecting flowering. For this several double and triple mutants were constructed and analyzed under different photoperiodic and quality light conditions.

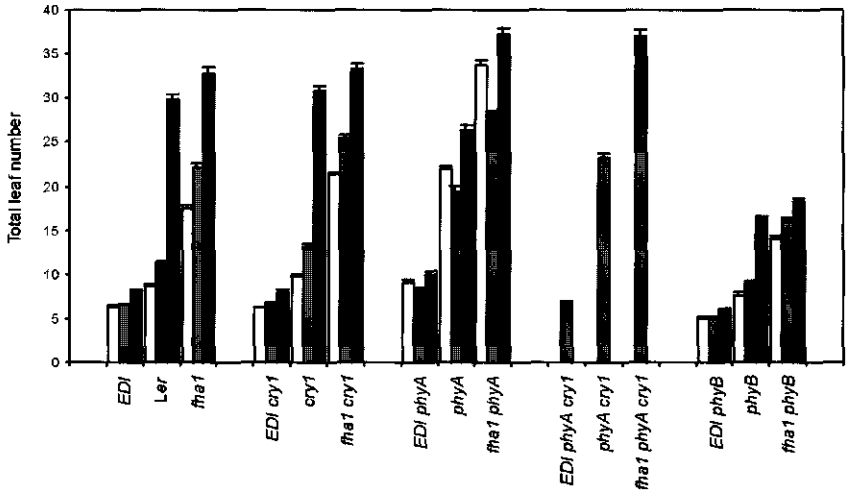

Figure 4-1. The effect of different daylength treatments on the total leaf number (TLN) of the L*er* wild type, different photoreceptor monogenic, double and triple mutants. Plants were grown in three different photoperiod conditions: Extended-LD conditions (white bars), standard-LD conditions (grey bars), and SD (black bars) climate chamber conditions. The standard error of the mean of 24 plants is indicated on each bar.

Interactions between cry2 and other photoreceptor genes

Interaction between cry1 and cry2

As shown in figure 4-1 the *CRY2* loss of function mutant *fha1* flowers later than L*er* wild plants mainly under extended-LD, standard (greenhouse)-LD and SD conditions, while the gain of function allele *CRY2*-Cvi = *EDI* flowers much earlier than L*er* under all three photoperiodic conditions, behaving as almost daylength insensitive. In contrast, *cry1* plants, deficient in the cry1 photoreceptor, were hardly delayed in any photoperiodic condition, in agreement with previous observations (Bagnall et al. 1996). However, the *fha1 cry1* double mutants flowered slightly later than *fha1* in LD conditions, indicating some redundancy in function of cry1 and cry2 in promoting flowering time, whereby cry1 plays a minor role in flowering, which is not observed when the *CRY2*-L*er* (WT) allele is present. The *EDI* monogenic line and the *EDI cry1* double mutant flowered with about the same number of leaves and are very early both in SD and LD (Fig. 4-1), indicating that in this "strong" cry2 background, cry1 has no effect at all.

The various genotypes were also grown under different light qualities, namely, continuous RL or R+BL was added (Fig. 4-2). Under continuous RL, both cry1 and cry2 deficient mutants, *fha1* and *cry1*, flowered late, while the double mutant *fha1 cry1* was only slightly later than the single mutants. In these conditions *EDI cry1* was as early as *EDI*, and both were significantly earlier than *cry1* in RL. These results indicate an effect of *CRY1* and *CRY2* not only in the presence of BL, as previously reported (Mockler et al. 1998), but also in RL. The addition of BL to RL was able to abolish the effect of the absence of cry1, when the *CRY2* allele is functional since the more (BL) was provided, the more the flowering-time of *cry1* was reduced (Fig. 4-2).The *fha1 cry1* double mutant was always later than L*er* and even later than the *fha1* mutant under continuous R+BL conditions. However, the *fha* mutant flowered later than L*er* in these light conditions, indicating that the presence of *CRY1* functional alleles are not able to reciprocally compensate the loss of cry2. Summarizing, cry1 has, in addition to cry2-L*er* (WT), an effect on floral initiation, except when *EDI* is present, or in the presence of *CRY2*-L*er* alleles when sufficient BL is provided. However, the role of cry1 on flowering is minor compared to the role of cry2 since cry1 is unable to compensate the lack of *CRY2* functional alleles in any condition. In addition, the of lack of cry1 cannot be fully compensated by cry2 under RL, indicating a specific requirement of BL for some of the cry2 effects on flowering

Interaction between cry2 and phyA

As shown in Figure 4-1, the Arabidopsis *phyA* mutant flowers at the same time as WT in SD, while it was slightly delayed under LD conditions. Extended LD delays flowering of *phyA* mutants more than the standard LD conditions provided by white light in the greenhouse, which has been also shown by Johnson et al. (1994) and this delay may be due to the reduction of BL in the extended LD.

In a *fha1* background, the *phyA* mutation showed an additive effect in all three photoperiodic conditions used (extended-LD, standard-LD, and SD) (Fig. 4-1). In addition, the observation that the *EDI phyA* double mutant flowered with a relatively

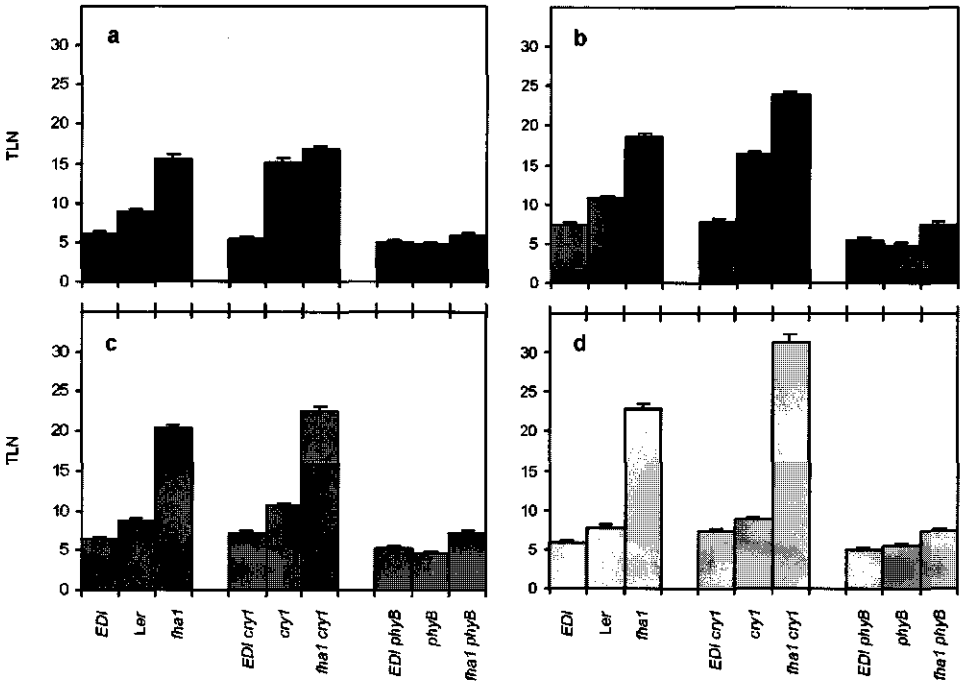

Figure 4-2. The effect of different wavelengths of light on the flowering time as measured by total leaf number (TLN) in Arabidopsis photoreceptor monogenic and double mutants grown in different light conditions: **a**, 24 h red light (RL); **b**, RL for 22 h plus 2 h blue + red light (B+RL) ; **c**, 16 h a day in RL plus 8 h B+RL; **d**, R+BL for 24 h. Means of 18 plants with standard errors of the mean are shown.

small delay compared to the monogenic *EDI* line, suggests that although phyA promotes flowering in LD, this photoreceptor is not required for cry2 function.

We also analyzed triple mutants affected in both cry1, cry2 and phyA. In standard-LD conditions, the triple mutant *fha1 cry1 phyA* flowered later than the monogenic parent lines, and than the *fha1 phyA* and *cry1 phyA* double mutants. In contrast, the *EDI phyA cry1* triple mutants flowered slightly earlier than *EDI phyA*, and as early as *EDI*, which is much earlier than the *phyA cry1* double mutants. These results indicate that the *CRY2* gene does not require functional phyA and that the gain of function of cry2 exhibited by the *CRY2*-Cvi=*EDI* allele may fully compensate the loss of phyA, and cry1 in LD conditions.

Interaction between cry2 and phyB

As shown in Figure 4-1, the *PHYB* gene inhibits floral initiation because phyB-deficient mutants flower earlier than the wild type, which is especially significant in SD conditions (Goto et al., 1991; Bagnall et al., 1995, Weller et al., 1997). The *fha1 phyB* double mutant flowered intermediate between the *phyB* and *fha1* monogenic mutants in LD. However, in SD conditions, the *fha1 phyB* double mutants flowered at about the same time as the *phyB* single mutant, and significantly earlier than wild type. *EDI phyB* double was significantly earlier than both *EDI* and *phyB* monogenic mutants in all 3 photoperiodic conditions, behaving even earlier than the *EDI* monogenic line. These experiments confirm the earliness of the phyB-deficient mutants described by Goto et al. (1991), which implies that the phyB inhibits flowering. In addition, it is also confirmed that the daylength response is not controlled exclusively by the phyB because the phyB-deficient mutant still shows a photoperiod response. Moreover, because the flowering promotion accounted for by phyB is almost absent in the *phyB* background in SD, the *fha1 phyB* double mutants were less sensitive to daylength. However, this epistatic behaviour is not present in LD, where the *fha1* mutation effect is larger, showing that the phyB inhibitory effect is additive to the cry2 promotion, which is also observed in an *EDI* background. Therefore the flowering promotion of cry2 does not depend exclusively on phyB.

The phyB genotypes were also grown under various light quality conditions (Fig. 4-2). In continuous RL the *phyB* mutant flowered earlier than the wild type, while as described above, the *fha1* mutant flowered later. However, in these conditions, the *fha1 phyB* double mutant plants flowered at about the same time as the *phyB* deficient mutant and significantly earlier than the *fha1* mutant, and this epistatic behaviour was also observed when BL was added (Fig. 2). In addition, *EDI phyB* plants flowered at the same time as *phyB* plants. Therefore, the flowering delay observed in the *fha1* mutants requires *phyB* functional = WT alleles, suggesting that in these RL and R+BL conditions, the promotive effect of cry2 counteracts the inhibitory effect of phyB, as previously proposed by Mockler et al. (1999).

Interactions between cry2 and genes of the photoperiod promotion pathway

To study the genetic interactions between cry2 and genes of the so-called photoperiod promotion pathway to which, *CRY2* is ascribed, a number of genotype combinations between the two *CRY2* alleles with opposing effects (*EDI* and *fha1*), and mutants of the *CO, GI, FWA, FT* and *EBS* genes were made and analyzed (Fig. 4-3).

Interestingly, when the *EDI* allele was combined with the *co* and *gi* mutants, these genotypes flowered late and practically with the same number of leaves than the monogenic *co* and *gi* mutants, in both LD and SD conditions. In addition, the *fha1 gi* and *fha co* double mutants were only slightly later than the monogenic *gi* and *co* mutants (Fig. 4-3a). These results showed that the *co* and *gi* mutants are completely epistatic to *CRY2*. Furthermore, the double mutants *EDI* 35S::*CO* and *fha1* 35S::*CO* flowered as early as the monogenic 35S::*CO* line, in agreement with the epistatic relationship of the *co* mutant. This genetic interaction implies that cry2 acts upstream of *CO* and *GI* and therefore cry2 needs the product of *CO* and *GI* genes to promote flowering through the photoperiod promotion pathway.

The double mutants *EDI fwa* and *EDI ft* flowered with a TLN intermediate between *EDI* and the two monogenic *fwa* and *ft* mutants. In addition to that, the double mutants *fha1 fwa* and *fha1 ft* were later than the monogenic parents, showing that *FWA* and *FT* are not the direct target for cry2 or at least not the only target. In addition, the analysis of the *EDI ebs* and *fha1 ebs* double mutants suggested an independent action of *CRY2* and the *EBS* genes (Fig. 4-3a). Regarding the response to photoperiod of these double mutants, all of them showed a response very much reduced compared to L*er*, and similar to the reduced response of the parental monogenic mutants (Fig. 4-3a).

Since cry2 action depends on CO, it was important to analyze if this interaction occurs at the level of *CO* gene transcription, as suggested by Guo et al. 1998, taking into account the circadian rhythm reported for these genes (Suarez-Lopez et al. 2001; Harmer et al. 2000). For this, the abundance of the *CO* and *CRY2* mRNAs was determined using quantitative real time PCR, in 1-week-old plants of the wild type (L*er*) and the *EDI* line grown under extended-LD and SD at different times sampled at 2 h intervals. Moreover, we looked at the expression of *SOC1*, a gene known to be regulated by *CO*. These samples have been previously analyzed for the amount of

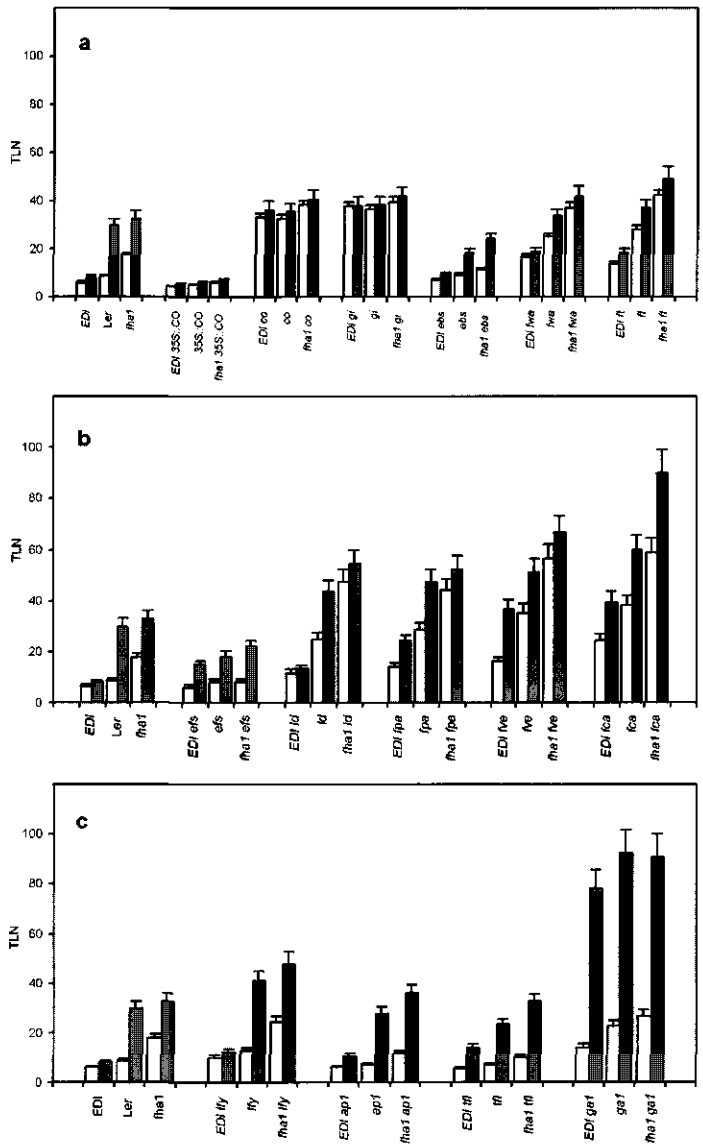

Figure 4-3. The effect of daylength on total leaf number (TLN): Different promotion pathway monogenic mutants and the double mutants with *EDI* and *fha1* were grown in extended-LD (white bars) and SD (grey bars) climate chamber conditions. **a**, photoperiodic promotion pathway and other related monogenic and the double mutants, **b**, autonomous pathway monogenic and the double mutants, **c**, meristem and gibberellin-deficient monogenic and the double mutants. Means of 24 plants per genotype and the standard error are shown.

CRY2 protein and it has been shown that a significant difference in CRY2 abundance exists between both genotypes exclusively in SD (El-Assal et al. 2001). As shown in figure 4-4, we found some differences in *CRY2*, *CO* and *SOC1* gene transcription between the two genotypes, which all showed variation in the expression of the 3 genes tested, similar to previous descriptions (Harmer et al., 2000; Tóth et al., 2001; Suarez-Lopez et al., 2001). The 3 genes showed a peak of expression at the beginning of the light period and the expression declined rapidly to reach the lowest value between 6 - 8 hours after the onset of light. The expression of *CO* increased after 8 h, both in LD and SD compared to the increase of the expression of *CRY2* and *SOC1*. The largest genotype difference between *CRY2*-L*er* (L*er* plants) and *CRY2*-Cvi = *EDI* was found for *CRY2* in SD at the end of the dark period, where the *EDI*-line showed a low amount that increased 2 h later before its level dropped rapidly, while L*er* showed high levels at the end of the dark period which decrease rapidly at the end of the dark period (Fig. 4-4).

Interactions between cry2 and the autonomous promotion pathway genes

The late-flowering genes, *LD, FPA, FVE*, and *FCA* are presumed to promote flowering constitutively, under extended-LD and SD, and are therefore involved in the so-called constitutive or autonomous promotion pathway. To study the genetic interactions between *CRY2* and the autonomous pathway, we constructed and analyzed combinations of *CRY2* alleles with the *ld, fpa, fve* and *fca* mutants (Fig 4-3b). All genotypes with the *CRY2*-Cvi allele showed intermediate flowering times between *EDI* and the corresponding mutant both in the extended-LD and SD indicating an additive effect of the *CRY2* allele and the autonomous pathway genes. Moreover, when the *EDI* and *fha1* alleles were combined with the autonomous pathway's related early flowering mutant, *efs*, also an additive effect with that mutation was also observed (Fig. 4-3b), which supports the previous results about the relationship between cry2 and the autonomous promotion pathway (Koornneef et al., 1998a).

Interestingly, and in contrast to the double mutants with the photoperiod promotion pathway genes, a partial restoration of the daylength response is observed in the genotypes with the *EDI* allele.

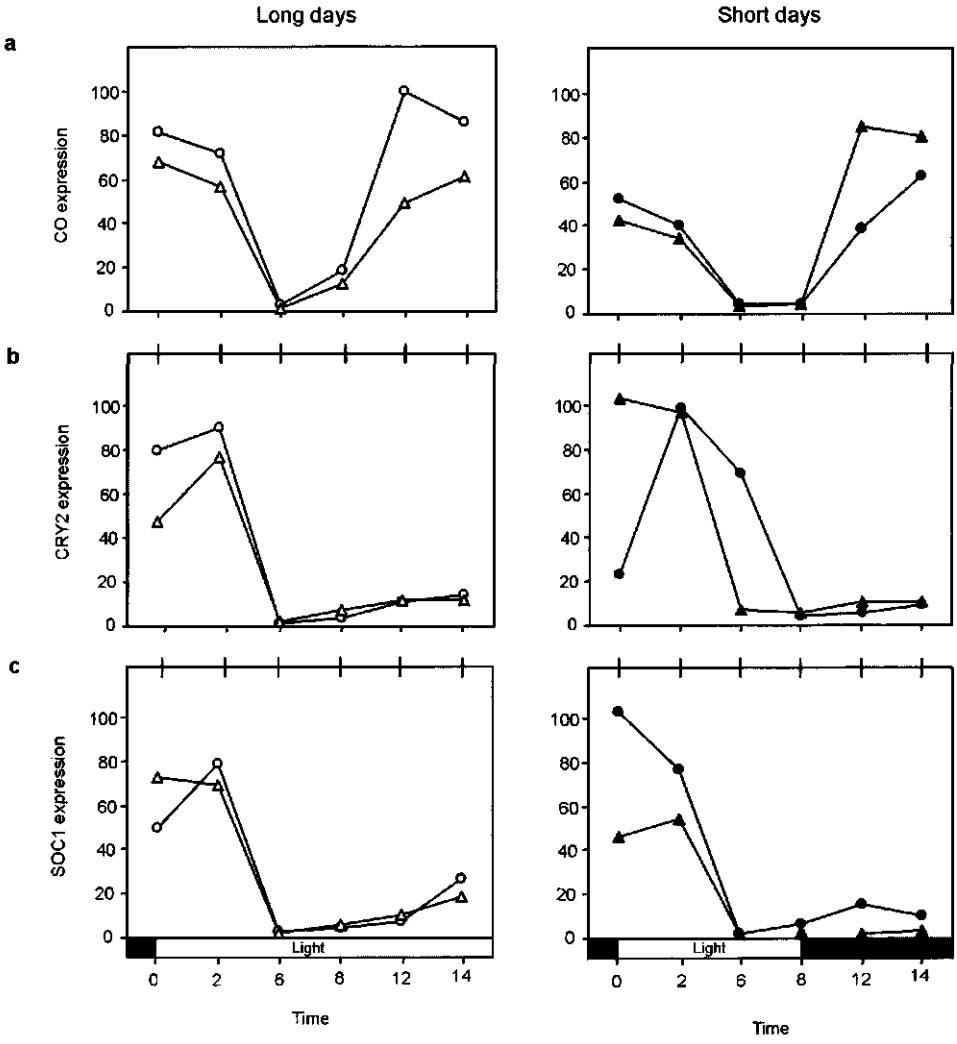

Figure 4-4. Relative gene expression levels of flowering time genes of 7-days-old seedlings growing in different day-length conditions. Seedlings of *EDI* were grown in extended-LD (open circles), or in SD (filled circles) and L*er* seedlings were grown in extended-LD (open triangle), or in SD (filled triangles), and sampled at the time indicated after the start of the light period. **a**, *CO* mRNA, **b**, *CRY2* mRNA, **c**, *SOC1* mRNA. Data presented as a % of the highest mRNA level for every gene.

This is especially the case for *fca* which is the latest mutant of this group (Fig 4-5). The only exception is the *ld* mutant, which in combination with the *EDI* allele flowers very early in SD. This daylength sensitivity is graphically illustrated in Figure 4-5, where different regression lines were fitted for the genotypes involving the photoperiod or the autonomous promotion pathways. The different intercept of both regression lines clearly shows an increased photoperiod response characterizing the genotypes with mutations in the autonomous promotion genes, which is due to a stronger delay of flowering under a SD photoperiod.

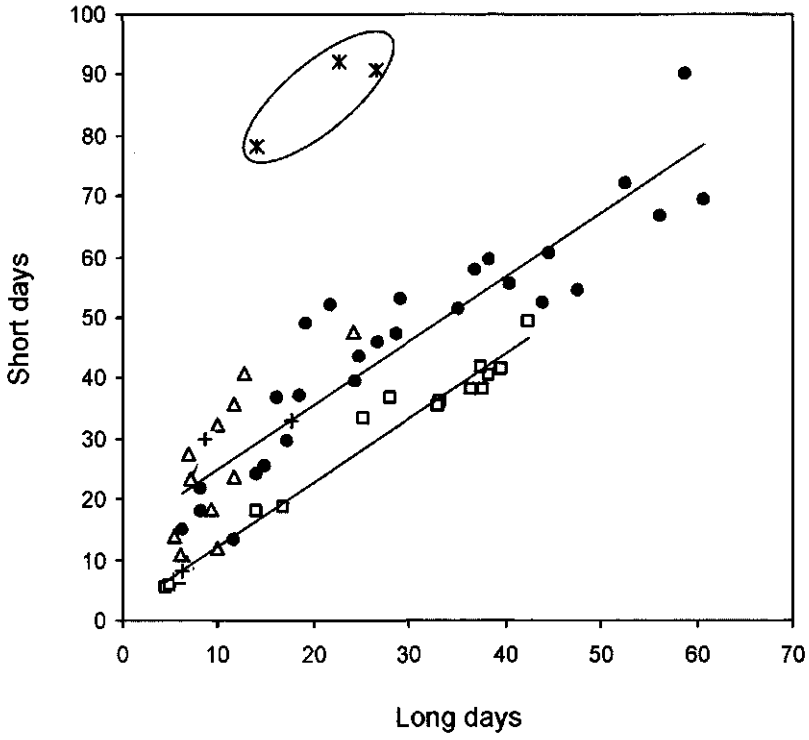

Figure 4-5. The correlation between flowering time in extended-LD and SD of the different flowering pathway monogenic and double mutants with *EDI* and *fha1*: Open squares represent the effect in photoperiodic mutants background; filled circles represent the genotypes in autonomous mutants background; open triangles represent the meristem-identity mutants background; stars represent the effect of the gibberellin-deficient mutant background and the cross symbols represent the WT (L *er*), *EDI* and *fha1*. The oval encircles the double mutants in the gibberellin-deficient mutant background and the regression lines are calculated for respectively the autonomous pathway mutants (filled circles) and photoperiod promotion pathway mutants (open squares).

Interaction between cry2 and the vernalization promotion pathway genes

To study the genetic interactions between *CRY2* and the vernalization pathway we analyzed the different *CRY2* alleles in genetic backgrounds with high *FLC* expression due to the presence of active *FLC-Sf2* and/or *FRI-Sf2* alleles. The various genotypes were grown in extended-LD and SD and without or with a vernalization treatment of 2 or 5 weeks.

As shown in Figure 4-6, the double mutants involving the *CRY2* alleles, *EDI* and *fha*, and *FLC-Sf2* or *FRI-Sf2*, were all intermediate between the monogenic parental lines in LD and SD (Fig. 4-6d) showing that the interaction of *EDI* with *FRI-Sf2* and *FLC-Sf2* is additive. However, as previously described by Lee et al. (1994a) the genotype *FRI-Sf2 FLC-Sf2* flowers much later than the parental monogenic mutations indicating a synergistic interaction between *FRI-Sf2* and *FLC-Sf2*. It is important to note that the *EDI FLC-Sf2 FRI-Sf2* and *fha1 FLC-Sf2 FRI-Sf2* lines flowered at about the same time as the *FLC-Sf2 FRI-Sf2* in both extended-LD and SD conditions. Furthermore, all genotypes were daylength sensitive, except the *EDI* containing lines carrying *FLC*-L*er* and *FRI*-L*er* alleles, or when the delay of flowering caused by active *FRI-Sf2* and *FLC-Sf2* alleles was reduced by a 5 week vernalization treatment (Fig. 4-6d). These results suggest that *CRY2* is not working in the presence of dominant alleles of *FLC-Sf2* and *FRI-Sf2* together, and this prompted us to look at the expression of these genes in these genotypes (Fig. 4-6b, 4-6c and 4-6d).

We analyzed the expression of *CRY2* and *FLC*, and in addition, the downstream gene *SOC1*, whose transcription has been shown to be negatively regulated by *FLC* (Samach et al., 2000; Michaels and Amasino, 2001). The mRNA abundance of these genes was determined by quantitative RT-PCR (see Material and Methods) at a single time point of 2 h after dawn, which we had previously determined to correspond to the highest mRNA levels of these genes in L*er* and *EDI*-NIL (Fig. 4). Given the circadian pattern of expression of these genes (Harmer et al., 2000), any change in the amount of their mRNAs at this single time point may be interpreted as either regulation of transcription strength or changes in the transcription rhythm.

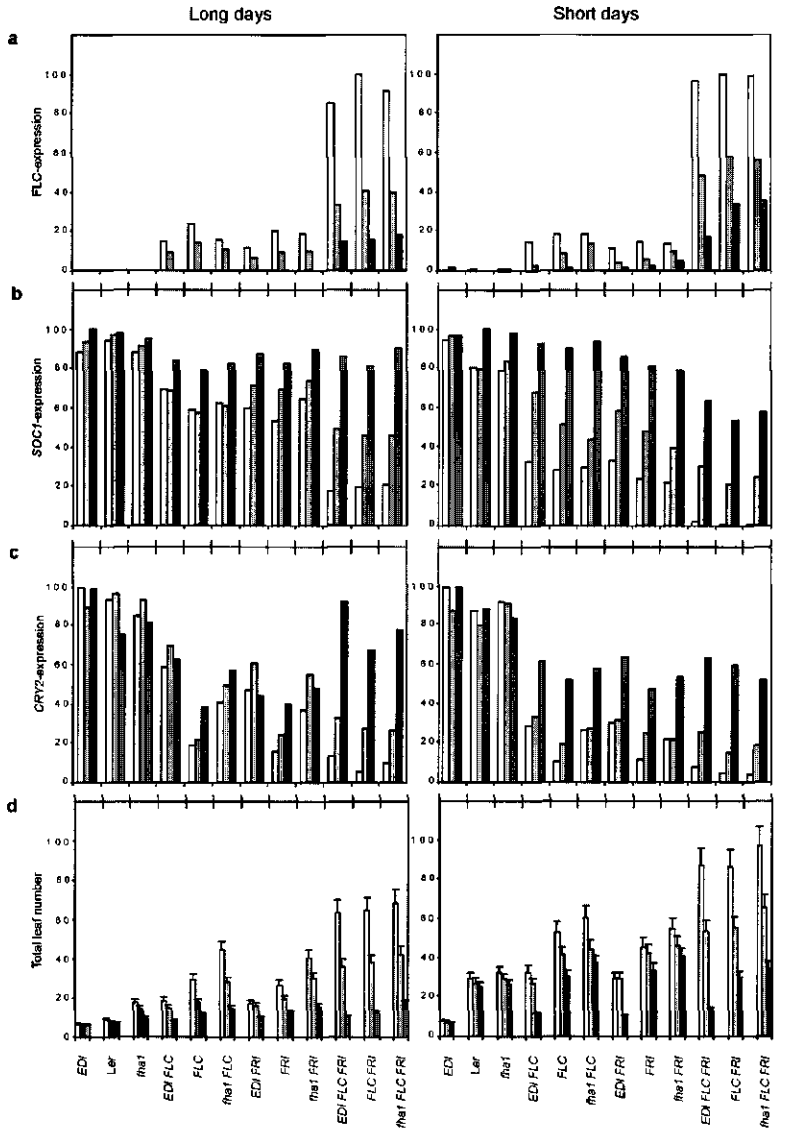

Figure 4-6. The levels of *FLC*, *SOC1*, *CO* mRNA and the total leaf number (TLN) in the vernalization pathway lines with genetic variation at the *CRY2* locus. Genotypes were grown in extended-LD and SD conditions. **a**, *FLC* ; **b**, *SOC1*; **c**, *CRY2*; **d**, Total leaf number (TLN). White bars represent plants without vernalization, gray bars show plants with 2 weeks of vernalization, and black bars represent plants with 5 weeks of vernalization treatment. Means of 18 plants and its standard error bars are shown. The mRNA abundance for every gene have relative to the highest expression value in the experiment for that specific gene.

As previously described (Sheldon et al., 1999, Michaels and Amasino, 2001) the mRNA levels of the *FLC* gene appear high in the presence of active *FLC-Sf2* and/or *FRI-Sf2* alleles, vernalization decreasing these levels strongly (Fig. 4-6a). In these genotypes with high *FLC* expression (all single, double and triple mutants carrying either *FRI-Sf2* and/or *FLC-Sf2*) there was hardly any difference in this amount between LD and SD conditions (Fig. 4-6a). Furthermore no effect of the different *CRY2* alleles on *FLC* expression was observed indicating that *FLC* mRNA levels are not controlled by cry2.

Regarding the expression of *SOC1*, no significant effect of the *CRY2* allele on the levels of its mRNA were found (Fig. 4-6b). The expression of the *SOC1* followed the opposite expression to *FLC,* its amount being reduced by the presence of active *FLC-Sf2* and *FRI-Sf2* alleles and increased by vernalization. However, in contrast to the expression of *FLC*, *SOC1* levels were significantly affected by daylength, being higher in the extended-LD compared with SD. This indicates that in addition to the negative control of its transcription by the *FLC* mediated pathway (Samach et al., 2000; Michaels and Amasino, 2001), there is a control by daylength apparently independent of cry2.

Regarding the expression of *CRY2* mRNA (Fig 4-6c), its amount was almost the same and was not affected by daylength in genotypes where *FLC* mRNA was practically absent, i.e. in *EDI,* L*er* and *fha1*, which was in agreement with previous observations (El-Assal et al. 2001). However, in the presence of active *FLC-Sf2* and *FRI-Sf2* alleles, *CRY2* expression is especially reduced in genotypes containing wild-type *CRY2*-L*er* alleles. When both *FRI-Sf2* and *FLC-Sf2* are present *CRY2* mRNA levels become even lower but increased strongly when vernalization is applied. These results indicate that the transcription of *CRY2* is negatively regulated by the level of *FLC* expression, *FLC* either reducing the level or changing the rhythm of the transcription of *CRY2*. In addition, another regulation by the CRY2 protein itself or by downstream flowering-time genes must occur in order to explain the minor differences in *CRY2* expression depending on the *CRY2* allele in *FLC-Sf2* and *FRI-Sf2* backgrounds. The similar lateness of *EDI FLC-Sf2 FRI-Sf2* and *fha1 FLC-Sf2 FRI-Sf2* in LD and SD (Fig 4-6d), with high *FLC* and low *CRY2* expression independent of the *CRY2* allele indicates that *FLC* regulates the expression of *CRY2* negatively (Fig. 4-6c).

From these gene expression analyses we conclude that there is a negative interaction, between FLC and SOC1 and cry2, which correlates with photoperiod response. This negative regulation of *CRY2* and *SOC1* by *FLC* can be direct between *FLC* and these other genes, or indirect through a downstream gene(s) of *FLC*. We have analyzed the expression of *CO* in the same plant materials to see if *CO* expression is controlled by *FLC* or *CRY2*. Interestingly, we found that the expression of *CO* was completely independent from the expression of *FLC* (data not shown), and as reported before by Suarez-Lopez et al. (2001) *CO* expression at this sample time showed correlation with photoperiod, but not any other relationship with the flowering time or vernalization response.

We confirmed the work of Michaels and Amasino (2000) and Samach et al. (2000), and found that *FLC* gene plays a role as a negative regulator to *SOC1* expression, because a negative association between levels of *FLC* expression and *SOC1* expression was found (Fig. 4-6a, b).

To investigate the effect of the expression of these 3 genes on flowering and on the photoperiod response we plotted TLN in LD and SD conditions against the expression of *FLC*, *SOC1* and *CRY2* (Fig. 4-7a, b and c respectively). There was a positive correlation between *FLC* expression and TLN (Fig. 4-7a), while a negative correlation was found between *SOC1* and *CRY2* expression and TLN (Fig. 4-7b and c). As described above, only plants carrying *CRY2*-Cvi and *FLC*-L*er* *FRI*-L*er* alleles did not respond to photoperiod indicating a double requirement for the control of this response. However, plants showing similar levels of *FLC* mRNA expression in LD than in SD flowered at different times. This absence of a complete quantitative relationship indicates that either FLC affects photoperiod response by a threshold relationship or FLC is a target of the photoperiod flowering control and a post-transcriptional regulation of *FLC* is involved. Similarly, plants flowered later in SD than LD at similar levels of *CRY2* mRNAs, and this difference between SD and LD is most pronounced for the *CRY2*-L*er* background genotypes. However, it has been previously shown that post-transcriptional regulation of cry2 is important, CRY2-L*er* protein being down regulated in SDs (El-Assal et al., 2001). Finally, *SOC1* expression showed similar regression lines for LD than SD data indicating that the mRNA level of this gene correlated the best with photoperiod response, which suggests that is one of the final targets of the various mechanisms regulating the photoperiod response.

Interactions between cry2 and the gibberellin pathway genes

The *EDI* and *fha1* mutants, the gibberellin deficient *ga1-3* mutant and the double mutants *EDI ga1* and *fha1ga1* were grown in the extended-LD and SD in order to study the genetic interactions between *CRY2* and the gibberellin promotion pathway (Fig. 4-3c).

In extended-LD, the *ga1* mutant shows flowering buds (but no bolting) with leaf numbers around 20, indicating that the *ga1* mutation has little effect on flowering in LD. The double mutant *EDI ga1* shows intermediate leaf number between both *EDI* and *ga1*, and the *fha1 ga1* double mutants showed also an intermediate phenotype between the parental monogenic mutants (Fig. 4-3c).

In contrast, in SD conditions, the *ga1* mutant initiated only a few flower buds after six months, when having more than 90 leaves (Fig. 4-3c and Fig. 4-5). Thereafter the *ga1* mutant died and never bolted. The *fha1 ga1* double mutants also died and some plants showed small flower buds on plants with about 90 leaves. However, the double *EDI ga1* flowered significantly earlier than the *ga1* mutant (Fig. 4-3c), suggesting that cry2 controls flowering time independently from the GA promotion pathway. As previously described by Wilson et al. (1992), this lateness of *ga1* in SD suggests a requirement of gibberellins in SD.

Interactions between cry2 and meristem identity genes

In order to investigate the genetic relationships between *CRY2* and the floral meristem identity genes, we analyzed double mutants involving some mutants of these genes, such as *lfy*, *ap1* and *tfl1* (Fig. 4-3c). In general, all combinations of *EDI* with *lfy*, *ap1* and *tfl* flowered earlier than the monogenic mutants, at intermediate times between *EDI* and mutations, in LD and SD (Fig. 4-3c). Also the double mutants between *fha1* and *lfy*, *ap1* and *tfl* were later than the parental monogenic mutants in both LD and SD conditions (Fig. 4-3c) indicating no specific interaction.

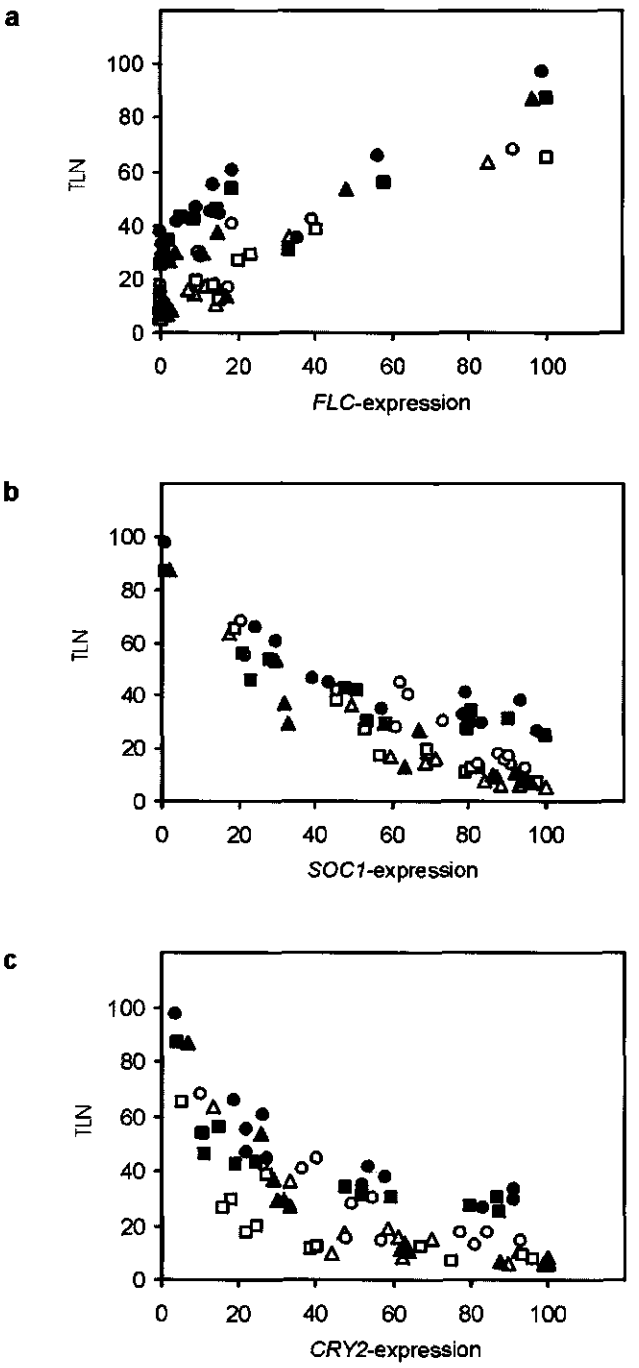

Figure 4-7. The correlation between TLN and the relative mRNA expression level : **a** *FLC* expression and TLN; **b**, *SOC1* and TLN; **c**, *CRY2*. Open symbols represent plants grown in extended-LD conditions and the filled symbols show the plants grown in SD conditions. Triangles, represent the genotypes of mutants in *EDI* background; squares, genotypes in L*er* background; circles, genotypes in *fha1* background.

The responses to photoperiod of these genotypes were in all case similar to the response of the corresponding *CRY2* allele, so very much reduced in the *EDI* background and less reduced in the *fha* genotypes.

Discussion

In this work, we have investigated the genetic interactions with respect to flowering between cry2 and other photoreceptors and the various floral-induction pathways, in relation to light-quality and photoperiod. For this, we combined the 3 available alleles of *CRY2* with different mutants representing the various photoreceptors and flowering pathways.

The flowering analysis of various *CRY2* and *CRY1* genotypes in LD and SD conditions showed that in the presence of the *fha1* allele (absence of cry2), cry1 plays a minor role in promoting flowering (Bagnall et al. 1996; Mockler et al. 1999). In addition, the role of cry1 is not detectable when the *CRY2*-L*er* (wild type) allele is present and the *CRY2*-Cvi allele also completely masks the deficiency of cry1.

The light quality experiments show that in RL, mutations in either cry1 or cry2 delayed flowering, and the presence of only one wild-type cryptochrome, was unable to fully compensate the absence of the other cryptochrome. This result indicates that, in addition to *CRY2*-L*er* (WT), *CRY1*-L*er* has an effect one floral initiation except when the gain of function allele *CRY2*-Cvi is present. The addition of BL to the RL was able to abolish late-flowering due to the absence of cry1, indicating that in BL a cry1 deffect on flowering is compensated by cry2. Further analysis might reveal if this diffferential effects of cry1 and cry2 on flowering depend on fluence rate as shown for hypocotyl elongation (Lin et al., 1998).

As reported by Johnson et al. (1994), our analysis showed that the Arabidopsis *phyA* mutant flowers at the same time as WT in SD, but extended LD conditions failed in promoting flowering. The additive effect of the mutations in the various double and the triple mutants of *CRY2* and *PHYA* in LD conditions, in addition to the observation that *CRY2*-Cvi allele may fully compensate the loss of phyA and cry1 in LD conditions, suggested that although phyA is important in promoting the flowering time in LD conditions, the effect of the cry2 does not depend on phyA.

The earliness of the phyB-deficient mutants described before by Goto et al. (1991), implies that the phyB inhibits flowering but does not control the photoperiod response exclusively, because SD still delays flowering in the *phyB* mutant. However, it cannot be excluded that phyD and phyE are responsible for this residual effect because plants impaired in both *PHYB* and the *PHYD* genes flowered earlier than the *phyB* monogenic mutation in both LD and SD conditions, and the double mutants *phyB phyE* flowered earlier than the *phyB* mutant in SD conditions (Devlin et al., 1999a,b).

The present work shows that the double mutants of *CRY2* and *PHYB* conditions were less sensitive to photoperiod, while the extra delay of the phyB mutant in the *fha1* background and the earlier flowering in *EDI* background in LD indicates that *PHYB* is not fully epistatic to *CRY2* and that the flowering promotion by *CRY2* gene does not depend only on the phyB inhibition. Apparently, this phyB-mediated inhibitor does not act through *CO* as indicated by the observation of Blazquez and Weigel (1999), who found that the mRNA expression of *CO* and *FT*, the 2 genes acting downstream in the photoperiod pathway, was hardly affected in *phyB* plants in SD, suggesting that the early-flowering phenotype of *phyB* mutants under SD is not caused by overexpression of genes in the photoperiod promotion pathway. The FCA-dependent autonomous pathway, known to repress the *FLC* expression, is most likely the target of phyB as is suggested by the epistasis of *fca* to *phyB* (Koornneef et al.,1995). Since this epistasis of *fca* is much stronger in SD, it appears that the flowering inhibitory effect of phyB via the autonomous pathway is much more important in SD probably because no suppression of the phyB inhibition by cry2, which is rapidly degraded in SD (El-Assal et al., 2001), can take place.

The light-quality experiment showed that the *phyB* mutation could suppress the late flowering phenotype of the *fha1* mutant in RL and R+BL. These results agreed with the epistasis of *phyB* over *fha1* in both of RL and R+BL also shown by Mockler et al., 1999. Apparently, the flowering effects of these photoreceptor mutants in the specific light quality treatments tested do not fully mimic their effects in different photoperiods of white light which suggest more complex interactions than the observed in the RL and BL tested. Interestingly, the lateness in RL depends strongly on the genetic background since Col and mutants in this background are much later than L*er* and L*er* background mutants (Mockler et al., 1999 and present

work), which might be due to the active *FLC* gene in the Col accession. However, these authors did not observe an RL effect of cry2 deficiency in either Col or L*er*, the reasons for it being unclear but may depend on the RL sources used. Under these specific light conditions an effect of photosynthetic limitations might occur since this also affects flowering in Arabidopsis (Bagnall, 1992).

The flowering effect of *CRY2* variants in the genetic background of meristem identity gene mutants shows that these genes are not exclusively dependent on a cry2-mediated pathways. Such an independence of pathways was also observed between cry2-dependent pathways and the gibberellin-promotion pathway as indicated by the floral promoting effect of *EDI* on the *ga1-3* mutant, which is extremely late flowering in SD conditions.

The flowering time analysis of combinations of *CRY2* alleles with the photoperiod promotion pathway mutants *co* and *gi* showed that in LD and SD conditions the *co* and *gi* mutants are completely epistatic to all *CRY2* variants. Moreover, the double mutants *EDI* 35S::*CO* and *fha1* 35S::*CO* flowered as early as the monogenic line 35S::*CO*, in agreement with the epistatic relationship described above. The additive phenotype of the double mutants of *CRY2* alleles with the photoperiod promotion pathway related genes, *FT* and *FWA* indicates that *FT* or *FWA* are not controlled exclusively by cry2 and also that cry2 functioning does not depend on these genes. That *FT* is not an exclusive target of CO was also suggested by Samach et al (2000). The independent effect of *CRY2* and the *EBS* gene in addition suggested the independence of the *CRY2* and *FT* genes since Gomez-Mena et al. (2001) have shown that the *EBS* gene mediates the repression of flowering through *FT*, probably independently from the *CO* gene.

The molecular and genetic analysis of the *FLC* gene, which is a common target of the autonomous and the vernalization pathaways provide important results on the control of the photoperiod response and its relationship with the *FLC* mediated pathways. We observed that an active *FLC* allele such as *FLC-Sf2*, which is characterized by higher expression than the inactive *FLC*-L*er* allele, restores photoperiod sensivity of *CRY2*-Cvi genotypes in a L*er* background. This is in agreement with the previous analysis of the L*er*/Cvi RILs in which lines carrying *CRY2*-Cvi, *FLF*-Cvi (which we think is *FLC*) and *FLG*-Cvi respond to photoperiod (Alonso-Blanco et al., 1998b).

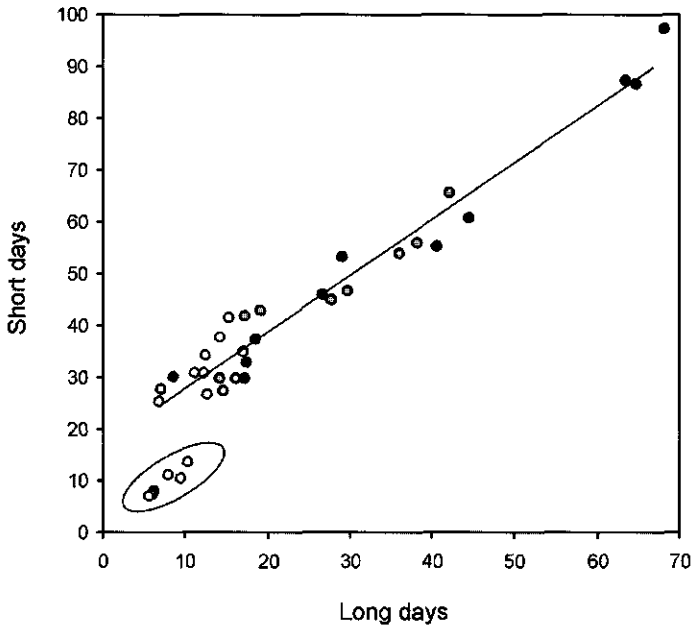

Figure 4-8. The correlation between the total leaf number (TLN) in extended-LD and SD conditions of the various genotypes differing for *FLC*, *FRI* and *CRY2* alleles. Filled circles represent the non vernalized lines, grey circles show the 2 weeks vernalized plants, and the open circles for the 5 weeks vernalized plants. The oval encircles the daylength insensitive genotypes, wile the regression line is calculated for the other genotypes.

Therefore, plants with increased levels of FLC respond more to photoperiod, i.e. FLC increases photoperiod sensitivity (Fig. 8). Several arguments support this observation such as the fact that *CRY2*-Cvi combined with mutations in the autonomous pathway, which are known to increase the *FLC* mRNA levels (Michaels and Amasino, 1999a, 2000; Sheldon et al., 1999) recover photoperiod sensitivity (present work) and the fact that mutants deficient in the photoperiod response pathway like *co* and *gi* respond to photoperiod when combined with mutations in the autonomous pathway, especially with *fca* (Koornneef et al., 1998a). This effect of FLC on photoperiod response might be through its effects on downstream genes such as *FT* and *SOC1*, or by interacting with a central regulator of the photoperiod pathway such as CO. Interactions between pathways may occur by the regulation of gene transcription of one pathway by the other pathway (by changing either its amount or its cyclic pattern) or by post-transcriptional regulation affecting, for instance, protein stability. Our results, indicate that FLC affects the transcriptional regulation of *CRY2* by either reducing its transcription or changing the time of its highest expression during the day. In genetic backgrounds with high *FLC* expression (such as *FLC-Sf2*, *FRI-Sf2* or a mutation in the autonomous pathway), there are reduced levels of cry2, and therefore, the specific

features of the *CRY2*-Cvi allele that causes reduction of photoperiod sensitivity by increasing its effect in SD because of its higher protein stability, becomes less relevant. In addition, we do not know if FLC might affect also photoperiod response through the regulation of other genes.

The observation that at low levels of cry2 (in *FLC* expressing lines and in the *fha1* mutant background) a photoperiod effect is observed, suggests that LDs are not exclusively perceived by cry2. Interestingly, Suárez-López et al., (2001) proposed that CO mediates between the circadian oscillator and the activation of the flowering time gene *FT*, supporting the hypothesis that CO mediates the daylenght perception from different sources parallel to cry2. Also it has been shown by Samach et al. (2000) that CO and FLC control the transcription of *SOC1 and FT*, which we have confirmed for SOC1. Thus, it has been suggested that the expression level of *SOC1* and *FT* may be determined by a balance of CO and FLC activity. This is in agreement with our observation that *SOC1* transcription is altered by photoperiod independently of the *CRY2* genotype, indicating that another sensor affects also *SOC1* transcription, and as suggested above might be through CO. A candidate for this is phyA, which we found to act independently of cry2. In addition, phyA may also act on the suppression of the phyB flowering repression, which might be related to *FLC* through *FCA*.

In figure 4-9 a schematic representation of the different effects of the various flowering pathways, deduced from previous observations and from the genetic interactions between *CRY2* alleles and the flowering promotion pathways described in this work is shown. This scheme indicates that cry2 is affecting flowering through its effect on CO (which acts downstream of *GI*) probably by affecting its activity (Suarez-Lopez et al., 2001) and not by promoting *CO* transcription as suggested by Guo et al. (1998). On the other hand, cry2 is also postulated to participate in removing the phyB induced flowering inhibitor (Mockler et al., 1999). In view of this model, the autonomous and the photoperiod pathways are suggested to regulate photoperiod sensitivity and response.

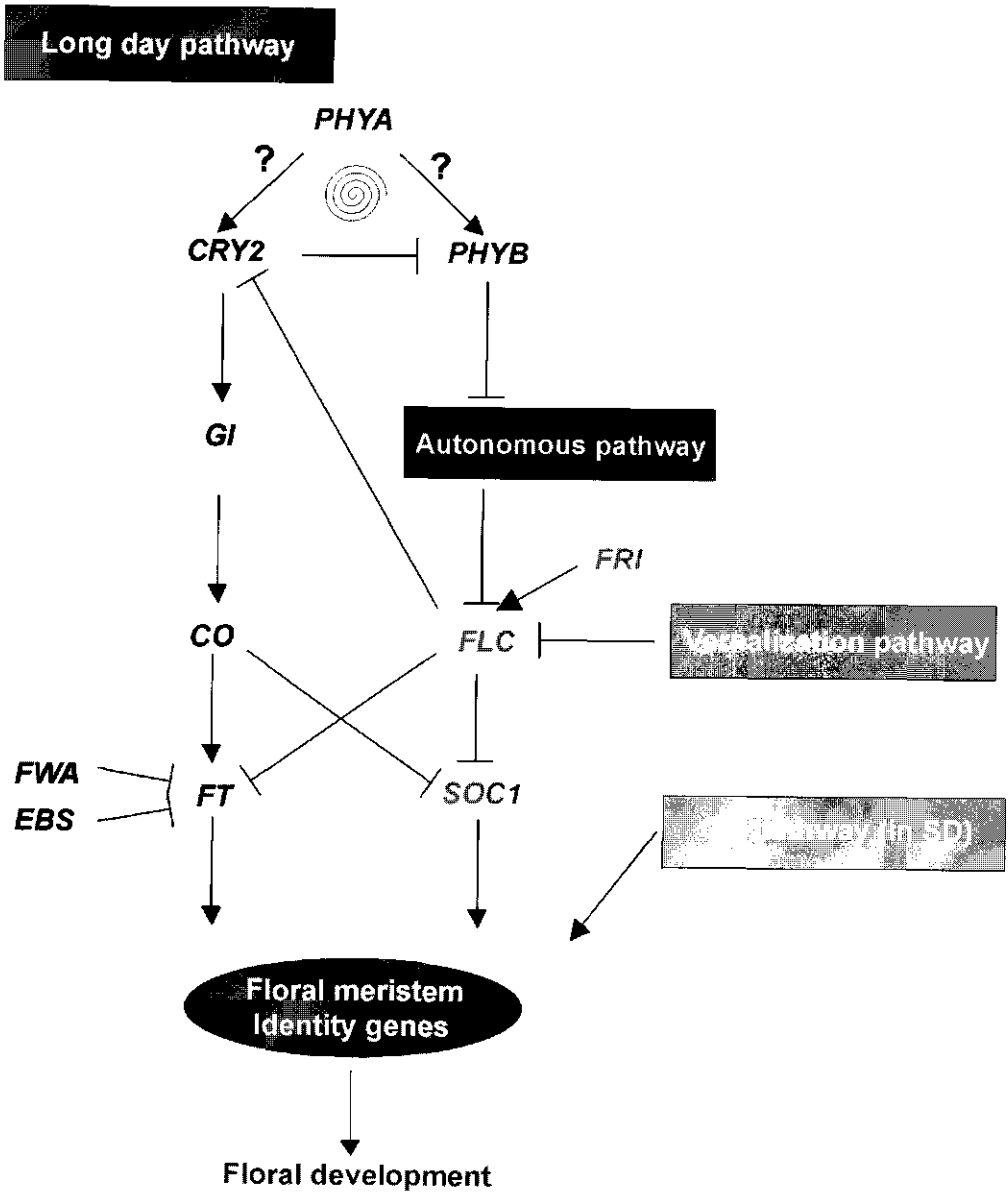

Figure 4-9. A schematic representation of the interaction of various components of the flowering pathway. The different flowering pathways are shown in boxes → indicates promotive effects and ⊥ the inhibitory effect.

The photoperiod pathway is speculated to promote flowering mainly in LDs with the participation of *CRY2*, *PHYA*, *GI* and *CO* genes, while the autonomous pathway is speculated to affect photoperiod response by its partial repression mainly in SDs controlled by phyB, and involving *FCA* and *FLC* genes. Cross regulation between both pathways is becoming clear as shown with the repression of the phyB inhibition by cry2, and reciprocally, with the repression of *CRY2* transcription by FLC. Furthermore, integrators of both mechanisms must involve the circadian clock, which might regulate the differential expression of both pathways in different photoperiods. A detailed analysis of the expression of *FLC* and target genes such as *SOC1*, *FT* and *LFY* as well as several other genes involved in photoperiod sensitivity such as *LHY*, *CCA* and *CO* in genotypes specifically constructed for this purpose might shed more light on these complex interactions of flowering pathways.

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Chapter 5

General discussion

Arabidopsis is an excellent experimental model for flowering plants, since it has a small size, a short life cycle and a small genome of approximately 130 megabases, estimated to contain around 25000 protein-coding genes, organized into 5 chromosomes. The sequence of almost its complete genome was published at by the end of the year 2000 (Arabidopsis Genome Initiative, 2000) and provided the basic information to search for the function of plant genes. In addition is the efficiency by which Arabidopsis can be transformed a very useful property for this functional analysis of genes either by reverse and/or forward genetics.

In the past, late flowering mutants were isolated and analyzed in Arabidopsis as early as the nineteen sixties. (Rédei, 1962; Hussein, 1968). The further genetic and physiological analysis later on, led to identification of more than 80 genes involved in control of flowering time (Levy and Dean, 1998) and showed a role of the various loci in the regulation of environmental and endogenous factors controlling this process. A conclusion from the many genetic studies is that the transition from the vegetative grown to flowering initiation in Arabidopsis occurs through a complex network of genetic pathways with the long-day and vernalization pathway as two important pathways mediating environmental responses (Simpson et al., 1999). Plant physiologists had identified the latter two processes in the early twentieth century.

The function of most of the flowering-time genes in Arabidopsis is deduced from mutant versions of the genes and the DNA sequences. Mutants have been and are used to clone the respective genes (forward genetics) and to connect the function (altered in the mutant) with the protein encoded by the gene. With the availability of the almost complete sequence of genes, sequence data can also become the starting material for the analysis of gene function (reverse genetics), which revealed a role, for instance, of the *SVP* (Hartmann et al., 2000) and *MAF1* genes (Ratcliffe et al., 2001).

As an alternative to laboratory-induced mutants, the genetic variation found among and within naturally occurring populations of Arabidopsis, which are collected from different geographical regions (Rédei, 1970; Alonso-Blanco and Koornneef, 2000) provides another source of genetic variation that can be used to study the function of genes. The geographic distribution of Arabidopsis embraces substantial variation in growth environments; hence, phenotypic variation among accessions is expected to reflect genetic variation that is important for the adaptation to specific conditions. Flowering time is a clear example of an adaptive trait for which extensive

variation is found among Arabidopsis accessions. It has already been shown that the analysis of flowering time variation in the naturally late-flowering accessions has complemented the mutagenic approach in the genetic dissection of the floral-induction pathways by the identification of two important repressors of the floral transition. The *FRI* (Johanson et al., 2000) and *FLC* (Michaels and Amasino, 1999a) genes are responsible for much of the natural variation for flowering time and the responsiveness to vernalization in Arabidopsis. Since the wild-type accessions used for most mutagenesis experiments had defective or very weak alleles for these loci the mutant approach had not revealed these important genes. The cloning of the latter two genes had shown the feasibility of isolating the genes responsible for natural variation which genetically was not too complex because only two major genes could be related to large genetic differences in flowering time (Koornneef et al 1994; Lee and Amasino 1995). In many other situations, including flowering time, natural variation is controlled by a larger number of genes, and the traits are also influenced strongly by environmental and random factors. This means that many traits that show genetic variation in nature behave as quantitative traits, which until recently were difficult to analyse. However, with the advent of efficient molecular marker technologies and specific statistical methods, the map position and the effect of quantitative trait loci (QTL) can presently be accurately established (Tanksley, 1993; Jansen, 1996; Kearsey and Farquar, 1998, Doerge 2002). If individual loci can be identified their cloning is possible. This was shown recently for the *Hd1* and *Hd6* genes, which are major QTLs controlling responses to photoperiod in rice and which were found to be homologues of the Arabidopsis *CONSTANS* and *CK2α* genes respectively (Yano et al., 2000; Takahashi et al., 2001).

Although several genes that play important roles in flowering time in Arabidopsis have now been genetically and molecularly identified and an increasing amount of information about their mutual interactions has been obtained, the total picture of flowering-time genes in Arabidopsis is still far from complete. A summary of the present state of knowledge about the genetic control of flowering time in Arabidopsis is described in chapter 1.

The aim of the presented work of this thesis is the genetic and molecular analysis of the differences at flowering time genes between the Arabidopsis accession Landsberg erecta (L*er*) and Cape Verde Islands (Cvi) (Alonso-Blanco et al. 1998b). These two accessions originate from very different locations and showed that natural

variation is a powerful genetic resource in Arabidopsis (chapter 2, Alonso-Blanco and Koornneef 2000).

To analyse the natural allelic variation at flowering-time loci in the L*er* and Cvi, a set of 162 recombinant inbred lines (RIL) derived from a cross between these homozygous parent lines was grown under in 3 environments, differing in day-length and/or vernalization treatment. The analysis of the data indicated that most of the flowering-time differences observed could be attributed to four QTL designated as *Early flowering and Daylength Insensitive* (*EDI*), and *flowering loci F, G,* and *H* (*FLF*, *FLG*, and *FLH*). At the *EDI* and *FLH* loci Cvi alleles resulted in earliness whereas at *FLF* and *FLG* Cvi alleles caused lateness. These 4 loci have been further characterized in relation to the flowering responses to daylength and vernalization. For that, a set of near isogenic lines (NILs) and RILs carrying the Cvi early and late alleles in L*er* background, have been analysed. This study showed that the Cvi allele of *EDI* locus, located near the top of chromosome 1, was largely dominant, and the *EDI*-Cvi plants flowered with almost the same total leaf number (TLN) under both photoperiod length conditions, indicating that *EDI* containing lines behave as an almost daylength neutral genotype. The allelic effect at *EDI* and *FLH* were basically additive since the *EDI*-Cvi *FLH*-Cvi line flowered earlier than the monogenic introgression lines in all environments. Lines with Cvi alleles at the *FLF* and *FLG* loci, located on the upper part of chromosome 5, at which Cvi alleles has to be present at both loci to cause lateness, respond strongly to both daylength and vernalization. It was shown that the daylength sensitivity, which is only slightly reduced in Cvi compared to L*er* depends on the presence of Cvi alleles at these loci. In addition, the *FLF*-Cvi allele behaved as a late allele of *FLC* in its synergistic interaction with *FRI*-M73, and with *ld*, although it must be a weaker allele than the San Feliu (Sf2) and Col alleles at the *FLC* locus (Koornneef et al., 1994; Lee et al., 1994a). Moreover, the *FLF* and *FLG* accounted for much of the vernalization response.

The cloning and molecular characterization of the *EDI* QTL is described in chapter 3 (El-Assal et al., 2001). The *EDI* locus was isolated by map based cloning. As a starting point for this, we generated a mapping population by crossing NIL45, carrying a 25 cM Cvi genomic region on the top of chromosome 1 in a L*er* background, with L*er*. NIL45 flowered earlier than Ler under SD and was practically insensitive to daylength (Alonso-Blanco et al., 1998b), which resulted in an F2 population segregating as a single Mendelian inherited locus (Alonso-Blanco and

Koornneef, 2000, El-Assal et al., 2001). This mapping population was screened for recombinants between the *EDI* locus and 6 molecular markers spanning the 10 cM interval between the *CER1* and *PHYA* genes, for which markers were available. The genetic analysis localized *EDI* to BAC F19B19, and fine mapping with additional molecular markers further refined the position of EDI to a 45 kb region containing 15 ORFs, including the *CRY2* gene. *CRY2* encodes the blue light photoreceptor cryptochrome 2, which was known to be involved in the control of flowering time and the perception of daylength (Guo et al., 1998) and which was therefore considered as a likely candidate gene for *EDI*. To confirm this, a 4.6 kb genomic fragment containing the complete *CRY2* coding region from Ler and Cvi was isolated by PCR and used for plant transformation to the L*er* background. Transgenic L*er* lines carrying the *CRY2*-Cvi construct showed the same early flowering and daylength-insensitive phenotype as the near-isogenic line *EDI*-NIL, a L*er* line derived from NIL45 but containing only a 7 cM genomic region from Cvi at the top of chromosome 1, including the *CRY2*-Cvi allele. However, plants carrying the *CRY2*-L*er* transgene flowered similarly to the untransformed L*er* controls, and exhibited normal daylength sensitivity. To determine the molecular basis for the functional difference of the *CRY2* allele from Cvi with the L*er* allele, we sequenced the 4.6 kb *CRY2* genomic fragment from Cvi and L*er* and compared it with the published Col accession sequence. The sequence analysis showed 12 single nucleotide polymorphisms between L*er* and Cvi. Two amino acid substitutions located in exons and specific for Cvi were possible candidates for the functional difference. Of these 2 changes the Cvi-specific substitution at position 476 was considered less likely to account for the *EDI* phenotype because this residue is poorly conserved across plant CRY proteins. However, the second Cvi-specific amino acid difference, which was the substitution of valine (L*er*) for methionine (Cvi) at position 367 was analysed in detail as a more likely molecular basis for the difference in flowering phenotype. This valine residue is highly conserved among the CRY proteins in both higher and lower plants. Genomic fragments differing only at the 367 position between the L*er* and Cvi were obtained and transformed into L*er*. These transformants showed unambiguously that only the plants with a methionine at this 367 position were early flowering in both LD and SD conditions.

To further understand the molecular mechanisms of the V367M amino acid substitution on function of cry2 in relation to photoperiod, we analysed its effects on

CRY2 expression. We found that the level of *CRY2* mRNA was similar in light and dark-grown, and there was also no difference between L*er* and the *EDI*-NIL under either condition. The pattern of CRY2 protein accumulation throughout a 24-h SD cycle was analysed and showed that in lines containing the wild-type L*er* *CRY2* allele and L*er* expressing *CRY2*-Cvi containing the M367V substitution, the CRY2 protein was strongly reduced within 2 h after dawn, and remained at a low level before gradual re-accumulation during the second half of the night. In contrast, lines expressing the *CRY2*-Cvi substitution showed a higher level of CRY2 throughout the cycle compared with the level in L*er*. Interestingly, under LD, diurnal variation in the level of CRY2 was not observed, either in plants containing the L*er* or Cvi *CRY2* allele. We also obtained evidence indicating that the light-induced down-regulation of CRY2 is developmentally affected. The extent of SD light-induced CRY2 depletion was small in 3-day-old seedlings, but increased significantly in 4 - 8-day-old seedlings before gradually diminishing by 10 to 14 days.

These results showed that the early-flowering phenotype under SD conferred by the V to M substitution in *CRY2*-Cvi is associated with a substantial increase of the amount of CRY2 protein in SD conditions, which is particularly evident early in the light period.

The cloning and characterization of the *EDI* QTL provides one of the first published examples in which a natural phenotypic variant is demonstrated to be caused by a single amino acid substitution altering the protein function (chapter 3, El-Assal et al., 2001). At the same time Maloof et al. (2001) have demonstrated that also for another photoreceptor, phytochrome A (phyA), specific alleles altering protein stability can be found among wild Arabidopsis accessions. Furthermore the analysis showed for the first time that photoperiod specifically may affect the protein stability of a photoreceptor protein of whose abundance correlated with early flowering.

In chapter 4, the genetic interaction between cry2 and gene products of other loci involved in the different flowering promotion pathways is described. For this the 3 different types of *CRY2* alleles (*CRY2*-Cvi = *EDI*, *CRY2*-L*er* and the *CRY2* null mutants *fha1-1*) were combined with and mutants representing the different flowering pathway components. Double mutants that involved phytochrome and cryptochrome photoreceptor mutants were studied to understand the role of photoreceptors in flowering and daylength and light-quality perception. It was shown that in red light (RL) cry2 is also active since the EDI line, as well as the *EDI cry1* genotype was

significantly earlier than *cry1*. The more blue light (BL) provided, the more flowering-time of *cry1* was reduced. The requirement for cryptochromes apparently depends on the duration and irradiance of BL. The data obtained suggest that *EDI* compensates very well both the *CRY2-Ler* and *CRY2* requirements. The analysis of double mutants involving phyA- and phyB-deficient mutants showed that although phyA promotes flowering in LD, this photoreceptor is not required for cry2 functioning and this consequently indicate that the *EDI-CRY2* allele may compensate fully the loss of phyA, cry1 and cry2 in LD conditions. On the other hand, because the LD promotion by the *CRY2* gene is almost absent in the phyB-deficient mutant background, whereas the *fha1-1* single mutant still showed some delay by SD, we conclude that the extra delay in *fha1 phyB* in LD compared to the *phyB* mutant is due to the absence of cry2. This indicates that phyB is not fully epistatic to *fha1-1* and that the LD promotion by cry2 does not depend only on phyB, although in the light quality experiments the *phyB* mutant is fully epistatic to *CRY2* locus. The latter experiments suggested that in BL no phyB inhibition occurs, but in B+RL the inhibition is present but removed by cry2, because we could see the delayed flowering-time only in *fha1-1* background confirming the data of Mockler et al. 1999. The *EDI-CRY2* allele is more active than the *Ler-CRY2* allele in all light conditions.

The study of genetic interaction between *CRY2* gene and the LD promotion pathway showed that the *co* and *gi* mutants were almost completely epistatic to *CRY2*. This implies that *CRY2* acts upstream of *CO* and *GI* and therefore *CRY2* needs the product of *CO* and *GI* genes to promote flowering through the LD promotion pathway. Moreover, the genotypes *EDI* 35S::*CO* and *fha1* 35S::*CO* flower as early as the monogenic line 35S::*CO*, in agreement with the epistatic relationship described above. In addition, the double mutants of *CRY2* with *FWA*, *FT* and *EBS* flowered intermediate between the monogenic mutant parents, concluding that *FWA*, *FT* and *EBS* are not the direct targets for cry2 or at least not the only target.

The effect of *CRY2* variants in the background of meristem-identity or autonomous-promotion pathway genes, show that these genes are not exclusively dependent on cry2-mediated pathways. Such an independency of pathways was also indicated by the floral-promoting effect of *EDI* on the *ga1-3* mutant, which is extremely late flowering in SD conditions.

The combination of the various *CRY2* alleles with active *FRI* and *FLC* alleles that confer lateness and a strong vernalization response showed that this extreme

lateness is epistatic to the *CRY2* alleles when high expression of the *FLC* gene occurs. In the double mutants involving the autonomous pathway and in genotypes carrying only the *FLC* or *FRI* wild type alleles an additive effect is observed. This corresponds to intermediate levels of *FLC* mRNA. Vernalization restored earliness and the effect of *CRY2* alleles becomes visible again after this treatment.

The analysis of the mRNA levels by real time PCR performed on samples of 2-week-old plants 2 h after the onset of the light period showed that in the presence of the dominant alleles of *FLC* and *FRI,* when *FLC* expression is very high, the expression of *CRY2* gene reduced. The similar lateness of the lines *EDI FLC FRI* and *fha1 FLC FRI* in LD and SD, with high *FLC* and low *CRY2* expression, independent of the *CRY2* allele, indicates that *FLC* regulates the expression of *CRY2* negatively. This negative regulation of *FLC* to *CRY2* can be direct between *FLC* and *CRY2* or indirect through a downstream gene(s) of *FLC.* In the absence of CRY2 expression the specific features of the *EDI* allele, that is it effectiveness in SD because of its higher protein stability (El-Assal et al., 2001), become irrelevant

The positive correlation between TLN and the *FLC* expression, and the negative correlation between the TLN and both *SOC1* and *CRY2* expression indicated the negative control of both floral promoters by *FLC*, which was shown before for SOC1 by Samach et al. (2000). The observation that flowering was later in SD at similar levels of *CRY2* mRNA most likely relates to the increased protein stability of L*er CRY2* in LD. Although CO acts downstream of cry2 we showed that in agreement with the findings of Suarez-Lopez et al. (2001), the expression of *CO* is not affected by cry2, but its mRNA levels depend on the time during the day which differs between LD and SD. This implies that the regulation of CO is not at the level of transcription but very likely, it may affect the activity or stability of the CO protein.

The observation that at very low levels of *CRY2* an effect of photoperiod suggests that in addition to cryptochromes the length of the light period can be measured by other factors which might include phytochrome A that behaves independently from *CRY2* in double mutant analysis.

The cloning of a flowering-time QTL was achieved and revealed a novel variant of the cryptochrome 2 gene. The effect of daylength and the stability of the cry2 protein is a new finding relevant for understanding the role of daylength in the initiation of flowering. In addition, the results have led to the discovery a new

relationship between the daylength perception and the vernalization promotion pathway as shown in the flowering schematic representation in chapter 4.

References

Ahmad, M. and Cashmore, A.R., (1993). *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature.* **366**, 162-166.

Ahmad, M., Jarillo, J.A. and Cashmore, A.R. (1998). Chimeric protein between cry1 and cry2 *Arabidopsis* blue light photoreceptors indicate overlapping functions and varying protein stability. *Plant Cell* **10**, 197-207

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P. and Kay, S.A. (2001). Reciprocal regulation between *TOC1* and *LHY/CCA1* within the Arabidopsis Circadian clock. *Science* **293**, 880-883.

Alonso-Blanco, C., Blankestijn-de Vries H, Hanhart C.J. and Koornneef, M. (1999). Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A.* **96**, 4710-4717.

Alonso-Blanco, C. and Koornneef, M. (2000). Naturally occuring variation in Arabidopsis: an underexploited resource for plant genetics. *Trends in Plant Science* **5**, 22-28.

Alonso-Blanco, C., Peeters, A. J. M., Koornneef M., Lister, C., Dean C., van den Bosch, N., Pot, J. and Kuiper, M.T. (1998a). Development of an AFLP based linkage map of L*er*, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a L*er*/Cvi recombinant inbred line population. *Plant J.* **14**, 101-113.

Alonso-Blanco, C., El-Assal, S.E-D., Coupland, G. and Koornneef, M. (1998b). Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana. Genetics* **149**, 749-764.

Amasino, R. M. (1996) Control of flowering time in plants. *Curr. Opin. Genet. Dev.* **6**, 480-487.

Arabidopsis Genome Initiative (2000). Analysis of the gene sequence of the flowering plant *Arabidopsis thaliana. Nature* **408**, 796-815.

Araki, T. and Komeda, Y. (1993). Analysis of the role of the late-flowering locus, *GI* in the flowering of *Arabidopsis thaliana. Plant J.* **3**, 231-239.

*At*DB. Arabidopsis thaliana database. http://www.genome.stanford.edu. (Recently, TAIR DB. The Arabidopsis Information Resource. http://www.arabidopsis.org.).

Aukerman, M.J., Lee, I., Weigle, D., and Amasino, R.M. (1999). The Arabidopsis flowering-time gene *LUMINIDEPENDENS* is expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates *LEAFY* expression. *Plant J.* **18**, 195-203.

Bagnall, D.J. (1992). The control of flowering of *Arabidopsis thaliana* by light, vernalization and gibberellins. *Aust. J. Plant Physiol.* **19**, 401-409.

Bagnall, D.J., King R.W. and Hangarter, R.P. (1996). Blue-light promotion of flowering is absent in *hy4* mutants of Arabidopsis. *Planta* **200**, 278-280.

Bagnall, D.J., King, R.W., Whitelam, G.C., Boylan, M.T., Wagner, D. and Quail, P.H. (1995). Flowering responses to altered expression of phytochrome in mutants and transgenic lines of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* **108**, 1495-1503.

Barton, N.H. and Keightley, P.D. (2002). Understanding quantitative genetic variation. *Nature Reviews Genetics* **3**, 11-21.

Bechtold, N., Ellis, J. and Pelletier, G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *CR Acad. Sci. (Paris)* **316**, 1194-1199.

Bell, C. J. and. Ecker, J. R (1994) Assignement of 30 microsatellite loci to the linkage map of Arabidopsis. *Genomics* **19**, 137-144.

Bentsink, L., Alonso-Blanco, C., Vreugdenhil, D., Tesnier, K., Groot, S.P.C. and Koornneef, M. (2000). Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of Arabidopsis. *Plant Physiol.***124**, 1595-1604.

Bernatzky, R. and Tanksley, S.D. (1986). Genetics of actin-related sequences in tomato. *Theor. Appl. Genet.* **72**, 314-324.

Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. (1993). Physiological signals that induce flowering. *Plant Cell* **5**, 1147-1155.

Blazquez, M. and Weigel, D. (1999). Independent regulation of flowering by phytochrome B and gibberellins in Arabidopsis. *Plant Physiol.* **120**, 1025-1032.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R. and Weigel, D. (1998). Gibberellins promote flowering of Arabidopsis by actvating the *LEAFY* promoter. *Plant Cell* **10**, 791-800.

Borner, R., Kampmann, G., Chandler, J., Gleibner, R., Wisman, E., Apel, K. and Melzer, S. (2000). A MADS domain gene involved in the transition to flowering in Arabidopsis. *Plant J.* **24**, 591-599.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D.R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* **119**, 721-743.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R. and Coen, E. (1997). Inflorescence commitment and architecture in *Arabidopsis*. *Science* **275**, 80-83.

Brown, J.A.M. and Klein, W.H. (1971). Photomorphogenesis in *Arabidopsis thaliana* (L.) Heynh. Threshold intensities and blue-far-red synergism in floral induction. *Plant Physiol.* **47**, 393-399.

Burn, J.E., Smyth, D. R., Peacock, W. J. and Dennis, E. S. (1993) Genes conferring late flowering in *Arabidopsis thaliana*. *Genetica* **90**, 147-155.

Carré, I.A., Ramsay, N. Schaffer, R. and Coupland, G., Characterisation of a putative circadian clock gene in *Arabidopsis*. European Symposium on Photomorphogenesis, Leicester, UK. (1997).

Casal, J.J. (2000). Phytochromes, cryptochromes, phototropin: photoreceptor interactions in plants. *Photochem. Photobiol.* **71**, 1-11.

Cashmore, A.R., Jarillo, J. A., Wu, Y.J. and Liu, D. (1999). Cryptochrome: blue light receptors for plants and animals. *Science* **284**, 760-765.

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J. and Kay, S.A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* **285**, 553-556.

Chandler, J., Wilson, A. and Dean, C. (1996). Arabidopsis mutants showing an altered response to vernalization. *Plant J.* **10**, 637-644.

Chase, K., Adler, F. R. and Lark, K. G. (1997) Epistat: a computer program for identifying and testing interactions between pairs of quantitative trait loci. *Theor. Appl. Genet.* **94**, 724-730.

Chou, I.T. and Gasser, C.S. (1997). Characterization of the cyclophilin gene family of *Arabidopsis thaliana* and phylogenetic analysis of known cyclophilin protein. *Plant Mol. Biol.* **35**, 873-892.

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E. and Briggs, W.R. (1998). Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science* **282**, 1698-1710.

Clack, T., Mathews, S. and Sharrock, R.A. (1994). The phytochrome apoprotein family in Arabidopsis is encoded by five genes: the sequences and expression of *PHYD* and *PHYE*. *Plant Mol. Biol.* **25**, 413-427.

Clarke, J. H. and Dean, C. (1994). Mapping *FRI*, a locus controlling flowering time and vernalization response in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **242**, 81-89.

Clarke, J. H., Mithen, R., Brown, J. K. M. and Dean, C. (1995). QTL analysis of flowering time in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **248**, 555-564.

Corbesier, L., Gadisseur, I., Silvestre, G., Jacqmard, A. and Bernier, G. (1996). Design in *Arabidopsis thaliana* of a synchronous system of floral induction by one long day. *Plant J.* **9**, 947-952.

Coupland, G. (1995). Genetic and environmental control of flowering time in *Arabidopsis*. *Trends in Genetics* **11**, 393-397.

Covington, M.F., Panda, S., Liu, X.L., Strayer, C.A., Wagner, D.R. and Kay, S. (2001). *ELF3* modulates resetting of the circadian clock in Arabidopsis. *Plant Cell* **13**, 1305-1315.

Devlin, P.F. and Kay, S.A. (2000). Cryptochromes are required for phytochrome signalling to the circadian clock but not for rhythmicity. *Plant Cell* **12**, 2499-2509.

Devlin, P.F., Patel, S.R. and Whitelam, G.C. (1999a). Phytochrome E influences internode elongation and flowering time in Arabidopsis. *Plant Cell* **10**, 1479-1488.

Devlin, P.F., Robson, P.R., Patel, S.R., Goosey, L., Sharrock, R.A. and Whitelman, G.C. (1999b). Pytochrome D acts in the shade-avoidance syndrome in Arabidopsis by controlling elongation growth and flowering time. *Plant Physiol.***119**, 909-915.

Doerge, R.W. (2002). Mapping and analysis of quantitative trait loci in experimental populations. *Nature Genetics Reviews* **3**, 43-52.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. *Cell* **96**, 271-290.

El-Assal, S.E-D., Alonso-Blanco, C., Peeters, A.J.M., Raz, V. and Koornneef, M., (2001). A QTL for flowering time in Arabidopsis reveals a novel allele of *CRY2*. *Nature Genetics* **29**, 435-440.

Emery, P., So, W.V., Haneko, M., Hall, J.C. and Rosbash, M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**, 669-679.

Eskins, K. (1992). Light-quality effect on Arabidopsis development. Red, blue and far-red regulation of flowring and morphology. *Plant Physiol.* **86**, 439-444.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B. and Coupland, G. and Putterill, J. (1999). *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. *EMBO J.* **18**, 4679-4688.

Frary, A., Nesbitt, T.C., Grandillo, S., Knaap, E., Cong, B., Liu, J., Meller, J., Elber, R., Alpert, K. B. and Tanksley, S.D. (2000). *Fw2.2*: A quantitative trait locus key to the evolution of tomato fruit size. *Science* **289**, 85-88.

Fridman, E., Pleban, T. and Zamir, D. (2000). A recombination hotspot delimits a wild-species quantitative trait locus for tomato sugar content to 484 bp within an invertase gene. *Proc. Natl. Acad. Sci. USA* **97**, 4718-4723.

Gallagher, S., Short, T.W., Pratt, L.H., Ray, P.M., and Briggs, W.R. (1988). Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections. *Proc. Natl. Acad. Sci. USA* **85**, 8003-8007.

Gendall, R.A., Levy, Y.Y., Wilson, A. and Dean, C. (2001). The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. *Cell* **107**, 525-535.

Gomez-Mena, C., Pineiro, M., Franco-Zorrilla, J.M., Salinas, J., Coupland, G, and Martinez-Zapater, J.M., (2001). *early bolting in short days*: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. *Plant Cell* **13**, 1011-1024.

Goto, N., Kumagai, T. and Koornneef, M. (1991). Flowering responses to light-breaks in photomorphogenic mutants of *Arabidopsis thaliana*, a long day plant. *Physiol. Plant.* **83**, 209-215.

Grbic, V. and Bleecker, A. B. (1996) An altered body plan is conferred on Arabidopsis plants carrying dominant alleles of two genes. *Development* **122**, 2395-2403.

Grbic, V. and Gray, J. (1997) Aerial rosette 1, *ART1*, is a new late flowering gene of *Arabidopsis thaliana*. 8th International Conference on Arabidopsis Research, Madison, Wisconsin.

Guan, K. and Dixon, J.G. (1991). Eukaryotic proteins expressed in *Escherichia coli:* An improved thrombin cleavage and purification procedure of fusion protein with glutathion S-transferase. *Anal. Biochem.* **192**, 262-267.

Guo, H., Duong, H., Ma, N. and Lin, C. (1999). The Arabidopsis blue light receptor cryptochrome 2 is a nuclear protein regulated by a blue light-dependent post-transcriptional mechanism. *Plant J.* **19**, 279-287.

Guo, H., Yang, H., Mockler, T.C. and Lin, C. (1998). Regulation of flowering time by *Arabidopsis* photoreceptors. *Science* **279**, 1360-1363.

Gustafson-Brown, C., Savidge, B. and Yanofsky, M. (1994). Regulation of the Arabidopsis floral homeotic gene *APETALA1*. *Cell* **76**, 131-143.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H, Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science* **290**, 210-213.

Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H. and Huijser, P. (2000). Molecular cloning of *SVP*: a negative regulator of the floral transition in Arabidopsis. *Plant J.* **21**, 351-360.

Haughn, G.W., Schultz, E.A. and Martinez-Zapater, J.M. (1995). The regulation of flowering in *Arabidopsis thaliana*: meristems, morphogenesis, and mutants. *Can. J. Bot.* **73**, 959-981.

Hempel, F.D. and Feldman, L.J. (1994). Bi-directional inflorescence development in *Arabidopsis thaliana*: acropetal initiation of flowers and basipetal initiation of paraclades. *Planta* **192**, 276-286.

Hempel, F.D. and Feldman, L.J. (1995). Specification of chimeric flowering shoots in wild-type Arabidopsis. *Plant J.* **4**, 901-913.

Hempel, F.D., Weigel, D., Mandel, M.A., Ditta, G., Zambryski, P.C., Feldman, L.J. and Yanofski, M.F. (1997). Floral determination and expression of floral regulatory genes in Arabidopsis. *Development* **124**, 3845-3853

Hicks, K.A., Millar, A.J., Carré, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R. and Kay, S.A. (1996). Conditional circadian dysfunction of the Arabidopsis *early-flowering 3* mutant. *Science* **274**, 790-792.

Hsu, D.S., Zhao, X., Zhao, S., Kazantsev, A., Wang, R.P., Todo, T., Wei, Y.F. and Sancar, A. (1996). Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. *Biochemistry* **35**, 13871-13877.

Hussein, H.A.S. (*1968*). Genetic analysis of mutagen-induced flowering time variation in Arabidopsis thaliana (L.) Heynh. PhD thesis, *Meded. Landbouwhogeschool*, 68-11, Wageningen, The Netherlands. pp 88.

Jansen, R.C, Van Ooijen, J.W., Stam, P., Lister, C. and Dean.C. (1995). Genotype by environment interaction in genetic mapping of multiple quantitative trait loci. *Theor. Appl. Genet.* **91**, 33-37.

Jansen, R.C. (1996). Complex plant traits: time for polygenic analysis. *Trends Pl. Sci.* **1**, 89-94.

Jansen, R. C., and Stam, P. (1994). High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* **136,** 1447-1455.

Jacobsen, S.E., and Olszewski, N.E. (1993). Mutations at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. *Plant Cell* **5**, 887-896.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R. and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. *Science* **290**, 344-347.

Johnson, E., Bradley, M., Harberd, N.P. and Whitelam, G.C. (1994). Photoresponses of light-grown *phyA* mutants of Arabidopsis. *Plant Physiol.* **105**, 141-149.

Kania, T., Russenberger, D., Peng, S., Apel, K. and Melzer, S. (1997). *FPF1* promotes flowering in Arabidopsis. *Plant Cell* **9**, 1327-1338.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999). Activation tagging of the floral inducer *FT*. *Science* **286**, 1962-1965.

Karlsson,B.H., Sills, G.R. and Nienhuis, J. (1993). Effect of photoperiod and vernalization on the number of leaves at flowering in 32 *Arabidopsis thaliana* (*Brassicaceae*) ecotypes. *Am. J. Bot.* **80**, 646-648.

Kearsey, M.J. and Farquar, G.L. (1998). QTL analysis in plants; where we are now ? *Heredity* **80**, 137-142.

Kendrick, R.E., and Kronenberg, G.H.M. (1994). Photomorphogenesis in plants. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp 828.

King, R.W. and Bagnall, D.J. (1996). Photoreceptors and the photoperiodic response controlling flowering time in Arabidopsis. *Sem. Cell Dev. Biol.* 7, 449-454.

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J. and Mitchell-Olds, T. (2001). Genetic control of natural variation in Arabidopsis Glucosinolate accumulation. *Plant Physiol.* **126**, 811-825.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960-1962.

Konieczny, A. and Ausubel, F. M. (1993). A procedure for mapping Arabidopsis mutations using co-dominan ecotype specific PCR-based markers. *Plant J.* **4**, 403-410.

Koornneef, M. and Van der Veen, J.H. (1980). Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* **58**, 257-263.

Koornneef, M., Rolff, E. and Spruit, C.J.P. (1980). Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. *Z. Pflanzenphysiol* **100**, 147-160.

Koornneef, M., Hanhart, C.J. and van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **229**, 57-66.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C.J., Soppe, W.J.J. and Peeters, A.J.M. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. *Plant J.* **6**, 911-919.

Koornneef, M., Hanhart, C., van Loenen-Martinet, P. and Blankestijn-de Vries, H. (1995). The effect of daylength on the transition to flowering in phytochrome-deficient, late-flowering and double mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **95**, 260-266.

Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C.J. and Peeters, A.J.M. (1998a). Genetic interactions among late-flowering mutants of Arabidopsis. *Genetics* **148**, 885-892.

Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M. and Soppe, W. (1998b). Genetic control of flowering time in Arabidopsis. *Annu. Rev., Plant Physiol.*, and *Plant Mol. Biol.* **49**, 345-370.

Kowalski, S. P., Lan, T. H., Feldmann, K. A. and Paterson, A. H. (1994). QTL mapping of naturally-occurring variation in flowering time of *Arabidopsis thaliana*. *Mol. Gen. Genet.* **245**, 548-555.

Krannitz, P.G., Aarssen, L.W. and Dow, J.M. (1991). The effect of genetically based difference in seed size on seedling survival in *Arabidopsis thaliana* (Brassicaceae). *Amer. J. Bot.* **7**, 446-450.

Kugler, I. (1951). Untersuchungen über das Keimverhalten einiger Rassen von *Arabidopsis thaliana* (L.) Heynh. Ein Beitrag zum problem der Lichtkeimung. *Beitr. Biol. Pflantzen* **28**, 211-243.

Kuittinen, H., Sillanpaa, M. J. and Savolainen, O. (1997). Genetic basis of adaptation: flowering time in *Arabidopsis thaliana*. *Theor. Appl. Genet.* **95**, 573-583.

Kunkel, B.N. (1996). A useful weed put to work: Genetic analysis of disease resistance in *Arabidopsis thaliana*. *Trends Genet.* **12**, 63-69.

Laibach, F. (1951). Über Sommer und Winterannuelle Rasse von *Arabidopsis thaliana* (L.) Heynh. Ein Beitrag zur Atiologie der Blutenbildung. *Beitr. Biol. Pflantzen* **28**, 173-210.

Larkin, J.C., Young, N., Prigge, M. and Marks, M.D. (1996). The control of trichome spacing and number in Arabidopsis. *Development* **122**, 997-1105.

Lazo, G.R., Stein,P.A. and Ludwig, R.A. (1991). A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology* **9**, 963-967.

Lee, I., Bleecker, A. and Amasino, R. M. (1993). Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **237**, 171-176.

Lee, I., Aukerman, M.J., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C. and Amasino, R.M. (1994a). Isolation of *LUMINIDEPENDENS*: A gene involved in the control of flowering time in Arabidopsis. *Plant Cell* **6**, 75-83.

Lee, I., Michaels, S.D., Masshardt, A.S. and Amasino, R.M. (1994b). The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDENS* is suppressed in the Landsperg *erescta* strain of Arabidopsis. *Plant J.* **6**, 903-909.

Lee, I. and Amasino, R.M. (1995). Effect of vernalization, photoperiod, and light quality on the flowering phenotype of *Arabidopsis* plants containing the *FRIGIDA* gene. *Plant Physiol.* **108**, 157-162.

Lee, I., Wolfe, D.S., Nilsson, O. and Weigel, D. (1997). A *LEAFY* co-regulator encoded by *UNUSUAL FLORAL ORGANS*. *Curr. Biol.* 7, 95-104.

Levy, Y.Y, and Dean, C. (1998). The transition to flowering. *Plant Cell* **10**, 1973-1989.

Li, B., Suzuki, J. and Hara, T. (1998). Latitudinal variation in plant size and relative growth rate in *Arabidopsis thaliana*. *Oecologia* **115**, 293-301

Lin, C., Ahmad, M., Gordon, D. and Cashmore, A.R. (1995). Expression of an Arabidopsis cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A, and green light. *Proc. Natl. Acad. Sci. USA* **92**, 8423-8427

Lin, C., Ahmad, M. and Cashmore, A.R. (1996). Arabidopsis cryptochrome 1 is a soluble protein mediating blue light-dependent regulation of plant growth and development. *Plant J.* **10**, 893-902.

Lin, C., Yang, H., Guo, H., Mockler, T., Chen, J. and Cashmore, A.R. (1998). Enhancement of blue-light sensitivity of *Arabidopsis* seedlings by a blue light receptor cryptochrome 2. *Proc. Natl. Acad. Sci. USA* **95**, 2686-2690

Lin, C. (2000). Plant blue-light receptors. *Trends Plant Science.* **5**, 337-342.

Livak, K.J., Flood, S.J.A., Marmaro, J., Giusti, W. and Deetz, K. (1995). Oligonucleotides with fluorescent dyes a opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Method and Applications*. **4**, 357-362.

Lobin W. (1983). The occurrence of *Arabidopsis thaliana* in the Cape Verde Islands. *Arab. Inf. Serv.* **20**, 119-123.

Lukowitz, W., Gillmoer, C.S. and Scheible, W.R. (2000). Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiol.* **123**, 795-805.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C. and Dean, C. (1997). *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* **89**, 737-745.

Maloof, J.N., Borevitzx, J.O., Dabi, T., Lutes, J., Nehring, R.B., Redfern, J.L., Trainer, G.T., Wilsonx, J.M., Asami, T., Berry, C.C., Weigel, D. and Chory, J. (2001). Natural variation in light sensitivity of Arabidopsis. *Nature Genetics* **29**, 441-446.

Mandel, M.A. and Yanofsky, M.F. (1995). A gene triggering flower formation in Arabidopsis. *Nature* **377**, 522-524.

Martinez-Zapater, J.M., Coupland, G., Dean, C. and Koornneef, M. (1994). The transition to flowering in Arabidopsis. In Arabidopsis. E.M. Meyerowitz and C.R. Smerville (eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, pp. 403-434.

McClung, C.R. and Kay, S. (1994). Circadian rhythms in Arabidopsis thaliana. In Arabidopsis (Meyerowitz, E.M. and Somervile, C.R., eds), Cold Spring Harbor, New York, USA, pp. 615-637.

McWatters, H.G., Bastow, R.M., Hall, A. and Millar, A.J. (2000). The *ELF3* zeitnehmer regulates light signaling to the circadian clock. *Nature* **408**, 716-720.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998). *Arabidopsis thaliana*: a model plant for genome analysis. *Science* **282**, 662-682.

Michaels, S.D. and Amasino, R.M. (1999a). *Flowering Locus C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956

Michaels, S.D. and Amasino, R.M. (1999b). The gibberellic acid biosynthesis mutant *ga1-3* of *Arabidopsis thaliana* is responsive to vernalization. *Developmental Genetics* **25**, 194-198.

Michaels, S.D. and Amasino, R.M. (2000). Memories of winter: Vernalization and the competence of flower. *Plant Cell Environ.* **23**, 1145-1154.

Michaels, S.D. and Amasino, R.M. (2001). Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-941.

Millar, A.J, Straume, M., Chory, J., Chua, N.H. and Kay, S.A. (1995). The regulation of circadian period by phototransduction pathways in Arabidopsis. *Science* **267**, 1163-1166.

Mitchell-Olds, T. (1995). The molecular basis of quantitative genetic variations in natural populations. *Trends Ecol. Evol.* **10**, 324-328.

Mitchell-Olds, T. (1996). Genetic constraints on life history evolution: quantitative trait loci influencing growth and flowering in *Arabidopsis thaliana*. *Evolution* **50**, 140-145.

Mitchell-Olds, T. and Pedersen, D. (1998). The molecular basis of quantitative genetic variation in central and secondary metabolism in Arabidopsis. *Genetics* **140**, 1105-1109.

Mizoguchi, T. and Coupland, G. (2000). *ZEITLUPE* and *FKF1*: novel connections between flowering time and circadian clock control. *Trends Plant Sci.* **5**, 409-411.

Mockler T.C., Guo, H.,Yang, H., Duong, H. and Lin, C. (1999). Antagonistic action of Arabidopsis cryptochromes and phytochrome B in the regulation of floral induction. *Development* **126**, 2073-2082.

Mozley, D. and Thomas, B. (1995). Developmental and photobiological factors affecting photoperiodic induction in *Arabidopsis thaliana* Heynh. Landsberg *erecta*. *J. Exp. Bot.* **46**, 173-179.

Murfet, I. C. (1997). Environmental interaction and the genetics of flowering. *Annu. Rev. Plant Physiol.* **28**, 253-278.

Nagatani, A., Reed, J.W. and Chory, J. (1993). Isolation and initial characterization of Arabidopsis mutants that are deficient in phytochrome A. *Plant Physiol.* **102**, 269-277.

Napp-Zinn, K. (1957). Untersuchungen zur Genetik des Kältebedürfnisse bei *Arabidopsis thaliana* L. Heynh. Zeitschrift Induktion Abstammungs und Vererbungslehre **88**, 253-285.

Napp-Zinn, K. (1969). *Arabidopsis thaliana* (L.) Heynh. In The induction of flowering: Some case histories. L.T. Evans (ed). Macmillan, Melbourne, pp. 291-304.

Napp-Zinn, K. (1987). Vernalization. Environmental and genetic regulation, pp. 123-132 in *Manipulation of flowering*, edited by J.G. Atherton. Butterworths, London.

Neff, M.M. and Chory, J. (1998). Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant physiol.* **118**, 27-35.

Neff, M.M., Neff, J.D., Chory, J. and Pepper, E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J.* **14**, 387-392.

Nienhuis, J., Sills, G.R., Martin, B. and King, G. (1994). Variance for water-use efficiency among ecotypes and recombinant inbred lines of *Arabidopsis thaliana* (*Brassicaceae*). *Amer. J. of Bot.* **81**, 943-947.

Nilsson, O., Lee, I., Blazquez, M.A. and Weigel, D. (1998). Flowering-time genes modulate the response to *LEAFY* activity. *Genetics* **150**, 403-410.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim. H.K., Soh, M.S., Kim, H.J., Kay, S.A. and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the Arabidopsis *GIGANTEA* gene. *Science* **285**, 1579-1582.

Peters, J.L., Schreuder M.E.L., Verduin, S.J.W. & Kendrick, R.E. (1992). Physiological characterization of a high-pigment mutant of tomato. *Photochem. Photobiol.* **56**, 75-82.

Pineiro, M. and Coupland. G. (1998). The control of flowering time and floral identity in Arabidopsis. *Plant Physiol.* **117**, 1-8.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995). The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factor. *Cell* **80**, 847-857.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y. and Wagner, D. (1995). Phytochromes: Photosensory perception and signal transduction. *Science* **268**, 675-680.

Ratcliffe, O.J., Nadzan, G.C., Reuber, L. and Riechmann, J.L. (2001). Regulation of flowering in Arabidopsis by an *FLC* homologue. *Plant Physiol.* **126**, 122-132.

Rashotte, A.M., Jenks, M.A., Nguyen T.D. and Feldmann, K.A. (1997). Epicuticular wax variation in ecotypes of *Arabidopsis thaliana*. *Phytochemistry* **45**, 251-255.

Raz, V. and Ecker, J.R. (1999). Regulation of differential growth in the apical hook of Arabidopsis. *Development* **126**, 3661-3668.

Rédei, G.P. (1962). Supervital mutants of Arabidopsis. *Genetics* **47**. 443-460.

Rédei, G.P. (1970). *Arabidopsis thaliana* (L.) Heynh. A review of the genetics and biology. *Bibliogr. Genet.* **20**, 1-151.

Rédei, G. P. (1992). A heuristic glance to the past of Arabidopsis genetics, in: *Methods in Arabidopsis research,* edited by C. Koncz, N. Chua and J. Schell. World Scientific, Singapore, New Jersey, London, Hong Kong, pp. 1-15.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M. and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. *Plant Cell* **5**, 147-157.

Reeves, P.H. and Coupland, G. (2001). Analysis of flowering time control in Arabidopsis by comparison of double and triple mutants. *Plant Physiol.* **126**, 1085-1091.

Roldán, M., Gómez-Mena, C., Ruiz-García, L., Martín-Trillo, M., Salinas, J. and Martnez-Zapater, J.M. (1997). Effect of darkness and sugar availability to the apex on morphogenesis and flowering time of Arabidopsis. *Flowering Newsletter* **24**, 18-24.

Roldán, M., Gomez-Mena, C., Ruiz-Garcia, L., Salinas, J. and Martnez-Zapater, J.M. (1999). Source availability on the aerial part of the plant promoters morphogenesis and flowering of Arabidopsis in the dark. *Plant J.* **20**, 581-590.

Ruiz-Garcia, L., Madueno, F., Wilkinson, M., Haughn, G.W., Salinas, J. and Martinez-Zapater, J.M. (1997). Different roles of flowering time genes in the activation of floral initiation genes in Arabidopsis. *Plant Cell* **9**, 1921-1934.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000). Distinct roles of *CONSTANS* target genes in reproductive development of Arabidopsis. *Science* **288**, 1613-1616.

Sanda, S.L. and Amasino, R.M. (1996a). Interaction of *FLC* and late-flowering mutations in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **251**, 69-74.

Sanda, S. L., and Amasino, R.M. (1996b). Ecotype-specific gene expression of a flowering mutant phenotype in *Arabidopsis thaliana*. *Plant Physiol.* **111**, 641-644.

Sanda, S. L., John, M. and Amasino, R. M. (1997). Analysis of flowering time in ecotypes of *Arabidopsis thaliana*. *J. Heredity* **88**, 69-72.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I.A. and Coupland, G. (1998). The *late elongated hypocotyl* mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93**, 1219-1229.

Schomburg, F.M., Patton, D.A., Meinke, D.W. and Amasino, R.M. (2001). *XSFPA*, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. *Plant Cell* **13**, 1-11.

Sessa, G., Yang, C.-X., Raz, V., Eyal, Y. & Fluhr, R. Dark induction and subcellular localization of the pathogenesis related PRB-1b protein. *Plant Mol. Biol.* **28**, 537-547 (1995).

Sharrock, R.A. and Quail, P.H. (1989). Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. *Genes Dev.* **3**, 1745-1757.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999). The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* **11**, 445-458.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J. and Dennis, E.S. (2000). The molecular basis of vernalization: The central role of *FLOWERING LOCUS C* (*FLC*). *Proc. Natl. Acad. Sci. USA* **97**, 3753-3758.

Simon, R., and Coupland, G. (1996). *Arabidopsis* genes that regulate flowering time in response to day-length. *Sem. Cell Dev. Biol.* **7**, 419-425.

Simon, R., Igeno, M.I. and Coupland, G. (1996). Activation of floral meristem identity genes in Arabidopsis. *Nature* **384**, 59-62.

Simpson, G.G., Gendall, A.R. and Dean, C. (1999). When to switch to flowering. *Ann. Rev. Cell Dev. Biol.* **99**, 519-550.

Somers, D.E. (1999). The physiology and molecular bases of the plant circadian clock. *Plant Physiol.* **121**, 9-20.

Somers, D.E., Devlin, P.F. and Kay, S.A. (1998). Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science* **282**, 1488-1490.

Soppe, W.J.J, Jacobsen, S.E., Alonso-Blanco, C., Jackson, J.P., Kakutani, T., Koornneef, M. and Peeters, A.J.M. (2000). The late flowering phenotype of *fwa* mutant is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol. Cell* **6**, 791-802.

Soppe, W.J.J., Bentsink, L. and Koornneef, M. (1999). The early-flowering mutant efs is involved in the autonomous promotion pathway of *Arabidopsis thaliana*. *Development* **126**, 4763-4770.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homologue. *Science* **289**, 768-771.

Súarez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F. and Coupland, G. (2001). *CONSTANS* mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature* **410**, 1116-1120.

Swarup, K., Alonso-Blanco, C., Lynn, J.R., Michaels, S.D., Amasino, R.M., Koornneef, M. and Millar, A.J. (1999). Natural allelic variation identifies new genes in the Arabidopsis circadian system. *Plant J.* **20**, 67-77.

Takahashi, Y., Shomura, A., Sasaki, T. and Yano, M. (2001). *Hd6,* a rice quantitative trait locus involved in photoperiod sensitivity, encodes the α subunit of protein kinase CK2. *Proc. Natl. Acad. Sci. USA* **98**, 7922-7927.

Tanksley, S.D. (1993). Mapping polygenes. *Annu. Rev. Genet.* **27**, 205-233.

Thomas, B. and Vince-Prue, D. (1997). Photoperiodism in plants. *Academic Press, New York.*

Todo, T., Ryo, H., Yamamoto, K., Toh, H., Inui, T., Ayaki, H., Nomura, T., and Ikenaga, M. (1996). Similarity among the Drosophila (6-4) photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. *Science* **272**, 109-112.

Tóth, R., Kevei, E., Hall, A., Millar, A.J., Nagy, F. and Kozma-Bognár, L. (2001). Circadian clock-regulated expression of phytochrome and cryptochrome genes in Arabidopsis. *Plant Physiol.* **127**, 1607-1616.

Van der Horst, G.T.J., Muijtjens, M., Kobayashi, K., Eker, A.M., Leenen, D.V., Buijs, R., Bootsma, D., Hoeijmakers, J.H. and Yasui, A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398**, 627-630.

Van der Schaar, W., Alonso-Blanco, C., Léon-Kloosterziel, K.M., Jansen, R.C., van Ooijen, J.W. and Koornneef, M. (1997). QTL analysis of seed dormancy in Arabidopsis using recombinant inbred lines and MQM mapping. *Heredity* **79**, 190-200.

Van der Veen, J. H. (1965). Genes for late flowering in *Arabidopsis thaliana*, in *Arabidopsis Research, Proceedings of the Göttingen Symposium*, ed. G. Röbbelen,. Wasmund, Gelschenkirchen, Germany, pp. 62-71.

Van Ooijen, J. W. (1992). Accuracy of mapping quantitative trait loci in autogamous species. *Theor. Appl. Genet.* **84**, 803-811.

Van Ooijen, J. W. and Maliepaard, C. (1996). MapQTL (tm) version 3.0: Software for the calculation of QTL positions on genetic maps. CPRO-DLO, Wageningen.

Van Ooijen, J. W. and Maliepaard, C. (1996). MapQTL (tm) version 3.0: Software for the calculation of QTL positions on genetic maps. Plant Genome IV Abstracts.

Van Tuinen, A., Kerckhoffs, L. H. J., Nagatani, A., Kendrick, R. E. and Koornneef, M. (1995). Far-red light-insensitive, Phytochrome A-deficient mutants of tomato. *Mol. Gen. Genet.* **246**, 133-141

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407-4414.

Wang, R.L., Stec, A., Hey, J., Lukens, L. and Doebley, J. (1999).The limits of selection during maize domestication. *Nature* **398**, 236-239.

Wang, Z.Y. and Tobin, E.M. (1998). Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* **93**, 1207-1217.

Weigel, D. (1995). The genetics of flower development: from floral induction to ovule morphogenesis. *Annu. Rev. Genet.* **92**, 19-39.

Weigel, D. and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature* **377**, 495-500.

Weigel, D., Alvarez, J., Smyth, D.R. Yanofsky, M.F. and Meyerowitz, E.M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. *Cell* **69**, 843-859.

Weller, J.L., Murfet, I.C. and Reid, J.B. (1997). Pea mutants with reduced sensitivity to far-red light define and important role for phytochrome A in day-length detection. *Plant Physiol.* **114**, 1225-1236.

Weller, J.L., Beauchamp, N., Huub, L., Kerckhoffs, J., Platten, J.D. and Reid, J.B. (2001). Interaction of phytochromes A and B in the control of de-etiolation and flowering in Pea. *Plant J.* **26**, 283-294.

Westerman, J.M. (1970). Genotype-environment interaction and developmental regulation in *Arabidopsis thaliana*. *Heredity* **25**, 93-106.

Whitelam, G.C. and Harberd, N.P. (1997). Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. *Plant Cell Environ.* **17**, 615-625.

Wilson, A. and Dean, C. (1996). Analysis of the molecular basis of vernalization in *Arabidopsis thaliana*. *Semin. Cell Dev. Biol.* 7, 435-440.

Wilson, R.N., Heckman, J.W. and Somerville, C.R. (1992). Gibberellin is required for flowering but not for senescence in *Arabidopsis thaliana* under short days. *Plant Physiol.* **100**, 403-408.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. and Sasaki, T. (2000). *Hd1*, a major photoperiod senstivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* **12**, 2473-2483.

Zagotta, M.T., Hicks, K.A., Jacobs, C.I., Young, J.C., Hangarter, R.P. and Meeks-Wagner, D.R. (1996). The Arabidopsis *ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *Plant J.* **10**, 691-702.

Zeng, Z-B. (1994). Precision mapping of quantitative trait loci. *Genetics* **136**, 1457-1468.

Samenvatting

Arabidopsis is een uitstekende modelplant voor moleculair en genetisch onderzoek aan hogere planten. Dit komt door verschillende eigenschappen zoals een korte generatietijd, geringe afmetingen en een relatief klein genoom waarvan de volledige DNA sequentie recent is gepubliceerd. Arabidopsis komt van nature voor in een groot aantal verschillende gebieden van de wereld, waardoor binnen de soort voor veel eigenschappen een aanzienlijke genetische variatie bestaat. Bloeitijd is een belangrijke eigenschap die van planten plaatsgebonden aanpassingen vraagt, doordat deze sterk beïnvloed wordt door omgevingsfactoren zoals daglengte en temperatuur (vernalisatie). Uit de isolatie van bloeitijdmutanten en klonering van de respectievelijke genen is gebleken dat het tijdstip waarop planten gaan bloeien een complexe kwantitatieve genetische eigenschap is. Naast mutanten als bron van genetische variatie is ook de in de natuur voorkomende genetische variatie zeer geschikt om de functie van genen te onderzoeken. De genen die deze genetische variatie bepalen worden 'Quantitative Trait Loci' (QTL) genoemd. Met behulp van moleculaire merkers kan van QTL vastgesteld worden waar die op de chromosomen liggen en wat hun effect is.

Uitgaande van 162 zogenaamde 'recombinant inbred lines' die ontwikkeld zijn uit een kruising tussen de Arabidopsis accessies (ecotypen) Landsberg *erecta* (L*er*) en Cape Verdi Islands (Cvi) konden significante verschillen in bloeitijd gevonden worden voor 4 loci, welke *EDI* (*e*arly *d*aylength *i*nsensitive), *FLH*, *FLF* en *FLG* (*fl*owering time loci *H*, *F* en *G*) werden genoemd. Voor *EDI* en *FLH*, respectievelijk gelokaliseerd boven aan chromosoom 1 en onderaan chromosoom 5, bleek L*er* allelen te bezitten met een bloeiverlatend effect ten opzichte van Cvi. Voor *FLF* en *FLG*, gelokaliseerd in de bovenste helft van chromosoom 5, bleek dat Cvi de late allelen bezat. Met name het *EDI* locus leek zeer interessant voor verdere analyse omdat lijnen met het Cvi allel op dit locus in aanwezigheid van L*er* allelen op *FLF* en *FLG* loci zowel onder lange als onder korte dag omstandigheden vroeg bloeiden.

Voor een exacte locatie van het *EDI* locus werd een 'Near Isogenic Line' (NIL) ontwikkeld waarvan alleen 24 centimorgan van de top van chromosoom 1 van Cvi afkomstig was terwijl de rest van het genoom uit L*er* bestond. Deze lijn werd met L*er* gekruist en de hieruit voortkomende F2 populatie werd voor bloeitijd en

moleculair merkers geanalyseerd. Hieruit kon worden vastgesteld dat het *EDI* locus moet liggen in een gebied van 45 kb op BAC ('Bacterial Artificial Chromosome') F19B19. In dit gebied bevindt zich ook het *cryptochroom 2* (*CRY2*) gen, waarvan eerder is aangetoond dat het bij de bloeibevordering door lange dag betrokken is. DNA fragmenten van 4.6 kb waarop alleen het *CRY2* locus ligt, werden uit Cvi en L*er* geïsoleerd en via Agrobacterium transformatie overgebracht naar L*er*. Transformanten met het *CRY2-Cvi* gen bloeiden net zo vroeg als de *EDI* NIL in lange en korte dag, terwijl transformatie met het L*er* fragment de bloeitijd van de transgene planten niet veranderde. Hiermee werd aangetoond dat de *EDI* QTL codeert voor het *CRY2* gen. Van het 4.6 kb fragment van zowel Cvi als L*er* werd de DNA basepaar volgorde bepaald en vergeleken met de gepubliceerde DNA sequentie van de accessie Columbia (Col). Er werden verschillen gevonden tussen L*er* en Cvi voor 12 nucleotiden. Twee hiervan waren het meest waarschijnlijk verantwoordelijk voor het bloeitijdverschil omdat ze in een exon gelokaliseerd waren en leiden tot aminozuur verschillen tussen L*er* en Cvi. Door middel van plant transformatie met een construct waarin het gengedeelte dat één van beide nucleotideverschillen bevat (welke een aminozuur verschil op positie 476 van Cvi en L*er* veroorzaakt) uitgewisseld werd tussen L*er* en Cvi, kon bewezen worden dat de methionine op deze positie in Cvi verantwoordelijk was voor het verschil in bloeitijdstip tussen het Cvi en L*er* *CRY2* allel. Analyse van de mRNA niveaus onder verschillende omstandigheden liet geen verschil in niveau zien tussen L*er* en de *EDI*-NIL. Een gedetailleerde analyse van de eiwitniveaus met behulp van western blotting, gebruikmakend van tegen CRY2 gerichte antilichamen, liet zien dat het CRY2 eiwit in een lange dag cyclus met 16 uur licht en 8 uur donker constant bleef, terwijl dit eiwit in een korte dag cyclus met 8 uur licht en 16 uur donker in de lichtperiode snel werd afgebroken. Onder deze korte dag omstandigheden bleek echter het CRY2 eiwit dat gecodeerd werd door het Cvi allel veel minder snel afgebroken te worden dan het eiwit gecodeerd door het L*er* allel. Dit effect bleek veroorzaakt te zijn door het bovengenoemde verschil in aminozuur positie 476.

De analyse van de *EDI* QTL liet niet alleen zien dat dergelijke loci gekloneerd kunnen worden, maar ook dat in de natuur heel specifieke allelen aanwezig zijn met verschillende functies als gevolg van een veranderde eiwit stabiliteit.

Een verdere analyse van de rol die het *CRY2* gen speelt in de bloeitijd was mogelijk door gebruik te maken van allelen van *CRY2* die functioneel verschilden van

het standaard L*er* allel doordat ze effectiever waren (met name in korte dagen) (het *CRY2*-Cvi allel) of hun functie verloren hadden (de *fha1* mutant). De interactie van deze drie *CRY2* varianten met 16 genen die de bloeitijd van Arabidopsis bepalen werd geanalyseerd. Van de hiervoor gemaakte 'dubbelmutanten' werd de bloeitijd bepaald in lange en korte dag. De epistatische interacties die gevonden werden, lieten zien dat CRY2 onafhankelijk werkt van phytochroom A. Bevestigd kon worden dat CRY2 de remming van bloei door phytochroom B kan remmen, omdat er slechts een gering effect werd gevonden van het *CRY2* allel type in een phytochroom B deficiënte mutant. Combinaties van alle *CRY2* allelen met de *co* en *gi* mutanten lieten zien dat de werking van *CRY2* volledig afhangt van een functionerend *CO* en *GI* gen. In combinatie met mutanten van andere bloeitijd genen daarentegen bleek het effect van deze mutaties additief ten opzichte van het *CRY2* allel. Het bleek dat ook in aanwezigheid van een *CRY2 –Cvi* allel een effect van de daglengte behandeling duidelijk waarneembaar is in de meeste dubbelmutanten. Dit was ook het geval wanneer *CRY2* allelen gecombineerd werden met functionerende *FLC* en *FRI* allelen. In de combinatie met zowel *FRI* als *FLC* was het effect van verschillende *CRY2* allelen niet meer waarneembaar. Na een vernalisatie behandeling bloeiden de planten echter vroeger en werd het effect van verschillende *CRY2* allelen weer zichtbaar. Een analyse van de mRNA niveaus van een aantal bij de bloei betrokken genen in twee weken oude planten liet zien, dat het niveau van *FLC* expressie vooral bepaald werd door het *FLC* en *FRI* genotype en door een vernalisatie behandeling, maar niet door het *CRY2* genotype. De expressie van *CRY2* was echter sterk verlaagd in de genotypen en behandelingen waarin *FLC* expressie relatief hoog was. Dit betekent dat *FLC* de expressie van *CRY2* onderdrukt en verklaart waarom het er niet toe doet welk *CRY2* allel aanwezig is bij hoge *FLC* expressie. Het *SOC1* gen, waarvan bekend is dat de expressie ervan positief door lange dagen en negatief door *FLC* gereguleerd wordt, bleek ook bij afwezigheid van *CRY2* (in de *fha1* mutant) beïnvloed te worden door daglengte condities, wat suggereert dat er naast *CRY2* andere factoren een rol spelen bij de daglengte perceptie.

Het in dit proefschrift beschreven onderzoek heeft laten zien dat natuurlijke variatie toegankelijk is voor moleculaire karakterisering en heeft bovendien belangrijke nieuwe informatie opgeleverd over de interactie tussen, tot voor kort als onafhankelijk geziene, routes die het bloeitijdstip beïnvloeden.

Curriculum vitae

Salah El-Din Sayed Mohamed El-Assal was born on April 14th 1963 in Cairo, Egypt. He graduated with a BSc degree in animal breeding in 1985 and an MSc in Genetics in 1990 from the faculty of Agriculture of Cairo University, Egypt. At this faculty he worked as demonstrator and assistant lecturer in the Genetics department, from where he obtained a fellowship from the government of Egypt to do his PhD research, starting in 1997 in the group of Professor M. Koornneef in the laboratory of Genetics, department of Plant Sciences, Wageningen University, which resulted in a PhD thesis entitled: 'A genetic and molecular analysis of flowering time in *Arabidopsis thaliana* using natural variation'.

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El-Assal, S.E-D., Alonso-Blanco, C., Peeters, A.J.M., Raz, V. and Koornneef, M. (2001). A QTL for flowering time in Arabidopsis reveals a novel allele of *CRY2*. *Nature Genetics* **29**, 435-440.

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وباستخدام البرنامج الإحصائي الخاص بتحديد المواقع الوراثية للصفات الكمية (QTL - mapping) تم التعرف على أربعة مواقع وراثية تتحكم في الاختلاف في عمر التزهير بين السلالتين الأبويتين أطلق عليها الأسماء التالية (*EDI* *FLF, FLG,* and *FLH*) و تمت العديد من الدراسات الوراثية المستفيضة على هذه المواقع الأربعة كل على حدة حتى يتم التعرف على الأهمية و الخصائص الوراثية لكل موقع من هذه المواقع الأربعة حيث أن الموقعان الوراثيان (*EDI* and *FLH*) يحتويان على الأليلات المبكرة في التزهير بينما الاثنان الآخران يحتويان على الأليلات المتأخرة في التزهير و وجد أن موقع (*EDI*) يعتبر من أكثر المواقع المتحكمة في التباينات الوراثية في فترة التزهير و يقع في قمة الكروموسوم الأول و أن الأليل (Cvi) في هذا الموقع أظهر عدم حساسية لطول اليوم حيث أنه يزهر مبكرا في نفس اليوم الطويل و اليوم القصير وهو سائد وراثيا على الأليل (*Ler*) في هذا الموقع.

في الفصل الثالث من هذه الرسالة قمنا بعمل فصل (*EDI* - QTL) على المستوى الجزيئي (Cloning) و هذا باستخدام عشيرة تم زراعتها في اليوم القصير حيث الاختلاف في التزهير يكون أكثر وضوحا بين النباتات التي عندها حساسية لطول اليوم وتلك التي ليس عندها هذه الحساسية. وعن طريق حالات العبور الوراثي تم عمل تحديد لموقع (*EDI* - QTL) في منطقة تبلغ طولها ٤٥ كيلو قاعدة وهذه المنطقة تحتوي على مجموعة (ORFs) فيما بينهما الجين المسؤل عن استقبال طيف الضوء الأزرق (*CRY2*) *Cryptochrome 2* .
من أجل التأكد أن (*EDI* - QTL) هو الأليل *CRY2*-Cvi [الأليل الأكثر نشاطا لهذا الموقع الوراثي]. تم عمل (PCR) لحوالي ٤و٦ كيلو قاعدة بها (*CRY2* - ORF) وتم وضعها داخل بلازميد واستخدم هذا المركب في عمل تحويل وراثي للاب لاندسبرج اركتا [المتأخر في التزهير في اليوم القصير]. كانت نتائج الجيل الأول والثاني من التحويل الوراثي مدهشة حيث أعطت نباتات مبكرة في التزهير في اليوم القصير. في دراسة أخرى أكثر دقة للتعرف على الطفرة الموضعية المسئولة عن هذه الصفة . تم عمل دراسة تحليلية لتتابعات القواعد في شريط (DNA) و تم حصر عدد من الطفرات الموضعية كان من أهمها وأكثرها ترشيحا هو طفرة في أحد القواعد الوراثية أدت الى تحويل في الحمض الاميني ثالين الى ميثيونين . تم بناء مركب وراثي حيث استبدلت فقط هذه القاعدة وباقي المركب عبارة عن تتابع يشبه الطراز البري (*Ler*) . واستخدمت مرة أخرى في التحويل الوراثي على السلالة البرية وأعطت ايضا صفة التزهير المبكر مما يدل بشكل قاطع على أن هذه الطفرة الموضعية هي المسئولة عن صفة التزهير المبكر. أيضا قمنا في دراسة مهمة اضافت كثيرا الى قيمة هذا البحث بعمل تتبع للتغيير الجيني على مستوى البروتين بين الطراز البري و الطفرة الطبيعية (*EDI*) وأيضا بعض التحولات وراثيا في هذا الموقع الوراثي. وجد أن الطراز البري في اليوم القصير لا يحتفظ بكمية البروتين بعد بداية اليوم بساعتين [بعد التحويل من الإظلام التام الى الإضاءة المعتادة] في حين ان الطفرة الطبيعية احتفظت بكمية البروتين الى وقت أطول بالمقارنة بالطراز البري وبينما في اليوم الطويل لم نجد أي فرق في كمية البروتين بين الطراز البري و الطفرة الطبيعية مما يتوافق مع الفرق في التزهير بين الطراز البري و الطفرة الطبيعية في اليوم القصير حيث ان وجود بروتين (*CRY2*) لفترة أطول مهم للإسراع في عملية التزهير.

في الفصل الرابع من هذه الرسالة قمنا بدراسة التفاعل الوراثي بين *CRY2* gene و معظم الجينات المهمة التي تنتمي الى المجاميع الوراثية المختلفة المتحكمة في التزهير. لذلك قمنا بعمل تواليف وراثية بين [لأليلات المختلفة لـ (*CRY2* gene) وطفرات هذه الجينات المختلفة وتم زراعة السلالات الناتجة التي تحتوي على هذه التواليف الوراثية بين كريبتوكروم و كل هذه الطفرات كل اثنين على حده و أحيانا قمنا بعمل سلالات مؤلفة من ثلاث طفرات مختلفة لثلاث جينات مختلفة من بينها احدى البيلات (*CRY2*) . تمت زراعة السلالات التي تحتوي على طفرتين أو ثلاث طفرات في حضانات خاصة بها مصدر ضوء أحمر أو أزرق أو ازرق مختلط مع أحمر و أشادت النتائج المختلفة لهذه النباتات الى انه بالرغم من اهمية (*CRY2*) لنباتات كمستقبل لطيف الضوء الأزرق الا أنه ثبت نشاطه أيضا و اهميته في وجود الضوء الاحمر بالإضافة الى اهمية أليلات في ازالة التاثير المثبط لـ *Phytochrome B* (*PHYB*) في عملية التزهير. وأن (*CRY2* - Cvi) هو الأليل من (*CRY2*) الأكثر نشاطا في تحليز عملية التزهير في مختلف التجارب التي تم استخدام الضوء الملون بها. بينما الطفرات الثنائية بين البيلات (*CRY2*) المختلفة وطفرات الجينات التي تتوسط في تحكم اليوم الطويل في فترة التزهير كانت النتائج المتحصل عليها في غاية الأهمية حيث أدت الى وضع (*CRY2*) في تسلسل الجينات التي تتحكم في التزهير من خلال احساسها لطول اليوم وان (*CRY2*) في قمة هذه المجموعة و يليه الجينات (*CO* and *GI*) و هما من الجينات التي لها أهمية كبيرة حيث تتوسط اليوم الطويل و تتحكم في التزهير بالإضافة الى تداخلها مع الجينات التي تظهر نشاطا يتوقف على الفترة الضوئية اليومية المختلفة المعتادة (Circadian clock genes) .
أخيرا الطفرات المزدوجة والثلاثية مع جينات (*FLC* and *FRI*) و ترجع أهمية هذه الجينات التي توسطها في الاستجابة بالمعاملة الباردة وانها تنقل هذا التأثير في صورة التزهير المبكر . وهذه التجربة أضافت الكثير من القيمة العلمية لهذا البحث حيث أسفرت نتائجها عن اكتشاف خط تفاعل مباشر يربط بين جينات اليوم الطويل و جينات المعاملة الباردة ولكن نتائج الطفرات الثنائية و الثلاثية بين البيلات (*CRY2* gene) والبيلات جينات (*FLC* and *FRI*) حيث وجد ان الأليلات النشطة من (*FLC* and *FRI* genes) تقوم بعمل تثبيط للأليل (*CRY2*-Cvi) [النشط] من (*CRY2* gene) مما يشير الى انه يوجد اتصال مباشر بين خط التفاعل الذي يتوسط الإستجابة للمعاملة الباردة وخط التفاعل الذي يشمل الجينات التي تتوسط اليوم الطويل [استجابة لطول اليوم] .

الملخص العربي

يعتبر الارابيدوبسيس نموذج تجريبي ممتاز للنباتات الزهرية حيث يتميز بأنه صغيرة الحجم و قصر دورة حياته و صغر حجم الهيئة الوراثية حيث انها تحتوي على حوالي ٢٥٠٠٠ جين موزعة على ٥ كروموسومات. و قد تم التعرف على تتابع المادة الوراثية مع نهاية عام ٢٠٠٠ مما ساهم بشكل كبير في التعرف على موقع و وظيفة عدد كبير من الجينات بالإضافة الى كفاءة استخدام هذا النبات في تجارب التحول الوراثي.
في أوائل الستينات تم التعرف على العديد من الطفرات المتأخرة في التزهير مقارنة بالطراز البري وقد أعقب ذلك بعض الدراسات الفسيولوجية المتخصصةالتي أدت الى التعرف على أكثر من ٨٠ جين تتحكم في عملية التزهير في الارابيدوبسيس. وقد أشارت العديد من الدراسات الوراثية المختلفة الى أن عملية الإنتقال من الحالة الخضرية الى الحالة الزهرية [فترة التزهير] في نبات الارابيدوبسيس تتم من خلالشبكة معقدة من التفاعلات الوراثية مع الوضع في الاعتبار أن طول فترة الاضاءة و درجة الحرارة المنخفضةيعتبرا من اهم العوامل البيئية التي تتداخل مع العوامل الوراثية من أجل التحكم في عملية التحول من الحالة الخضرية الى الحالة الزهرية.

قد تم استخدام الطفرات المستحدثة معمليا كاسلوب ناجح على مدار الاعوام السابقة من اجل التعرف على مواقع و وظيفة الجينات المتحكمة في فترة التزهير و قد تم استخدام التباينات الوراثية التي تظهر بين العشائرالطبيعية من نباتات الارابيدوبسيس التي تنتمي الى مواقع جغرافية مختلفة كبديل ناجح للطفرات المستحدثة معمليا من اجل دراسة وظائف الجينات المختلفة.
هناك العديد من الامثلة الوراثية الناجحة على استغلال المعلومات المتوافرة من التباينات الوراثية الطبيعية في بعض سلالات الارابيدوبسيس المتأخرة التزهير من أجل عزل بعض الجينات المهمة و التي تلعب دورا رأيسيافي التحكم في عملية التزهير مثل جينات (*FLC* and *FRI*) . وهذين الموقعين الوراثيين يعتبران مسئولان بدرجة كبيرة عن التغيرات الطبيعية في فترة التزهير بالإضافة الى استجابتهما الكبيرة لدرجة الحرارة المنخفضة التيتؤدي الى التزهير المبكر في هذه النباتات التي تحمل الأليل السائد من هذه الجينات [النشطة وراثيا]. وأن عملية الفصل الوراثي لهذين الجينين أعطت الاشارة الى امكانية الفصل الوراثي للجينات المسئولة عن التغيرات الوراثية الطبيعية في العديد من الصفات و خصوصا صفة التزهير. وجد أنه يتم التحكم في التغيرات الوراثية الطبيعية بواسطة عدد كبير من الجينات التي بدورها تتأثر ببعض العوامل البيئية و هذا مما جعل العديد منالصفات التي تظهر تغيرات وراثية طبيعية تسلك سلوك الصفات الكمية التي حتى وقت قريب كانت دراستها غاية في الصعوبة.
مع تقدم مجال الوراثة الجزيئية و استخدام بعض المعلمات الوراثية و بعض الطرق الاحصائية المتخصصة تم التعرف على مواقع الجينات التي تتحكم في صفة التزهير بطرق كمية (QTL - mapping) . بالاضافة الى استخدام بعض التقنيات الوراثية بحيث يمكن تتبع و فصل أحد المواقع المتحكمة في هذه الصفة الكمية و التعامل معها باعتبار انها صفة مندلية و من ثم يمكن دراستها باستفاضة.
بالرغم من انه تم التعرف وراثيا و جزيئيا عل العديد من الجينات التي تلعب دورا مهما في عملية التزهير في نبات الارابيدوبسيس وأيضا تم التعرف على كيفية التفاعلات الوراثية بين هذه الجينات و بعضها البعض الاانه ما تزال الصورة الوراثية عن الجينات التي تتحكم في صفة التزهير مازالت لم تكتمل بعد.
وتهدف الدراسة الحالية في هذه الرسالة الى الدراسة الجزيئية و الوراثية لبعض العوامل الوراثية المسؤلة عن الاختلافات الطبيعية في صفة التزهير بين السلالتين الطبيعيتين لنبات الارابيدوبسيس و هما لاندسبرج اريكتا (*Ler*) و جزر الرأس الاخضر (Cvi) . و من المعروف ان هاتين السلالتين نشأتا في منطقتين جغرافيتين شديدتا الاختلاف في المناخ مما يعني ان هيئتهما الوراثية تحتوي على العديد من التباينات الوراثية الطبيعية.

في الفصل الثاني من هذه الرسالة و من اجل دراسة التباينات الوراثية الطبيعية التي تتحكم في فترة التزهير تم الحصول على عشيرة مرباه داخليا لمدة عشرة اجيال حتى نضمن عدم وجود مواقع خليطة وراثيا . و تم زراعة هذه العشيرة في ثلاث بيئات مختلفة من حيث طول اليوم و المعاملة في درجة الحرارة المنخفضة حيث انه من المعروف أن النباتات التي تنمو في اليوم الطويل تزهر أسرع من تلك التي تنمو في اليوم القصير و أيضا الحرارة المنخفضة تشجع على التزهير المبكر و تم تدوين الإختلافات في فترة التزهيرفيما بين أفراد هذه العشيرة بعضها البعض و فيما بينها و بين السلالتين الابويتين.